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Directing dendritic cell immunotherapy towards successful cancer treatment

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Abstract

The use of dendritic cells (DCs) for tumor immunotherapy represents a powerful approach for harnessing the patient's own immune system to eliminate tumor cells. However, suboptimal conditions for generating potent immunostimulatory DCs, as well as the induction of tolerance and suppression mediated by the tumors and its microenvironment have contributed to limited success. Combining DC vaccines with new approaches that enhance immunogenicity and overcome the regulatory mechanisms underlying peripheral tolerance may be the key to achieving effective and durable anti-tumor immune responses that translate to better clinical outcomes.

Keywords

dendritic cell; tumor immunotherapy; vaccination

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs), capable of activating both naive and memory immune responses, and maintaining the delicate balance between immunity and tolerance. Several studies have demonstrated that antigens expressed by tumors, including tumor-specific antigens, can be loaded on DCs to trigger an immune response *in vitro*. Clinical trials of antigen-pulsed DCs have been conducted in patients with various types of tumors, including breast cancer, multiple myeloma, prostate cancer, renal cell carcinoma, malignant melanoma, colorectal cancer and non-small-cell lung cancer [201]. While these studies have demonstrated that antigen-loaded DC vaccines are a safe and promising therapy for tumors, their clinical efficacy remains to be established. The first part of this review discusses human DC biology and the second part focuses on the application of DCs for harnessing for anti-tumor immunity.

DC biology

The combined action of both the innate and adaptive immune system is required for the development of protective immune responses. The innate immune response serves as the initial defense against pathogenic organisms or neoplastic cell growth by providing a local and

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immediate response, and allows for the development of the adaptive immune response. The adaptive immune response is required for the eventual clearance and the generation of immunologic memory. APCs, which consist of monocytes, macrophages, B cells and DCs, are central to this system as they provide the important link between innate and adaptive immune responses.

It is generally thought that DCs are the most potent APCs owing to their superior capacity for acquiring and processing antigens for presentation to T cells, and their potential to express high levels of costimulatory/coinhibitory molecules that drive immune activation or anergy. In addition, DCs have the capacity to modulate immune responses by instructing T-cell differentiation and polarization. Therefore, DCs play a significant role in the overall generation of immune responses against antigens [1].

DC subsets

There are two major subsets of human DCs: myeloid and plasmacytoid. DCs originate from $CD34^+$ bone marrow precursors and are distinguished from other peripheral white blood cells by their lack of expression of lineage markers (CD3, CD14, CD16, CD19, CD20 and CD56) and high expression levels of MHC II molecules on their surface. The myeloid DCs (mDCs), which include the dermal DCs, Langerhans cells, interstitial DCs and interdigitating DCs, are distinguished by their expression of CD11c and differentiate from myeloid progenitors. The plasmacytoid DCs (pDCs), distinguished by their expression of CD123, were first thought to have come from lymphoid progenitors. However, studies in mice demonstrated that pDCs may also differentiate from myeloid progenitors [2]. Thus, myeloid progenitors may have the ability to differentiate into both mDCs and pDCs.

The two DC subsets have distinct properties. pDCs are primarily found in blood and lymphoid organs such as the thymus, bone marrow, spleen, tonsils and lymph nodes. pDCs are the principal producers of type I interferons (IFNs) in response to microbial and viral infection, with the ability to produce up to 1000-fold more type I IFNs in response to viral infections than other cell types [3]. pDCs may also be involved in immune responses against tumors. *In vitro*, they have been shown to prime melanoma-specific $CD8^+$ T-cell responses [4]. However, pDCs may also contribute towards suppressing anti-tumor immune responses. For example, pDCs with diminished capacity to produce IFN-α have been found in many tumors [4,5]. Type I IFNs, in addition to their antiviral functions, are critical for preventing the establishment of tumors through their ability to enhance both innate and adaptive immune responses [6]. Furthermore, pDCs expressing indoleamine 2,3-dioxygenase (IDO), an enzyme implicated in the generation of regulatory T cells, have also been located in tumor-draining lymph nodes [7–10]. mDCs are more prevalent than pDCs. They are found in peripheral tissues, lymphoid organs and in the blood. mDCs secrete large amounts of IL-12 upon activation and are therefore important for generating immune responses against pathogenic organisms or to suppress neoplastic cell growth [11]. They are thought to be similar to DCs generated from blood monocytes, the latter constituting the main source of DCs used for vaccination trials.

Antigen uptake, processing & presentation

In their immature state, DCs are thought to be highly specialized in antigen uptake. DCs take up antigens through phagocytosis, macropinocytosis or endocytosis using Fc receptors (Fc-γ receptor types I [CD64] and II [CD32]), integrins (αVβ3 or αVβ5), C-type lectins (mannose receptor DEC205) and scavenger receptors. Upon uptake, DCs process protein antigens into peptides that are loaded onto MHC molecules for presentation to T cells.

Endogeneous antigens are degraded into peptides by the proteasome in the cytosol. These peptides are then transported into the endoplasmic reticulum (ER) using transporters for antigen

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presentation (TAP) molecules and loaded on MHC class I molecules. Peptide–MHC class I complexes are transported out of the ER, through the trans Golgi network, and out to the surface for presentation to $CD8^+$ T cells. DCs and other cell types can process peptides using this pathway.

Exogenous protein antigens are engulfed and processed in endosomes. Endosomes contain proteases that degrade the protein antigens into peptides before loading onto MHC class II molecules. Peptide–MHC class II complexes are then transported to the cell surface for presentation to $CD4^+$ T cells. Owing to the limited expression of MHC class II molecules, only APCs can process peptides using the exogenous pathway. Exogenous protein antigens can also be processed by DCs and loaded onto MHC class I molecules using cross-presentation. While the precise mechanism of cross-presentation remains controversial, the ability of DCs to utilize this process to activate $CD8^+$ T cells is well established [1,12].

Dendritic cells can also process and present lipid antigens. Lipid antigens, expressed on pathogens or self-tissues, are processed in specialized intracellular compartments and presented on CD1d molecules via several mechanisms. Surface receptors internalize pathogens into phagosomes, where they are processed by lysosomal hydrolases to produce lipid antigens, and are then loaded onto CD1d molecules. Membrane-bound lipids are internalized in clathrin-coated vessicles and transported to late endosomes where lipid antigens are then loaded onto CD1d molecules. Lipid antigens are extracted from internalized apoptotic bodies or exosomes by lipid transfer proteins and loaded onto CD1d molecules [13]. Lipid antigens complexed with CD1d molecules on the surface of APCs activate natural killer T (NKT) cells. Clinical studies using α-galactosyl ceramide (α-GalCer) to activate NKT cells resulted in the specific expansion of the NKT cell population [14]. NKT cells have been shown to be important for mediating anti-tumor immune responses through direct cytotoxicity on tumor cells as well as activation of both innate and adaptive immune responses [15].

In addition to lipid antigens, DCs can also recognize and uptake antigens based on their carbohydrate structures. DCs express C-type lectin receptors (CLRs) such as the macrophage mannose receptor, DEC-205 and DC-specific ICAM-3-grabbing nonintegrin (DC-SIGN), all of which can function as endocytic receptors to internalize antigens for degradation for antigen processing and presentation [16,17]. Each CLR recognizes and binds specific carbohydrate moieties expressed on pathogens or self-tissues to mediate its uptake. MMR has been shown to deliver antigen to early endosomes while DEC-205 and DC-SIGN deliver antigens to late endosomes or lysosomes for degradation [18]. Endocytosed antigens are loaded onto MHC class II molecules to stimulate $CD4^+$ T-cell responses. Endocytosed antigens are also presented on MHC class I molecules for stimulation of $CD8^+$ T-cell responses [19–21]. Although less understood, this process presumably occurs via cross-presentation whereby small quantities of antigen escape from the endosome to the cytosol and gain access to the MHC class I processing pathway [18]. In addition, increasing evidence suggests the existence of cross-talk between CLRs and Toll-like receptors (TLRs), which may have important implications in the balance between immune tolerance and activation.

Maturation

Maturation of DCs is characterized by reduced phagocytic capacity, enhanced processing and presentation, enhanced ability to migrate to lymphoid tissues, and increased ability to stimulate B and T cells. Maturation is often induced by stimuli or ‘danger signals,’ which can be classified as exogenous or endogenous. Exogenous stimuli refer to microbial products and are mediated through activation of pattern recognition receptors such as TLRs [22], or via activation of endogenous sensors such as RIG-I [23] or members of the inflammasome [24]. Endogenous stimuli refer to inflammatory molecules (TNF-α, IL-1, IL-6 and IFN-α), produced by the cells of the immune system or by damaged tissues (Figure 1) [25].

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Maturation is accompanied by increased expression of chemokine receptors, adhesion molecules and costimulatory molecules that are involved in the migration of DCs to lymphoid tissues and are necessary for optimal activation, proliferation, and differentiation of B and T cells. DCs upregulate expression of CCR7, which directs DCs to lymphoid tissues in response to CCL19 (MIP-3β, ELC) and CCL21 (SLC) [26]. Adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1 or CD54) are upregulated upon maturation and bind lymphocyte-function-associated antigen 1 on T cells to form stable long-lasting contacts necessary for T-cell activation [27]. DCs also upregulate surface expression of costimulatory molecules that function either to augment or attenuate antigen-specific T-cell responses. DCs express various members of the B7 superfamily that function to costimulate or coinhibit T-cell activation upon binding to CD28 and CTLA-4 on T cells, respectively. DC maturation induces the expression of B7-1 (CD80) and B7-2 (CD86) and binds to CD28 on T cells to provide the requisite secondary signal for optimal T-cell activation. T-cell activation induces the expression of CTLA-4, and upon binding to B7-1 and B7-2 leads to inhibition of T-cell stimulation [28]. DCs, along with B cells and macrophages, constitutively express CD40, which is further upregulated upon DC activation. Binding of CD40 to CD154 (CD40L), expressed by activated $CD4^+$ and $CD8^+$ T cells, results in the upregulation of MHC molecules, enhanced DC survival, and increased production of inflammatory cytokines such as TNF and IL-12 [29].

In addition, maturation of DCs also involves the production of cytokines and chemokines, which influence the type of immune response generated. Cytokines secreted by activated DCs and by other immune cells play a role in differentiating subtypes of $CD4^+$ T cells such as T helper (Th)1, Th2, Th17 and regulatory T cells. Th1 cells are important for immune responses against intracellular pathogens. Th2 cells are important for immune responses against parasites. The recently described Th17 cells are important for immune responses against extracellular bacteria and fungi, but at the same time can also mediate autoimmunity [30]. Regulatory T cells are important for control of immune responses [31]. The requirements for the differentiation of Th2, Th17 and regulatory T cells remain the subject of controversy. The lack of Th1 cytokines, specifically IL-12, appears to direct development of Th2 cells. IL-4 and thymic stromal lymphopoietin, possibly from basophils, may also play an important role in differentiating Th2 cells [32,33]. TGF-β, IL-1β, and IL-6, IL-23 or IL-21 have been implicated in the differentiation of Th17 cells [34]. However, it has been established that IL-12, produced primarily by activated DCs, is critical for the differentiation of Th1 cells [11]. In addition, chemokines produced by DCs, such as RANTES, MIP-1α, MIP-1β and IP-10, recruit NK cells, T cells, monocytes, as well as other DCs into the local environment. Thus, by activating and recruiting specific cell types into the local environment, DCs qualitatively and quantitatively orchestrate the type of immune responses that develop.

Immune stimulation

Upon maturation, DCs migrate to the secondary lymphoid tissues such as the lymph nodes (capturing antigen from the skin and solid organs), spleen (capturing antigen from the blood), or the Peyer's patches (capturing antigen from the gut lumen) where they come into contact with T and B cells [1]. Through their T-cell receptors (TCRs), T cells specifically recognize antigens bound to MHC molecules on the surface of the DCs. Peptides bound on MHC class I molecules are recognized by $CD8^+$ T cells while peptides bound on MHC class II molecules are recognized by $CD4^+$ T cells.

Activation of T cells is dependent upon the intensity and length of DC–T cell interactions, mediated through the immunological synapse (IS). The IS results from cytoskeletal reorganization within the T cells, leading to the dynamic clustering of T-cell surface receptors and signaling molecules into supramolecular activation clusters and providing an optimal

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environment for signaling molecules downstream of the TCR. The upregulation of costimulatory molecules during the maturation of DCs is critical for making stable long-lasting contacts with T cells. It is this interaction within the IS that is required for clonal expansion and differentiation into memory and effector T cells. In addition to the activation of T cells, DCs can also activate naive and memory B cells [35], NK cells via IL-12, IL-15, and type I IFNs [36], and NKT cells through expression of invariant CD1 molecules and presentation of glycolipid molecules [37]. Therefore, DCs play an important role in mobilizing multiple arms of both innate and adaptive immune responses.

Immune tolerance

Dendritic cells play an important role in balancing the immune system between immunity and tolerance [38]. DCs mediate peripheral tolerance using several mechanisms. In their immature state, owing to the low expression levels of MHC and costimulatory molecules, DCs do not activate T cells. DCs may also induce the expression of indoleamine 2,3-dioxygenase (IDO) leading to T-cell deletion. IDO is responsible for the degradation of tryptophan, an amino acid essential for T-cell proliferation. Degraded tryptophan drives T cells to undergo cell cycle arrest or apoptosis. In addition, the tryptophan metabolites (kynurenine, 3-hydroxy-kynurenine and 3-hydroxy-anthranilinic acid) produced can exert direct cytotoxic effects on T cells [39,40]. DCs can also induce the differentiation of regulatory T cells [41,42] that exert their effects through the activities of TGF-β, IL-10 and CTLA-4, amongst other mechanisms, to inhibit proliferating T cells [31,38]. Regulatory T cells have been shown to infiltrate several tumors [43,44] and their depletion leads to enhancement of tumor antigen-specific immune responses [45]. In addition to mDCs, pDCs have also been demonstrated to induce regulatory T cells [9,46]. DCs also play an important role in mediating tolerance through the uptake of apoptotic cells. Under steady-state conditions, DCs recognize and capture apoptotic cells using different receptors including LOX-1, CD36, αVβ3, αVβ5 and the complement receptors (CRs) CR3 and CR4 to mediate peripheral tolerance to self-antigens. Ligation of CR3 by apoptotic cells on DCs led to impaired maturation in response to stimulation with LPS. Furthermore, the ability of DCs to prime and activate memory T-cell responses was impaired [47]. Therefore, DCs play an important role in mediating peripheral tolerance and prevention of autoimmunity [48].

Manipulating DC biology for tumor immunotherapy

The scientific rationale for developing immunotherapeutic approaches aimed at enhancing tumor-specific immune responses came from several studies indicating that the immune system may occasionally eradiate tumors. There have been reports of spontaneous regression of both primary and metastatic melanoma [49]; animal and clinical studies revealed the existence of a wide variety of tumor-associated or specific antigens that are immunogenic [50]; knockout mice lacking components of the IFN-γ signaling pathways, perforin, or recombination-activating gene 1/2 (*RAG-1/2*) had a significantly higher risk of developing tumors [51]; and the adoptive transfer of tumor-infiltrating lymphocytes has been shown to induce the objective regression of tumors in melanoma patients [50].

These studies led to the development of the cancer immunosurveillance hypothesis. However, the discovery that the immune system plays a dual role; both protecting the host against the establishment of tumors as well as promoting tumor growth, led to a new concept termed cancer immunoediting [52]. The failure of the immune system to eliminate all precancerous or cancerous cells favors the outgrowth of tumors with reduced immunogenicity, thereby accounting for the fact that tumor progression or ‘escape’ can occur despite the induction of immune responses. Therefore, administration of vaccines that boost endogenous anti-tumor responses may be beneficial and may lead to effective eradication of tumors.

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Given that the presentation of tumor antigens by DCs is a central step in the induction of anti-tumor responses, DCs serve as an ideal tool for boosting endogenous anti-tumor responses. In support of this, early clinical trials testing vaccination with *ex vivo*-generated DCs pulsed with tumor antigens demonstrated that immune responses could indeed be induced. These early studies fuelled further studies designed to manipulate DCs to enhance immune responses *in vivo*, and these are summarized in the following sections.

Preparation of DCs

The development of *ex vivo* methods of generating DCs paved the way for studies to use DCs for immunotherapy. As there is no current consensus on the optimal method of generating DCs for immunotherapy use, several methods for generating DCs are currently used in clinical trials. These include differentiation from monocyte precursors, $CD34^+$ hematopoietic precursors and *in vivo* expansion of circulating DCs. Although no direct comparison of all the different methods of DC generation exists in clinical trials, DCs derived using these different methods have been demonstrated to stimulate antigen-specific T-cell responses in both preclinical and clinical studies.

Monocyte-derived DCs

The most commonly used approach is the differentiation of DCs from peripheral blood mononuclear cells (PBMCs) obtained from whole blood or leukapheresis procedures. These DCs are called monocyte-derived DCs (moDCs). To obtain sufficient numbers of DCs for vaccinations, PBMCs are usually obtained from leukapheresis procedures. $CD14^+$ monocytes are first selected from PBMCs either by plastic adherence or positive selection using immunomagnetic beads [53–57]. The monocytes are induced to differentiate into immature $CD14^-CD83^-$ DCs by culturing for several days in the presence of IL-4 and GM-CSF. The immature DCs are stimulated to become mature DCs by culturing for an additional 1–2 days in the presence of a maturation stimulus. Mature DCs are $CD14^-$and $CD83^+$ cells that express high levels of MHC class I and II molecules, the costimulatory molecules CD40, CD80 and CD86 [56].

More recently, a novel faster method of differentiating DCs from monocyte precursors has been developed. Owing to the speed with which these DCs can be derived (2 days vs 5–7 days), these DCs are termed FastDCs. Monocytes are enriched from PBMCs by $CD14^+$ selection using CD14 immunomagnetic beads and subsequently cultured for 48 h with GM-CSF and IL-4. After 24 h of culture with GM-CSF and IL-4, the monocytes downregulated expression of CD14 and upregulated expression of MHC class II, characteristic of immature DCs. Addition of proinflammatory cytokines (TNF-α, IL-1β and IL-6) and PGE_2 for an additional 24 h led to the differentiation of the immature DCs into phenotypically mature DCs [58]. Comparison of FastDCs with proinflammatory cytokine-matured moDCs revealed a similar efficiency in inducing antigen-specific T-cell proliferation [59–61]. Further studies are required to determine the effectiveness of DCs generated using this method in stimulating tumor-specific immune responses in clinical trials.

DCs derived from $CD34^+$ hematopoietic progenitors

Dendritic cells can also be propagated from $CD34^+$ precursors. $CD34^+$ precursors are first mobilized from the bone marrow by treatment of patients with GM-CSF prior to leukapheresis procedures [62]. The harvested cells are further expanded in culture for 1 week or more in the presence of GM-CSF, Flt3L and TNF-α. The DCs obtained from this culture are a mixture of moDCs, DCs that are phenotypically similar to epidermal Langerhans cells, and a large proportion of myeloid cells at different stages of differentiation. It is worth noting the Langerhans cells from this mixture may be the cell type responsible for stimulating T-cell

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responses instead of the DCs; whereas the DCs may be more important for inducing B-cell responses akin to the dermal DCs of the skin [63]. Similar to moDCs, $CD34^+$-derived DCs that are matured and loaded with antigens have been used in clinical trials [64,65].

DCs enriched from peripheral blood

Dendritic cells can also be directly isolated from circulating DCs. Circulating DC subsets comprise less than 1% of PBMCs. *In vivo* expansion of these rare cells can be achieved by administration of hemopoietic growth factors such as Flt3L followed by leukapheresis [66]. Daily administration of Flt3L for 10 days led to a 48-fold expansion of mDCs and 13-fold expansion of pDCs. DC subsets expanded *in vivo* upregulated maturation markers and produced cytokines upon stimulation and stimulated T-cell responses [67].

Dendreon's Provenge (sipuleucel-T), currently awaiting approval by the US FDA for treatment of hormone-refractory prostrate cancer, is an example of a DC vaccine using DCs enriched from peripheral blood. Erythrocytes, granulocytes, platelets, lymphocytes and low-density monocytes were removed from leukapheresis collections, resulting in a DC-enriched product that also includes B cells, monocytes and NK cells. This DC-enriched product is then processed, cultured *ex vivo* with a recombinant fusion protein containing prostatic acid phosphatase and GM-CSF, and then administered back to patients within 48 h of the leukapheresis collection [68].

Maturation stimuli

Maturation of DCs prior to vaccination is important. *In vitro* studies have shown that mature DCs have enhanced expression of costimulatory molecules and increased production of important cytokines and chemokines necessary for the efficient activation of T-cell responses [1]. Furthermore, immature DCs fail to induce antigen-specific responses [69] and have the potential to induce the differentiation of regulatory T cells [38,70]. A direct comparison of peptide-loaded immature and mature DCs in patients with metastatic melanoma established that only the peptide-loaded mature DCs induced antigen-specific T-cell responses [71]. Furthermore, effective migration of DCs to lymphoid tissues requires the maturation of DCs [72]. However, determining the appropriate maturation stimulus for the generation of potent immunostimulatory DCs that would stimulate the desired quality of T-cell responses *in vivo* is complex.

The majority of clinical trials using DCs for immunotherapy have used a cocktail of proinflammatory cytokines to mature the DCs [73]. The DC maturation stimulus used in the past has been a combination of autologous cytokines (TNF-α, IL-1β, IL-6 and sometimes IFN-α) secreted by adherent monocytes in culture (monocyte-conditioned medium [MCM]). However, this cocktail of cytokines induced maturation that was highly variable from donor to donor. Therefore, a standardized cocktail of proinflammatory cytokines consisting of TNF-α, IL-1β, IL-6 and PGE_2 (MCM-mimic), which promotes a more uniform maturation in moDCs after 2 days of culture and is more compatible for clinical applications, was developed [73]. moDCs matured with the standardized cytokine cocktail upregulated expression of MHC class I and II, CD40, CD80 and CD86, consistent with a mature DC phenotype. In addition, moDCs matured with the cytokine cocktail expressed elevated levels of CCR7, necessary for trafficking to lymphoid tissues. One notable deficiency in the cytokine cocktail-matured DCs is the lack of IL-12p70 production. Despite the lack of IL-12p70 production, side by side comparison with other DC maturation stimulus (CD40L trimer, MCM, poly I:C and LPS) demonstrated that maturation with MCM mimic resulted in the most uniform maturation in terms of upregulation of phenotypic markers associated with DC maturation; the highest DC yields and recovery; it stimulated the highest levels of allogeneic T-cell proliferation and cytokine production; and induced the priming of Th1 responses [73]. Therefore, this standardized

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cytokine cocktail became the maturation stimuli of choice for DCs for clinical applications. However, the efficacy of MCM-mimic in generating potent immunostimulatory DCs for use in clinical trials has been questioned. Recent *in vitro* studies indicate that DCs matured with the current standard cytokine cocktail, in particular the presence of PGE_2, may induce the differentiation of regulatory T cells and Th2 responses [74], express IDO [75], and are deficient in IL-12p70 production [76]. They may, therefore, inhibit rather than prime the development of anti-tumor responses. On the other hand, PGE_2 is critical for the migration of DCs into lymphoid tissues through the upregulation of CCR [77]. Furthermore, PGE_2 may be important for enhancing T-cell proliferation through induction of OX40L, CD70 and 4-1BBL on DCs [78]. Recently, a novel cytokine cocktail was introduced, which included TNF-α, IL-1β, polyinosinic:polycytidylic acid, IFN-α and IFN-γ. DCs matured using this novel cytokine cocktail, termed α-type 1-polarized DCs, produced high levels of IL-12p70, migrated in response to CCR7 ligands, and induced CTL responses against TAA [79,80].

CD40 ligand (CD40L or CD154) has also been used to mature DCs. CD40L is a principal component of $CD4^+$ T-cell help that binds to CD40 on DCs and licenses DCs to mature, and therefore has the capacity to prime $CD8^+$ T cells into effector cells. CD40L is expressed primarily by activated T and B cells and induced in other cells during inflammatory conditions [29]. CD40L has been used to mature DCs for vaccination in clinical trials [54] and in combination with IFN-α or TNF to elicit maximal activation of DCs.

Toll-like receptors are pattern-recognition receptors that bind pathogen-associated molecular pattern molecules. Activation of TLRs on DCs induces DC maturation and the upregulation of costimulatory molecules, and the production of cytokines and chemokines [81]. In addition, the simultaneous triggering of different TLRs on DCs can mediate synergistic effects on DC function, resulting in the production of supramolecular levels of IL-12 [82,83]. Thus, TLR agonists have the potential for inducing optimal DCs for stimulating effective immune responses.

Strategies for loading of antigens

In addition to the activation of DCs, the method of delivery for antigens is important as this determines whether $CD4^+$ T cell and/or $CD8^+$ T cells are activated. In general, delivery of antigens directly to the cytosol results in presentation on MHC class I molecules and activation of $CD8^+$ T cells. Antigens that are endocytosed are preferentially presented on MHC class II molecules and activate $CD4^+$ T-cell responses. In certain circumstances, endocytosed antigens may also be presented on MHC class I molecules via cross-presentation. As both $CD4^+$ and $CD8^+$ T cells are required to mediate protective immunity, antigen-loading strategies that target presentation on both MHC class I and II molecules are ideal.

Pulsing of peptides, proteins & tumor cells

The most common mechanism by which DCs are loaded is through pulsing of DCs with peptides, proteins or actual irradiated tumor cells. The preparation for loading with peptides, proteins or tumor cells is relatively straightforward and involves incubation of DCs, before or after maturation, with the chosen peptides, proteins or tumor cells [57]. Peptides are loaded directly onto MHC class I and II molecules on the surface of the DCs whereas the use of proteins and tumor cells requires the initial processing and presentation by the DCs to stimulate $CD4^+$ and $CD8^+$ T cells. Once loaded with antigens, the DCs can be frozen in aliquots and thawed as required for vaccinations [57].

Tumors express antigens that are recognized by the immune system. There are now a number of characterized tumor-associated antigens (TAAs) for both $CD4^+$ and $CD8^+$ T cells from a variety of tumors that can be exploited for immunotherapy. TAAs can be the products of

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mutations (inactivating mutations on CDK4 or activating mutations on Ras), differentiation antigens (tyrosinase, tyrosinase-related protein [TRP]1, TRP-2, gp100 or Melan A/MART-1), antigens derived from oncogenic viruses (human papilloma virus E6 and E7 proteins in cervical cancer), or self-antigens specifically upregulated on tumors. A major disadvantage of using peptides is that it necessitates knowledge of patient's haplotypes and the defined peptides that would bind these specific haplotypes. Thus, the use of peptides may not be applicable for all patients. Recent studies using synthetic long peptides (SLPs) given with adjuvant demonstrated that they could be used to activate both $CD4^+$ and $CD8^+$ T cells. Owing to their length, SLPs are unable to bind directly on MHC class I and, therefore, require processing. SLPs are presumably taken up, processed, and presented by DCs through cross-presentation [84,85].

Dendritic cells loaded with proteins [84] and autologous or allogeneic tumors/tumor-cell lines [54,87–90] have also been used for DC immunotherapy in numerous cancers. A major advantage of this strategy is that it is not limited to a selected haplotype. Multiple epitopes are presented on different haplotypes and therefore have the potential to induce immune responses against a broad spectrum of antigens. In addition, the requirement for processing results in prolonged antigen presentation [91]. A major disadvantage of using proteins or tumor cells is that the MHC class I pathway is not specifically targeted, although studies using proteins or tumor cells have noted the activation of $CD8^+$ T-cell responses, indicating that the cross-presentation pathway in DCs is activated. However, conclusive evidence that using proteins or tumor cells have an overall benefit in inducing immune responses over peptides is lacking.

Bacterial & viral vectors

The use of recombinant bacterial or viral vectors is an attractive choice for loading tumor antigens on DCs. Nonreplicating bacterias such as bacille Calmette–Guerin, *Listeria monocytogenes*, Salmonella and Shigella and viruses such as the Canarypox, Newcastle disease, Vaccinia, Sindbis, Yellow fever virus, human papillomavirus, adenovirus, adeno-associated virus and lentiviruses are currently explored for use as vectors in the setting of DC immunotherapy against infectious diseases and tumors [53,92–97]. Genes encoding tumor antigens or whole proteins are inserted into the vector while genes encoding virulence or replication factors are deleted out. The use of vectors for delivery of antigens to DCs have the advantage of eliciting natural immune responses against the vector of choice and, therefore, enhancing the immunogenicity of TAAs. In some cases, the vector may also induce the maturation of DCs, thereby bypassing the need for a separate maturation stimuli. In a recent study, DCs infected with a killed but metabolically active (KBMA) *L. monocytogenes* encoding an epitope of the melanoma-associated antigen MelanA/MART-1 induced the phenotypic and functional maturation of the DCs and induced the priming of MART-1-specific $CD8^+$ T cells and lysis of patient-derived melanoma cells *in vitro*. Furthermore, the KBMA efficiently targeted APCs *in vivo* to induce protective antitumor responses in a mouse therapeutic tumor model [98]. In addition to the tumor antigens, genes encoding for cytokines and costimulatory molecules that enhance DC function can also be added to the vector. However, pre-existing immunity against the chosen bacteria or virus vector may reduce their ability to induce immune responses *in vivo* and the safety of the patients becomes a primary concern when using these vectors.

The use of lentiviruses to load TAAs on DCs for tumor immunotherapy has recently received attention. Lentivirus-based vectors have several properties that offer distinct advantages over current vectors used in clinical trials [99,100]. Lentivirus-based vectors are typically less immunogenic as most genes encoding viral proteins have been removed, thereby allowing for the repeated use of these vectors for immunization without inducing pre-existing immune responses against the vector. Although lentivirus-based vectors are less immunogenic, they retain the potential to trigger endosomal or cytoplasmic sensors (i.e., TLRs, RIG-I, PKR, etc.)

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for the activation of the innate immune system. Lentivirus-based vectors have the additional capacity to transduce quiescent and non-dividing cells, which is especially applicable as moDCs are usually propagated from quiescent $CD14^+$ or $CD34^+$ progenitors. Numerous studies have demonstrated the ability of lentiviruses to successfully transduce MDCCs [101–103]. Indeed, studies using lentivirus-based vectors as immunotherapeutic agents against tumors in mouse models have demonstrated promise by inducing strong $CD8^+$ T-cell responses and inhibiting the growth of pre-existing tumors [104,105]. Furthermore, lentivirus-based encoding TAAs have been shown to prime both tumor-specific $CD4^+$ and $CD8^+$ T-cell responses *in vitro* [106]. These distinct properties of lentiviral-based vectors make them attractive for use in loading TAAs on DCs for tumor immunotherapy. In addition to these properties, lentiviral-based vectors have the potential for use as a vehicle for direct therapeutic vaccination. Lentiviral-based vectors can be pseudotyped with glycoproteins to target specific cell types and therefore can directly target DC subsets *in vivo*. A group established the *in vivo* targeting of DCs using a lentiviral vector pseudotyped with glycoprotein from Sindbis virus engineered to be specific for DC-SIGN. The *in vivo* use of the lentiviral vector resulted in the efficient transduction and maturation of DCs, and the activation of both humoral and cell-mediated responses [107]. Therefore, with improved biosafety, these vectors have the potential to deliver antigens to DCs both *in vitro* and *in vivo* and to induce effective tumor-specific immune responses.

RNA transfection of DCs

Antigen loading of DCs by mRNA coding for TAA represents an attractive strategy for loading DCs. RNA can be transfected directly on DCs without the use of bacterial or viral vectors. Similar to using proteins, use of mRNA obviates the need to identify patient's haplotypes and determine which epitopes can bind the specific haplotypes and multiple epitopes can be presented. Furthermore, DCs loaded with mRNA encoding TAAs have been demonstrated to induce potent tumor antigen-specific $CD4^+$ and $CD8^+$ T-cell responses [108–111]. As noted above, this technique may also be used to load with maturation stimuli or cytokines. Thus, RNA transfection serves as another attractive option for loading tumor antigens as well inducing maturation of DCs on DCs.

Transfection of DCs with mRNA can be accomplished with the use of a cationic lipid (i.e., DOTAP [lipid-mediated transfection], mRNA alone [passive transfection], or electroporation). Lipid-mediated transfection requires careful optimization as lipids can be quite toxic. Furthermore, the choices of lipids for clinical applications are limited. The use of lipid-mediated transfection to load DCs has been successful in eliciting CTL responses [108]. Passive transfection refers to the uptake of mRNA by DCs by incubation with mRNA in medium alone. Passive transfection has been demonstrated to elicit CTL responses that were similar to those stimulated with lipid-transfected DCs. However, both of these methods are limited by their low efficiency of transfection into DCs. Electroporation of mRNA has been shown to be a more efficient method to introduce mRNA into DCs than lipid-mediated or passive transfection. Electroporation allows the mRNA to enter the cell by temporarily increasing permeability [112]. Electroporation is more ideal as it obviates the need for additional reagents, and is therefore more compatible for clinical use. Electroporation of DCs has been successfully used in preclinical [113–115] and clinical [116] studies for tumor immunotherapy. Transfection of CD40L can also be used to induce the maturation of DCs

In vivo targeting of DCs

A novel strategy for loading antigens involves the direct targeting of antigens to DCs *in vivo* to induce tumor-specific immune responses [117,118]. *In vivo* targeting of DCs represents a lucrative option for DC immunotherapy as it bypasses the expensive and labor-intensive *ex*

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vivo DC generation process. Vaccines may be produced on a larger scale, doing away with the customized vaccine for each patient. More importantly, *in vivo* targeting allows for the stimulation of natural DC subsets at multiple sites *in vivo*.

Early approaches for targeting DCs *in vivo* involved engineering irradiated tumor cells to secrete GM-CSF [119,120]. The presence of GM-CSF is thought to stimulate the recruitment and enhance the function of APCs. These approaches involved autologous tumor cells engineered to secrete GM-CSF by retroviral- or adenoviral-mediated gene transfer, or allogeneic tumor cell lines stably transfected with expression plasmids encoding GM-CSF, or the mixing of autologous tumor cells with GM-CSF-secreting cell lines. Clinical trials using these approaches have demonstrated the recruitment of DCs, granulocytes, macrophages and T cells into the vaccination sites. Most patients developed delayed-type hypersensitivity reactions, which typically involved $CD4^+$ and $CD8^+$ T cells, eosinophils and macrophages in response to the vaccinations [121–123]. Furthermore, tumor biopsies of vaccinated patients revealed extensive tumor necrosis and the presence of $CD4^+$ and $CD8^+$ T cells and plasma cells that displayed potent cytotoxicity and polyfunctional cytokine profiles upon restimulation, indicative of the successful induction of tumor-specific immune responses. However, prolonged GM-CSF production in the tumor microenvironment has been associated with disease progression in some experimental models, and most recently in a Phase III trial of immunization with irradiated, GM-CSF-secreting allogeneic prostrate carcinoma cells in patients with hormone therapy-refractory metastatic disease [120]. Disease progression may be a result of immune tolerance induced by the prolonged GM-CSF administration. Prolonged GM-CSF may lead to the recruitment of myeloid suppressor cells or differentiation of myeloid precursors into immature tolerogenic DCs [124,125].

Newer approaches involve the targeting of DC-specific molecules. Candidate receptors include Fc receptors, CD40 and C-type lectin receptors (CLRs). CLRs are the most attractive target as different DC subsets are known to express different CLRs such as DEC205, DC-SIGN, mannose receptor (MR) and Dectin-1. Furthermore, CLRs are involved in the recognition and capture of many glycosylated self-antigens and pathogens for antigen presentation, DC trafficking and DC–T-cell interactions, and consequently the activation of immune responses [126]. Early studies targeting antigen to DCIR2 and DEC205 revealed that immune tolerance is elicited in the absence of DC-activating stimuli whereas co-administration of DC-activating stimuli is required to induce immune responses [20]. Subsequent studies targeting antigens to a range of CLRs with a DC-activating stimulus resulted in the effective generation of $CD4^+$ and $CD8^+$ T-cell responses [127–129]. In addition, targeting antigens to CLRs also resulted in the enhancement of antibody responses [130]. Although the majority of these studies are performed in mice, a number of studies in humans using DC-SIGN [19] and MR [131] have emerged, and demonstrated successful induction of naive and memory tumor-specific T-cell responses. However, further studies are still required to translate this new strategy to clinical applications in humans.

Routes of administration

Effective migration of DCs to secondary lymphoid organs is essential for DCs to stimulate immune responses. Thus, the ability to induce effective migration of DC vaccines is critical for their success. DC vaccines are typically administered intradermally, subcutaneously or intravenously. Intravenous injection of DCs leads to their transient lung uptake before redistribution to the liver, spleen and bone marrow, whereas subcutaneous or intradermal vaccination leads to improved migration of DCs to lymph nodes [132]. For the treatment of solid tumors, over 50% of DC vaccines were administered intradermally or subcutaneously [202]. However, the optimal route of administration for DC vaccines has yet to be established.

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The limited success of DC vaccines may also be in part attributed to the less-than-optimal migration of DC vaccines. A recent study using ^{111}indium-labeled moDCs loaded with melanoma peptides and administered intradermally demonstrated that large numbers of injected DCs remained at the injection site, lost viability and cleared within 48 h. Furthermore, less than 5% of DC vaccines reached the draining lymph nodes [133]. Therefore, given that the interaction of DCs and T cells within lymphoid tissues is critical for stimulating immune responses, the need to improve the number of DC vaccines that reach these sites is critical.

Strategies currently employed to enhance migration of DCs include administration of DC vaccines via multiple routes (i.e., intradermally and intravenously) to induce a more systemic response. Alternatively, DC vaccines can be administered directly to the lymph nodes (intranodally); however, this requires ultrasound guidance of the injection needle by an experienced radiologist. When delivered correctly, intranodal administration of moDCs loaded with melanoma peptides resulted in the redistribution of the injected DCs to multiple lymph nodes within 30 min of injection. Otherwise, incorrect delivery can result in highly variable redistribution. It is worth noting that despite the direct delivery of DC vaccines into the lymph nodes, the immunologic responses elicited were comparable to those of intradermally administered DC vaccine [133]. Thus, further studies are required to determine the most effective method to enhance migration of DC vaccines, and thus translate into better clinical responses.

DC vaccines for tumor immunotherapy

The clinical success of DC vaccines has been limited. Despite the induction of tumor-specific T-cell responses in many patients and occasional complete tumor regressions, DC vaccines have not translated into meaningful therapeutic responses. Almost 200 DC vaccine trials have been reported to date [202] with melanoma as the most frequent type of cancer treated with DC vaccines. Overall, clinical trials have demonstrated the feasibility and safety of DC vaccines. Side effects were relatively mild and transient, and include fever, injection site reactions, adenopathy and fatigue. The most common DCs used for these studies were moDCs generated from monocytes with GM-CSF and IL-4. Figure 2 is a typical representation of DC immunotherapy used in the majority of current clinical studies. A small subset of DCs was generated from $CD34^+$ hematopoietic precursors or *in vivo*-expanded circulating DCs. Although a small number of studies used immature DCs, the most commonly used DCs were matured with proinflammatory cytokines. DC vaccines were administered intravenously, intradermally, subcutaneously or intranodally using different vaccination schedules (weekly, biweekly and monthly). Sources of antigen included peptides, proteins and tumor lysates. TAAs delivered using bacterial and viral vectors were also used. Despite the numerous ways in which DCs were manipulated to enhance their function, and the promising preliminary results from Phase I and II trials, immune responses that correlate with the clinical responses have failed to materialize in large Phase III trials [134,135].

As the plethora of conditions used for DC vaccines indicates, there is no consensus on the optimal DC for immunotherapeutic use. This may be a major contributing factor to the lack of success for DC vaccines. Source of antigen, effective delivery of antigen to DCs, appropriate activation stimuli of DCs, optimal timing for activation and delivery of antigens, enhanced migration of DC vaccines to lymphoid tissues, and overcoming immune tolerance/suppression, are some of the aspects of DC vaccination that require further optimization to improve efficacy *in vivo*. Studies focused on optimizing these aspects will likely improve the efficacy of DC vaccines *in vivo*.

The lackluster results of DC vaccines may also be partially attributed to the fact that the majority of DC vaccines were administered to patients with advanced metastatic disease, when all other treatment modalities had failed. Vaccines aimed at enhancing tumor-specific immune

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responses may be more effective in the prevention of tumors. Prophylactic cancer vaccines have been demonstrated to be effective in many cancer models [136]. In support of this, patients that underwent nephrectomy for renal cell carcinoma and were injected with autologous tumor cells had a progression-free and overall survival benefit over the control group [137]. However, once the tumors become established, the efficacy of immune-modulating therapies in eradicating the tumors may be limited. At this point, vaccines face the challenge of overcoming immune regulatory mechanisms that have already led to tumor tolerance and may be too insurmountable to overcome. It is likely that using DC vaccines as therapy in the early stages of cancer, before tumor metastasis or directly after conventional treatment modalities, may lead to the long-awaited success of DC vaccines.

New strategies

Despite the expression of antigens by tumor cells and the presence of tumor-specific T cells, clinical responses remain limited. This may be due to several mechanisms mediated by the tumor or tumor microenvironment to evade immune surveillance [138] that include loss of tumor antigen expression, alteration of MHC molecules, lack of costimulation, expression of inhibitory ligands, induction of regulatory T cells, expression of IDO and the production of immunosuppressive cytokines [40,138–141]. These mechanisms undermine the ability of the immune system to mount effective immune responses against the tumor. Therefore, developing new approaches for overcoming the tolerance/suppression induced by the tumor or tumor microenvironment and enhancement of immunogenicity may potentiate the efficacy of DC vaccines *in vivo*, and translate to overall improved clinical outcomes.

Inhibition of inhibitory molecules

Antibodies blocking the interaction of PDL-1, overexpressed on a variety of tumors [142] and DCs, with PD-1 on activated T cells have been shown to lead to enhancement of tumor-specific immune responses *in vitro* [143–145]. A Phase I trial of the humanized antibody against PD-1 (CT-011) was recently completed and was shown to be safe and well-tolerated in patients with advanced hematologic malignancies. Furthermore, the antibody induced elevations in the percentage of peripheral blood $CD4^+$ lymphocytes, possibly as a result of inhibition of apoptosis of effector T cells [146].

Antibodies against CTLA-4 that target both effector and regulatory T cells have also been shown to enhance immune responses [147]. Clinical trial results of two CTLA-4-blocking antibodies – ipilimumab and tremelimumab – for treatment in melanoma produced promising clinical responses [148]. In addition, anti-CTLA-4-blocking antibodies are currently being investigated for other malignancies, such as prostrate cancer, renal cell carcinoma and non-Hodgkin's lymphoma [149].

Other strategies include targeting components of signaling pathways that inhibit DC function. The suppressors of cytokine signaling (SOCS) family represent an attractive target for inhibition [150]. SOCS have been shown to inhibit JAK/STAT signaling, an important signaling pathway for DC function in response to TLR agonists. Furthermore, numerous cancers exhibit dysregulation of the JAK/STAT signaling pathway [151]. siRNA-mediated inhibition of the SOCS1-enhanced antigen presentation [152]. Furthermore, inhibition of SOCS1 may be important for breaking tolerance against self tumor-associated antigens [153]. Targeting of glucocorticoid-induced leucine zipper (GILZ) may also be beneficial as it has been shown to alter the maturation of DCs in response to TLR agonists and CD40L [154]. Furthermore, its expression in macrophages infiltrating Burkitt lymphomas has been postulated to contribute to the failure of the immune system to reject the tumor [155].

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Inhibition of regulatory T cells

The IL-2/diptheria toxin fusion protein denileukin diftitox (ONTAK) has been shown to transiently deplete regulatory T cells and has been approved for treatment of cutaneous T-cell lymphoma [156]. Other regulatory T-cell-targeting therapies including cyclophosphamide [157] and anti-GITR [158–160] are currently under investigation.

Agents targeting IL-10 and TGF-β are also currently being explored. Combination therapy of TLR9 agonist CpG and IL-10R antibodies enhanced the tumor-specific immune response and triggered *de novo* IL-12 production [161]. In addition, inhibition of TGF-β suppressed the proliferation of regulatory T cells and increased the number of tumor antigen-specific T cells [162]. Furthermore, the prolonged survival of DC-vaccinated melanoma patients correlated with the reduction of TGF-β-expressing T cells [163]. Therefore, targeting these immunosuppressive cytokines will more likely also add to a vaccine's effectiveness.

Enhancement of T-cell costimulation

4-1BB/CD137 is expressed by activated T cells and responds to a ligand on APCs. 4-1BB is thought to play an important role in the survival of activated and memory $CD8^+$ T cells [164]. An antibody against 4-1BB was shown to enhance rejection of large tumor by promoting survival of $CD8^+$ T cells [165], and this anti-tumor effect of antibody against CD137 was mediated through DCs [166]. Furthermore, combination therapy with anti-CTLA-4 enhanced tumorspecific immunity while reducing autoimmune responses [167].

Dendritic cells expressing CD40 engage its cognate ligand CD40L on $CD4^+$ T cells and this leads to enhancement of expression of costimulatory molecules and cytokines and the subsequent activation of $CD8^+$ T cells. CD40 and CD40L interactions also lead to more efficient cross presentation by DCs [29]. A novel approach for extending the activated state of DCs was developed using a recombinant receptor containing the cytoplasmic domain of CD40 fused to ligand-binding domains and a membrane targeting sequence. Activation of CD40 signaling by this recombinant receptor is regulated with a lipid-permeable dimerizing drug. Activation of DCs with this recombinant receptor resulted in prolonged activation of DCs, induction of more potent effector $CD8^+$ T-cell responses, and the eradication of established tumors [168]. Morever, combining this recombinant receptor with the TLR4 agonists LPS or monophosphoryl lipid A (MPL®) led to syngergistic production of IL-12 and IL-6, high expression of DC maturation markers, and induction of $CD4^+$ and $CD8^+$ T-cell responses [169].

Addition of immune potentiators

Cytokines such as IL-15 and IL-7 have been demonstrated to enhance T-cell survival and function. In vaccine settings, IL-15 has been shown to promote the induction of longer-lived and higher avidity $CD8^+$ T cells [170] with the capacity to effectively kill tumor cells [171]. In addition, IL-15 has been shown to reverse T-cell anergy [172]. IL-7 is required for T-cell development and naive T-cell survival in the periphery [173]. Phase I trials established that administration of IL-7 led to dramatic increases in total $CD4^+$ and $CD8^+$ T-cell populations, without increasing regulatory T-cell numbers [174]. IL-12, a critical cytokine for development of Th1 responses, can also be used to enhance immune responses. Transfection of DCs with the gene encoding IL-12 was able to skew Th2 responses from melanoma patients into Th1 responses [175]. A new strategy of providing a preconditioned burst of IL-12p70, which is activated upon ligation of CD40L within lymph nodes, has led to high rates of T-cell activation and generation of complement-fixing, tumor-lytic antibodies [176].

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The use of TLR agonists to potentiate immune responses is actively pursued. The TLR7 agonist imiquimod [177] and TLR9 agonist CpG ODN [178] with tumor antigens for adjuvant therapy resulted in enhanced tumor antigen-specific immune responses. Topical application of TLR7 agonist imiquimod induced infiltration of mononuclear cells including T cells, monocytes, NK cells, mDCs and, to a lesser extent pDCs, and resulted in the activation of both humoral and cellular responses [177]. Furthermore, as the simultaneous triggering of multiple TLR agonists mediates synergistic effects [82], combining TLR agonists may lead to further enhancement of immune responses. The combination of TLR3 and TLR7 agonists induced rapid and sustained activation of DCs as well as enhanced CTL effector functions [179]. Furthermore, the combination of TLR3 and TLR7/8 agonists with PGE_2 induced DCs to produce high levels of IL-12 and migrate in response to CCR7 ligands [81]. The combination of the TLR4 agonist MPL and the TLR9 agonist CpG induced both humoral and B-cell responses [180]. A recent study demonstrated that TLR3 agonist Poly IC was superior to other TLR agonists (CpG, R848, LPS, Pam3cys and Malp-2) in inducing $CD4^+$ T cells [181]. Therefore, further studies exploring the uses of TLR agonists are likely to be beneficial.

Although the ability of adjuvants to induce vaccine-specific immune responses has long been recognized and used, the mechanism by which many of these adjuvants activate the immune system is unclear and may be partially TLR-dependent [182]. Recent studies indicate that particulate adjuvants, including aluminum hydroxide and saponins, activate DCs via NALP3 inflammasome leading to enhanced production of IL-1β, IL-18 and IL-1α [183]. The discovery of an alternate pathway besides TLRs provides another potential target for enhancing immune responses. Another adjuvant that is currently investigated is montanide. Montanide is a water-in-oil emulsion containing mannidemono-oleate as an emulsifier. It is similar in physical character to incomplete Freund's adjuvant but biodegradable. Montanide is currently used in a number of clinical studies [184,185] and has been demonstrated to enhance immune responses. Furthermore, combination of montanide with TLR agonists led to expansion of tumor antigen-specific $CD8^+$ T cells in melanoma patients [186].

Additional strategies

The use of heterologous prime and boost immunizations may also improve efficacy of DC vaccines. Each vaccine can carry multiple TAAs and enhance different effectors of the immune system. The successful use of multiple vaccine therapies have been demonstrated in cancer patients who have received different prime-and-boost vaccination protocols [187,188].

As protective immune responses likely result from the coordinated efforts of multiple arms of the immune system, strategies that target pDCs, NK cells and NKT cells may be beneficial. NKT cells are of particular interest. In addition to its direct anti-tumor effects, DC–NKT cross-talk may further potentiate DC activation and IL-12 production. Furthermore, the observation of dysfunctional NKT cells in several advanced malignancies underscore the need for approaches to target these cell types [189]. In a Phase I study, α-Gal-Cer loaded mature DCs injected into patients with advanced cancer led to *in vivo* expansion and activation of NKT cells and was associated with increases in serum levels of IL-12p40 and IP-10 and increases in antigen-specific T-cell responses [190].

In addition, combining the use of standard chemotherapeutic agents with DC vaccines may also enhance efficacy by altering the phenotype of tumor cells and rendering them more susceptible to lysis by T cells [191,192].

Conclusion & future perspective

Although the important role that DCs play in immune responses is well established, the ability to harness this capacity for immunotherapy against tumors requires further optimization before

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it can be used as standard treatment for cancer. Clinical studies using *ex vivo* DCs demonstrated that immune responses against TAAs can be induced. Furthermore, the use of DC vaccines for HIV therapy has yielded exciting results. Patients vaccinated with autologous moDCs pulsed with aldrithiol-2-inactivated autologous HIV suppressed viral loads for at least 1 year, and this positively correlated with HIV-specific $CD4^+$ and $CD8^+$ T cells [193]. However, the limited clinical responses in the majority of DC vaccine studies emphasize the need for further optimization of DC vaccine protocols. This is exemplified in a recent unpublished study by our group, whereby comparison of *ex vivo* cytokine-matured DCs and montanide with melanoma peptides demonstrated that montanide was better, in terms of frequency and magnitude, at stimulating immune responses than DCs in melanoma patients [O'Neill *et al.*, Unpublished Data]. Therefore, the use of new strategies outlined above in combination with DC vaccines will likely enhance immunogenicity and efficacy *in vivo* and be the key to achieving effective, durable, anti-tumor immune responses in a larger population of patients.

Executive summary

Dendritic cell biology

- Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs).
- There are two major DC subsets: myeloid (mDC) and plasmacytoid (pDC).
- DCs take up and process antigens for presentation on MHC class I, MHC class II and CD1d molecules.
- DCs mature in response to proinflammatory cytokines, Toll-like receptor (TLR) agonists, or activation via endogenous sensors (RIG-I and MDA5) or members of the inflammasome.
- Maturation of DCs leads to the upregulation of costimulatory molecules and production of cytokines and chemokines necessary for optimal T-cell activation.
- In addition to stimulating immune responses, DCs also mediate tolerance via different mechanisms including expression of indoleamine 2,3-dioxygenase and induction of regulatory T cells.

DCs for tumor immunotherapy

- Given that the presentation of tumor-associated antigens (TAAs) by DCs is a central step in the induction of anti-tumor responses, DCs serve as an ideal tool for boosting endogenous anti-tumor responses.
- DCs can be generated *ex vivo* under good manufacturing practice conditions from monocytes, $CD34^+$ hematopoietic progenitors, or enriched from peripheral blood in large quantities for vaccination.
- The most common maturation stimuli used is a cocktail of proinflammatory cytokines, although the use of TLR agonists and CD40L is also utilized.
- TAAs can be loaded on DCs as peptides or proteins, using bacterial or viral vectors, or RNA transfection.
- *In vivo* targeting of DCs for loading of TAAs is a novel method that is currently under investigation.

DC vaccination trials

- Immune responses have been noted in a majority of patients, however few correlations with clinical responses have been documented.

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- Source of antigen, effective delivery of antigen to DCs, appropriate maturation stimuli, optimal timing for activation and delivery of antigens, enhanced migration of DCs to lymphoid tissues, and overcoming immune tolerance/suppression are some of the aspects of DC vaccination that requires further optimization to improve efficacy *in vivo*.

New strategies

- Combination therapy may be the key to achieving effective and durable anti-tumor immune responses in clinical trials.
- Combining DC vaccines with strategies that target tolerance/suppression (i.e., inhibitory molecules, suppressive cytokines and regulatory T cells), potentiate immune responses (i.e., TLR agonists or cytokines), heterologous vaccine strategies or conventional treatment modalities may further enhance clinical responses.
- Use of DC vaccines as a prophylactic vaccine may also improve their efficacy.
- Studies comparing the various types of DC in use for vaccination are required to optimize their application.

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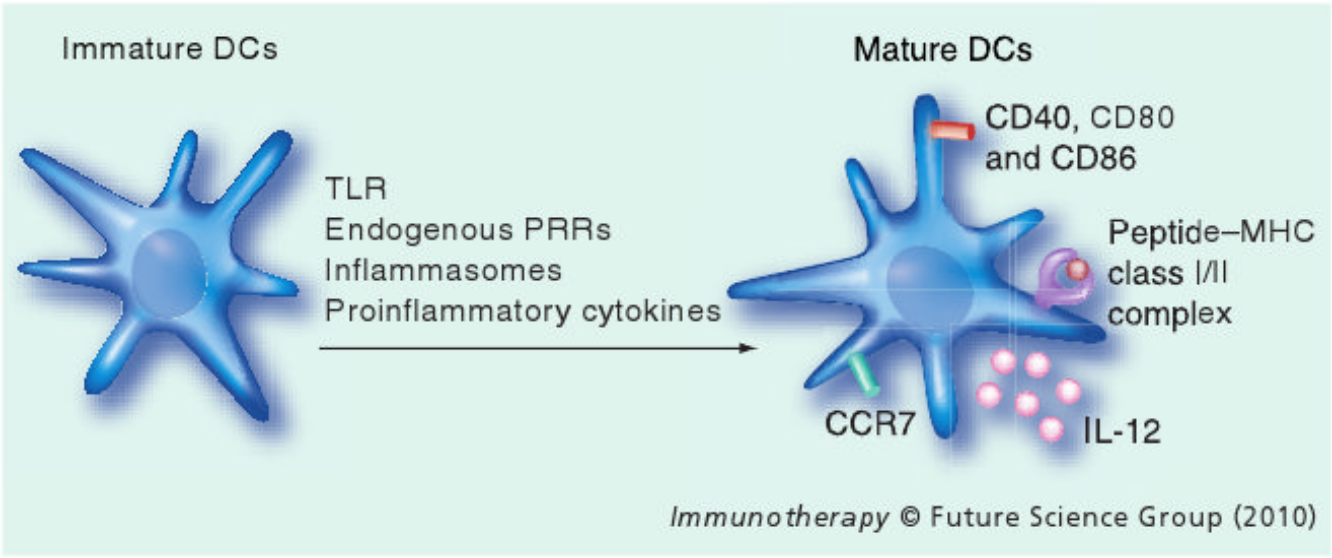

Figure 1. Dendritic cell functional states

DCs are the most potent antigen-presenting cells, capable of activating both naive and memory immune responses and maintaining the delicate balance between immunity and tolerance. In their immature state, DCs are thought to be highly efficient in antigen uptake. Antigen is taken up through endocytosis, phagocytosis and macropinocytosis followed by processing and presentation on MHC molecules. DCs are stimulated to mature via interactions through TLRs, endogenous PRRs, inflammasomes or the presence of proinflammatory cytokines or pathogens. DCs undergo a maturation process involving the upregulation of costimulatory molecules CD40, CD80 and CD86, and the production of inflammatory cytokines such as IL-12. CCR7 is also upregulated, thereby allowing DCs to migrate into lymphoid tissues to stimulate humoral and cell-mediated immune responses. DC: Dendritic cell; PPR: Pattern recognition receptor; TLR: Toll-like receptor.

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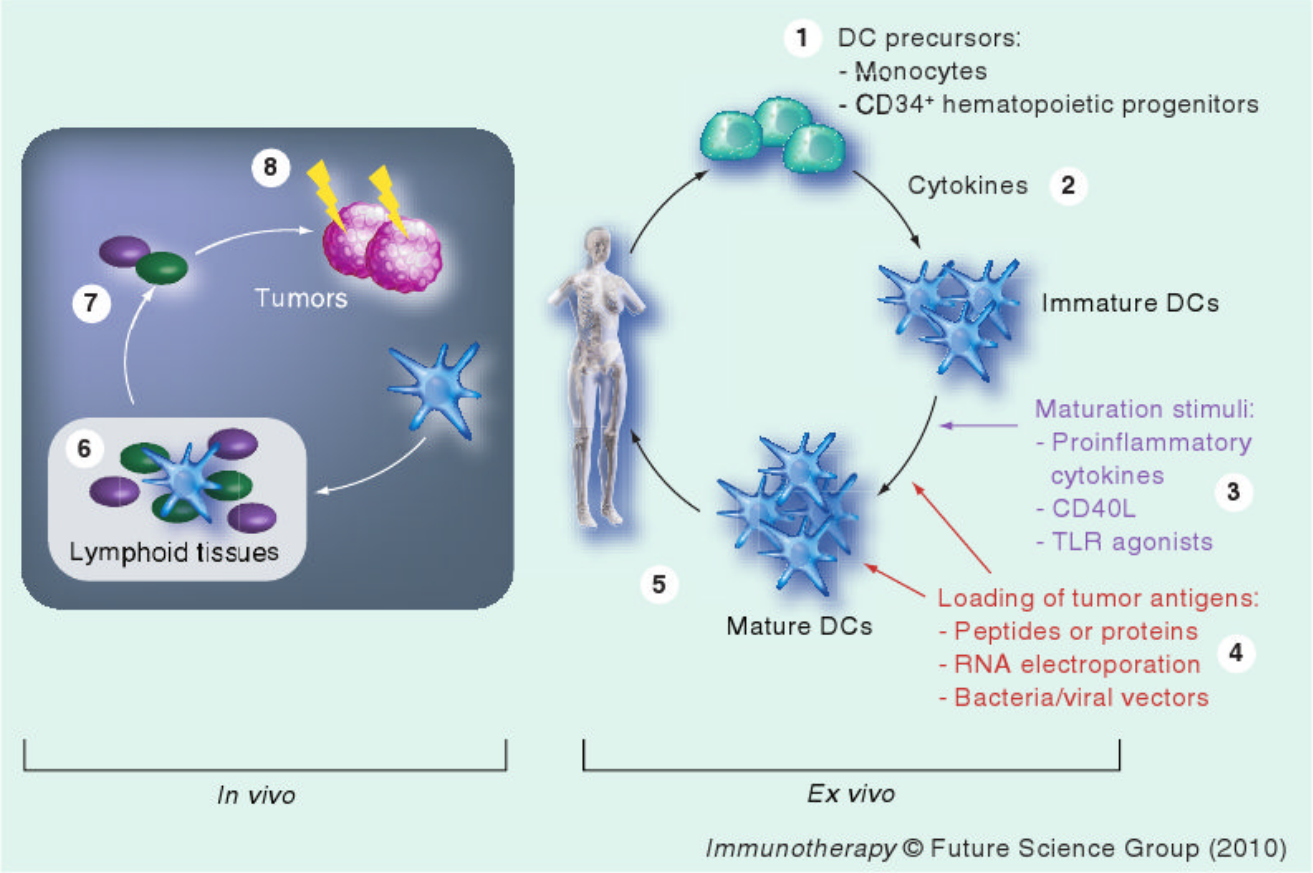

Figure 2. Dendritic cell immunotherapy

Most DC-based vaccines currently explored for tumor immunotherapy in clinical trials consist of mature antigen-loaded autologous DCs that are administered to patients with the intention of enhancing tumor-specific immune responses. The figure illustrates the procedures involved in generating DC vaccines for clinical applications. **(1)** DC precursors (monocytes or $CD34^+$ hematopoietic progenitor cells) are typically isolated from patients from leukapheresis collections. **(2)** Incubation with GM-CSF and IL-4 differentiates precursor monocytes, and with GM-CSF, FLt3L and TNF-α differentiates $CD34^+$ hematopoietic precursor cells to become immature DCs. **(3)** Immature DCs are induced to become mature using proinflammatory cytokines, CD40L or TLR agonists. **(4)** Loading of tumor antigens can occur simultaneously with the maturation stimuli or after DCs are matured. **(5)** Matured and tumor antigen-loaded DCs are injected back into the patient. **(6)** Injected DCs migrate to lymphoid tissues and ideally activate tumor antigen-specific $CD4^+$ and $CD8^+$ T cells in addition to stimulating innate immune responses. **(7)** Activated T cells traffic out of the lymphoid tissues and back to the tumor to **(8)** inhibit its growth. Overall, clinical trials have demonstrated the feasibility and safety of DC vaccines. More importantly, these trials have demonstrated that DC vaccination can induce immune responses in many of the patients. CD40L: CD40 ligand; DC: Dendritic cell; TLR: Toll-like receptor.