

## Review Article

# Vaccines against Human Carcinomas: Strategies to Improve Antitumor Immune Responses

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Multiple observations in preclinical and clinical studies support a role for the immune system in controlling tumor growth and progression. Various components of the innate and adaptive immune response are able to mediate tumor cell destruction; however, certain immune cell populations can also induce a protumor environment that favors tumor growth and the development of metastasis. Moreover, tumor cells themselves are equipped with various mechanisms that allow them to evade surveillance by the immune system. The goal of cancer vaccines is to induce a tumor-specific immune response that ultimately will reduce tumor burden by tipping the balance from a protumor to an antitumor immune environment. This review discusses common mechanisms that govern immune cell activation and tumor immune escape, and some of the current strategies employed in the field of cancer vaccines aimed at enhancing activation of tumor-specific T-cells with concurrent reduction of immunosuppression.

## 1. Introduction

The role of the immune system in limiting tumor growth, designated as cancer immunosurveillance [1], has been first elucidated in mouse models of immune deficiency characterized by a high incidence of spontaneous and chemically induced tumors [2]. Those studies have identified several components of the innate and adaptive immune response being responsible for tumor elimination, including  $\alpha\beta$  and  $\gamma\delta$  T-cells [3], and NK cells [4]. Reinforcing the role of cytotoxic T lymphocytes (CTLs) in the eradication of malignant cells, transgenic mice deficient in perforin, a component of the cytolytic granules of T and NK cells, are more susceptible to spontaneous and chemically induced tumors than their wild type counterparts [5]. In humans, evidence on the role of the immune system in limiting tumor growth and progression is linked to observations indicating a positive correlation between the presence of tumor infiltrating CD8+ T-cells and good prognosis in various types of cancer. In colorectal cancer, for example, significantly higher levels of early memory and effector memory CD8+ T-cell infiltrates positively correlate with good clinical outcome, defined as

absence of metastatic invasion, less advanced pathological stage, and increased survival [6, 7]. Similarly, the presence of intraepithelial tumor infiltrating CD8+ T-cells has been associated with the lack of tumor metastases in the draining lymph nodes of cervical cancer patients [8]. In non-small cell lung carcinoma patients, increasing numbers of tumor infiltrating CD8+, CD20+, and CD4+ T lymphocytes have also been shown to significantly correlate with improved disease-specific survival [9]. Altogether, these observations support a role for the immune system in controlling tumor burden and form the rationale for the development of vaccine-based interventions against cancer that rely on the stimulation of an effective antitumor immune response in the host.

The immune system, however, has two paradoxical roles in cancer. While various components of the innate and adaptive immune response are able to mediate tumor cell destruction, specific types of immune cells can also induce a protumor environment that favors tumor growth and the development of metastasis [10]. Among the latter are, for example, regulatory T (Treg) cells [11, 12], tumor associated macrophages (TAM) [13, 14], and type 2 helper CD4+ (Th2)

T-cells [15, 16]. These various immune cells have been shown to accumulate at the site of the tumor, negatively impacting the establishment of antitumor T-cell responses, that is, creating an immunosuppressive tumor environment.

Cancer cells themselves are also equipped with mechanisms that allow them to evade recognition by the immune system or to negatively affect the functionality of effector T-cells. In order to avoid immune recognition, tumor cells have been shown to downregulate antigen expression, components of the antigen-processing and presentation machinery, and expression of major histocompatibility complex (MHC) molecules [17]. Decreased expression of costimulatory molecules of crucial importance to T-cell activation, and enhanced surface expression of molecules that negatively regulate activation of T-cells, such as PD-L1/B7-H1 and B7-H4, have also been demonstrated in various types of tumors [18–20]. Cancer cells can also restrain the function of the immune system by secreting a milieu of soluble factors that ultimately inhibit the activation, proliferation, and differentiation of the various components of the immune response. Among these molecules are TGF- $\beta$  [21], IL-10 [22], IL-13 [23], and VEGF [24].

The goal of vaccine-based cancer immunotherapy approaches is to induce a tumor-specific immune response that ultimately will reduce tumor burden by tipping the balance from a protumor to an antitumor immune environment (Figure 1). This review discusses strategies employed in the field of cancer vaccines aimed at enhancing activation of tumor-specific T-cells with concurrent reduction of immunosuppression. Specifically, vaccine design, immune adjuvants, and multimodal approaches using vaccines in combination with other treatment modalities will be discussed here, with a particular emphasis on studies conducted at the National Cancer Institute, NIH.

## 2. Vaccine Design: Choice of Vaccine-Delivery System(s)

Depending on the vaccine-delivery system of choice, cancer vaccines can elicit an immune response against an individual or multiple tumor antigens. A list of the various types of vaccine-delivery systems under investigation in the field, either at the preclinical or clinical stages, is presented in Table 1. Multiple studies have demonstrated that combinations of some of the strategies in the form of diversified prime/boost regimens may enhance the outcome of the intervention against the tumor [25]. Moreover, it has also been shown that concurrent vaccination with two distinct vaccine platforms targeting the same antigen can elicit a more diverse population of antigen-specific T-cells thus resulting in higher antitumor immunity [26].

Among the various types of vaccine delivery systems, there are strategies based on the use of whole tumor cells, dendritic cells-(DCs-) tumor cell fusions, or preparations of DCs loaded with tumor protein lysates or tumor-derived RNA (Table 1, left column). These vaccine platforms induce an immune response against multiple tumor targets, either known or unknown. Other vaccine modalities (Table 1, right

TABLE 1: Vaccine-delivery systems.

Vaccine-delivery systems	
Immunization against multiple antigens*	Immunization against specified antigen(s)
<b>Cell-based</b>	<b>Cell-based</b>
Autologous whole-tumor cells	DCs pulsed with peptide
Allogeneic whole-tumor cells	Genetically-modified DCs
Genetically-modified tumor cells	
DCs-tumor fusion	<b>Protein/Peptide based</b>
DCs loaded with tumor lysate	Protein
DCs transfected with tumor-derived RNA	Peptide
	Agonist peptide
	Antidiotype MAb
	Mab fusion proteins
<b>Protein/Peptide based</b>	
Tumor lysates	<b>Vector-based</b>
Heat shock proteins-tumor peptides	<i>Plasmid DNA</i>
	<i>Bacterial vectors</i>
	Listeria
	Salmonella
	<i>Yeast vectors</i>
	<i>Viral vectors</i>
	Adenovirus
	Vaccinia
	Avipox (fowlpox)
	MVA

\*Vaccine formulation includes known and unknown antigens.

column) are based on the previous characterization of tumor antigens to be used as “specified” targets in the vaccine formulation.

## 3. Tumor Antigens

Tumor antigens are molecules either exclusively expressed in the tumor cells, designated as “tumor-specific antigens”, or molecules that are overexpressed in cancerous versus normal tissues, designated as “tumor-associated antigens”. A comprehensive list of tumor antigens and their corresponding T-cell epitopes can be found at [27, 28]. Table 2 shows a brief list of selected examples for either type of antigen. Tumor-specific antigens appear *de novo* after cancer cells acquire mutations within the coding regions of certain genes, for example the oncogene *ras* [29] and the tumor suppressor *p53* [30], or novel fusion proteins are generated as in the case of the Bcr-Abl fusion in chronic myeloid leukemia [31]. Moreover, in tumors driven by infectious agents like human papillomavirus (HPV) or Epstein-Barr virus (EBV), virally-derived products become *de novo* targets of T-cell immune responses directed against the tumor. The majority of tumor-associated antigens so far identified have a certain level of expression in normal tissues and thus tolerance to these antigens often exists.

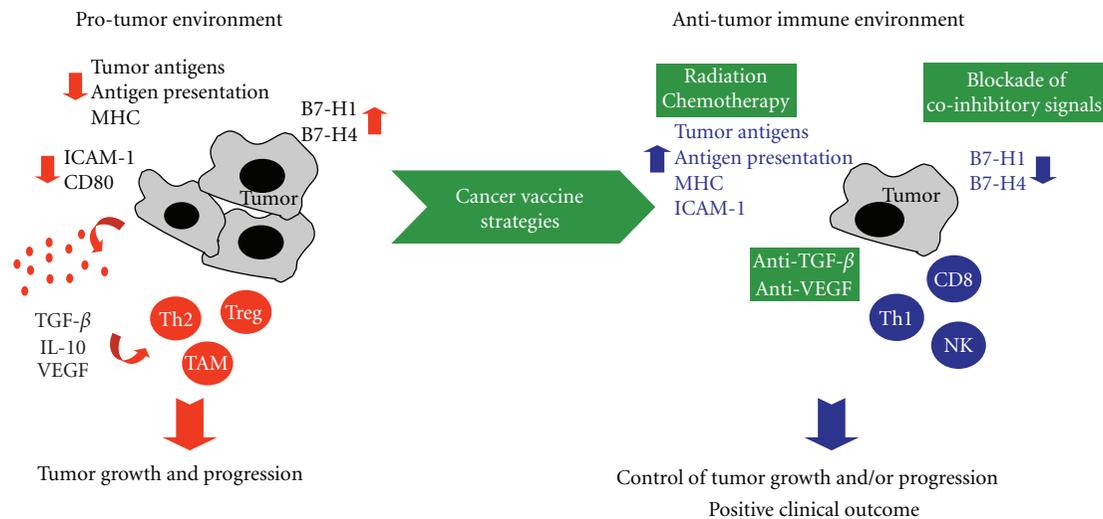


FIGURE 1: Cancer vaccine strategies aimed at shifting the immune environment of a tumor from protumorigenic to antitumorigenic.

A strategy used by tumors to escape immune recognition and destruction is the complete or partial loss of an antigen(s) [17]. Both in experimental animal models [32, 33], and in human cancer [34], it has been shown that “antigen-negative tumor variants”, characterized by the loss of the targeted antigen, can emerge subsequently to an immune intervention. An approach to overcome this problem is the targeting of “functionally relevant antigens”, which are proteins with an essential role during tumor initiation, growth, survival, or metastasis [35]. It can be hypothesized that an immune intervention directed against a functioning tumor antigen would greatly reduce the emergence of antigen-negative variants, since cells that lose the antigen will fail to grow, survive, or metastasize.

#### 4. Targeting of Molecules that Control Metastatic Dissemination

In order to achieve the various steps along the metastatic cascade, epithelial tumor cells may need to undergo a phenotypic conversion into mesenchymal cells via a process designated as epithelial-to-mesenchymal transition (EMT) [36]. The EMT program involves the downregulation of epithelial proteins such as E-cadherin and cytokeratins, and the upregulation of mesenchymal proteins including Fibronectin, N-cadherin, and Vimentin. Various genes normally expressed in the early embryo have been implicated in the control of the EMT triggered during tumor progression, including *Twist*, *Snail*, *Slug*, *Gooseoid*, and *SIP1* [36, 37]. The transcription factors encoded by these genes can impart to tumor cells the traits of mesenchymal cells, including motility and the ability to invade the extracellular matrix (ECM). The expression of *Twist*, for example, has been found to be elevated in various types of cancer, including breast, prostate, and cervical cancer, with higher levels of *Twist* protein being detected in prostate cancer tissues of high Gleason score [38, 39]. Since the EMT process

TABLE 2: Human carcinoma antigens.

Carcinoma antigens*	
Tumor-specific antigens	Tumor-associated antigens
<b>Mutated molecules</b>	Carcinoembryonic antigen (CEA)
K-RAS	Mucin 1 (MUC-1)
p53	Prostate-specific antigen (PSA)
<b>Fusion molecules</b>	Prostate acid phosphatase (PAP)
BCR-ABL	Prostate stem-cell antigen (PSCA)
	Brachyury
<b>Virally-derived molecules</b>	TERT
HPV-16 E6, E7	Wilm's tumor 1 (WT1)
EBNA1, LMP1 and LMP2	Her-2/neu
	Sox-2
	NY-ESO-1
	Cyclin D1
	Mesothelin
	Survivin

\*Included is only a partial list of antigens for human carcinomas.

appears to be a necessary step for tumor cells to initiate the metastatic cascade [37, 40], interfering with EMT in early stages of the disease is likely to prevent tumor cell spreading and might also be effective in treating established metastatic lesions (Figure 2). An example of a tumor antigen with a functionally relevant role in the EMT program is the T-box transcription factor Brachyury, highly expressed in various human tumors of epithelial origin, but not in most human normal adult tissues [41]. It has been recently demonstrated that Brachyury overexpression in epithelial tumor cells induces an EMT, promoting the expression of mesenchymal markers and downregulation of epithelial markers, with concomitant increase in tumor cell migration and invasion [42]. Additionally, stable silencing of Brachyury expression in Brachyury-positive human carcinoma cells has been shown to downregulate mesenchymal markers

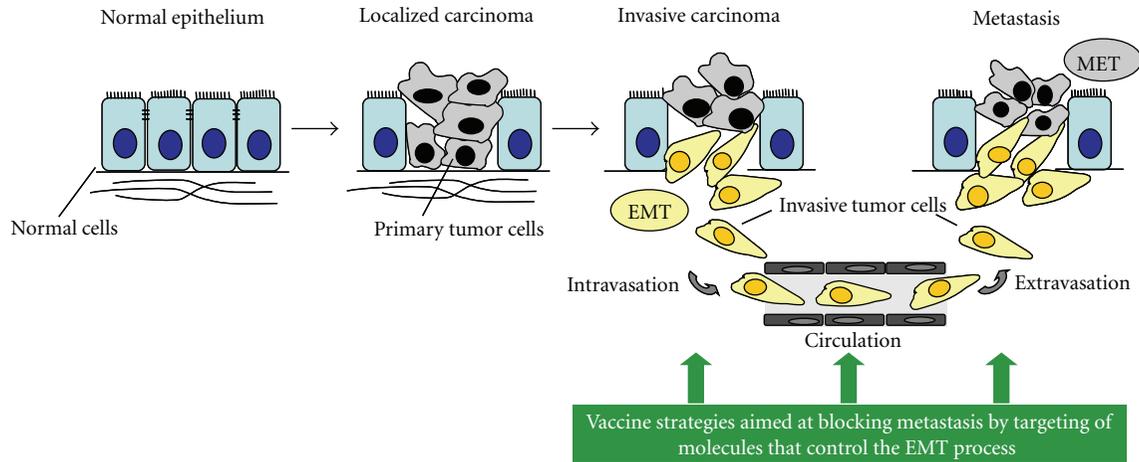


FIGURE 2: The epithelial-to-mesenchymal transition (EMT) in tumor progression: an opportunity to target metastatic tumor cells. The epithelial-to-mesenchymal transition (EMT) and its reverse process, designated as mesenchymal-to-epithelial transition (MET), are involved in the progression of epithelial tumors towards metastasis. Vaccine strategies targeting molecules that control the EMT process, for example, the transcription factor Brachyury, could be used to block tumor spreading.

and upregulate epithelial markers with simultaneous loss of cell migration and ECM invasion. In vivo, Brachyury-inhibited human tumor cells had a decreased ability to form experimental lung metastases after intravenous injection, as well as to disseminate from the primary, subcutaneous tumor to the site of metastases [42]. A CD8 T-cell epitope of Brachyury capable of expanding Brachyury-specific T-cells from the peripheral blood of cancer patients was recently identified [41]; Brachyury-specific T-cells have been used to efficiently lyse Brachyury-positive tumor cells in vitro. The successful expansion of T-cells directed against the transcription factor Brachyury exemplifies the ability of T-cell mediated immunotherapies to target (a) highly conserved tumor proteins, and (b) tumor proteins irrespective of their cellular localization. Because of its relevant role during tumor progression, Brachyury is an appealing tumor antigen for interventions aimed at interfering with the metastatic spreading of tumor cells (Figure 2). Additionally, the transcription factors Twist, Snail, and Slug, among others, which are critically involved in the control of EMT during tumor progression, could also be explored as novel tumor antigens for the targeting of metastatic disease.

## 5. Antigen Cascade

A phenomenon observed with cancer vaccines is the induction of immune responses to tumor antigens that are not present in the vaccine formulation. For example, it has been shown that CEA-transgenic mice cured of CEA-positive tumors by a CEA/TRICOM vaccine regimen were able to subsequently reject CEA-positive as well as CEA-negative tumors, and that this effect was mediated by the generation of specific T-cell immune responses directed against gp70, an antigen expressed by the tumor but not present in the vaccine [43]. The same phenomenon has also been reported in clinical studies [44, 45]. Altogether these studies showed that the immune response initiated against a tumor antigen included

in the vaccine formulation is then followed by cross-priming and initiation of an “antigen cascade” that expands the immune response to additional antigens expressed on the tumor, thus potentiating antitumor immunity.

## 6. Enhancing Activation of Tumor-Specific T-Cells: The Use of Costimulation in the Vaccine Formulation

Optimal activation of T-cells is known to require at least two signals. The first signal is mediated by the interaction between the peptide/MHC complex on the surface of antigen presenting cells (APC) and the T-cell receptor (TCR) on the surface of T-cells. The second signal, also designated as costimulation, is mediated by the interaction between accessory molecules located on the surface of the APC and their corresponding ligand(s) on the T-cells [46]. Activation via the TCR in the presence of adequate costimulatory signals results in the clonal expansion, differentiation, and expression of effector functions by antigen-specific T-cells. Optimization of the mechanism of T-cell activation is critical to achieve a successful immune response to an antigen included in a vaccine formulation. A list of various strategies being explored in the field of cancer vaccines to achieve enhanced antitumor immune responses is presented in Table 3. One of them is the delivery of a single or multiple costimulatory molecules along with the tumor antigen as part of the vaccine formulation. One of the most studied T-cell costimulatory molecules is B7-1 [47, 48]. In preclinical studies it has been shown that mice immunized with an admixture of recombinant vaccinia (rV-) viruses encoding for a tumor antigen (rV-CEA or rV-MUC-1) and the costimulatory molecule B7-1 (rV-B7-1) generate effective antigen-specific T-cell immune responses that translate into successful antitumor immunity [49]. Furthermore, it was demonstrated that combinations of various costimulatory

molecules act synergistically to further enhance antigen-specific T-cell responses. Using recombinant vaccinia and fowlpox viruses encoding for the tumor antigen CEA and three different costimulatory molecules (B7-1, ICAM-1, LFA-3, designated as TRICOM), T-cell responses against the tumor antigen were further enhanced above the level observed when only one costimulatory molecule was used [50]. In preclinical studies with human T-cells in vitro, enhanced activation of antigen-specific T-cells was observed against DCs modified by infection with rF-TRICOM [51]. Moreover, the same vector was successfully used to enhance the antigen-presentation potency of freshly isolated B cells, resulting in enhanced activation of antigen-specific T-cell responses in vitro [52]. Results from a Phase II randomized clinical trial in 125 metastatic prostate cancer patients were recently reported [53]; patients were randomized to receive either a vaccine regimen consisting of two recombinant viral vectors each encoding for prostate specific antigen (PSA) and the TRICOM molecules (rV-PSA/TRICOM and rF-PSA/TRICOM), or control empty vector (control arm). The results from this trial demonstrated a 44% reduction in the death rate and an 8.5 month improvement in median overall survival (OS) in men in the vaccine compared to the control arm [53]. Additionally, NCI also reported the results from a randomized Phase II trial in 32 patients with metastatic castrate-resistant prostate cancer, who received a prime with rV-PSA/TRICOM and booster vaccinations with rF-PSA/TRICOM. Twelve of 32 patients showed declines in serum PSA post-vaccination; patients with greater PSA-specific T-cell responses showed a trend toward enhanced survival. In general, there was evidence of enhanced median overall survival, particularly among patients with more indolent type of disease [54]. Overall, cancer vaccine strategies incorporating costimulatory molecules have demonstrated the generation of antitumor immunity and evidence of clinical benefit in cancer patients.

## 7. The Use of Cytokines as Vaccine Adjuvants

**7.1. Cytokines that Affect the APCs.** Biological adjuvants are agents generally used for improving the immunogenicity of an antigen in a vaccine formulation. Several cytokines have the ability to enhance immune responses by either (a) promoting the differentiation, activation, or recruitment of APC, therefore enhancing antigen presentation and activation of antigen-specific T-cell responses, or (b) by directly acting on various subsets of T-cells. Within the first group, one of the most studied cytokines is the granulocyte-macrophage colony-stimulating factor (GM-CSF). It has been demonstrated in preclinical studies that subcutaneous injections of GM-CSF at the vaccination site can significantly increase the infiltration of DCs in regional lymph nodes that drain the site of vaccination [55, 56]. In several preclinical studies, tumor cells or DCs genetically engineered to secrete biologically active GM-CSF have been used to generate a systemic antitumor immune response [57, 58]. The use of GM-CSF at high doses, however, could be detrimental in the context of vaccines since it may result

TABLE 3: Strategies to enhance antitumor T-cell responses\*.

Strategy
Use of costimulation in vaccine formulation
Cytokines
Effect on APC (GM-CSF)
Effect on T-cells (IL-2, IL-7, IL-15, IL-12)
Radiation
Chemotherapy
Small molecule targeted therapies
Inhibition of coinhibitory signals
At the tumor site (B7-H1, B7-H4)
Directly on T-cells (CTLA-4)
Depletion/inhibition of Treg cells
Inhibition of immunosuppressive cytokines
AntiTGF- $\beta$
AntiVEGF

\* Only a partial list is included here.

in immune suppression via the activation and expansion of myeloid-derived suppressor cells [59]. In the clinical setting, two placebo-controlled Phase III trials in patients with hormone-refractory prostate cancer, for example, have been performed with an autologous DC-based vaccine, designated Sipuleucel-T, which is genetically modified to express prostatic acid phosphatase (PAP) as a tumor antigen, and GM-CSF. Results from the initial trial with 127 patients [60] demonstrated a 4.5-month improvement in median survival in the vaccine versus placebo group, though without meeting the primary endpoint of time to progression. Results from a subsequent Phase III, placebo-controlled trial measuring overall survival as the primary endpoint have been recently reported [61], indicating a statistically significant survival advantage in patients in the vaccine versus placebo group.

**7.2. Cytokines that Affect the T-Cell Compartment.** The second group includes cytokines that directly affect the T-cell compartment by promoting T-cell proliferation, activation, and effector function. Among these, the cytokines IL-2 [62, 63], IL-7 [64], IL-15 [65, 66], and IL-12 [67] are currently under investigation to enhance antitumor immune responses elicited by a vaccine. The most used of these cytokines is IL-2, a T-cell growth factor which, as a single agent, has demonstrated clinical responses in patients with metastatic renal cell carcinoma [68] and metastatic melanoma [69]. A disadvantage, however, in the use of IL-2 therapy in vivo, particularly with high-dose IL-2, is the rate of associated toxicities [70]. Moreover, IL-2 mediates not only the proliferation of activated, effector T-cells and NK cells, but also the development and homeostasis of regulatory T-cells (Tregs), which constitutively express elevated levels of the IL-2R alpha (CD25) [71]. In vitro, IL-2 has also been used to expand tumor-specific T-cells to be used for adoptive immunotherapy [72].

The cytokines IL-15 and IL-7 are also T-cell growth factors; their function, however, is different to that of IL-2 in

vivo. IL-15 is necessary for the development and homeostasis of memory CD8 T-cells and NK cells [65]. It has also been demonstrated a role for IL-15 in the induction of long-lived, high avidity CD8 T-cells [73, 74] and, unlike IL-2, there is no role for IL-15 on the proliferation of Tregs. Up to date, studies with IL-15 as an adjuvant for cancer vaccine strategies have only been conducted in preclinical models, with encouraging results [67]. The cytokine IL-7, another T-cell growth factor, targets a different population of T-cells, promoting the expansion of naïve T-cells and thus increasing the diversity of T-cell repertoire after lymphopenia [64, 65]. IL-7 has also demonstrated positive results as an adjuvant cytokine for cancer vaccine interventions in preclinical studies [67].

Another cytokine that is under investigation as a vaccine adjuvant is IL-12, which promotes Th1 polarization, proliferation of activated T-cells and NK cells, and cell-mediated immunity. IL-12 has been shown to have potent antitumor effects in preclinical models [75]. In humans, however, the systemic delivery of IL-12 has resulted in elevated toxicities [76], hence leading towards the investigation of alternative modes for local delivery of IL-12. For example, in preclinical studies, a coformulation of IL-12 with chitosan intravesically delivered was well tolerated and very efficient at curing mice with superficial bladder cancer. A durable antitumor immune response was also generated in mice receiving IL-12/chitosan, providing them with complete protection from intravesical tumor rechallenge [77]. Overall, the use of cytokines as vaccine adjuvants to enhance the immune response to a tumor is a very promising and active field of investigation. Current research is focused on understanding the proper ways of delivery for each particular cytokine in order to maximize the immune adjuvant effects while reducing potential toxicities, when used in combination with various types of vaccine platforms.

## 8. Vaccine Plus Radiation

As it was mentioned above, a mechanism by which tumor cells escape immune recognition and attack is through the downregulation of tumor antigens, MHC expression, or various components of the antigen processing/presentation machinery. A strategy to overcome these obstacles is the use of radiation on tumor cells. Radiation is the standard of care for many types of cancer because of its direct cytotoxic effect on the tumor or its palliative effects on the patient. It has been recently reported that local irradiation of tumors with doses insufficient to induce tumor cell death could result in changes on the phenotype of the tumor cells that include the upregulation of MHC, Fas, ICAM-1, and various tumor associated antigens [78–80]. As a result of these changes, irradiated tumor cells are more susceptible to T-cell mediated immune attack. In preclinical studies with a murine colon carcinoma tumor model, sublethal, local tumor irradiation significantly improved the therapeutic efficacy of a recombinant rV-/rF-CEA/TRICOM vaccine regimen against CEA-positive tumors in CEA-transgenic mice [81], while radiation alone or vaccine alone had no effect on tumor growth. In the clinical setting, the approach

has been investigated in a phase II clinical trial in patients with localized prostate cancer, randomized to receive a PSA-based poxviral vaccine plus radiotherapy versus radiotherapy alone [82]. The results from this trial indicated increases in PSA-specific T-cell responses of at least 3-fold in patients in the combination arm; the authors also reported evidence of de novo generation of T-cells to well-described prostate-associated antigens not found in the vaccine, providing indirect evidence of immune-mediated tumor killing. These studies thus demonstrated a new paradigm for the use of local tumor irradiation in combination with active vaccine therapy to elicit an effective antitumor immune response.

## 9. Vaccines Plus Cytotoxic Drugs

Because of the widespread use of chemotherapy for the treatment of most malignancies, it is rational to design combinatorial approaches using vaccines plus standard chemotherapeutic agents. Like radiotherapy, the use of various types of chemotherapy in combination with vaccines has resulted in enhanced antitumor immune responses. Although the mechanisms involved vary among the various types of cytotoxic drugs employed, in general, drugs can: (a) induce “immunogenic death” of tumor cells, leading to activation of DCs followed by antigen presentation to T-cells [83, 84], or (b) modulate the phenotype of the tumor cells making them more susceptible to immune-mediated killing. For example, it has been shown that treatment of human colon carcinoma cell lines with 5-fluorouracil or cisplatin enhances their lytic sensitivity to antigen-specific CD8+ cytotoxic T lymphocytes, by inducing expression of ICAM-1 and Fas [85]. Similarly, treatment of renal cell carcinoma cells with subtoxic concentrations of adriamycin has been shown to upregulate the expression of ICAM-1 and LFA-3, as well as to enhance T-cell mediated killing, Fas-mediated, and TRAIL-mediated killing of tumor cells [86]. Taxanes, on the other hand, are a widely used type of chemotherapeutic agents known to have various effects on the immune system, promoting macrophage activation and release of inflammatory cytokines at the tumor site, thus enhancing tumor lysis [87]. In preclinical studies with CEA-transgenic mice transplanted with CEA-positive tumor cells, enhanced antitumor effect was achieved by using a combination of a rV-/rF-CEA/TRICOM vaccine regimen plus docetaxel, compared to that of vaccine or docetaxel alone [88]. In preclinical models as well, cyclophosphamide, doxorubicin, and paclitaxel have all been shown to enhance the antitumor immune response elicited with a GM-CSF-secreting, HER2/neu-expressing whole-tumor cell vaccine in tumor bearing neu-transgenic mice [89]. The authors were able to demonstrate that the increased antitumor effects in the combination group (vaccine plus drugs) were due to enhanced vaccine efficacy rather than a direct cytolytic effect of the drugs on cancer cells [89].

Altogether, these studies demonstrated that, if used in appropriate schedule and at the correct doses, chemotherapeutic agents could enhance antitumor responses when used in combination with cancer vaccine modalities. Therefore,

further studies investigating optimum schedules and dosing for various chemotherapeutic agents are needed in order to optimize the use of different cytotoxic agents in combination with cancer vaccines.

## 10. Vaccine Plus Small Molecule Targeted Therapies

As the molecular pathways involved in the various steps of carcinogenesis and tumor progression are being elucidated with the advent of sophisticated genetic and molecular techniques, a novel group of therapeutic cancer drugs aimed at inhibiting specific molecular pathways is emerging, designated as small molecule targeted therapies. These drugs are also now being investigated for their immune-modulatory functions to be potentially used in combination with cancer vaccines. For example, the anticancer agent lenalidomide (Revlimid, Celgene Corp., NJ, USA), which is FDA approved for the treatment of patients with multiple myeloma, has been shown to have several immune-modulatory effects that include costimulatory effects on CD3-activated T-cells, augmentation of NK cell cytotoxicity, and suppression of Treg cells proliferation and function [90, 91]. Results from a study combining a small molecule BCL-2 inhibitor and a rV-rF-CEA/TRICOM vaccine regimen were recently reported [92]. It has been shown that, when administered after the vaccine, the BCL-2 inhibitor GX15-070 was able to increase the intratumoral ratio of activated, CD8+ T effector to Treg cells, thus resulting in significant reduction of pulmonary tumor nodules in a mouse model of experimental lung tumors [92]. As with chemotherapeutic agents, the use of small molecule targeted therapies could also be associated with potential toxicities and, in particular, with negative effects on the immune compartment. Thus, further studies investigating optimum schedules and dosing for various small molecule targeted drugs are needed in order to optimize their use in combination with cancer vaccines.

## 11. Inhibition of Coinhibitory Signals

An alternative strategy to enhance the outcome of an antitumor immune approach is to eliminate negative signals imparted to T-cells by coinhibitory molecules such as B7-H1, B7-H4, and cytotoxic T-lymphocyte antigen-4 (CTLA-4), among others. B7-H1 is constitutively expressed in many types of human tumors and has been shown to promote evasion of tumor immunity by promoting apoptosis of activated effector T-cells [18] and tumor resistance to T-cell mediated lysis [93]. In preclinical studies, blockade of B7-H1 with a specific monoclonal antibody has resulted in enhanced antitumor immune responses [93, 94]. B7-H4 is another member of the B7 family that has been implicated as a negative regulator of T-cell immunity [95]. It has been demonstrated that B7-H4 can inhibit T-cell proliferation and IL-2 production, and that blockade of B7-H4 in preclinical animal models results in enhanced cytotoxic T-cell responses against an alloantigen [19]. The expression of B7-H4 has been observed in many types of human cancer, such as breast

[96], ovary [97], and lung [98]. In renal cell carcinoma, its expression has been correlated with more aggressive tumors, particularly in those cases where both B7-H1 and B7-H4 are aberrantly overexpressed [99]. Therefore, blocking of molecules such as B7-H1 and B7-H4 expressed on tumor cells can reduce coinhibitory signals directly at the site of the tumor (Table 3), resulting in enhanced antitumor immune responses.

A different strategy involves blocking of inhibitory molecules directly expressed on T-cells. An example is CTLA-4, a negative regulator of T-cell activation, which is expressed on the T-cells and, like its homolog CD28, binds to B7 on the surface of the APC. Binding of CTLA-4 to B7 initiates a negative signal cascade that leads to downregulation of the T-cell response [100, 101]. The blockade of CTLA-4 with specific, monoclonal antibodies (mAb) has been explored as a monotherapy or in combination with vaccine therapy in preclinical and clinical studies. In preclinical studies, the use of antiCTLA-4 mAb as a monotherapy has shown antitumor activity with immunogenic tumors [100, 101], but not with poorly immunogenic tumors, such as MC38 [102]. In combination with vaccines, antiCTLA-4 mAb has been used to enhance antitumor T-cell responses elicited by the vaccine, thus resulting in effective antitumor effects [102]. In the clinic, antiCTLA-4 has been used also as a monotherapy or in combination with other immune-mediated anticancer modalities [103]. Results from a phase I clinical trial with antiCTLA-4 mAb (ipilimumab) in patients with metastatic melanoma or renal cell cancer recently reported a 14% response rate and multiple, and at times severe, immune-mediated toxicities such as nephritis, panhypophysitis, and enterocolitis, among others [104, 105], this last point constituting a potential disadvantage of the approach.

## 12. Depletion/Inhibition of Regulatory T-Cells

Naturally occurring regulatory T (Treg) cells, characterized by expression of IL-2R alpha, the transcription factor Foxp3, CTLA-4, and glucocorticoid-induced tumor necrosis factor (TNF) receptor (GITR), constitute 5% to 10% of peripheral CD4<sup>+</sup> T-cells [11, 106]. Treg cells represent an important mechanism of peripheral T-cell tolerance through their inhibition of self-reactive effector T-cells [107], and have also been implicated in the lack of effective antitumor immunity as the number of Treg cells are increased in the tumors and peripheral blood of cancer patients [108, 109]. Previous studies in several models of mouse tumors have demonstrated that deletion of Treg cells by using an antiCD25 mAb enhanced the development of antitumor immunity leading to tumor rejection [110, 111]. Furthermore, it has been previously shown that antigen-specific T-cell responses induced by poxviral vaccines can be augmented by simultaneous administration of antiCD25 mAb in mice [112]. Among alternative modalities that can be used in humans to delete Treg cells is denileukin diftotox, (DAB<sub>389</sub>IL-2), a fusion protein of IL-2 and diphtheria toxin, previously shown in mouse preclinical studies to reduce Treg cells and to enhance antigen-specific immune responses induced by poxviral

vaccines [113]. A potential drawback of both approaches is that activated, effector T-cells also transiently upregulate the expression of CD25 on their cell surface, therefore being at risk of depletion by strategies that target CD25. In the clinic, denileukin difitox was previously shown to reduce Treg cells and to lead to objective clinical responses in patients with ovarian cancer [12, 114]. Moreover, antitumor immunotherapy approaches combining denileukin difitox-mediated deletion of Treg cells followed by vaccination with RNA-transfected DCs, tumor antigen peptides, or DCs modified by infection with rF-CEA(6D)/TRICOM, have been shown to improve tumor-specific T-cell responses in patients with renal cell cancer, melanoma, or CEA-positive malignancies, respectively, [115–117].

### 13. Inhibition of Immunosuppressive Cytokines

Tumors can also evade immunosurveillance by directly secreting a number of inhibitory cytokines or by inducing various types of immune cells to secrete cytokines associated with reduced immune responses. One of these cytokines is the transforming growth factor beta (TGF- $\beta$ ), which can be directly secreted by many types of tumor cells, including breast, prostate, colon, liver, lung, and melanomas, among others [118, 119]. TGF- $\beta$  exerts its negative effects on T-cells, NK cells, macrophages and DCs [120]. Several preclinical studies have demonstrated that blockade of TGF- $\beta$  can reverse the immunosuppressive effects of the tumor microenvironment. For example, a small molecule TGF- $\beta$  inhibitor was used in a mouse tumor model to rescue the functionality of infiltrating CD8+ T-cells (TILs), which are usually hyporesponsive [121]. Recently, it was also reported a synergistic improvement of a peptide vaccine modality in combination with a monoclonal antibody against TGF- $\beta$ , in a murine tumor model [122]. Although no adverse effects have been observed in studies so far conducted, the dual role of TGF- $\beta$  on normal versus tumor cells, where it can function as a suppressor or promoter of tumor development, respectively, may constitute a potential problem for this approach and indicates the necessity for detailed studies aimed at optimizing the use of antiTGF- $\beta$  reagents while minimizing the potential for adverse effects.

Another cytokine that has a negative impact on the development of antitumor T-cell responses is the vascular-endothelial growth factor (VEGF). A major role of VEGF is to induce the process designated as tumor angiogenesis, which involves the development of an adequate tumor vasculature that will support a blood supply to the growing tumor [123, 124]. Additionally, VEGF contributes to tumor immune escape by inducing the development of immune cell populations with immunosuppressive functions, like immature DCs [24] and the recruitment of tumor-associated macrophages (TAM) to the tumor stroma [13, 14, 125]. Strategies aimed at inhibiting VEGF or its receptors, therefore, will not only disrupt the tumor vasculature thus impairing tumor growth, but will also improve antitumor immunity by eliminating inhibitory cell populations, resulting in enhanced responses to cancer vaccines. In preclinical

studies, for example, an antiVEGF antibody enhanced the efficacy of a peptide-pulsed DC-based vaccine that resulted in prolonged and pronounced antitumor effect [126]. Therefore, inhibition of VEGF may be a valuable adjuvant in the immunotherapy of cancer. A disadvantage of antiVEGF therapies in the clinic has been the emergence of toxicities that included wound healing complications as well as adverse vascular effects.

### 14. Conclusions

Progress in understanding the molecular mechanisms that govern immune activation as well as the mechanisms used by tumor cells to evade surveillance by the immune system are advancing the development of immune-mediated therapies that could be effectively used against a range of human cancers. The combination of cancer vaccines with other therapeutical modalities, in particular established therapies such as radiation and chemotherapy, as well as small molecule targeted therapies, provides an opportunity to further improve the outcome of vaccine interventions against cancer. Moreover, several studies also indicated that patients who receive a cancer vaccine have an enhanced outcome to subsequent therapies, thus providing another possible approach for the use of cancer vaccines prior to other cancer interventions. A prospective randomized trial is being initiated to substantiate these findings. Unlike other modalities, cancer vaccines have so far demonstrated no associated toxicities and therefore their use could not only result in improved patient survival but also in improvements in quality of life.

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