# DC-based cancer vaccines

**Eli Gilboa**

Department of Microbiology and Immunology, Sylvester Comprehensive Cancer Center, Miller School of Medicine, University of Miami, Miami, Florida, USA.

**Because of the large preexisting antigenic load and immunosuppressive environment within a tumor, inducing therapeutically useful antitumor immunity in cancer patients requires the development of powerful vaccination protocols. An approach gaining increasing popularity in the tumor vaccine field is to immunize cancer patients with their own DCs loaded ex vivo with tumor antigens. The underlying premise of this approach is that the efficiency and control over the vaccination process provided by ex vivo manipulation of the DCs generates an optimally potent APC and a superior method for stimulating antitumor immunity in vivo compared with the more conventional direct vaccination methods, offsetting the added cost and complexity associated with this form of customized cell therapy.**

The adaptive immune system evolved to protect the host from infectious pathogens in part by evolving mechanisms to generate a diverse repertoire of antigen-specific T and B cells that can respond to almost any antigen to which they are exposed. The downside of such diversity is that the antigen-specific T and B cell repertoire contains cells able to recognize and attack the host's own tissues. However, such cells are controlled and prevented from responding to self tissues by several mechanisms that collectively render the immune system tolerant of these self antigens.

Both immunity and tolerance are controlled by a network of professional APCs, the most important of which are known as DCs (1, 2). Tissue-resident DCs that capture pathogen-encoded antigens are activated by stimuli generated in the course of a pathogen-induced inflammatory response. Activation of DCs occurs in two phases, maturation and licensing, and is an essential step that enables the antigen-loaded DCs to migrate to the draining lymph nodes where they can activate T cells that recognize the antigens they are presenting (cognate T cells) (3). Unlike infectious pathogens, tumors do not induce an effective inflammatory response conducive for optimal activation of DCs, and as a result the ensuing immune response is weak and ineffective. The primary purpose of vaccinating individuals with cancer is to overcome this "defect" by channeling tumor antigens into DCs and providing the conditions for their optimal maturation into potent immunostimulatory APCs.

The age-old protocols for vaccinating individuals against infectious diseases, of injecting antigen mixed with adjuvant, targeted and activated DCs in situ long before the existence of professional APCs was suspected. Such in vivo or direct vaccination approaches, although simple, cost effective, and broadly applicable, have not been effective in the setting of cancer (4). One thing likely to contribute to such failures is that vaccines against infectious agents are administered prophylactically to healthy individuals as a protective measure against future exposure, whereas cancer vaccines are administered therapeutically in the cancer patient in the face of a preexisting antigenic load (the tumor). Other factors contributing to the limited efficacy of early cancer vaccination protocols include the need to stimulate the cellular arm of the immune response and the fact that immune responses are suppressed in cancer patients. Such failures underscore the need to develop increasingly potent cancer vaccination strategies (5).

One approach that is gaining increasing popularity among tumor immunologists, and the primary focus of this Review, is to immunize cancer patients with autologous, patient-derived DCs loaded with tumor antigens ex vivo. The underlying premise of this approach is that the efficiency and control provided by ex vivo manipulation of the DCs generates an optimally activated APC and a superior method for stimulating immunity in vivo as compared with more traditional vaccination methods. Recent years have witnessed rapid and remarkable progress in developing DC-based vaccines, yet the promise remains just that, a promise. This Review discusses these advances and whether they can be successfully applied to induce clinically significant antitumor immunity.

## Ex vivo generation of immunocompetent DCs

The era of ex vivo DC vaccines was ushered in by the pioneering work of Inaba, Steinman, and colleagues, demonstrating that mouse DCs can be cultured ex vivo from bone marrow precursors (6). In a similar fashion, human DCs can be generated in culture from $CD34^+$ hematopoietic progenitors and, more commonly, from peripheral blood–derived monocytes (reviewed in refs. 7–9). For cancer vaccination, the goal is to generate ex vivo a population of antigen-loaded DCs that stimulates robust and long-lasting $CD4^+$ and $CD8^+$ T cell responses in the patient with cancer, with the emphasis on "long-lasting". What seems to be the rate-limiting step at present is the inability to fully recapitulate ex vivo the development of immunocompetent DCs, in particular the process of DC activation. In what is undoubtedly an oversimplification, DC activation can be divided into two stages (Figure 1). In the periphery, quiescent (immature) DCs undergo a maturation process in response to inflammatory stimuli originating from pathogens (pathogen-associated molecular patterns [PAMPs]) or from dying cells, collectively referred to as "danger signals" or "danger-associated molecular patterns (DAMPs)" (10). One important consequence of the maturation process is that DCs acquire the capacity to home to lymph nodes. DCs receiving the appropriate maturation stimuli upregulate expression of CC chemokine receptor 7 (CCR7) and become responsive to CC chemokine ligand 19 (CCL19) and CCL21, chemoattractants produced in the afferent lymphatics and the lymph node. DC migration is also controlled by leukotrienes (such as $LTD_4$ and $LTE_4$), which act downstream of CCR7 signaling (11, 12). When reaching the lymph node, antigen-loaded mature DCs undergo an additional activation step,

**Nonstandard abbreviations used:** CD40L, CD40 ligand; DTIC, dacarbazine; PAMP, pathogen-associated molecular pattern; PolyI:C, polyinosinic-polycytidylic acid; PSA, prostate-specific antigen; TERT, telomerase reverse transcriptase.

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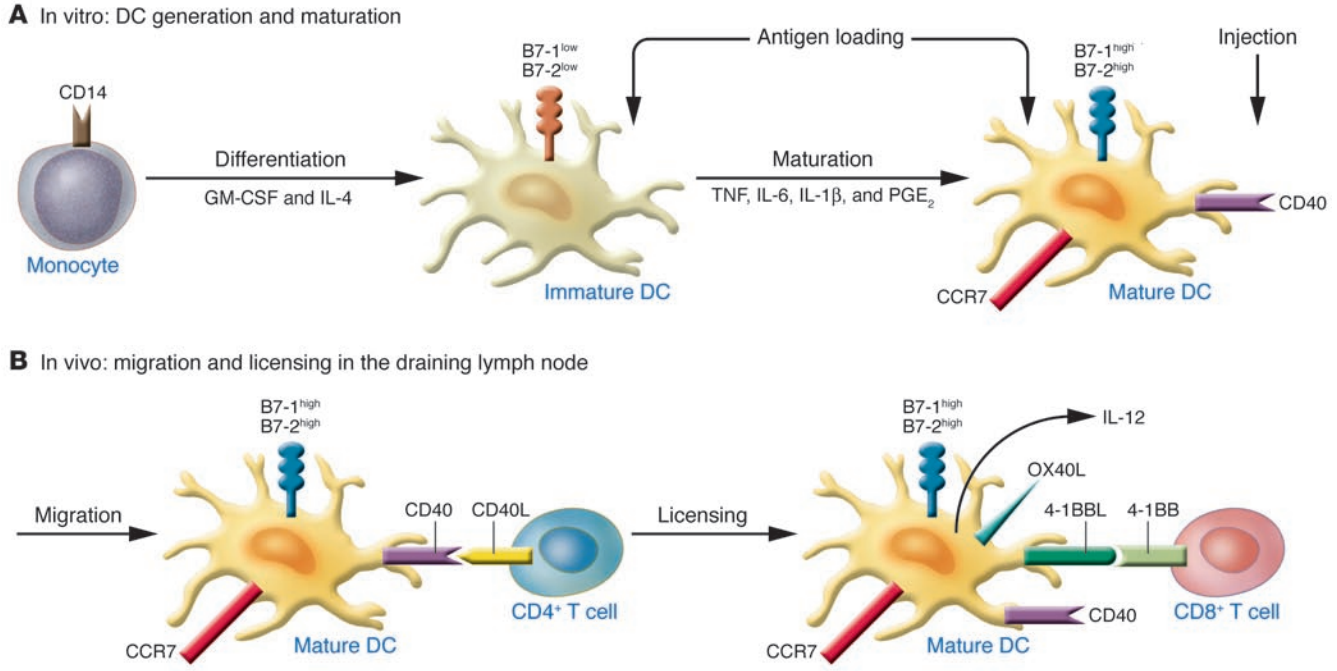


**Figure 1**

Ex vivo differentiation and activation of DCs for cancer immunotherapy. (**A**) The most common method used to generate DCs for clinical trials is to culture CD14+ monocytes in serum-free media in the presence of GM-CSF and IL-4. Following 5–7 days in culture, the monocytes differentiate into immature DCs, which lose CD14 expression and express moderate to low levels of CD40 and the costimulatory ligands B7-1 and B7-2. DC maturation is accomplished by culturing the immature DCs for an additional 24–48 hours in the presence of several biological agents, the most popular combination being TNF, IL-6, IL-1β, and $PGE_2$ (41). Mature DCs further upregulate CD40, B7-1, and B7-2 and induce the de novo expression of the lymph node homing receptor CC chemokine receptor 7 (CCR7). Antigen loading occurs at either the immature or mature DC stage. (**B**) Mature antigen-loaded DCs are injected into patients subcutaneously, intradermally, or intravenously. They migrate to the draining lymph node, where they encounter and present antigen (not shown) to cognate CD4+ T cells. Cross-linking CD40 on the DCs by CD40L, which is expressed on the antigen-activated CD4+ T cell, induces the mature DCs to differentiate further, a process known as licensing. Licensed DCs upregulate additional cell surface products, notably the ligands for OX40 and 4-1BB (OX40L and 4-1BBL, respectively). The licensed DCs present antigen to cognate CD8+ T cells. 4-1BBL–mediated costimulation through 4-1BB on the antigen-activated CD8+ T cells enhances the survival and proliferative capacity of the activated CD8+ T cells. Likewise, OX40L-mediated costimulation enhances the survival and proliferation of the activated CD4+ T cells (not shown).

termed "licensing," in response to various stimuli, notably CD40 ligand (CD40L) which is expressed on cognate CD4+ T cells. In addition to antigen loading, which will be discussed in the next section, DCs need to be generated in vitro such that they undergo optimal maturation but not licensing because full activation of DCs ex vivo might be counterproductive, as discussed below. The goal, therefore, is to differentiate antigen-loaded DCs only to the point that they have acquired lymph node migratory capacity and become responsive to licensing stimuli when they reach the lymph node and encounter cognate T cells (Figure 1).

*Enhancing DC maturation*. Recent insights in DC biology have provided some guidelines as to how optimally matured DCs might be generated ex vivo so that when administered to a patient with cancer they have the ability to migrate to a lymph node and respond to licensing stimuli. These include new information regarding the role of TLRs in sensing danger signals, the identity of molecular mediators of feedback mechanisms that attenuate DC function, the identity of DC-derived costimulatory signals that potentiate T cell activation, and the recognition that DC viability can affect their immunogenicity.

Pathogen-mediated maturation of DCs is mediated mainly through the TLRs that are expressed on immature DCs and activated in response to distinct microbial compounds, PAMPs (10, 13). Culture of DCs with such compounds or their pharmacological analogs (such as the TLR4 ligand LPS, the TLR3 ligand polyinosinic-polycytidylic acid [polyI:C], the TLR9 ligand oligodeoxynucleotide containing one or more unmethylated CpG dinucleotides [CpG ODN], and the TLR7/8 ligands R848 and imiquimod) can induce the phenotypic and functional maturation of cultured DCs. Importantly, functional maturation of DCs is markedly augmented by using certain combinations of TLR agonists (14–16). Cytokines, such as TNF, IL-1, and IL-6, are also capable of promoting DC maturation but cannot substitute for TLR stimulation (17). This should raise concern because most cancer DC vaccine clinical trials use cytokine-only maturation protocols that do not include TLR ligands (18). The remarkable discovery of two phagosome autonomous uptake mechanisms that discriminate in their ability to process antigens for MHC class II presentation depending on the presence or absence of TLR ligands in the phagosome (19) strongly argues, as was indeed shown (20), that fusion of antigens with TLR ligands should enhance the presentation of antigens in the context of MHC class II and thereby potentiate CD4+ T cell immunity. Optimal ex vivo DC maturation might therefore require a combination of both cytokines and TLR

ligands, with enhanced antitumor immunity and clinical efficacy being achieved by physically linking TLR ligands with the tumor antigen. In a recent study, Gavin et al. have raised some questions regarding the physiological role of TLRs, introducing the possibility that engaging other pathogen-sensing receptors might be more useful in DC vaccination (21).

Ex vivo DC maturation protocols attempt to recapitulate a complex biological process that has evolved in response to infection with pathogens, but these have so far had limited success in generating DCs that elicit effective antitumor immunity. An alternative and complementary approach is to inhibit negative regulatory pathways that attenuate DC maturation (22). This approach was pioneered by Shen and colleagues, who showed that siRNA inhibition of the function of SOCS1 (which negatively regulates cytokine signaling in DCs and T cells) in DCs potentiates DC immunogenicity (23). Another attractive target is glucocorticoid-induced leucine zipper (GILZ), which is the common effector of suppressive signals mediated by glucocorticoids, IL-10, and TGF-β (24).

Survival and proliferative signals are provided to activated T cells through costimulatory molecules such as OX40 and 4-1BB, which are cross-linked by ligands expressed on activated DCs (25, 26). Current strategies to enhance costimulation include the systemic administration of agonist antibodies or soluble ligands at the time of DC vaccination (25). An alternative approach, transfecting the genes encoding the corresponding ligands into the antigen-loaded DCs, is a simple procedure using readily available reagents that provides added specificity by limiting costimulation to cognate T cells. For example, transfection of mRNA encoding OX40 into DCs has been shown to potentiate the ability of mouse DCs to induce antitumor immunity in vivo and to enhance the activation of human DCs in vitro (27).

Recent studies have also shown that extending the persistence and presentation of antigen by DCs in the lymph node enhances the ensuing immune response (28, 29). However, DCs that are presenting antigen in the lymph node are prone to elimination by their cognate T cells (30). Therefore methods to enhance DC viability, such as generating DCs expressing antiapoptotic proteins (31, 32) or using siRNA to decrease the expression of proapoptotic proteins (33) should also potentiate their immunogenicity.

*To license or not to license?* Licensing of antigen-loaded DCs in the T cell zone of lymph nodes is mediated by local stimuli, notably CD40L expressed by cognate $CD4^+$ T cells, IFN-γ, and surely other stimuli yet to be identified (34). Signaling through CD40 has multiple effects on DCs, including inducing the upregulation of costimulatory molecules, the secretion of cytokines (notably IL-12), and the upregulation of several antiapoptotic molecules, all of which cumulatively potentiate the ability of DCs to optimally activate cognate T cells, especially $CD8^+$ T cells (35, 36). However, premature licensing of DCs prior to their encounter with cognate T cells in the lymph node might be counterproductive. IL-12, an important licensing cytokine that mediates the polarization of activated $CD4^+$ T cells to a Th1 phenotype such that they provide help for the generation of potent $CD8^+$ CTL responses, is a case in point. DCs can be induced in vitro and in vivo to secrete IL-12, but IL-12 expression is transient and DCs become refractive to subsequent induction of IL-12, a phenomenon termed "exhaustion" or "paralysis" (37, 38). These observations strongly suggest that ex vivo DC maturation protocols should avoid conditions that induce DCs to express IL-12, and should instead use conditions that induce the DCs to acquire responsiveness to IL-12 induction (39). But can one enhance the licensing potential of ex vivo–generated DCs, that is, specifically augment CD40 signaling after they have been injected into the patient? An ingenious solution has been offered by the work of Hanks et al. (40), who developed a drug-inducible CD40 expression system whereby the trimerization-dependent activation of ectopically expressed engineered CD40 molecules in ex vivo–generated DCs is delayed until they reach the lymph node. This was achieved by fusing a membrane-localized cytoplasmic domain of CD40 to a drug-binding domain and injecting the appropriate bivalent drug into mice once the DCs arrived at the lymph node, thereby mediating CD40 trimerization .

*DC generation and maturation protocols*. The most widely used maturation protocol for human monocyte–derived DCs consist of four reagents, TNF, IL-1β, IL-6, and $PGE_2$, also known as monocyte-conditioned media mimic or cytokine cocktail (41). A recent phase III clinical trial failed to show that vaccinating melanoma patients with cytokine cocktail–matured DCs provided benefit over standard dacarbazine (DTIC) chemotherapy (42). It is not inconceivable that the suboptimal nature of the maturation conditions, and hence the suboptimal immunogenicity of the DCs, was a primary reason for the failure. It is tempting to speculate that the main culprit in the cytokine cocktail formula was $PGE_2$. The rationale for including $PGE_2$ in the maturation protocol is to endow the ex vivo–generated DCs with the capacity to migrate (43, 44), but $PGE_2$, in the context of the tumor microenvironment, can mediate Th2 polarization and promote the differentiation of DCs secreting the immunosuppressive cytokine IL-10 (45). Therefore, the key negative impact of $PGE_2$ on the function of ex vivo–generated DCs is probably that $PGE_2$ abolishes both the responsiveness of mature DCs to stimulation through CD40 and their ability to synthesize IL-12 when they reach the lymph node and encounter cognate T cells (44). $PGE_2$ notwithstanding, the elegant study of Sporri and Reis e Sousa has shown that optimal activation of DCs requires TLR signaling, which this maturation protocol does not provide (17). If all that is not enough, a recent study comparing several maturation protocols found that cytokine cocktail–matured DCs were most effective, even more than immature DCs, at expanding a population of immunosuppressive Tregs expressing the forkhead box transcription factor FOXP3 (46).

The cytokine cocktail protocol is not the only human DC maturation protocol used. Kalinski and colleagues have designed a "megacytokine cocktail" protocol consisting of 5 reagents, TNF, IL-1β, PolyI:C, IFN-α, and IFN-γ (47). In vitro megacytokine cocktail–matured DCs exhibited superior immunogenicity to cytokine cocktail–matured DCs, that is, they stimulated more potent CTL responses (43). Furthermore, the megacytokine cocktail–matured DCs were responsive to stimulation through CD40, able to produce IL-12, and, notably, despite the absence of $PGE_2$ in the megacytokine cocktail the DCs retained lymph node migratory capacity in vitro. Promising as this might be, judging from in vitro analysis, clinical trials are necessary to determine the value of this novel approach in vivo. It is thought, based on in vitro studies, that maturing DCs in TNF alone or omitting $PGE_2$ from the cytokine cocktail will not generate DCs able to induce therapeutically effective antitumor immunity (41). However, the results of a study in which rhesus macaques infected with SIV were vaccinated with DCs matured using TNF and a follow-up clinical trial in which patients infected with HIV were vaccinated with DCs matured using IL-1β, IL-6, and TNF (but in the absence of $PGE_2$) were nothing short of spectacular, resulting in the induction of T cell responses and

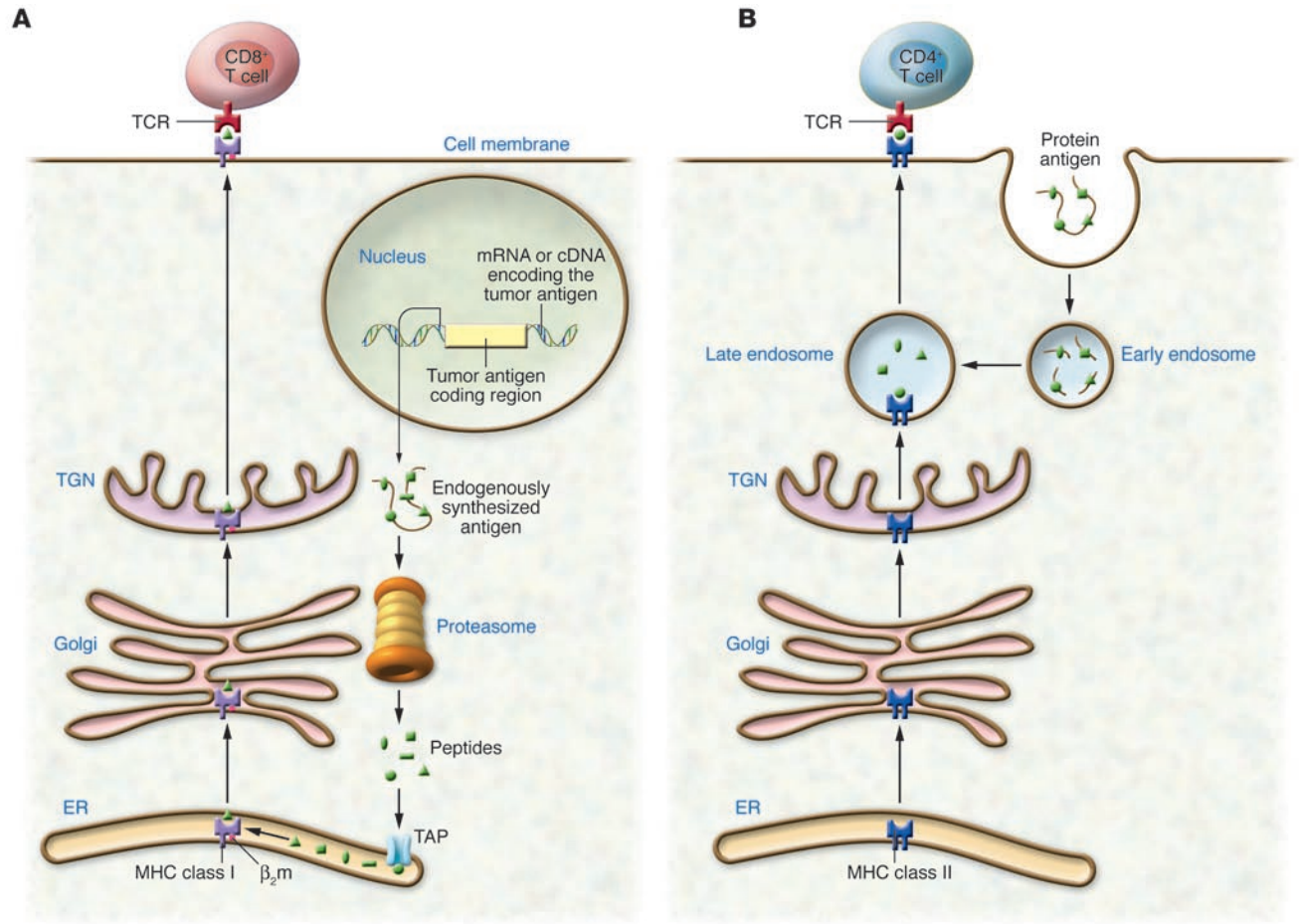


**Figure 2**
The MHC class I and class II antigen presentation pathways. (**A**) The endogenous MHC class I presentation pathway leads to the activation of CD8+ T cells. Antigens expressed in the cells or introduced into the cytoplasm are degraded by the proteasome complex to generate short peptides that are translocated into the ER through special pores controlled by the transporter associated with antigen processing (TAP) proteins. In the lumen of the ER, the peptides associate with newly synthesized MHC class I molecules and the peptide–MHC class I complex is transported to the cell surface, where it is presented to CD8+ T cells expressing the cognate TCR (that is, the TCR that recognizes the particular peptide-MHC complex). Therefore, delivery into DCs of nucleic acid–encoded antigen, which needs to be translated in the cytoplasm, favors the generation of MHC class I–restricted CD8+ T cell responses. TGN, trans-Golgi network. (**B**) The exogenous MHC class II presentation pathway leads to the activation of CD4+ T cells. Antigens captured by professional APCs such as macrophages and DCs are routed through the endosome, where they undergo partial proteolytic degradation to generate peptides that associate with nascent MHC class II molecules. The peptide–MHC class II complexes are transported to the cell surface and presented to cognate CD4+ T cells. Therefore, generation of CD4+ T cell responses is favored by vaccination with whole protein–based antigens.

substantial reductions in viral titer in most vaccinees (48, 49). In addition, several rapid, two-day "fast-DC" protocols have been developed that generate DCs able to stimulate T cell responses in vitro as effectively as DCs generated by standard protocols, which usually require 7–9 days of culture. (50–52). In a recently published clinical trial, HER2/neu-positive breast cancer patients vaccinated with peptide-loaded DCs generated in a two-day culture of monocytes incubated with IFN-γ and LPS induced HER2/neu-specific CD4+ and CD8+ T cell responses and measurable decreases in tumor volume (53). Importantly, in vitro analysis suggests that DCs generated in such a manner are mature, as judged by phenotypic analysis, and transiently secrete IL-12, but are not "exhausted" because they are able to respond to CD40 signaling by producing more IL-12 (50, 53). Clearly, our understanding of the immunobiology of DCs is still evolving, and in vitro observations, notwithstanding their critical importance in guiding the development of improved DC-based vaccines, as well as encouraging data from early clinical trials need to be interpreted with caution.

An alternative to optimizing the ex vivo DC maturation process is to circumvent this step altogether and mature the DCs in situ. This can be achieved by injecting antigen-loaded ex vivo–generated immature DCs into sites that have been pretreated with adjuvant to induce a local inflammatory reaction (54, 55). In addition to simulating more closely the maturation process that occurs following infection with a pathogen, in situ maturation dispenses with the need to use reagents that are expensive and often hard to get, especially for clinical applications. For example, my group has shown that immature ex vivo–generated DCs injected into the skin (the ear pinna) of mice pretreated with the adjuvant imiquimod (a TLR7/8 ligand) showed lymph node migration and CTL and antitumor

immunity induction comparable, if not superior, to that of DCs generated and matured ex vivo. In addition, in cancer patients, in situ–matured, ex vivo–generated DCs acquired lymph node migratory capacity comparable to that of DCs generated and matured ex vivo (54). Clinical trials are currently ongoing to assess the therapeutic efficacy of this simplified method of DC vaccination.

Lastly, an approach that does not pertain directly to the maturation issue but represents a radical departure from the standard vaccination protocols using ex vivo–generated and ex vivo–matured DCs is the use of subcellular vesicles, known as exosomes or dexosomes, derived from antigen-loaded ex vivo–generated DCs (56). In mice, injection of exosomes derived from antigen-loaded immature DCs has been shown to stimulate protective antitumor immunity (57). One concern with using exosomes is that because they are derived from immature DCs (as opposed to mature DCs) they might induce tolerance instead of immunity if they are not subjected to additional manipulation. Apart from this concern and the practical problems of using exosomes, these vesicles might hide an important biological phenomenon — exosomes might be a (or the) conduit of antigens in instances in which antigen-capturing DCs transfer antigen to lymph node–resident DCs for presentation to cognate T cells (58). Of note, in a recent publication, Kovar et al. have described a different type of vesicle, derived from mature DCs by sonication, that seems to be more potent than exosomes in stimulating immune responses (59). The bottom line is that strategies such as using in situ–matured, ex vivo–generated DCs or subcellular vesicles derived from ex vivo–generated DCs represent fertile avenues for further exploration that could improve the potency of the DC vaccination approach and simplify this otherwise complex protocol.

## Loading DCs with tumor antigens

Which tumor antigen we vaccinate against and how the antigen is presented to the immune system are also critical components of an effective cancer vaccination strategy. The form of antigen used to load the DCs is one of the most important things to consider when designing cancer vaccination strategies because this has a big impact on the ability of the antigen to access the MHC class I and MHC class II presentation pathways and thereby to induce $CD8^+$ and $CD4^+$ T cell responses, respectively (Figure 2). However, there are other things that need to be considered, such as how much antigen to load, the efficiency of loading, the length of time the antigen will persist and be presented, and the potential adverse effects of the loading technique.

*The form of the antigen loaded onto DCs*. The antigen(s) to be presented by the DCs can be provided in many forms. First, it can be added exogenously, as peptides, whole protein, tumor lysate, or apoptotic debris or complexed with antibody. Second, the DCs can be engineered to synthesize it endogenously by transfection with mRNA or cDNA encoding the antigen. Exogenous provision of short peptides corresponding to the epitopes presented by the MHC class I and MHC class II molecules used to be the favorite form of antigen to load DCs (8, 9). Such peptides are synthesized by chemical means and are readily available for clinical use. However, the logistical advantages of using peptides is offset by the need to determine the MHC haplotype of the patient (that is, the set of MHC alleles an individual expresses, which determines the MHC molecules expressed and is important because different MHC molecules bind and present different peptide repertoires), the paucity of known tumor-specific peptides (especially MHC class II restricted peptides), and the limited persistence of peptide-MHC complexes on DCs. I favor the use of whole antigen because it is likely to contain peptides that can be effectively presented by most MHC molecules (8, 9, 60, 61). This is supported by the observations that CTL responses specific for the tumor antigens carcinoembryonic antigen (CEA), prostate specific antigen (PSA), and telomerase reverse transcriptase (TERT) were stimulated in vitro and induced in vaccinated patients independently of MHC haplotype in over 200 PBMC samples and patients, respectively (refs. 62–66, and our unpublished observations). Use of protein-based antigens to load DCs, which allows peptides to be channeled into both the MHC class I and MHC class II presentation pathways, is limited by access to clinical grade reagents. A "marriage made in heaven" that lies somewhere between the peptide and protein approach is to use overlapping long (20–25 aa) peptides covering most, but not necessarily all, of the coding sequence of the tumor antigen. This approach should provide both MHC class I and MHC class II epitopes, does not require knowledge of an individual's MHC haplotype, and seems to be highly effective (67). Generating clinical grade libraries of overlapping peptides is more feasible than generating clinical grade proteins but is not inexpensive, and the regulatory requirements might prevent the use of this approach if each peptide component has to be validated separately.

The use of antigen encoded by nucleic acid, either cDNA or mRNA, is attractive because their isolation and use in clinical settings is more straightforward than the use of exogenously provided peptides and proteins. However, transfection of DCs with cDNA encoding antigen has not proven effective for loading DCs with the antigen (68). By contrast, transfection of DCs with mRNA that encodes antigen has turned out to be an efficient method of loading DCs with antigen; in mice, vaccination with mRNA-transfected DCs stimulated robust CTL responses and antitumor immunity, and in phase I/II prostate cancer and renal cancer clinical trials, vaccination with mRNA-transfected DCs induced tumor antigen–specific $CD8^+$ T cell responses in the majority of patients (61). One main drawback of transfection as an approach to expressing tumor-specific antigens in DCs is that antigen is channeled primarily into the MHC class I presentation pathway, limiting the generation of effective $CD4^+$ T cell responses (Figure 2) (69). One approach to rechannel cytoplasmic antigens into the MHC class II presentation pathway is to engineer the antigen such that is has a lysosomal targeting signal fused to its carboxyl end (70). Transfection of DCs with mRNA encoding an antigen modified in this fashion has been shown to enhance $CD4^+$ T cell stimulation in vitro (62) and, compared with nonmodified antigen, augmented the induction of $CD4^+$ T cell responses in vaccinated patients, albeit to a limited extent (66). In view of the important role of $CD4^+$ T cell responses in antitumor immunity (71), additional means of augmenting MHC class II presentation of nucleic acid–encoded tumor antigens need to be developed, such as fusing the antigens to products that traffic to the endosomal compartment.

Enhancing MHC class I antigen presentation could also benefit from further improvement. When loading DCs with exogenous protein-based antigen (which enter the MHC class II antigen presentation pathway), methods and agents that disrupt endosomes improve access of the antigen to the cytoplasm and thereby enhance MHC class I presentation (62). In addition, fusing antigens to peptide translocation domains derived from proteins that have evolved to escape the endosomal compartment and to enter the cytoplasm can enhance MHC class I presentation (72). When

using nucleic acid–based antigen, the considerations are different because the tumor antigens generated by these approaches are already considered by the DCs as endogenous antigens. Unlike MHC class II epitopes, which originate from full-length translation products, MHC class I epitopes are derived from unstable polypeptides, most probably representing aborted translation products (known as defective ribosomal products [DRiPs]) (73). Therefore, approaches designed to destabilize the antigen, for example fusing the antigen to ubiquitin, can augment MHC class I antigen presentation (72). However, such attempts have not always been successful, probably because the fine rules of ubiquitin-mediated protein destabilization in mammals were not appreciated until recently (74).

*Antigen quantity, persistence, and timing.* Several studies (refs. 62, 75, and our unpublished observations) have shown that, at least in some cases, there is a discrepancy between the amount of antigen expressed by the DC or processed for MHC class I presentation and the stimulatory capacity of the DC in vivo; undetectable to low levels of antigen can stimulate effective CTL responses, and increasing the amount of antigen expressed by the DC does not seem to improve their immunostimulatory capacity, at least as measured in short term in vitro stimulation assays. These findings are indicative of the high sensitivity of the immune system in detecting antigen at very low concentrations and the ability of the immune system to respond to antigen at low concentrations that our measurements can barely detect.

Therefore, in this instance, more is not necessarily better, underscoring the importance of using relevant functional end points and suggesting that optimizing antigen loading of DCs is not necessarily time well spent. Furthermore, a high density of peptide-MHC complexes on the APC surface translates into enhanced TCR signaling, which in turn increases the propensity of the high avidity T cells to undergo activation-induced cell death (76–80), in effect selecting for low-avidity T cells that are less effective at conferring protective immunity (76, 81, 82). Therefore, when antigen loading of DCs, which is proportional to the density of the peptide-MHC complexes detected on the cell surface, is increased above a certain threshold, protective immunity in the patient might be diminished.

The preoccupation with enhancing antigen loading of DCs has obscured the fact that persistence of antigen presentation in the ex vivo–loaded DC might be a more critical parameter determining DC immunogenicity. It takes at least several hours for the injected DCs to reach the lymph nodes, and even then, continued presentation of antigen is necessary for inducing an effective antitumor response (83, 84). Since turnover of peptide-MHC complexes is slowed but not abolished upon DC maturation, especially for peptide-MHC class I complexes (85), the density of peptide-MHC complexes can be substantially reduced before the ex vivo antigen–loaded DCs reach the lymph node. Indeed, several studies have demonstrated a correlation between antigen persistence in the DC and magnitude of the immune response (28, 29, 86). In this regard, use of nucleic acid–encoded antigens offers the advantage of providing a supply of antigen over an extended period of time.

Another as yet unresolved and potentially important issue is whether to load DCs with antigen when they are immature or following maturation. The latter would be desirable from a logistical standpoint. However, to the extent that antigen loading is mediated by DC-specific antigen capture pathways, and since such pathways and/or the antigen-processing pathways are, by and large, downregulated in mature DCs, antigen loading of immature DCs followed by maturation should be more effective. However, the published data on this issue are conflicting. Several studies from my laboratory (62, 87) have suggested that loading immature DCs is superior to loading mature DCs, as measured by the ability of the DCs to stimulate T cell responses in vitro, but a more recent study from another group has concluded otherwise (88). Notwithstanding the need to resolve the discrepancy between these opposing observations, all three studies suffer from the deficiency that DC function was assessed by measuring the ability of the DCs to induce $CD8^+$ CTL responses, and their impact on the induction of $CD4^+$ T cell responses was not tested.

*The quintessential question: which antigens?* Much effort has been directed toward identifying potent tumor antigens that induce an effective antitumor response (89), but this a topic beyond the scope of this Review. The effectiveness of a cancer vaccine is the product of the potency of the antigen and the method of vaccination. I am leaning toward the view that the current limiting factor in developing cancer vaccines is more the latter. One debated issue is whether to vaccinate individuals with broadly expressed, well defined antigens (usually self-antigens) or with antigenic mixtures, namely the total unfractionated antigenic content of tumor cells. It has been argued, supported by some evidence from preclinical models, that the latter represents a more potent form of antigen because it contains the tumor-specific immunodominant antigens (89). Isolating antigens from autologous tumor adds another layer of unwelcome complexity in the design of a cancer vaccine, and it is not clear whether the differences seen in the preclinical models, of increased potency when vaccinating with antigenic mixtures compared with single, defined antigens, will translate to a substantially enhanced clinical response in patients. It is possible, although it remains to be tested, that the additive effect of combining several weak antigens could match the potency of tumor-derived antigenic mixtures. The demonstration that antigens such as TERT and survivin are expressed in virtually every type of cancer, making them "universal" antigens (63, 90), and that products expressed in the normal constituents of the tumor stroma, such as fibroblasts, endothelial cells, and macrophages, are capable of stimulating antitumor immunity (see ref. 5) further argues for adopting this more straightforward approach of using defined antigens.

## Clinical trials with DC-based vaccines

Multiple clinical trials have been carried out to date targeting different cancers using different methods of generating DCs, different antigens, and different antigen-loading techniques (91). At this early stage of clinical development, no indication or evidence has been obtained that DC vaccines represent a method of stimulating protective immunity in cancer patients that is superior to other vaccination strategies. In most studies, a fraction of patients, often half or less, exhibited immune responses against the vaccinating antigen (92). Despite occasional correlations between immunological and clinical responses in such single-arm clinical trials (see for example ref. 93), we don't know whether the modest clinical responses were caused by the vaccination or whether they reflect patients with better prognoses capable of mounting immune responses. The field of DC vaccines suffered a serious setback when a phase III clinical trial in patients with stage IV melanoma failed to demonstrate that DC vaccination provided increased benefit compared with standard DTIC chemotherapy (42). This was especially disappointing because the bar was set very low — melanoma is thought to be highly responsive

to immunotherapy, and the clinical impact of DTIC treatment is minimal at best. Nevertheless, the overall response was low in both patient groups (DTIC, 5.5%; DC vaccinated, 3.8%); if anything, a statistically nonsignificant trend suggested that the DC-vaccinated individuals were worse off (42). The study was therefore discontinued. This did not help an already skeptical audience of oncologists and immunologists wary of cancer immunotherapy and the complicated DC vaccination strategy touted by many of us developing DC-based vaccines. Why did this clinical trial fail? Is the concept and promise of vaccination with ex vivo–generated DCs misplaced? I don't believe so. It is conceivable that the suboptimal nature of the cytokine cocktail maturation protocol discussed above might have had an important role in the failure of this trial. In addition, it is important to appreciate the fact that a DC vaccination protocol is a complex, multi-step process and that a myriad of seemingly trivial steps such as how the cells are frozen and thawed, how long the cells are matured, at what speed they are centrifuged, the mechanics of their administration, and the time intervals between boostings can have a critical impact on the outcome of the treatment. A failure can be the foundation of success if we make a good faith effort to learn from it through discussions in open forums rather than sweeping it under the rug.

The short history of clinical DC vaccines also has a success story to tell. As mentioned above, Andrieu and colleagues showed, first in rhesus macaques infected with SIV (48) and subsequently in patients chronically infected with HIV (49), that DC vaccination induced robust T cell responses in most vaccinees and that this correlated with marked reduction in viral titers. It is puzzling why the field has essentially shrugged these studies off and ignored an opportunity to counter the negative publicity of the above-mentioned phase III clinical trial and to learn from it. Another example offering a glimpse at what could be the promise of DC vaccines is the clinical experience of Vieweg and colleagues, in which patients with prostate cancer vaccinated with DCs transfected with mRNA encoding tumor antigens such as PSA or TERT and patients with renal cancer vaccinated with DCs transfected with unfractionated tumor-derived mRNA developed tumor antigen–specific $CD8^+$ T cell responses (65, 66, 94, 95). The hallmark of the early clinical experience from this group is that virtually all vaccinated patients responded immunologically with the induction of measurable T cell responses. Furthermore, clinically related responses, such as reduction in PSA levels, were often seen in the vaccinated patients in the prostate cancer trials (65, 95). As a harbinger of things to come, in a recent phase I/II clinical trial, Dannull et al. were able to show that partial removal of Tregs can further potentiate DC vaccine–induced immune responses in cancer patients (95).

How can DC vaccines be improved? Improving the maturation protocol is, in my opinion, a central challenge, and suggestions as to what can be done are discussed above. Above I also allude to the need to pay attention to more "trivial" matters that could have a critical impact on the effectiveness of DC vaccines. One such example is the question, How are DCs delivered? DCs are usually injected as a bolus of suspension cells into one or several sites, usually subcutaneously or intradermally. The result is that most DCs sense other DCs in the vaccination mixture and don't contact the microenvironment, which would provide the necessary cues for their optimal differentiation. Therefore, most DCs are probably lost, from a therapeutic standpoint. This is perhaps the reason why so few DCs from the vaccination mixture migrate to the lymph node (54, 96). Administering DCs in a manner that results in better dispersal and contact with the microenvironment, for example by using multineedle devices, could make a huge difference. Another supposedly trivial question is, What is the optimal boosting frequency? The current consensus is that longer time intervals between boostings are better, and protocols vary among weekly, biweekly, and monthly intervals. However, recent mouse studies suggest that more frequent vaccinations, provided excessive inflammation is avoided, are superior, especially in generating a long-lasting response (83, 84, 97).

## Food for thought

I conclude this article with two questions, questions which, if we (meaning the DC vaccine diehards) don't ask, others will.

*Are DCs the ultimate professional APC to use in the setting of cancer vaccination with ex vivo–derived APCs?* What about monocytes, B cells, or γδ T cells? A recent study has provided provocative evidence that monocytes loaded with antigens can do just about everything that DCs do — migrate to the lymph node and stimulate potent T cell immunity (98). Even more intriguing are B cells. An increasing number of studies have shown that B cells loaded with antigens can stimulate robust T cell response in vitro and, more tellingly, in vivo in mice (99). A key advantage that B cell–based APCs have over DCs and monocytes is that B cells can be easily expanded in vitro in the presence of CD40L. Human γδ T cells have also been shown to function as potent APCs in vitro (100). Notwithstanding the need to demonstrate their APC function in small animal tumor models, γδ T cells are an attractive alternative to DCs because they can home to lymph nodes and are easily expanded ex vivo.

*Vaccination with ex vivo–generated DCs: is it worth the trouble?* The underlying premise of DC vaccination is that the added complexity and cost associated with this customized form of cell therapy will be offset by the substantial added benefit to the patient. Clearly we are not there yet. How long will it take to get there? And how critical is it, anyway — how important is it, and how much effort should we devote to developing increasingly effective means of stimulating an immune response in cancer patients using DC-based vaccination or other approaches? The goal of cancer immunotherapy, after all, is not to induce antitumor immunity but to engender protective antitumor immunity. This is not semantics. Protective antitumor immunity is not only a function of how many tumor-specific effector T cells we can generate but also of how long they will persist and how well they can overcome tumor-induced immunosuppression. It is beyond the scope of this Review, but the more I think about it, in the overall scheme of engendering protective antitumor immunity, inducing immunity per se becomes less and less important. Which raises the question of whether the development of complex forms of customized cell therapy such as DC vaccines are worth the trouble. Perhaps new strategies that target antigens to DCs in vivo, accompanied by means of specifically activating the targeted DCs, will replace the need for using complex patient-specific approaches. Alternatively, perhaps existing vaccination strategies are sufficient and our focus needs to be redirected.

On a final note, the goal of cancer vaccination, whether using ex vivo–generated DCs or other protocols, is to generate a pool of tumor antigen–specific activated immune cells. This, however, might not be sufficient to contain tumor growth. Additional treatments need to be developed and combined with vaccination to enhance the persistence of the vaccine-induced immune responses. For example, one approach would be to enhance T cell

costimulation either by administering agonistic antibodies specific for 4-1BB or OX40 or by administering antagonistic antibodies specific for coinhibitory receptors such as CTLA4 or PD-1 (101). Another approach, to potentiate the immunogenicity of ex vivo–generated DCs would be to transduce the DCs with ligands for costimulatory receptors such as OX40 (27) or use RNAi technology to block the immune response–attenuating functions of molecules such as SOCS1 (23). Equally important would be to develop interventions that counter the propensity of tumors to evade immune elimination, such as removing Tregs or immunizing against the tumor stroma (5, 102–104).

Address correspondence to: Eli Gilboa, Miller School of Medicine, 1550 NW 10th Avenue Medical Campus, Papanicolau Building 211, University of Miami, Miami, Florida 33136, USA. Phone: (305) 243-1767; Fax: (305) 243-4409; E-mail: egilboa@med.miami.edu.

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