Engineering Dendritic Cells to Enhance Cancer Immunotherapy

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Cancer immunotherapy aims to establish immune-mediated control of tumor growth by priming T-cell responses to target tumor-associated antigens. Three signals are required for T-cell activation: (i) presentation of cognate antigen in self MHC molecules; (ii) costimulation by membrane-bound receptor-ligand pairs; and (iii) soluble factors to direct polarization of the ensuing immune response. The ability of dendritic cells (DCs) to provide all three signals required for T-cell activation makes them an ideal cancer vaccine platform. Several strategies have been developed to enhance and control antigen presentation, costimulation, and cytokine production. In this review, we discuss progress toward developing DC-based cancer vaccines by genetic modification using RNA, DNA, and recombinant viruses. Furthermore, the ability of DC-based vaccines to activate natural killer (NK) and B-cells, and the impact of gene modification strategies on these populations is described. Clinical trials using gene-modified DCs have shown modest results, therefore, further considerations for DC manipulation to enhance their clinical efficacy are also discussed.

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INTRODUCTION

The idea of treating cancer patients with antigen-specific immunotherapy has matured over the past 120 years. At the end of the 19th century, Coley noticed that some tumors could regress in cancer patients who contracted bacterial infections.[1] About 15 years later Ehrlich suggested that transformed cells continuously arise in our bodies which the immune system is able to recognize and eliminate before they are clinically detectable.[2] In the mid-20th century, Burnet and Thomas provided experimental evidence for the concept of immune surveillance, showing that tumors could be repressed by the immune system in tumor transplantation models.[3] This concept was later substantiated by the identification of tumor-associated antigens (TAAs)[4] and the isolation of TAA-specific tumor-infiltrating lymphocytes.[5] These findings logically led to the hypothesis that the immune system could be further primed for the treatment of cancer.

The induction of antigen-specific immune responses requires potent interactions between antigen-specific T-cells and professional antigen-presenting cells (APCs), including monocytes, macrophages, and dendritic cells (DCs). It is generally accepted that three signals are required for the induction of robust T-cell responses; all three can be delivered by DCs. The first signal consists of the recognition of an antigenic peptide in the context of MHC molecules on APCs through a specific T-cell receptor.[6] The second signal is given by interactions between costimulatory ligands on the T-cells and their receptors on APCs.[7] In the absence of signal two, antigen-specific T-cells will become anergic. The third signal, established by the local cytokine milieu, influences T-cell polarization.[8,9] When all the necessary signals are present, the interaction between DCs and T-cells leads to T-cell activation, clonal expansion, and differentiation into effector and memory cells.

Immature DCs residing in the peripheral tissues are specialized in antigen capture and processing from invading pathogens. In the presence of ongoing inflammatory immune responses, immature DCs respond to inflammatory and pathogen-derived signals by differentiating into a mature state. At this stage, DCs reduce their antigen uptake/processing capacity and transform into efficient APCs capable of stimulating both $CD4^+$ and $CD8^+$ T-cells. To this end, DCs undergo several morphological, phenotypical, and functional changes: (i) they become more motile and increase their CCR7 expression, which controls the migration from the periphery to the lymphoid organs; (ii) they increase their expression of MHC class I and II molecules and of costimulatory molecules (CD40, CD80, CD83, CD86); (iii) upon arrival in the secondary lymphoid organs, they secrete chemokines to recruit macrophages, natural killer (NK) cells, B-cells, additional DC subsets, and specific T-cell subsets to the local environment; and (iv) they secrete cytokines which are critical for determining the nature of the ensuing immune response. Over the years, multiple protocols have been developed for *in vitro* generation of mature DCs[10] and for their genetic modification,[11] both through viral and nonviral approaches.

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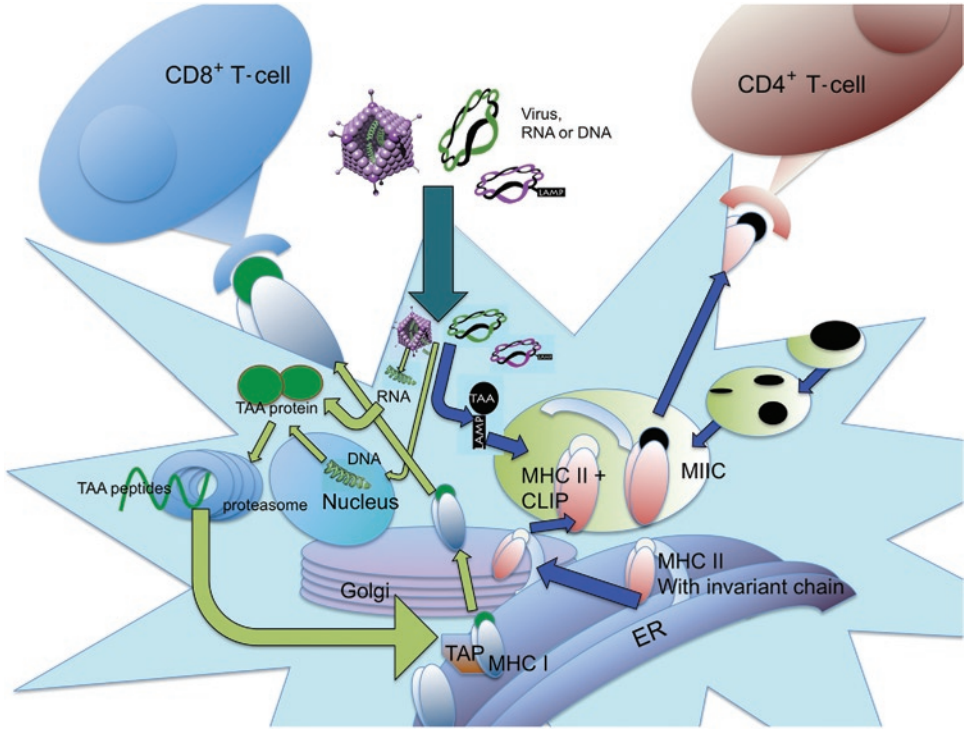

Figure 1 Pathways of antigen processing and presentation. Endogenous proteins are degraded in the cytoplasm by the proteasome. Cleaved peptides are ushered into the endoplasmic reticulum by TAP (transporter associated with antigen processing), where they are loaded onto preformed MHC I/β2m complexes. Stable MHC I:peptide binding allows the complexes to traffic *via* the Golgi to the cell surface for antigen presentation to $CD8^+$ T-cells. MHC II molecules are formed in the endoplasmic reticulum (ER) and traffic through the Golgi. The invariant chain is used to prevent binding of "self" peptides and to stabilize the MHC II complex. Upon entry into the MHC II compartments (MIIC), the invariant chain is degraded, leaving a small, class II-associated peptide (CLIP). Within the MIIC, the CLIP is replaced with peptides resulting from degradation of endocytosed pathogens. For ectopic expression, genes can be introduced by virus infection or RNA/DNA transfection. Unless otherwise modified, proteins expressed by either strategy are typically processed by the proteasome and presented on MHC I molecules. However, proteins can be also targeted to the MHC II pathway by tagging with sorting signals, including lysosome-associated membrane protein-1 (LAMP-1). TAA, tumor-associated antigen.

DCs are being modified to express TAAs or immune-potentiating molecules, or to downregulate negative modulators of DC functioning, with the goal of strengthening the three distinct signals required for $CD4^+$ and $CD8^+$ T-cell activation. This review will focus on the genetic modification of DCs to enhance each of these three pathways.

GENETIC MODIFICATION TO ENHANCE ANTIGEN DELIVERY FOR T-CELL RECEPTOR STIMULATION (SIGNAL 1)

A major advantage of engineering DCs for expression of TAAs is that it allows multi-epitope presentation of full-length TAAs without requiring knowledge of the patient's human leukocyte antigen (HLA) type, unlike widely used peptide vaccination strategies. Secondly, presentation of TAA-derived peptides might be intrinsically enhanced due to their endogenous expression within DCs. Finally, high-efficiency of gene transfer ensures a sufficient, continuous supply of natively processed antigen. Besides these inherent enhancements, several approaches have been utilized to further improve antigen delivery for T-cell receptor stimulation. They can be subdivided into methods that enhance $CD8^+$ T-cell stimulation or $CD4^+$ T-cell stimulation (**Figure 1**).

When DCs are genetically modified for TAA production, these proteins are generated in the cytoplasm. In order to obtain presentation of TAA derived peptides to $CD8^+$ T-cells, these proteins must be degraded by the proteasome (**Figure 1**). In most cases, this occurs through protein ubiquitinylation and subsequent targeting to the proteasome. Several proteins such as ornithine decarboxylase, p53, and thymidylate synthase can additionally target proteins for proteasomal degradation through an ubiquitinylation-independent system.[12] Antigens are degraded by the proteasome into short peptides which are subsequently transported into the endoplasmic reticulum (ER) by transporter associated with antigen processing. There, newly synthesized HLA class I heavy chains assemble with β2m and peptide and this complex is transported to the cell surface for presentation to $CD8^+$ T-cells, as depicted in **Figure 1**.

The MHC I pathway has been exploited to enhance antigen presentation. For instance, linking the mRNA for pp65 to ubiquitin or ornithine decarboxylase to enhanced stimulation of $CD8^+$ T-cells. Moreover, when the antigen was linked to both ubiquitin and ornithine decarboxylase, immunogenicity was further increased.[13] Recently, we have observed that the immunogenicity of a given TAA can be greatly enhanced by deleting its nuclear localization signal (D. Benteyn, S. Anguille, A.M.T. Van Nuffel, C. Heirman, J. Corthals and W. Waelput, unpublished results), demonstrating that further manipulation of the TAA-encoding sequence can result in favorable induction of potent antitumor immune responses.

It is generally believed that the induction of $CD4^+$ T-cells is necessary to obtain robust and long-lasting $CD8^+$ T-cell responses, especially against weakly immunogenic antigens like TAAs. However, the transgenic proteins produced by genetically modified DCs are located in the cytoplasm and they are less efficient at accessing the endocytic pathway to be processed for presentation to $CD4^+$ T-cells. Consequently, additional measures must be taken to obtain presentation of the introduced gene in the context of MHC II molecules.

In the ER, MHC II molecules are assembled with invariant chain (Ii) bound to the antigen binding groove to stabilize the MHC class II complexes and prevent binding of self-peptides present in the ER (**Figure 1**). Ii contains two sorting signals in its cytoplasmic tail which regulate the transport of the MHC/Ii complexes from the ER through the Golgi network into the endosomal and lysosomal compartments, called MHC class II compartments (MIIC).[14] Ii is degraded in the MIIC, leaving the MHC class II binding groove free to bind peptides derived from antigens present in the endocytic compartments (derived from exogenous antigens). Besides Ii, many other proteins, including lysosome-associated membrane protein-1 (LAMP-1), DC-LAMP and lysosomal acid protease reach the MIIC by virtue of a targeting sequence. A number of studies have appended these sequences to TAA-coding regions to target whole antigens to the MIIC for presentation to $CD4^+$ T-cells (reviewed previously[15]).

For DC modification strategies, the most extensively used signal is the sorting sequence of LAMP-1. Lin *et al*. demonstrated that modification with the LAMP-1 sorting signal directs antigens to the endolysosomal compartments.[16] Wu and colleagues confirmed that targeting the HPV16 E7 protein to the endolysosomal compartments with the LAMP-1 sorting indeed results in an enhanced presentation of MHC class II/E7 derived peptide complexes.[17] Since then, the LAMP-1 sorting signal has been coupled to gene modification vectors, including vaccina virus encoding pp65,[18] retrovirus coexpressing HPV16 E7,[19] and through mRNA electroporation with carcinoembryonic antigen,[20] human telomerase reverse transcriptase (hTERT),[21] and Mage-A3.[22] DCs electroporated with the chimeric LAMP-1 hTERT were used to immunize patients with metastatic prostate cancer; these patients developed significantly higher frequencies of hTERT-specific $CD4^+$ T-cells than subjects receiving DC transfected with the unmodified hTERT template. Moreover, cytotoxic T-lymphocyte (CTL)-mediated killing of hTERT targets was enhanced in the LAMP-1 hTERT group.[21]

Improved CTL induction after vaccination with antigens linked to an MHC II targeting sequence has often been observed[18,19,23] and can be interpreted as a mechanism mediated by concomitant stimulation of $CD4^+$ cells. Nevertheless, several $CD4^+$ T-cell independent models also showed that enhanced MHC class I presentation of antigens can be observed when the antigen is linked to an MHC class II targeting sequence.[22,24] For instance, degradation of misfolded chimeric proteins after retranslocation from the ER into the cytosol could enhance their availability for presentation on MHC I.[24]

GENETIC MODIFICATION TO ENHANCE COSTIMULATION (SIGNAL 2)

T-cell activation and inhibition are calibrated by surface-bound costimulatory molecules. Therefore, the genetic modification strategies for costimulatory molecules can be divided into two categories: modifications aiming at the enhanced expression of activating molecules, and modifications aiming at the downregulation of inhibitory molecules (**Figure 2** and **Table 1**).

Enhancing expression of costimulatory molecules

CD40-CD40L is the costimulatory receptor/ligand pair whose expression has been most often enhanced for the purpose of improving DC function. Ligation of CD40 on DCs is normally provided by activated $CD4^+$ T-cells.[25] This "licensing" interaction is the mechanism through which $CD4^+$ type 1 T helper (Th1) cells provide help in generating primary $CD8^+$ T-cell responses, especially to noninflammatory antigens.[26] This process leads to DC maturation with upregulation of other costimulatory molecules and enhanced production of cytokines/chemokines. Licensing has been mimicked by engineering DCs to express CD40L through transduction with adenovirus,[27] lentivirus,[28] vaccinia virus,[29] or through mRNA electroporation.[30] These studies indeed provided evidence that CD40L-engineered DCs express higher CD80 and CD86, and produce more IL-12p70. Furthermore, T-cell responses against weak tumor antigens such as glycoprotein (gp)100 and MelanA were significantly enhanced when CD40L expressing DCs were used as antigen-presenting cells.[31,32]

In conjunction with delivering CD40L for licensing, modifying DCs to express a constitutively active form of toll-like receptor 4 (caTLR4) has been evaluated by Cisco *et al*. They have shown that electroporating DCs with mRNA encoding the caTLR4 mimics binding of lipopolysaccharide to TLR4, enhances DC maturation and IL-12p70 secretion, and leads to potent induction of MelanA specific T cells.[33] Further, we have combined CD40L and caTLR4 together with CD70 (called TriMix). TriMix DCs are matured through caTLR4 and CD40L signaling, and additionally provide stimulation to naive T-cells, *via* CD27-CD70 interactions, to inhibit activated T-cell apoptosis and support T-cell activation and proliferation. When loaded with an HLA-A2 restricted MelanA epitope or coelectroporated with full-length MelanA encoding mRNA, TriMix DCs are better in stimulating MelanA specific $CD8^+$ T-cells than cytokine cocktail matured DCs.[34] Moreover, TriMix DCs can induce T-cells against TAA with a lower precursor frequency, such as Mage-A3, Mage-C2, and tyrosinase.[32]

Besides CD40L and CD70, other members of the tumor necrosis factor (TNF) superfamily or their ligands have been introduced into DCs to enhance their function. Our group has introduced GITRL into DCs through mRNA electroporation.[35] Consistent with mouse data, we showed that human GITRL functions as a costimulator for responder T-cells, and priming with GITRL-expressing DCs increases the number of Melan-A-specific $CD8^+$ T-cells. However, in contrast to data obtained in mice, no significant abrogation of Treg suppression by GITRL-expressing human DC could be observed.[35] Grünebach *et al*. have shown that mRNA electroporation with 4-1BBL increased expression of CD80 and CD40.[36] Furthermore, cotransfection of 4-1BBL with HER-2/neu resulted in an increased specific lysis of target cells by *in vitro* induced CTL lines, indicating that 4-1BBL enhances the ability of DCs to elicit primary CTL responses.[36] These data confirmed results obtained by Wiethe *et al*. in a murine model where DCs were adenovirally transduced with 4-1BBL and the E7

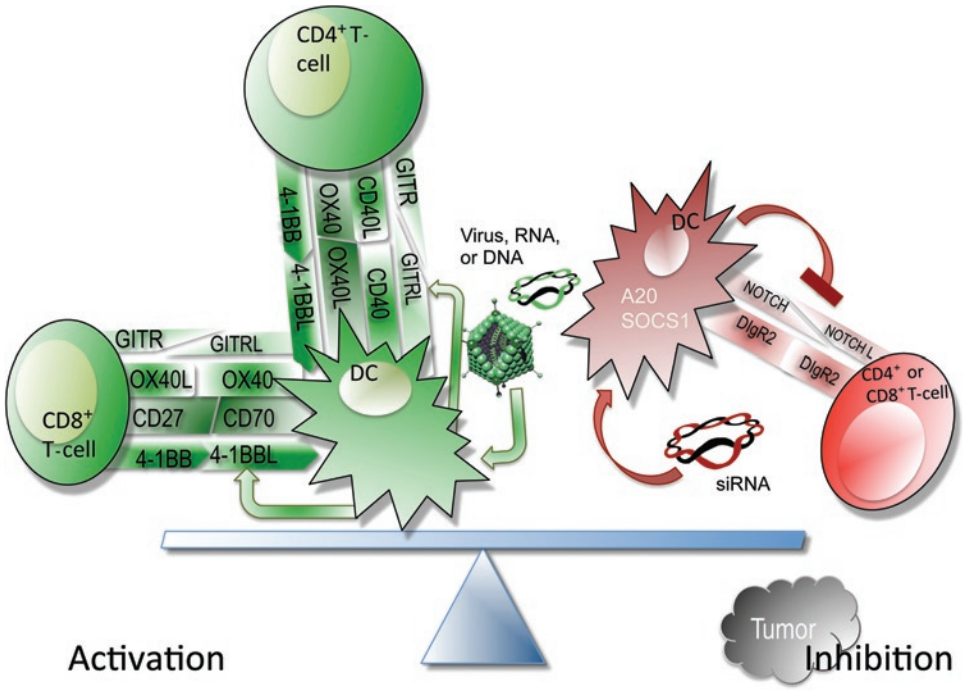

Figure 2 Pathways of activation and inhibition *via* costimulatory molecules. Dendritic cell (DC)-mediated T-cell activation requires a second, antigen nonspecific signal. Costimulatory molecules are not constitutively expressed by DCs; they are upregulated during maturation and as a consequence of environmental conditions. Similarly, DCs can inhibit T-cell activation or suppress T-cell activity, especially in the context of pre-existing tumors. Gene modification of DCs has been utilized both to enhance expression of activating costimulatory molecules and to inhibit expression of inhibitory molecules shown in this figure. As described in the text, costimulatory molecules can be delivered by gene transfection or recombinant viruses; inhibitory molecules can be knocked-down using siRNA. SOCS, suppressors of cytokine signaling.

oncoprotein of human papillomavirus.[37] Using the same model, this group also showed that adenoviral transduction with the costimulatory molecule receptor-ligand pair RANK/RANKL augmented E7-specific, interferon-γ (IFN-γ)-secreting effector and memory T-cells. Similar T-cell enhancement was observed upon cotransduction of DCs for coexpression of T-cell costimulatory molecules, receptor activator of nuclear factor κ-b ligand (RANKL) and CD40L, or for the coexpression of DC costimulatory molecules, RANK, and CD40.[37]

Another frequently studied costimulatory molecule is OX40L, which enhances stimulation of antigen-specific $CD4^+$ T-cells.[38] DCs transfected with OX40L mRNA facilitate generation of antigen-specific $CD4^+$ T-cell response and Th1 polarization, and as a result, improve the induction of antigen-specific CTL responses *in vitro*. Moreover, mice carrying pre-established B16 melanomas and vaccinated with OX40L-expressing DCs showed an enhancement of antitumor activity due to *in vivo* priming of Th1-type $CD4^+$ T-cells.[38]

Downregulating inhibitory molecules

DCs are capable of priming both proinflammatory and regulatory/suppressive T-cell responses based on the complement of costimulatory receptors (or lack thereof) that they express. The downregulation of suppressive molecules in DCs is therefore an attractive approach for generating therapeutic immunity against cancer. Although many molecules qualify for this purpose (reviewed by Mao *et al.*[39]), only a few have been investigated by genetic modification of DCs.

The zinc-finger protein, A20, is an ubiquitin-editing enzyme with de-ubiquitinase activity in its amino-terminal region and ubiquitinase activity in the zinc-finger domain of its carboxy-terminal region. Through this dual ubiquitin-editing function, A20 can negatively regulate the TLR and TNF receptor signaling pathways. It has been shown that A20-silenced murine DCs showed enhanced expression of costimulatory molecules and proinflammatory cytokines. These DCs were refractory to Treg-mediated suppression and effectively activated tumor-infiltrating CTLs and $CD4^+$ T-cells.[40] Our group subsequently reported that A20 silencing in human DCs results in activation of the transcription factors nuclear factor κ-B (NFκB) and activator protein-1, leading to increased and sustained production of interleukin (IL)-6, IL-10, and IL-12p70. Moreover, A20 downregulated DCs skew naive $CD4^+$ T-cells toward IFN-γ producing Th1 cells and have an enhanced capacity to prime MelanA/melanoma antigen recognized by T-cells (MART-1) specific $CD8^+$ T cells.[41]

SOCS1 (suppressor of cytokine signaling 1) is an immunosuppressive protein mediating negative-feedback inhibition of cytokine signaling. It is induced by cytokines such as IFN-γ, IL-12, IL-2, IL-7, and granulocyte-macrophage colony stimulating factor (GM-CSF), and subsequently inhibits their function by suppressing signal transducer and activator of transcription (STAT) molecules.[42] Vaccination of mice with SOCS1-silenced DCs strongly enhances antigen-specific antitumor immunity in *in vivo* murine models, likely due to the prolonged antigen presentation permitted by SOCS1 silencing.[43]

Table 1 Genetic modification of dendritic cells (DCs) for manipulation of costimulatory factors and their immunological outcomes

Costimulatory factor	Reference(s)	Immunological outcome
CD40 L	27–30	Induction of DC maturation
		5× increase in B-cell proliferation
		4× increase in antibody production
		Bypass $CD4^+$ T-cell requirement
		Enhanced $CD8^+$ T-cell activation
TriMix (CD40L + CD70 + caTLR4)	32–34	Increased IL-12 and TNF-α production
		Higher induction of $CD8^+$ T-cell activation against poorly immunogenic TAAs
		Enhanced DC surface maturation
GITRL	124	Enhanced $CD8^+$ T-cell activation against MART-1 and gp100
4-1BBL	36,125	Enhanced expression of surface maturation markers on DCs
		Enhanced $CD8^+$ T-cell activation by DCs
RANK/RANKL	41,125	Enhanced expression of surface maturation markers on DCs
		Enhanced $CD8^+$ T-cell activation by DCs
OX40L	38	Activation of anticancer immunity in tumor-bearing animals
		Enhanced DC maturation
		Enhanced $CD4^+$ and $CD8^+$ T-cell priming
A20 (siRNA knockdown)	126	Upregulation of DC surface activation and cytokine production
		Enhanced $CD4^+$ and $CD8^+$ T-cell activation
		Improved therapeutic anticancer activity
SOCS1 (siRNA knockdown)	43	Induced prolonged antigen presentation
		Greater CTL expansion
		Enhanced cytokine production by DCs
		Enhanced prophylactic tumor protection
NOTCH ligands (siRNA knockdown)	45	Enhanced DC cytokine production
		Enhanced $CD4^+$ T-cell activation
DIgR2 (siRNA knockdown or blockade)	46	Enhanced prophylactic antitumor activity
		Improved $CD4^+$ and $CD8^+$ T-cell activation

Abbreviations: caTLR4, constitutively active form of TLR4; CTL, cytotoxic T-lymphocytes; DigR2, dendritic cell-derived immunoglobulin receptor 2; gp, glycoprotein; IL-12, interleukin-12; MART-1, melanoma antigen recognized by T-cells; RANKL, receptor activator of nuclear factor κ-b ligand; siRNA, small interfering RNA; SOCS1, suppressor of cytokine signaling 1; TAAs, tumor-associated antigens; TLR4, toll-like receptor 4; TNF, tumor necrosis factor.

Surface molecules that have direct suppressive effects on T-cells are also attractive targets for silencing. To date, two surface molecules have been evaluated for this purpose: the Notch ligands and DC-derived immunoglobulin receptor 2 (DIgR2). The expression of Notch ligands (Delta1, Jagged1, or Jagged2) has been shown to deliver suppressive signals to T-cells.[44] Knockdown by small interfering RNA (siRNA) in human DCs leads to enhanced IFN-γ production in allogeneic mixed lymphocyte reaction. Moreover, Delta1 siRNA leads to enhanced cytokine production by $CD4^+$ T-cells in response to polyclonal T-cell receptor activation.[45]

The second inhibitory molecule that has been targeted in DCs, DIgR2, is a member of the immunogobulin superfamily. This family includes several molecules with key roles in the biology of innate and adaptive immune responses, some of which—like DIgR2—act as inhibitory receptors. Silencing of DIgR2 in murine DCs with specific siRNA enhances T-cell proliferation and antigen-specific T-cell responses.[46] Furthermore, immunization of mice with antigen-pulsed, DIgR2-silenced DCs elicits more potent antigen-specific $CD4^+$ and $CD8^+$ T-cell responses, thus protecting the vaccinated mice from tumor challenge more effectively.[46]

GENETIC MODIFICATION TO ENHANCE THE IMMUNE ENVIRONMENT (SIGNAL 3)

In addition to cognate antigen recognition and costimulation, DC-derived soluble factors create a critical third signal to condition the immune environment. The cytokine and chemokine milieu established during early innate reactions directs immune polarization and induces recruitment of accessory leukocyte populations. Priming and activity of anticancer T-cell responses occur ideally in Th1-polarized microenvironments, which are established by type I IFN (IFN I), IFN-γ, and IL-12p70, characterized by the presence of $CD8^+$ T-cells, Th1-polarized $CD4^+$ helper T-cells, and NK cells.[47,48] To facilitate development and maintenance of Th1 signaling after vaccination, DCs can be modified for constitutive production of Th1 cytokines and chemokines (Summarized in **Table 2**).

Table 2 Manipulation of chemokine and cytokine secretion and sensitivity in dendritic cell (DC)-based cancer vaccines

Cytokine/chemokine/chemokine receptor	Reference(s)	Reported immunological outcomes
IL-12p70	52,53,56	Increased costimulatory molecule expression
		Upregulation of antigen presentation machinery
		Greater $CD8^+$ T-cell activation and IFN-γ production
IL-2	55	Increased expression of surface costimulatory molecules
		Enhanced IFN-γ secretion by splenocytes
		Enhanced prophylactic and therapeutic anticancer activity
IL-18	54	Increased $CD8^+$ T-cell activation
		Increased NK cell activation
		Improved therapeutic and prophylactic anticancer activity
GM-CSF	122	Induced upregulation of Bcl-xL
		Decreased sensitivity to TGF-β
		Upregulation of DC costimulatory molecules
		Enhanced T-cell cytotoxicity
TGF-β (dominant-negative receptor)	58	Upregulation of costimulatory molecules on DCs
		Increased CTL activation
		Therapeutic anticancer immunity
CCR7	64	Enhanced DC migration to LN
		Enhanced antigen presentation and CTL activation
		Enhanced prophylactic antitumor activity
CXCL10	65	Enhanced DC migration
		Increased CTL activation and IFN-γ production
CCL17 and CCL22 (siRNA knockdown)	66	Inhibition of Treg and Th2 $CD4^+$ T-cell migration
		Enhanced Th1 T-cell activation
CCL21	67	Enhanced DC and T-cell activation
		Generation of therapeutic antitumor immunity

Abbreviations: Bcl-xL, B-cell lymphoma-extra large; CCL, chemokine (C-C motif) ligand; CTL, cytotoxic T-lymphocytes; CXCL10, C-X-C motif chemokine 10; GM-CSF, granulocyte-macrophage colony stimulating factor; IFN-γ, interferon-γ; IL-12, interleukin-12; LN, lymph node; NK, natural killer; siRNA, small interfering RNA; TGF, transforming growth factor; Th, T helper cells.

Manipulating Th1 cytokine secretion

IL-12p70, produced by DCs after stimulation, initiates Th1 polarization by inducing upregulation of TNF-α, IFN-γ, IL-2, and IL-18 from neighboring leukocytes.[49–51] On its own, IL-12 has potent anticancer effects attributable to its ability to activate T- and NK cells, and has been used in clinical trials.[47,50] Repeated systemic delivery of IL-12 has potent anticancer effects, unfortunately, however, it is also associated with severe toxicity.[47] Modification of DCs for cytokine production provides a continuous supply of IL-12 that is restricted to the immune environment and eliminates the requirement for systemic administration. DCs transduced using recombinant adenovirus carrying IL-12 demonstrate increased antigen presentation and costimulatory molecule expression, and induce increased numbers of activated T-cells.[52,53] Similarly, modification of DCs for coexpression of antigens and Th1 cytokines downstream of IL-12, including IL-2 or IL-18, supports development of CTL responses to prevent tumor growth.[54,55] Finally, DCs transduced for IL-12 production can reprogram primed T-cells isolated from melanoma patients to produce IFN-γ,[56] suggesting that IL-12-engineered DCs can induce Th1 immune polarization and favor the development of tumoricidal T-cell responses, even in tumor-bearing hosts.

Inhibiting responsiveness to regulatory cytokines

Tumor growth is associated with establishment of an immunosuppressive environment, characterized by the presence of regulatory cytokines including transforming growth factor-β (TGF-β) and IL-10. These conditions favor recruitment of immature myeloid suppressor cells and support *in situ* priming of regulatory T-cells.[57] Moreover, TGF-β and IL-10 can induce apoptosis of cytotoxic T-cells and DCs.[57,58] T-cells primed in the periphery, including those activated by DC vaccines, demonstrate diminished cytotoxic activity within the tumor environment.[52,59] Using genetically modified DCs, it may be possible to overcome the suppression imposed by pre-existing tumors (**Table 2**). Transduction for expression of GM-CSF induced upregulation of the anti-apoptotic molecule, B-cell lymphoma-extra large (Bcl-xL), in DCs and increased their resistance against TGF-β-induced apoptosis.[60] To directly reduce DCs' sensitivity to TGF-β, Wang and colleagues infected DCs using a retrovirus encoding a dominant-negative mutant of the TGF-β receptor.[58] Consequently, DCs became less sensitive to TGF-β and produced greater concentrations of IL-12. [58] *In vivo*, TGF-β receptor knocked-down DCs induced significantly stronger CTL activity and effected greater tumor rejection than green

fluorescent protein-transduced controls.[58] DCs producing proinflammatory cytokines may also be useful to directly establish a proinflammatory microenvironment when injected intratumorally. For instance, intratumoral delivery of DCs overexpressing IFN I recruited and maintained cytotoxic T-cells and extended survival of their hosts.[61]

Manipulation of chemokine secretion and responsiveness

Upon sensing pathogens, DCs upregulate chemokine production and receptor expression to facilitate T-cell recruitment and homing to draining lymph nodes. However, following subcutaneous injection of *ex vivo* cultured DCs, fewer than 5% of inoculated DCs can be recovered from the draining lymph node.[62,63] To enhance their recruitment to the lymph node, DCs have been modified to respond to chemokines that are constitutively expressed in the lymphatic system, including chemokine (C-C motif) ligand 21 (CCL21).[64] For example, following transduction with adenovirus encoding *CCR7*, the receptor for CCL21, DCs accumulated in draining lymph nodes 5.5 times more efficiently than those infected with control adenovirus.[64] Similarly, DCs have also been modified by retroviral transduction to extend their natural production of C-X-C motif chemokine 10 (CXCL10), a chemoattractant for naive T cells.[65] CXCL10-producing DCs could induce recruitment of T-cells in *in vitro* cultures and enhanced $CD8^+$ T-cell priming *in vivo*.[65]

The elimination of established tumors will require recruitment of cytotoxic effectors, however, the chemokine milieu within the tumor environment favors recruitment of Treg. Accordingly, DCs can be modified for controlled chemokine expression prior to intratumoral inoculation. Activated DCs express an array of chemokines, including CCL17 and CCL22, which favor recruitment of primed Th2 and Treg T-cells, and CCL23, which favors $CD8^+$ T-cell recruitment.[65,66] When CCL17 and CCL22 were knocked-down using siRNA, DCs became potent recruiters of $CD8^+$ T-cells, and intratumoral injection of these DCs lowered the ratio of Treg:CTL within the tumor environment.[65] Similarly, the inoculation of DCs transduced using adenovirus expressing CCL21 led to enhanced recruitment and activation of T-cells and increased concentrations of Th1 cytokines within the tumor microenvironment.[67]

GENETIC MODIFICATION OF DCs TO INDUCE RECRUITMENT OF OTHER CELL TYPES

The development of a Th1 immune environment is not only conducive to cytotoxic T-cell priming; recapitulation of a Th1 scenario actually facilitates recruitment and activation of additional effector cell populations. Whether intentionally or collaterally targeted, B-cells and NK cells can contribute significantly to the outcome of DC-based cancer vaccines.[68–74] A schematic representation of DCs' interaction with these cell types is shown in **Figure 3**.

Activation of B cells by DC-based vaccines

Because most of the identified TAAs are intracellular antigens, cancer immunotherapies have primarily aimed at generating T-cell responses. However, several extracellular TAAs have been identified, therefore, B-cells and antibodies can provide an additional avenue for tumor targeting. In fact, monoclonal antibody

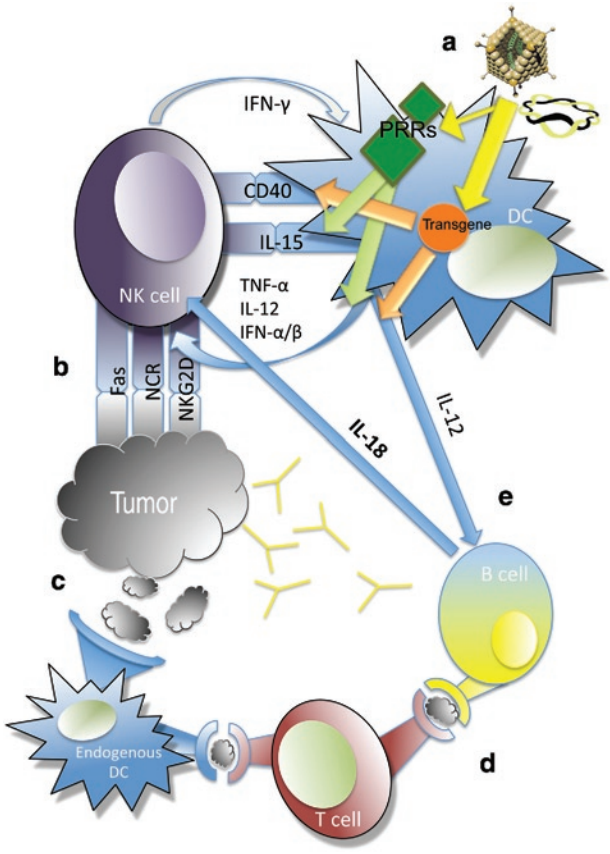

Figure 3 Complex interactions between dendritic cells (DCs) and other cell populations. **(a)** DCs are modified by RNA or DNA transfection, or infection using recombinant viruses. In addition to supporting T-cell priming, these factors can additionally support natural killer (NK) and B-cell activation. Viral components or foreign DNA/RNA can activate pattern recognition receptor, leading to upregulation of DC maturation factors and cytokine production, which, in turn, support NK cell activation. Bidirectional interaction between DCs and NK cells leads to interferon (IFN)-γ production and cytotoxicity from NK cells, and additional interleukin-12 (IL-12) from DCs. **(b)** NK cells initiate direct tumor cell lysis and additionally support T-cell activation by releasing tumor components. **(c)** Released tumor antigens can be processed and presented by endogenous DCs. This process facilitates further T-cell priming against an array of tumor-associated antigens. **(d)** Activated T-cells can additionally support B-cell activation. **(e)** Additionally, B-cells can support NK cell activation by releasing IL-18 in response to DC-derived IL-12.

therapies, including herceptin and rituxumab are among the most successful immunotherapeutic drugs available for clinical use.[74] B-cell activation and production of antibodies is not directly stimulated by DCs, however, their capacity to deliver antigen and support $CD4^+$ T-cell priming are critical for B-cell activation. Therefore, strategies to prime T-cell responses may collaterally activate B-cells. For instance, vaccination using DCs transduced for production of erbB2 (the murine homologue of HER2/neu), led to antibody production and T-cell activation which mediated downstream tumor protection.[75]

Few investigations have targeted DCs to initiate interaction with B-cells, however, proof-of-principle does exist to demonstrate that genetically modified DCs can activate B-cells for cytokine and antibody production. For instance, DCs transduced with recombinant adenovirus encoding the HER-2/neu oncogene delayed the

onset of spontaneous mammary tumor growth in neuT transgenic mice in a manner dependent on antibodies and $CD4^+$ T-cells.[73,76] Surprisingly, B-cells can also contribute to polarization of the Th1 immune environment by producing IL-18 in response to DC-derived IL-12.[49] In turn, IL-18 has been shown to activate NK cells for IFN-γ production and cytotoxicity.[49]

Recently, Boczkowski *et al*. have reported on a novel approach that might circumvent the need of B-cell activation by DC vaccines: they have engineered the DCs to secrete antibodies.[77] By electroporating murine DCs with genes for the heavy and light chains of a rat anti-mouse GITR mAb, they engineered DCs to secrete anti-GITR antibodies. They showed that treatment with DCs secreting anti-GITR and expressing TAA was comparable to administering TAA-expressing DCs plus systemic delivery of 1 mg of anti-GITR mAb, even though the DCs that were injected secreted only 2–3 ng of antibody.

Activation of NK cells by DC-based vaccines

Without prior stimulation, NK cells provide critical immunosurveillance for elimination of transformed, precancerous cells. NK cells are important as a major source of IFN-γ and for direct lysis of tumor cells through cytotoxicity receptors, including NKG2D, Fas, and the natural cytotoxicity receptor families.[78] Activated NK cells additionally provide help for B- and T-cell activation, bypassing their requirement for $CD4^+$ T-cell-mediated help.[73,79,80] In fact, the presence of activated NK cells in cancer patients correlates with improved prognosis, and may actually be a better predictor of DC vaccine efficacy than T-cells.[81–83] DCs support the development and activation of NK cells *in vivo* through bidirectional interactions,[84–86] and NK cells activated by DC vaccination can provide protection against tumor challenge, even when adoptively transferred to naive hosts.[69,70,79,86] Therefore, it is important to consider the impact of DC-based vaccines on NK cell activation and function.

NK cells can be primed beyond their "natural" state to provide enhanced antitumor activity by a variety of stimuli.[69,70,86,87] In response to inoculation with DCs, IFN-γ and granzyme B expression is upregulated by NK cells in the spleen and draining lymph node, and is indispensible for tumor protection.[53,60,69,70] Protocols for genetic modification of DCs may amplify NK cell activation and improve the overall outcome of DC-based vaccines. For example, modifications of DCs for Th1 cytokine production, including IL-12 or GM-CSF, have been demonstrated to induce NK recruitment and activation.[53,56,60] Similarly, DCs differentiated in the presence of polyI:C and IFN-β induce recruitment of activated NK cells, which support Th1 T-cell development.[87]

CREATING A STRONG INFLAMMATORY ENVIRONMENT

Maturation of DCs can be accomplished by exposure to cytokine cocktails, TLR ligands or virus infection, however, these stimulations activate different pathways.[88] TLR ligands and virus infection induce maturation by agonizing pathogen sensing pathways through activation of pattern recognition receptors, including the toll (TLR)-, NOD (NLR)-, and RIG-I (RLR)-like pathways [pattern recognition receptor (PRRs)].[89] In contrast, cytokine cocktails, which are most frequently used in clinical trials, actually recapitulate events that occur downstream of pathogen sensing in DCs by binding to cytokine receptors and inducing activation of STAT molecules. PRR or cytokine receptor engagement triggers downstream signaling that can lead to activation of AP-1, NFκB, and the innate interferon, MAP kinase, and inflammasome pathways.[89] Although cytokine cocktails, TLR ligands and virus infection can each induce upregulation of costimulatory molecules and cytokines conducive to T-cell activation, they may differentially influence the longevity of immune responses or the activation of additional cell populations.

Similar to cytokine cocktails, TLR ligands are generally washed from DC preparations before inoculation. The extension of TLR signaling, however, may assist in generating immune responses. As mentioned earlier, transfection of constitutively active mRNA for TLR4 alongside costimulatory molecules and TAAs enhanced T-cell activation *in vivo* by providing continuous stimulation.[32,34] Transfection with RNA or infection with recombinant, replication-incompetent viruses provides a continuous supply of cytoplasmic RNA and DNA which is sensed by RLRs and TLRs 3, 7, 8, and 9.[48,70,87,90,91] This continued stimulation leads to upregulation of prototypical DC maturation markers, including CD83 and CD86.[92,93]

Engagement of multiple receptors can synergize to tailor DC maturation, migration, and cytokine production against a given pathogen.[89,94] Recombinant viruses activate components of multiple pathways, and lead to potent, persistent DC activation. For instance, DCs infected with vesicular stomatitis virus activate tumoricidal NK cells in cancer-bearing mice, in a mechanism dependent on IFN I signaling and IL-15 signaling.[95] Similarly, DCs infected with adenovirus activate NK cells *in vivo via* cooperative TNF-α and IL-15 pathways.[96] Recombinant canarypox virus also elicits activation of the innate interferon signaling pathway in DCs, upregulates NFκB activity, induces CXCL10 production from DCs and primes IFN-γ production by NK cells.[97,98] Taken together, these experiments demonstrate that virus infection of DCs indeed leads to prolonged Th1 immune activation, which assists in activating NK cells in addition to T cells.

CURRENT PROGRESS IN CLINICAL TRIALS

Preclinical models of DC-based cancer vaccines provided significant optimism for translation to clinical application. Protocols for deriving DCs from $CD14^+$ and $CD34^+$ monocytes are established, and DCs are well-tolerated in phase I clinical trials.[92,99–104] DCs modified for expression of TAAs have been shown to activate antitumor T-cell responses in cancer patients and in therapeutic animal models.[48,70,105] A summary of a number of clinical trials using genetically modified DCs is shown in **Table 3**. Unfortunately, the success of experimental models of DC-based cancer vaccines has generally not translated into clinical efficacy.[88] Improvements in DCs' migration, immune polarization and ability to engage effector populations despite tumor-induced immunosuppression will likely be required to facilitate the widespread clinical use of DC-based vaccines. One of the strategies to accomplish this is to manipulate the microenvironment during DC differentiation or following *in vivo* injection. Recently, a DC-based vaccine for prostate cancer became the first FDA-approved cellular vaccine after reporting a 4.1-month extension of patient survival,[105] generating significant optimism for DC-based cancer vaccines. This vaccine,

Table 3 Summary of outcomes from a number of clinical trials employing gene-modified dendritic cell (DCs)

Reference	DC modification	Disease details	Type of trial	Disease outcome (frequency)	Immunological outcome (frequency)	Remarks
127	Autologous tumor RNA, transfected	2 Metastatic melanoma patients 1 Metastatic adenocarcinoma patient	N/A	N/A	Increased CTL responses against tumor-associated antigens	2/3 patient tumors were debulked by surgery prior to DC procedures
128	MUC1 cDNA, lipofected	7 Breast cancer patients 2 Pancreatic cancer patient 1 Papillary cancer patients All metastatic	Phase I/II	SD (1/10)	Increased anti-MUC1 $CD8^+$ T-cells (4/10)	
92	Autologous renal tumor RNA, transfected	7 Stage IV metastatic renal cell carcinoma patients	Phase I	Of those not receiving adjunct therapy, one died 2 months post-DC; One survived >22 months	Increased CTL response against RCC antigens (6/7)	8/10 patients in this study went on to receive adjunct therapy
129	CEA mRNA-transfected DCs	24 Patients with CEA-expressing hepatic metastases, various primary tumors	Phase I/II	CR (1/24) PR (2/24) SD (3/24)	3 patients had activated T-cells	
130	Autologous brain tumor RNA, pulsed	7 Recurrent brain cancer	Phase I	Stable disease (3/7) Progressive disease (4/7)	No detectable T-cell responses (7/7)	DCs were not matured prior to inoculation
131	Autologous tumor RNA, pulsed	11 Stage IV neuroblastoma patients	Phase I	Complete response (4/11) Partial response (4/11) Minor response (1/11) Progressive disease(2/11)	Nonsignificant increase in T-cell activation Increased antibody responsiveness	Most patients succumbed to disease post-follow-up (10/11) Immune measurements are against copulsed, DTH antigens
129	Infection using recombinant fowlpox encoding CEA, B7-1, LFA-3, and ICAM-1	11 Colorectal cancer patients 3 Non small-cell lung carcinoma patients	Phase I	SD (5/13) PD (8/13)	Generation of T-cell responses against CEA (13/14)	
117	DC infected with adenovirus carrying IL-12	17 Metastatic gastrointestinal cancer patients	Phase I	SD (2/10) PR (1/11)	NK cell activaiton (5/17)	
132	mRNA from allogeneic tumor cell lines, transfected	19 Prostate cancer patients	Phase I/II	Stable disease (11/19) Progressive disease (8/19)	Generation of antitumor T-cell responses (12/19)	Stable disease was strongly correlated with T-cell responses (10/11)
133	Autologous tumor RNA, amplified *ex vivo*, transfected	6 Stage IV metastatic melanoma patients	N/A	PD (5/6) SD (1/6)	No detectable immune responses	
81	Infection using recombinant fowlpox encoding CEA, B7-1, LFA-3 and ICAM-1	5 Colorectal cancer patients 3 Lung cancer patients 1 Urachal adenocarcinoma patients	Phase I	SD (5/9) PD (4/9)	Increased T-cell response (9/9) Increased NK frequency and cytotoxicity (4/9)	Stable disease correlated with increased NK activity
104	Autologous tumor mRNA, transfected	22 Advanced melanoma patients	Phase I/II	N/A	Increased T-cell response (9/19)	Intradermal DC inoculation elicited T-cell responses; intranasal delivery did not
104	Autologous tumor RNA, transfected	18 Stage IV metastatic malignant melanoma patients	Phase I/II	SD (2/20) PD (18/20)	Enhanced T-cell proliferation (10/19)	
134	DCs transduced with adenovirus encoding MART-1	14 stage IV metastatic melanoma patients	Phase I/II	SD (4/19)	Expansion of anti-MART-1 T-cells (4/14) preliminary evidence of NK cell activatin	Disease responses were temporary in all patients except for 1, who received surgery postvaccination Evidence of epitope spreading
99	Wilm's tumor antigen mRNA-transfected DCs	10 AML patients	Phase I	N/A	N/A	Dose escalation trial; DCs were well-tolerated *in vivo*
83	Wilm's tumor antigen mRNA-transfected DCs	10 AML patients	Phase I/II	CR (2/10 that started with SD) Maintained CR (2/10)	T-cell activation (9/9)	Followup to Van Dreissche 2009
105	PAP antigen + GM-CSF fusion, pulsed DCs	341 castration-resistant prostate cancer patients	Phase III	Mean 4.1 extension of patient survival	T-cell activation (46/63) Antibody production (100/151)	Results earned FDA approval for Sipuleucil-T

Abbreviations: AML, acute myeloid leukemia; CEA, carcinoembryonic antigen; CR, complete response; CTL, cytotoxic T-lymphocytes; DTH, delayed-type hypersensitivity; GM-CSF, granulocyte-macrophage colony stimulating factor; MART-1, melanoma antigen recognized by T-cells; N/A, information not available; PR, partial response; SD, stable disease.

Sipuleucil-T, utilizes DCs pulsed with a fusion protein of a prostate cancer-associated antigen and GM-CSF,[105] reinforcing the notion that the combination of DC-based vaccines and cytokines may improve therapeutic outcomes.

To date, the majority of clinical trials have used DCs pulsed with TAAs, and matured by exposure to cytokine cocktails that include PGE-2, IL-1β, IL-6, and TNF-α. PGE-2 is required for DC migration to lymph nodes,[106,107] however it also impairs DCs' production of IL-12,[108] and induces Th2 cytokine production, including IL-5.[56] More recently, protocols using IFN I have been established that favor development of "DC1" DCs. DC1 DCs upregulate CXCL10, recruit and activate NK cells, instruct Th1 cytokine production, and reduce Treg frequency.[9,87,109–111] Compared with standard myeloid DC culture protocols, DC1 DCs induce greater CTL activation and support superior antigen cross priming.[9,112] Moreover, DC1 DCs can induce primary antibody immune responses.[113] When DCs were transduced for IFN-α prior to *in vivo* inoculation, their ability to migrate and survive was enhanced compared with control-transduced DCs in human clinical trials.[114] Together, these findings support the use of type I IFN during DC differentiation.

IL-12 and TNF-α are also candidates for cytokine gene therapy with DCs, owing to their ability to polarize Th1 immune environments. The clinical utility of DCs genetically modified for IL-12 or TNF-α production is currently under investigation.[115,116] Intratumoral administration of DCs infected with adenovirus encoding IL-12 mediates increased recruitment of $CD8^+$ T-cells and activation of NK cells, however, objective clinical response rates remained low.[117] This failure may be attributable to impaired DC migration from the tumor to present antigen in draining lymph nodes. Indeed, Feijoó and colleagues have reported that the migration of IL-12-producing DCs was inhibited by tumor-derived IL-8, and their migratory capacity could be restored using an anti-IL-8 antibody.[118] An alternative strategy is to control DC interaction with naive T-cells by modifying DCs for expression of CCL21. This chemokine leads to the formation of lymphoid-like structures *in vivo*, where naive T-cells can be primed extranodally, eliminating the requirement for DCs to traffic to the local lymph nodes. DCs modified for CCL21 expression are the subject of an ongoing clinical trial.[115]

We have performed a clinical trial with DCs genetically modified through mRNA electroporation with TriMix mRNA, coelectroporated with Mage-A3, Mage-C2, gp100, or tyrosinase mRNA (S. Wilfenhof, A.M.T. Van Nuffel, J. Corthals, C. Heirman, S. Tuyaerts and D. Benteyn, unpublished results). Thirty-five metastatic melanoma patients received four biweekly vaccinations. Thereafter they could initiate interferon-α2b therapy and receive additional TriMix-DC vaccines every 8 weeks. Immune monitoring of T-cells infiltrating a delayed-type hypersensitivity reaction showed that almost 60% of the patients tested after the 4th vaccine had mounted an immune response against one or more of the vaccine antigens. Furthermore, disease control for >6 months with regression of metastases was observed in 7 of 20 patients (35%) with evaluable disease at baseline. For 15 patients without evaluable disease at baseline, recurrence-free survival is 23.0 months.

Clinical trials have demonstrated that the presence of activated NK cells correlates with improved anticancer activity following DC vaccination, and may actually be a better prognostic indicator than T-cell activation.[81,83] NK cells can kill tumor cells directly and support T-cell activity, both *via* cytokine secretion and through release of TAAs by tumor cell lysis. These TAA can subsequently be scavenged and presented by endogenous DCs.[78,119] Thus, future efforts may benefit from a focus on developing conditions conducive to NK cell activation and *in situ* T-cell priming, rather than focusing on selection of TAAs.

CONCLUSIONS AND FUTURE DIRECTIONS

DC-based vaccines have shown excellent promise in preclinical studies, but further improvements are required to amplify their therapeutic utility. Clinical trials have mainly employed DCs pulsed with TAAs and matured by exposure to cytokine cocktails. These DCs are sufficient for T-cell activation, but they may not be adequate to provide continuous costimulation to mount and maintain a proinflammatory immune environment and to recruit additional effector components. Since an existing tumor creates an immunosuppressive immune environment, successful DC-based cancer vaccines should be prepared to prime strong and persistent immune responses after immunization. Genetic modification of DCs will allow for a continuous supply of natively processed antigen and immune-stimulating molecules, and might therefore provide more robust and persistent anticancer immunity *in vivo*.

Several investigations have demonstrated that DCs can be simultaneously modified with multiple genes and/or immune factors.[11,33,34,48,108] It is unlikely that modification of DCs for a single factor, or engagement of a single effector population will be sufficient for successful cancer immunotherapy. Not only must DCs activate anticancer immune responses, they must also resist tumor-induced immunosuppression. Thus, DCs should be rationally engineered to simultaneously deliver the three signals required for T-cell activation, along with neutralizing factors for inhibitory components. To accomplish this, DCs can be genetically modified as described in this manuscript, and/or used in combination with other treatment approaches. For example, anthracyclin chemotherapy will facilitate tumor cell apoptosis conducive to DC-mediated antigen cross presentation to T-cells.[120] Moreover, DC immunization in combination with antibody therapies, such as anti-IL-8 or anti-CTLA4, will facilitate DC migration and presentation of tumor-derived antigens, or inhibit tumor-induced immunosuppression, respectively.[118,121]

Clinical trials of DC-based cancer vaccines have revealed that activated NK cells are better predictive of vaccine efficacy than CTL responses.[81–83] Accordingly, future clinical trials may benefit from the inclusion of strategies to target NK cells in addition to CTL. We, and others, have demonstrated that tumoricidal NK cells can be activated by DC-based vaccines, especially those modified by virus infection.[69,70,96] This success has been attributed to IL-15 and membrane-bound TNF-α.[96] Similarly, other cytokines, including GM-CSF or IL-12, have been shown to participate in recruitment and activation of NK cells following DC-based cancer vaccination.[53,56,122] In addition, delivery of ligands for NK-activating receptors, (*i.e.*, those that bind to NKG2D family receptors), could facilitate the activation of NK cells for IFN-γ production and cytotoxicity.[123] Existing DC-based vaccines would likely benefit from additional modification to provide NK-activating cytokines and

molecules. Further investigation will be required to determine the optimal combination of these factors to provide NK cell activation and support for CTL-mediated rejection of tumors.

Genetic modification is safe and sufficient for delivery of TAAs, costimulatory molecules, and the environmental signals. The use of DNA, mRNA, or viruses to introduce TAAs allows for endogenous expression and processing of full-length proteins, including tumor antigens and immune response factors. Moreover, chemokine, cytokine, and costimulatory molecule expression can be made continuous by delivering the relevant genes under the control of constitutive promoters.

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