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Review

Towards progress on DNA vaccines for cancer

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Abstract. Cancer immunotherapy faces many obstacles that include eliciting immune reactions to self antigens as well as overcoming tumor-derived immunosuppressive networks and evasion tactics. Within the vaccine arsenal for inhibiting cancer proliferation, plasmid DNA represents a novel immunization strategy that is capable of eliciting both humoral and cellular arms of the immune response in addition to being safely administered and easily engineered and manufactured. Unfortunately, while DNA vaccines have performed well in preventing and treating malignancies in animal models, their overall application in human clinical trials has not impacted cancer regression to date. Since the establishment of these early trials, progress has been made in terms of increasing DNA vaccine immunogenicity and subverting the suppressive properties of tumor cells. Therefore, the success of future plasmid DNA use in cancer patients will depend on combinatorial strategies that enhance and direct the DNA vaccine immune response while also targeting tumor evasion mechanisms.

Keywords. DNA vaccines, cancer, immunotherapy, non-human primates, animal models.

Introduction

The use of plasmid DNA to elicit the immune system against disease provides a variety of practical benefits for large scale vaccine production that are not as easily manageable with other forms of vaccines including recombinant protein or whole tumor cells [1, 2]. DNA vectors are capable of encoding a number of needed immunological components and are easily engineered and produced for administration using bacterial expression systems. Their safety in terms of adverse reactions after injection has also been demonstrated in animal models and human clinical trials. More importantly, neutralizing immune responses to plasmid DNA is rarely observed, making repeated injections possible; however, continued use of viral vectors such as vaccinia and adenovirus can direct the immune response to viral coat proteins and produce anti-vector responses, limiting the vaccine's efficacy. Viral vaccines of these types have been used in prime-boost strategies or, if available, been constructed from less common serotype backgrounds [3].

In its simplest description, immunization with a naked DNA vector prompts the host cell harboring the vector to express the gene constructs of the plasmid. The expressed protein enters into proper immunological presentation pathways that cause specific antibody and cell-mediated immune responses, which may prove necessary for alleviating disease. These vaccination outcomes are in contrast to immunizing with recombinant soluble protein that enters

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into exogenous presentation pathways and predominately induces humoral immunity [1]. Historically, Wolff and colleagues [4] first demonstrated that long-term gene expression in mouse skeletal muscle could be achieved with direct intramuscular injection of plasmid DNA. This and other early studies demonstrating the feasibility of DNA vaccination propelled the first vaccination studies utilizing plasmid DNA in protection scenarios involving influenza [5] and HIV-1 [6]. Years later, with an accumulation of plasmid DNA studies in animal models, the first human clinical trail was initiated to monitor the safety and efficacy of a DNA vaccine against HIV-1 infection [7].

DNA immunization studies in animal models involving cancer and infectious disease have demonstrated preventative and therapeutic success [8]. In contrast, the crossover application of DNA vaccines in humans has faced many obstacles and difficulties, leading to their less-than-desired efficacy in the clinical setting. Although human administration of DNA vaccines as prophylactic and therapeutic tools is in its infancy, much understanding and progress has been made concerning the use of DNA vectors to target specific illnesses. The scope of this review focuses on the advancements and challenges facing DNA vaccines, particularly against human cancer.

Inherent difficulties associated with cancer

It is estimated by the American Cancer Society that over 550 000 individuals in the United States will die from some form of cancer in 2007 [9]. As, on a whole, standard therapeutic procedures currently in practice, including surgery, radiation, and chemotherapy, have not greatly impacted the spread and recurrence of progressive malignancies, newer strategies are needed to improve upon the current treatment success rate [10]. Immunotherapeutic strategies including the use of DNA vaccines hold great promise as an alternative or additive agent to the standard treatment regime. The nature of immunotherapy is designed to specifically target cancer types using components of the immune system. However, the inherent properties of tumorigenic cells pose problems for immunological-based vaccines.

The idea of self

The cellular pathways that ultimately lead to cancer could be initiated by intrinsic genetic abnormalities [11] or extrinsic factors such as viral infection or carcinogen exposure. Indeed, oncogenic viruses, such as Epstein-Barr virus and human papilloma virus, have been found to be etiological agents for certain human neoplasms [12]. These viral infections result in tumor cells that express foreign viral proteins and represent ideal targets for vaccine development. In contrast, for the broader cancer types, the majority of tumors arise from other defined factors that do not impart an evident immunogenic phenotype based on the host's central tolerance system. Such tolerance to self antigens expressed by the host is primarily achieved through early immune processes that remove self-reacting lymphocytes from the bone marrow and thymus [13, 14]. Nevertheless, self-reacting lymphocytes do survive central tolerance mechanisms of the host and are present in the periphery, allowing self tolerance to potentially be broken. These populations, for example, represent positively selected lymphocytes that react to self antigen weakly or foreign antigen, which cross-reacts with naturally occurring proteins [15].

Many obstacles exist with regard to choosing the appropriate DNA vaccine to target a specific type of malignant cell. The nature of the antigen and its tissue expression profile within the body are important guiding principles for vaccine candidacy. Ideally, one would wish to target an antigen both presentable to the immune system and expressed only by a particular neoplasm. Unfortunately, these preferences are not always achievable as many tumor self antigens are also expressed by normal cells [16–18]. Therefore, in a relatively broad outlook, DNA vaccines are faced with the difficult task of (i) breaking self tolerance to generate an appropriate immune response, and (ii) not initiating therapeutically uncontrollable autoimmune reactions within the body.

To date, a growing list of tumor self-antigens has been compiled by investigators and provides potential vaccine targets against specific human cancers [16, 18]. Many of the more common self antigens are described in Table 1 and are classified based upon antigen type. For example, MAGE-A1 was the first reported gene to encode a human tumor antigen recognized by T lymphocytes and is characterized as a cancer/testis (CT) antigen [19]. This antigen class is denoted by expression in tumor cells and germline tissues (*e.g.*, testis, placenta, ovary). CT antigens represent ideal conditions for vaccine use since the antigen is typically not transcriptionally active in normal adult cells and germline tissues do not express the proper receptors for antigen presentation to the immune system [20]. Interestingly, antibodies and T cells specific to CT antigens such as NY-ESO-1 have been found in cancer patients [21]. Alhough the protein is derived from the host, the increased immunogenicity to NY-ESO-1 is hypothesized to result from the primary expression in immune-privileged sites such as the testis, thereby, evading central tolerance mechanisms that take place in somatic

Table 1. Common examples of tumor self antigens recognized by T lymphocytes.

Antigen category	Antigen	Selected cancer type expression
Tumor specific	CDK-4	Melanoma
	β-catenin	Melanoma
	Caspase-8	Head/neck
CT antigen	MAGE-A1	Melanoma, myeloma, breast, lung
	NY-ESO-1	Melanoma, myeloma, breast, lung
Overexpression	MUC1	Breast, ovarian
	HER-2/neu	Breast, melanoma, ovarian
	PSMA	Prostate
Differentiation	CEA	Colon
	Gp100	Melanoma
	MART-1/Melan-A	Melanoma
	Tyrosinase	Melanoma
	PSA	Prostate

Abbreviations: CDK, cyclin-dependent kinase; CT, cancer/testis; MAGE, melanoma-associated antigen; NY-ESO, New York esophageal squamous cell carcinoma; MUC, mucin; HER/neu, human epidermal receptor/neurological; PSMA, prostate-specific membrane antigen; CEA, carcinoembryonic antigen; MART/Melan-A, melanoma antigen recognized by T cells/melanoma antigen-A; PSA, prostate specific antigen.

tissues. Overexpressed self proteins such as HER-2/neu represent targets that are widely distributed among tissue types and are noted for overexpression in tumors *versus* the normal cell counterpart. On the other hand, MART-1/Melan-A is a differentiated antigen that is only expressed in tumor and normal cells of a specific lineage. Finally, tumor-specific antigens represent targets against tumorigenic cells since the molecular alteration of the noted antigen normally results in the progression of the cell to a transformed state. Viral oncoproteins are further represented by this class of antigen.

Murine studies have extensively studied the efficacy of DNA vaccines against many of the above tumor antigens. Collectively, protective immune responses from tumor challenge can be achieved when DNA vaccines are appropriately designed and delivered. The specific results of these animal models have been discussed in detail previously [8, 22].

Table 2. Tumor-associated mechanisms of immune escape.

Type of evasion	Examples
Immune activation	
	MHC/HLA defect Mutational effects
	MHC/HLA loss Gene regulation
	Antigen loss
	Antigenic drift
	Reduced costimulation B7–1, B7–2
	Inhibitory molecules HLA-G
Resistance to killing	
	Fas-FasL directed apoptosis
	Apoptosis prevention Decoy molecules, mutations, caspase inhibitors
Immune suppression	
	Chronic inflammation
	DC modulation VEGF, TGF-β, IL-10
	Treg activation TGF-β, IL-10

Abbreviations: MHC, major histocompatibility complex; HLA, human leukocyte antigen; DC, dendritic cell; VEGF, vascular endothelial growth factor; TGF, transforming growth factor; IL, interleukin; Treg, T regulatory.

Immune evasion

Malignant cells within the tumor microenvironment are well-adept at preventing immune cell function to achieve growth and systemic dissemination. These evasive mechanisms are numerous and represent obstacles to generating an appropriate immune response against cancer (Table 2).

Loss of activation. The activity of the immune system is built upon a system of checks and balances. For instance, devastating autoimmune diseases (*e.g.*, systemic lupus erythematosus, multiple sclerosis) can occur without restriction of overactive lymphocytes through self-tolerance pathways [23]. Antigen presentation and recognition is another such example of immune restraint that involves activation and inhib-

itory elements during initiation of the immune response to a particular target.

Naïve T lymphocytes first recognize protein peptides expressed by professional antigen-presenting cells (APCs) such as dendritic cells (DCs) within the context of major histocompatibility (MHC) class I or II molecules [24, 25]. In the case of $CD8^+$ T cells, the T cell receptor along with the CD8 co-receptor bind the APC MHC I:peptide complex. Costimulatory receptor ligation is required to induce T cell activation and includes among others B7 and CD28 binding by the APC and $CD8^+$ cell, respectively [26]. Upon antigen recognition and costimulation, the T lymphocyte proliferates and differentiates into effector cells that exert cytolytic functions on tissues that express the targeted protein peptide via MHC class I receptors. Due to their genetically unstable nature, tumor cells are able to circumvent T cell recognition with a low immunogenic phenotype. Loss of tumor antigen expression [27–29] and antigenic drift [30] have been characterized as evasive mechanisms that lead to the clonal expansion of non-immunogenic tumor cells. The disruption of human leukocyte antigen (HLA; *i.e.*, MHC designation in humans) presentation machinery has also been observed in many types of patient cancer specimens causing protein peptides to not be properly presented for T cell destruction [31]. This aberrant HLA surface expression on the cell may be a result of structural alterations due to mutations [32, 33] or regulation of the protein subunits at the genetic level [34, 35]. Although HLA-negative malignant cells should signal their destruction by natural killer (NK) cells, tumor cells have employed further escape mechanisms as discussed below. Lastly, reduced costimulatory receptor expression on tumor cells might confer an additional T cell escape phenomenon. Experimental evidence demonstrates that tumor cell lines become more immunogenic and responsive to immune destruction after *in vitro* transfection of costimulatory molecules such as B7–1 (CD80) and B7–2 (CD86) [36–38]. In this sense, tumors that present antigen within the context of MHC class I receptors and express the B7 costimulatory molecules function as APCs that are capable of activating $CD8^+$ T cells.

Tumor cells might also be equipped with inhibitory receptors that contribute to the loss of immune activation. For example, MICA is a stress-induced ligand that is expressed by many types of neoplasms and binds the NKG2D-activating receptor on NK and T cells. NKG2D-mediated tumor destruction has been shown to be abrogated with the shedding of soluble MICA by the tumor [39, 40] or loss of MICA on the cell surface [41]. Additionally, inhibitory receptors such as the non-classical HLA molecule, HLA-G, are expressed in cancer tissues and associated with down-regulating the activities of NK and T cells [42]. Therefore, HLA-loss tumor variants can escape NK cell surveillance by modulating the activity of NK cells through specific receptor interactions.

Resistance to killing. Malignant cells may resist overall destruction by causing it themselves. In one scenario termed activation-induced cell death, $CD8^+$ T cells recognize tumor antigens, become activated, and express the Fas receptor and its ligand, FasL [43]. The interaction between Fas and FasL induces a signaling cascade involving caspases that leads to apoptosis or programmed cell death of the tumor-bound immune effector cell and other nearby $CD8^+$ T cells. In another example, tumor cell release of soluble HLA-G can interact with the CD8 co-receptor and cause FasL up-regulation on $CD8^+$ T cells and induce apoptosis via Fas-FasL interactions as described above [44, 45].

Immune evasion could also be the result of defective death receptor signaling [46]. Ideally, immune cells induce apoptosis via a direct pathway that involves binding death receptors including Fas or TRAIL expressed by tumorigenic cells [46, 47]. However, this killing mechanism can be subverted through tumor secretion of soluble ligands specific to these receptors. The signaling cascade from death receptor engagement could also be interrupted by way of mutations or expression of caspase inhibitory proteins within the malignant cell that prevent apoptosis. In terms of mutational effects, either death receptor surface expression or internal cellular signaling processes could be disrupted.

Immune suppression. The establishment of tumor growth has generally been described as a state of chronic inflammation that is a result of cancer-secreted inflammatory molecules such as cytokines and reactive oxygen species [48]. This constant period of inflammation appears to confer an advantage to tumor expansion by stimulating angiogenesis and preventing apoptosis among other pro-tumor growth properties. Yet, malignant cells also secrete soluble factors that work against inflammatory-driven pathways of the immune system. In all, tumor growth and progression set the stage for the establishment of immunosuppressive networks within the body that discount the activation and effector properties of immune cells.

The more commonly observed soluble factors secreted by tumor cells include vascular endothelial growth factor (VEGF), interleukin (IL)-10, and transforming growth factor (TGF)-β. High levels of these suppressive molecules ultimately affect the maturation,

differentiation, and activity status of APCs such as DCs [49]. Therefore, within the tumor microenvironment, DC antigen presentation to immune effector cells does not exist at the level needed for a robust anti-tumor response. For example, VEGF production by malignant cells initiates chemotactic signals for the migration of immature DC progenitor cells that can become further manipulated by the neoplasm to develop into immunosuppressive DCs [48, 49]. The downstream effects of DC-directed suppression include T lymphocyte inhibition and the activation of T regulatory cells (Tregs) as discussed below. VEGF also promotes tumor angiogenesis by binding the FLK-1 receptor that is up-regulated by endothelial cells within the tumor microenvironment [50]. The establishment of angiogenesis is a necessary factor for cancer growth and metastasis.

Tumor secretion of TGF-β and IL-10 further fuels the potency of malignantly derived immunosuppressive networks within the body. Elevated serum levels of TGF-β and IL-10 have frequently been found in human cancer patients. TGF-β promotes tumor spread [51] as well as inhibits the maturation stages of adaptive immune cells [52]. TGF-β is also capable of stimulating a naturally occurring population of $CD4^+$ T cells into functioning $CD4^+CD25^+$ Tregs that suppress other T lymphocyte responses [53]. On the other hand, IL-10 is capable of preventing T helper (Th)1-driven cytotoxic T lymphocyte (CTL) pathways by producing a Th2 dominating response as well as activating IL-10-producing Tregs (Tr1 cells). Although the exact details of Treg suppression are unclear, possible mechanisms appear to involve cell-to-cell inhibition of effector cells mediated through the inhibitory molecule, cytotoxic T-lymphocyte antigen (CTLA)-4, or secretion of additional suppressive cytokines. Taken together, TGF-β and IL-10 thwart proper immune recognition and mediate T cell inhibition within the tumor microenvironment.

Immunoediting

The cancer immunoediting hypothesis is a relatively recent proposal and was developed largely from observations comparing the immunogenic phenotypes of tumor cells. These studies clarified that protein expression profiles of tumors were different when compared between immunocompetent and immunodeficient mice [54].

Immunoediting is a process described whereby immune pressure drives the selection and outgrowth of tumor cells that are non-responsive to host protection mechanisms [55]. First, the host innate and adaptive immune components provide continual "surveillance" protection against malignant cells. This might result in complete protection from spontaneous cancer development or lead to some form of equilibrium where cancer outgrowth is prevented. However, the equilibrium reached could eventually shift in favor of tumorigenesis through situations that might include the selection of non-immunogenic tumor variants that have altered their cell surface expression (*e.g.*, decreased MHC class I receptor expression) and tumor cells that are prone to secreting suppressive molecules. Altogether, the end result is the selection of a malignant mass that can progress to systemic disease in the presence of an activated immune response.

The tumor microenvironment can be viewed as a heterogeneous collection of cells with protein expression profiles above or below the antigenic threshold required to initiate an effective immune response [56]. Therefore, targeted immunotherapy against a particular tumor self antigen would be effective against many but not all cells. Considering the above principles of immunoediting, a DNA vaccine would have to be comprised of several antigenic components to reduce the selection and recurrence of a tumor cells non-responsive to immunotherapy. Formulating a vaccine in this manner could prove troublesome if multiple self-protein targets are unknown for a particular cancer type. This may, on the other hand, represent an unnecessary concern as a vaccine encoding an immunodominant target can cause determinant spreading of unknown tumor-associated antigens [57]. This process works once a vaccine-encoded antigen initiates lysis of malignant cells. Unknown components released at the time of tumor destruction are taken up by APCs and presented for a polyclonal immune response.

Immunological mechanisms of activation

Plasmid DNA immunization has been shown to elicit robust humoral and cellular immune responses in animal models. Broad-based immunity in this sense has been found to be beneficial in preventing metastasis, particularly in our own studies, where specific antibody and $CD8^+$ T cells are required to immune components to achieve anti-tumor mechanisms against a tumor-specific antigen [58]. Although the elucidation of all immunological components involved following DNA immunization has not been entirely achieved, two overarching models have been proposed and involve the direct and indirect presentation of antigen to APCs.

At the site of DNA injection (*e.g.*, muscle, skin), the surrounding cells are transfected with plasmid DNA and begin to express the vector's components. APCs such as DCs might become directly transfected with

plasmid DNA and present endogenously expressed protein peptides within the context of MHC class I receptors [59–61]. Upon traveling to the nearest draining lymph node, DCs can activate $CD8^+$ T cells to initiate a specific cell-mediated response.

A second possible mechanism is APC acquisition of protein indirectly. In this scenario, muscle or skin transfected with plasmid DNA produces and secretes the vector's encoded products [62, 63]. As the muscle and skin do not express the necessary costimulatory molecules to present antigen to lymphocytes, APCs at the site of injection take up the exogenous protein and process and present the antigenic components through MHC class I (a process termed cross-presentation) and MHC class II receptors. $CD8^+$ and $CD4^+$ T cells can then become primed and activated by APCs to exogenous antigen produced by non-lymphoid cells.

There do remain uncertainties with specifics of these models. For example, the resident bone marrow-derived DC population in the muscle is low compared to the skin. Accordingly, it is hypothesized that other professional APCs such as macrophages mediate the bulk of antigen capture and presentation with intramuscular injection of plasmid DNA [64]. Another issue involved is whether APCs acquire antigen from transfected muscle and skin cells undergoing self or targeted destruction. However, gene expression after DNA vaccination in these tissue types has been shown to be stable over an extended period of time [4, 65].

Human clinical trials

A number of investigations related to developing and testing new treatment modalities have utilized rodent animal models, particularly murine systems. However, rodents may not represent the best experimental animal model to predict the human response to a specific treatment. For example, failed DNA vaccine attempts in human clinical trials may reflect the issue of scale and efficiency of DNA transfection achieved in murine models that do not accurately translate into appropriate dosages for humans. In general, those animals that most closely represent humans are likely to mimic more accurately the human situation, including the immunological response to an immunotherapeutic-based strategy and associated adverse events that may reflect a concern for safety issues.

Phylogenetically, the great apes are most closely related to humans (*Homo* species) and include chimpanzees (*Pan troglodytes*), orangutans (*Pongo pygmaeus*), gorillas (*Gorilla gorilla*), and gibbons (*Hylobates lars*). These animal models best reflect the human situation but are problematic for use as experimental animal models based on scarcity, as some are on the endangered species list, and other issues related to cost. Yet, a number of these hominoid nonhuman primates have been reported to develop cancers similar to those reported in humans that include, but are not limited to, liver, lung, brain, and a variety of hematological malignancies. Next in the evolutionary development line are the Old World monkeys of Africa and Asia, which include the drills and mandrills (*Mandrillus*), common or savannah baboons (*Papio*), gelada baboons (*Theropithecus*), mangabeys (*Cercocebus*), African green monkeys (*Ceropithecus*), and macaques (*Macacca*). The most distantly related to humans are the New World monkeys that are indigenous to South America. The most common New World monkeys that are used in biomedical research investigations include the cotton-topped marmoset or cotton-top tamarin (*Sanguinus Oedipus*), common marmosets (*Callithrix jacchus*), owl or aotus monkey (*Aotus trivergatus*), capuchin monkey (*Cebus*), and squirrel monkeys (*Saimiri sciureus*). Although these non-hominoid non-human primate species have been under investigated for use in models of cancer, a number of these species have been reported to develop a variety of malignancies similar to those observed in humans [66].

In contrast to animal model studies, a small number of published reports exist that detail DNA vaccine studies in cancer patients. Of these few, DNA immunization strategies have been reported in clinical trails involving melanoma [67–70], prostate [71–74], B cell lymphoma [75], and colorectal [76] cancers. The patient population of these studies normally consists of individuals with advanced metastatic disease who have previously undergone a variety of therapeutic strategies that might include surgery, radiation, chemotherapy, hormonal therapy, and other forms of immunotherapy. Generally, injection of the plasmid DNA construct is tolerated well in terms of safety in the patient population and rarely involves systemic toxicities. Common adverse events associated with DNA immunization include pain, swelling, and redness at the site of injection that is not related to dosage. In the majority of cases, though, the DNA vaccines utilized do not induce a sufficient immune response and the progression of disease is unaffected.

However, there are some trials that remark on the ability of certain DNA vaccination regimes to elicit a specific immune response to a desired antigen of interest in at least half of the participants. In the Phase I/II study carried out by Todorova and colleagues [74], prostate cancer patients were prime-boosted with alternate injections of recombinant adenoviral vector expressing PSMA and plasmid DNA encoding PSMA and CD86 alongside receiving GM-CSF protein. After

a 36-month observation period from the first vaccine injection, 86% of participants developed anti-PSMA antibody. Other investigators have also shown response to a Phase I dose escalation study of a DNA vaccine encoding PSA in conjunction with adjuvant injections of GM-CSF and IL-2 [72, 73]. With the highest DNA dose given, patients with hormone-refractory prostate cancer responded with the induction of both PSA-specific IgG antibody and cell-mediated reactions, as assessed by T cell IFN-γ secretion.

DNA vaccine strategies

Considering the hallmarks of cancer [11] and immunological evasion tactics employed for malignant growth, it is no surprise that DNA vaccine therapies have failed on a whole to achieve clinical benefit. Nonetheless, by taking into account the complexities of the tumor microenvironment and improving delivery of plasmid DNA, a new generation of DNA vaccine therapies alongside combinatorial treatments can be instituted to provide robust prophylactic and therapeutic strategies for patients facing metastatic disease.

Subverting immune evasion

As has already been described, vaccination will do little to halt metastatic growth if activated effector cells are unable to exert their functions at a targeted destination. Since immunosuppressive networks instilled by the neoplasm contribute to immunological attenuation, immunotherapeutic strategies should also approach tumor-derived suppression. As the tumor mass is composed of a heterogeneous collection of cells, not all cells will be responsive to specific therapies [56]. Therefore, the most efficacious strategies will include those combinatorial techniques that peel back negative layers shielding tumorigenic growth and allow better opportunities for vaccine-induced immune activation and tumor cell destruction. Reasoning of this fashion finds support in the success of controlling HIV-1 infection with a drug "cocktail" tailored to attack the virus at multiple points.

Breaking immunosuppressive networks

Removing suppressor cells such as Tregs may provide an excellent opportunity to generate activated effector T lymphocytes to tumor antigens through vaccination, but several difficulties exist with this form of therapy. There is concern that unmanageable autoimmune disorders might result by disrupting the balance of immune suppressors to achieve immune activation. Additionally, discrepancies between studies of Tregs in mice and humans are notably evident. Whereas the IL-2 receptor, CD25, provides a valid marker for $CD4^+$ Tregs in animals, the distinction of human Tregs based on CD25 is less precise. Activated effector T cells express CD25 as well as other markers such as CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor (GITR) that phenotypically define Tregs [53, 77]. Other Treg population types exist such as the IL-10-inducible Tr1 cells, which are CD25 negative and may play a role in tumor evasion, although the role of Tr1 cells within the overall framework of cancer and Treg suppression is not entirely known. In all, the issue of manipulating Tregs will remain clouded until appropriate therapies can be tailored to specifically target these cell types in cancer patients.

Animal studies as well as human trials related to the subject of Treg disruption do provide a hopeful outlook on the possibility of enhancing the immune response to tumors by reducing Treg function. Already in hand is the FDA approved drug, denileukin diftitox (Ontak), which is a recombinant IL-2 protein fused to diphtheria toxin and used to treat cutaneous T cell lymphomas expressing CD25. Denileukin diftitox works by being endocytosed into CD25-expressing cells where the diphtheria toxin protein becomes activated and inhibits cellular protein synthesis. There appears to be some indication that denileukin diftitox can suppress the functions of $CD4^+CD25^+$ Tregs in cancer patients, although one report downplays the drug's potential [78]. In a study performed by Dannull and colleagues [79], metastatic renal cell carcinoma patients were administered increasing doses of denileukin diftitox to assess levels of $CD4^+CD25^+$ Tregs. The dose-dependent escalation depleted Tregs from peripheral blood mononuclear cells of patients – without observable effects on other cell types – and led to enhanced stimulation of CTLs *in vitro*. This group further confirmed that prior to immunization with a DC-derived vaccine, denileukin diftitox treatment resulted in higher frequencies of tumor-specific $CD8^+$ T cells, although a link between Treg suppression and cancer remission was not detailed.

Antibody therapy remains an additional avenue to explore in diminishing the suppressive properties of Tregs. Animal models have extensively engaged this option with success in reducing tumor proliferation by targeting CD25, CTLA-4, and GITR molecules [80]. On the other hand, human application of these techniques is less well documented, but tumor responses have been characterized, although selective Treg depletion was not the focus of these studies. The anti-CD25 monoclonal antibody, daclizumab (Zenapax), is an additional FDA approved drug used for

transplant allograft rejection cases. In two separate investigations, daclizumab was fused to cytotoxic agents including a bacterial exotoxin [81] and radionuclide [82] for study in individuals suffering $CD25^+$ malignancies with objective tumor response rates of 40% (in the highest dosed participants) and 56%, respectively. Anti-CTLA-4 [83] and anti-GITR [84] monoclonal antibody treatments have been described alongside xenogeneic DNA vaccination in a murine B16 tumor model. Overall, animals vaccinated with DNA and treated against CTLA-4 and GITR developed enhanced antigen-specific T cell responses and tumor immunity when compared to groups receiving vaccine alone. Patients with metastatic melanoma immunized with peptide-based vaccines have also been tested for the benefit of CTLA-4 blockade [85, 86]. The success rates of cancer patients in these studies were low and the level of specific T cell response did not correlate with tumor regression. Yet, there is indication that patients demonstrating severe but controllable autoimmune reactions due to anti-CTLA-4 treatment experienced greater clinical benefit than non-autoimmune responders [86]. In a separate trial, metastatic melanoma patients undergoing anti-CTLA-4 therapy and previously immunized with autologous tumors engineered to secrete GM-CSF displayed tumor lymphocyte infiltration and necrosis [87]. Taken together, therapies engaged in disrupting molecules associated to regulatory T cells are encouraging and have merit in subverting the suppressive networks of tumor growth as well as initiating immune responses to self antigens expressed by human neoplasms. Again, much work remains on defining human Tregs from other immune cell components for targeted therapies as well as initiating human trials that specifically set out to disable Treg function since other immune cell populations express CD25, CTLA-4 and GITR, and may become adversely affected by such depletion regimes.

Blocking suppressive cytokines within the tumor microenvironment represents another area of interest to break tumor proliferation within the host. Tumor metastasis suppression has been achieved in murine cancer models evaluating techniques to disturb TGF-β binding its receptor [88–90]. Anti-IL-10 methods have also been the subject of investigations to reverse the suppressive nature of the tumor microenvironment by shifting a predominant Th2 response to achieve increased Th1-based CTL activity [91]. Inhibitors directed towards other tumor-secreted soluble factors such as VEGF represent additional avenues to explore in reversing the tide of immunological attenuation in cancer patients. In one report by Niethammer and colleagues [50], the VEGF receptor, FLK-1, was targeted through DNA vaccination in mice. The technique engaged an immune response to proliferating endothelial cells that up-regulate FLK-1 expression within the tumor microenvironment and aid in the progression of angiogenesis. By abrogating the tumor vasculature, DNA-vaccinated animals were protected from tumor challenge in prophylactic scenarios as well as demonstrated enhanced tumor immunity to resident malignant growth.

VEGF also has implications in attracting immature myeloid cells to the site of malignant growth where they can differentiate into immature DCs [48] and remain fixed in such a state by high levels of VEGF, TGF-β, and IL-10 [49]. Immature DCs at the site of tumor growth are thought to account for many of the failed attempts at immunological infiltration and activity. These cell types function as suppressor cells by inducing lymphocyte anergy upon binding T cells as well as activating Tregs through stimulation. Therefore, in combination with VEGF reduction, blockade of mechanisms that characterize suppressor DC function could have an effect on tumor proliferation. Indeed, many of the receptors that characterize tumor-induced DCs (*e.g.*, B7-H1, B7-H4) and potentiate their suppressive functions represent ideal targets for immunotherapeutic intervention [49, 80].

Enhancing DNA vaccines

The inability of DNA vaccines to induce specific immune responses in the patient population may be reflective of issues related to inefficient plasmid DNA uptake, plasmids encoding self antigens, and overall patient immunosuppression. In conjunction with combinatorial techniques that thwart evasive tactics of tumor cells, several additional strategies exist to circumvent non-immunogenicity of plasmid DNA in individuals suffering malignant disease.

Directing the immune response. The immune response to DNA vaccination can be modulated by directing plasmid DNA expression at specific sites within the body. Most animal studies have delivered DNA vaccines either through skin or muscle injections, and these locations have been successful at inducing systemic immunity. However, other DNA injection routes could be applied to prevent primary growth and expansion of particular cancer types. Intranasal and oral delivery of plasmid DNA represents one such alternative and would involve priming mucosal-associated lymphoid tissues to prevent the dissemination of mucosal-derived malignant growth. To date, much of the work surrounding mucosal-based DNA vaccination involves preventing infectious disease since many pathogens first initiate infection by way of mucosal routes, including the gastrointestinal, vaginal, and nasal tracts [92]. Nevertheless, DNA

vaccines directed to the mucosal epithelium have met success in preventing cancer development [50, 93, 94]. An advantage of mucosal priming is that CTLs can become activated at both mucosal-associated lymphoid tissues and systemic lymphoid sites [95]. In contrast, parenteral immunization predominately induces CTL activity systemically and leaves mucosal sites unprimed from vaccination. To achieve DNA transfection at mucosal sites, one popular transport system involves the oral administration of attenuated *Salmonella typhimurium* acting as carrier for specific plasmid DNA to M cells of the small intestine. Other DNA delivery methods to the mucosal epithelium have been developed and include microparticles and liposomes, although their use has been used exclusively in other disease models [96]. In all, the field of DNA vaccines and mucosa cancer development is an exciting area of research that requires additional study and development.

Improving the immune response. A variety of techniques are recognized to enhance DNA uptake and immunogenicity and include alternative injection systems, biological adjuvants, and prime-boost strategies. Incorporating modifications as these to the current use of plasmid DNA in humans may result in more effective vaccines.

Biolistic gene gun delivery represents an alternative to direct injection of plasmid DNA. This approach involves adhering naked DNA to gold beads and shooting the particles through a high-pressured instrument. This system delivers DNA directly into skin and Langerhans cells where immune priming is contributed by Langerhans cells and other immune-based components [97]. In terms of genetic expression, the direct delivery of DNA vectors into cells by a gene gun is a highly efficient process [98]. Gene gun immunization has been shown to induce a greater $CD8^+$ T cell response [99] as well as to require less vaccine to achieve tumor immunity when compared to other injection routes of plasmid DNA [98, 99].

Electroporation is an additional method that can be utilized to increase the immunogenicity of DNA vaccines. The technique involves subjecting an area of the body to an electrical current that enhances the cellular uptake of plasmid DNA and, thus, gene expression. Electroporation of plasmid DNA has shown early promise as a cancer vaccine delivery method by preventing the development of B16 melanoma tumor growth [100]. The superiority of plasmid DNA transfection into skeletal muscle over direct intramuscular injection has also been the subject of a number of studies. In one example, Quaglino and colleagues [101] undertook the task of testing a DNA vaccine treatment in transgenic BALB/c mice prone to developing HER-2/neu expressing mammary carcinomas. Once *in situ* carcinomas were observed in animals, mice were either injected directly or electroporated into the muscle with plasmid DNA specific to the extracellular and transmembrane domains of HER-2/neu. While electroporation resulted in protection from tumor development, mice directly injected with DNA vaccine were unable to prevent the progression of mammary lesions.

Plasmid DNA derived from bacterial expression systems naturally contain unmethylated DNA sequences, termed CpG oligodeoxynucleotides (ODNs), that stimulate innate immunity. CpG ODNs bind Toll-like receptor 9, expressed by professional APCs, and induce the secretion of pro-inflammatory cytokines and chemokines that help skew a Th1 response. Specific classes of CpG ODNs exist that preferentially activate different cell types such as DCs and B cells. CpG neutralizing sequences are also evident within the plasmid backbone and can play a role in inhibiting the activation of APCs that uptake DNA [102]. Several experimental examples point to the beneficial role of using CpG ODNs solely as prophylactic and therapeutic treatments to prevent malignant growth [103]. Its use as an adjuvant with DNA plasmid immunization has likewise demonstrated enhanced immune responses that provide protective tumor immunity. Therefore, it may prove advantageous to engineer plasmid vectors with optimal CpG sequences that target the maturation of particular immunological cell types, while downplaying resident inhibitory motifs. CpG ODNs could also serve as adjuvants by being injected alongside specific DNA vaccines to provide an environment conducive to immune activation.

Many cytokines, chemokines, and costimulatory molecules have been delivered as biological adjuvants to improve the immune response to DNA vaccines [104, 105]. Where IL-4 induces a Th2 bias and B cell activation, cytokines such as IL-12 and IFN-γ promote Th1 differentiation and CTL activity. Additionally, production of chemokines such as RANTES and MIP-1α at the site of DNA immunization would favor migration of professional APCs and increase antigen presentation. One of the more common cytokines employed in plasmid DNA studies is GM-CSF, a molecule that recruits and helps activate DCs. The costimulatory molecules, B7-1 and B7-2, have also been fused to DNA vaccines to enhance activation of $CD4^+$ and $CD8^+$ T cells. Considering the ease in design and construction of plasmid DNA, biological adjuvants such as cytokines, chemokines, and costimulatory molecules can be tailored and encoded within the same DNA vector used to target a particular neoplasm. In this sense, the adjuvant is being deliv-

ered at the site of DNA transfection where it can aid in the activation of a preferable immune response that enhances tumor cell lysis. This strategy, no doubt, would also reduce the cost and efforts associated with producing adjuvant compounds in their natural protein form.

A final approach to surmounting the low immunogenicity of DNA vaccines would involve immunizing with multiple vaccine moieties. Prime-boost strategies can involve initial immune priming with plasmid DNA and secondary injections of recombinant protein or viral vector encoding the appropriate target antigen. The technique has shown remarkable benefit in mouse studies by eliciting a robust immune response to tumorigenic challenge [106] that is long-lived and greater than injection of one or the other components of the prime-boost regime [107]. Human clinical trials of infectious disease have also engaged the option of priming patients with DNA vaccines and immunizing later with recombinant protein or modified viral vectors. For example, McConkey and colleagues [108] primed patients with a DNA vector specific to a malaria antigen and later boosted using a modified vaccinia virus vector expressing the malaria antigen. The results of the study indicated a statistically greater increase in IFN-γ $CD8^+$-specific T cells that persisted for several months than that observed in individuals immunized with plasmid DNA or viral vector alone. Although these and other studies remark on the ability of prime-boost to enhance the level of immune response to vaccination, the efficacy of prime-boost strategies in cancer patients has yet to be fully established.

Conclusions

DNA vaccines hold great potential as immunotherapeutic tools to prevent and treat human cancer. Their advantages as a vaccine modality include a culmination of safety, ease and cost of construction and mass production, and the ability to activate protective antibody and CTL immune reactions. To date, plasmid DNA-based studies in human clinical trials have been ineffective at generating tumor regression, but the strategy is far from being labeled an unrealistic strategy for cancer therapy. To push for success, the next generation of DNA vaccine use will have to incorporate multiple strategies that enhance plasmid DNA immunogenicity as well as target immune suppression within the tumor microenvironment. No doubt, DNA vaccine design and treatment optimization will take additional time and study, and may include routes that join conventional techniques such as surgery, chemotherapy, or radiation. Additional avenues to explore might also involve combining adoptive cell therapies [109] with DNA vaccines. For example, autologous DCs could be transfected with plasmid DNA *ex vivo* and infused back into the cancer patient for potent antigen presentation to tumor effector cells [110, 111]. Altogether, the potential success of plasmid DNA immunization, as observed in tumorigenic animal models, offers hope to individuals stricken with untreatable malignancies that current standard therapies cannot provide alone.

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