DNA vaccine for cancer immunotherapy

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DNA vaccination has emerged as an attractive immunotherapeutic approach against cancer due to its simplicity, stability, and safety. Results from numerous clinical trials have demonstrated that DNA vaccines are well tolerated by patients and do not trigger major adverse effects. DNA vaccines are also very cost effective and can be administered repeatedly for long-term protection. Despite all the practical advantages, DNA vaccines face challenges in inducing potent antigen specific cellular immune responses as a result of immune tolerance against endogenous self-antigens in tumors. Strategies to enhance immunogenicity of DNA vaccines against self-antigens have been investigated including encoding of xenogeneic versions of antigens, fusion of antigens to molecules that activate T cells or trigger associative recognition, priming with DNA vectors followed by boosting with viral vector, and utilization of immunomodulatory molecules. This review will focus on discussing strategies that circumvent immune tolerance and provide updates on findings from recent clinical trials.

Introduction

Cancer remains one of the leading causes of death in the modern world. Finding effective ways to combat cancer has been one of the main goals of scientists worldwide for decades and still poses tremendous challenges. The standard treatments currently practiced in the clinic, including surgery, radiation, and chemotherapy, have shown limited success. These therapies are usually only effective against early stage localized tumors and rarely against later staged, metastatic malignancies, leading to frequent relapse. Furthermore, various agents used in radiation and chemotherapy are damaging to normal tissues, which may lead to prominent side effects. Thus, there is an urgent need for new therapeutic strategies that can specifically eliminate cancer cells and induce long lasting protection.

The immune system is the natural defense mechanism that the human body uses to combat diseases. It has been observed that intratumoral pathogenic infections can lead to spontaneous tumor regression.[1] This observation has demonstrated the potential anti-tumor properties of the immune system and has inspired the development of cancer immunotherapies including therapeutic cancer vaccines. Traditionally, vaccines have been mainly used as a preventive measure against infectious diseases, triggering the immune system to produce neutralizing antibodies against specific antigens. More recently, vaccines have also been applied as therapeutic strategies, inducing the immune system to activate cytotoxic T cells against infected cells and cancers. It has been shown that mammalian cells are capable of expressing genes encoded on plasmid DNA after transfection.[2] Furthermore, it was also demonstrated that intramuscular injection of plasmid DNA can lead to long term gene expression and elicit both humoral and cellular immune responses against the encoded antigen.[2,3] These studies have sparked the development of DNA vaccines against various diseases including influenza and HIV-1 and have demonstrated protective immunity.[4,5] These findings along with the recent discovery and identification of cancer antigens have propelled the investigation and development of DNA vaccines against cancer.[6]

DNA vaccines emerge as a practical and attractive approach with great potential to translate to the clinics. Practically, they are more cost effective compared to other vaccines, such as recombinant protein, tumor cells, or viral vectors. Recent advancements in molecular biology and recombinant technologies along with the increasing identification of tumor antigens provide the tools for plasmid gene manipulation. Genes in DNA vaccines can be designed to encode different antigens as well as various other immunomodulatory molecules to manipulate the resulting immune responses. In addition, DNA vaccines allow for multiple administrations, and their safety profile have been well established in multiple studies.[7-9]

Despite all the advantages, DNA vaccines have had limited success in producing therapeutic effects against most cancers due

to poor immunogenicity. DNA vaccines have been most successfully used on cancer models where the etiological oncogenic agents are of foreign viral origin, for example, human papillomavirus-associated malignancies. However, most tumors arise from normal body tissues and express endogenous antigens that are either not recognized by or are only weakly reactive to the immune system. This is the body's natural mechanism to prevent autoimmunity in which the immune system reacts and attack the host's tissues. Furthermore, CD4+ CD25+ Foxp3 regulatory T cells inhibit the immune functions of lymphocytes capable of recognizing endogenous antigens. In addition, cytotoxic lymphocytes can be rendered anergic when the T cell receptors engage the MHC molecule: peptide complex on antigen presenting cells in the absence of costimulatory molecules. These mechanisms of central and peripheral immune tolerance limit the efficacy of DNA vaccines, which aim to exploit the host's immune system. Moreover, tumors may induce mutation or loss in the immunodominant epitopes capable in triggering the strongest T cell activation, which further hinders the therapeutic effects of DNA vaccines.

Various strategies have been investigated to enhance the potency of DNA vaccines. Plasmids encoding antigens have been designed to promote antigen expression and presentation. New vaccine delivery techniques have been explored. Immunomodulatory molecules have been used in tandem with DNA vaccines either to stimulate the immune system or reduce immunosuppression. This review focuses on the most recent developments in strategies to optimize plasmid design to enhance immunogenicity and circumvent central immune tolerance, as well as provides an update on the progress of DNA vaccines in human clinical trials.

Cancer Antigens

The advancement of genetic technologies, including genome sequencing and gene profiling, has helped with the rapid identification of tumor antigens, which has propelled the development of antigen-specific cancer immunotherapies. Cancer immunotherapies seek to use the host's immune system to eliminate cancer cells making cancer antigens critically important, as they are responsible for triggering a specific immune response. Although large numbers of cancer antigens have been discovered and are being studied in both labs and clinics, their difference in origin has resulted in great variation in immunogenicity.

Ideally, antigens for cancer vaccines should be highly immunogenic to induce strong immune responses, and only be expressed by malignant cells for specific tumor killing. However, the majority of neoplasm developments are caused by loss of growth control in normal tissues. Therefore, they present self-antigens and do not possess phenotypes that are capable of stimulating immune cells due to negative selection. During lymphocyte development, lymphocytes with high affinity toward the host's self-antigens are eliminated to prevent autoimmunity.[10,11] In general, the central tolerance of the host's immune system creates one of the greatest obstacles faced by scientists developing effective cancer vaccines. Investigators have been searching for

cancer antigens with profiles that are capable of inducing potent immune responses. A general guideline to determine the most appropriate cancer antigens for vaccines has been created in a NCI pilot prioritization project.[12]

Scientists have been working on identifying tumor antigens that are recognized by the immune system, and various tumor-associated antigens (TAAs) have been characterized.[13] The first cancer antigen reported that could be recognized by T cells, MAGE-A1, represents a class of TAAs known as the cancer-testis (CT) antigens.[14,15] CT antigens are good candidates for cancer vaccines as they are only expressed on particular tumor cells and immune privileged germ line tissues and not on normal adult cells.[16] Other CT antigens include NY-ESO-1 and SSX.[17,18] This class of TAAs is expressed on a number of tumors, and vaccines against NY-ESO-1 are currently in clinical trials.[19] New CT antigens are constantly identified and studied. Several investigations have used MAGE-C1 or MAGE-C2 as targets for multiple myeloma treatments.[20,21] The gene MAPE is also being assessed as a biomarker for various solid tumors.[22] Other CT antigens, such as HORMAD1, CXorf61, and ACTL8, as potential therapeutic targets are being evaluated.[23]

Another class of TAAs that can serve as targets for cancer vaccines are overexpressed self-proteins.[24] An example of this class of TAAs is HER-2/neu, an oncoprotein most commonly associated with breast cancer.[25] Expression of these proteins is significantly upregulated on neoplastic cells in a wide range of tissue types on such a level that can potentially surpass the recognition threshold of T lymphocytes and trigger an antitumor immune response.

Differentiation antigens are cell type specific and shared between tumors and the normal tissue of origin.[26] For example, both melanoma and normal melanocytes express GP100, Tyrosinase, and Melan-A/MART-1.[27-29] Other differentiation antigens include PSA,[30] Mammoglobin-A,[31] and carcinoembryonic antigen (CEA) overexpressed in prostate cancer, breast carcinoma, and colon cancer respectively.[32] Thus, differentiation antigens may serve as good targets for cancer vaccines?

Among different tumor self-antigens, unique tumor-specific TAAs are the most immunogenic. These antigens are expressed only on tumor cells and not on any normal tissue[33] as a result of somatic point mutations. Antigens from this class can be generated from aberrant gene expression, such as transcription of alternative open reading frame through alternate start codons and introns, incomplete splicing, and posttranslational modifications.[34-37] It has been reported that proteasomes can also be used to produce unique antigenic peptides by splicing precursor proteins.[38] These mutated proteins often play a critical role in the oncogenic process and therefore survive immune selection in order to maintain tumor growth and proliferation. Due to their unique nature of being expressed only on tumors and not on any other normal tissues, these tumor-specific TAAs can be recognized as non-self and not be subjected to central immune tolerance, leading to generation of the highest antitumor effect when incorporated into cancer vaccines. However, certain complications prevent vaccines incorporating unique tumor-specific antigens from being readily translated into the clinics. The same tumor type can be affected by different point mutations making the identification

of a widely applicable antigen for a vaccine difficult.[39] Furthermore, a single tumor can contain multiple mutations, whose roles in tumor proliferation would have to be characterized before the ideal vaccine antigen can be determined.[40] Recently, more and more unique tumor antigens have been slowly identified, such as CDK-4 and β-catenin.[24] Another class of unique tumor antigen is the immunoglobulin idiotype (Id) displayed on most malignant B cells. The Ids on B cell receptors are clone specific, making them unique to every B cell. This antigen represents another ideal target for cancer vaccines, in which multiple studies have been done on idiotypic vaccination.[41]

Despite extensive studies on various self TAAs showing promise in eliciting antitumor effects in animal models, these successes have yet to be replicated in humans, held back by their inherent low immunogenicity. In fact, the most ideal antigens for cancer vaccine come from oncogenic viruses, such as the human papillomavirus, the etiological cause of cervical cancer.[42,43] The integration of viral genes into the host's genome after infection leads to expression of viral oncoproteins, which are foreign proteins readily recognized by lymphocytes and subjected to effector mechanisms. Since most cancer antigens come from self-tissue, cancer vaccines have to be innovatively engineered to overcome inherent immune tolerance.

Immune Activation by DNA Vaccines

The principal concept of cancer immunotherapy is to introduce various tumor antigens into the host to facilitate immune system-mediated clearance of tumor cells. Thus, the ability of a particular therapy to induce robust immune responses has a direct and significant impact on its effectiveness. Various immunotherapies including antibody therapies, cytokine therapies, adoptive T cell therapies, and cancer vaccines have been studied both pre-clinically and clinically. Among the various forms of cancer vaccines, DNA vaccines represent a promising strategy to induce such potent immune responses. A plasmid DNA that encodes antigen and other genes of interest under the control of a mammalian promoter is delivered into the host's tissues, and subsequently transfected into the cells allowing for in vivo production and expression[44] by the host's protein expression machineries. DNA vaccines are shown to be able to trigger innate immune responses, and depending on their designs and sites of delivery, DNA vaccines can also elicit antigen specific humoral and cellular immune responses.[45]

The plasmids utilized in DNA vaccines are of bacterial origin and have been shown to stimulate innate immune responses.[45] The bacterial DNA appears to serve as a ligand that stimulates Toll-like receptors (TLRs), a class of membrane-spanning proteins on dendritic cells that plays an important role in the innate immune system by recognizing pathogen-associated molecular patterns. Specifically, the hypomethylated CpG dinucleotides motif that is common in bacterial DNA, but rare in mammalian DNA, interacts with TLR9.[46] TLR9 is expressed in a number of immune cells, such as dendritic cells, B cells, and natural killer cells, which get stimulated as the introduced DNA is picked up either by direct transfection or phagocytosis.[47] Activation of TLR9 leads to a cascade of pro-inflammatory responses and results in the production of various cytokines. The local inflammation and increased production of cytokines from the innate immune responses can attract and activate additional immune cells, such as lymphocytes, and enhance subsequent specific immune responses.[48] Particularly, activation of TLR9, through the signaling of MyD88, leads to activation of interferon regulatory factor (IRF) 7, resulting in expression of Type I interferons (IFNs) (**Figure 1**). Furthermore, the adjuvant effect of plasmid DNA was also found to be mediated by Tank-binding kinase 1 (TBK1). The presence of intracellular DNA plasmids in the cytosol can be sensed by DNA sensors, such as DAI, H2B, IFI16, DDX41, LRRFIP1, and cGAS, which activate TBK1 and stimulator of interferon genes (STING), leading to activation of IRF3 and production of Type I IFNs.[49] These signaling pathways were shown to be essential in the activation of antigen specific T cells and B cells.[50,51]

DNA vaccines can be delivered intradermally with devices like Gene Gun, leading to transfection of epidermal keratinocytes and Langerhans cells.[52-54] Langerhans cells are immature dendritic cells residing in the skin that actively participate in the capture and processing of antigens. Transfection of Langerhans cells with DNA leads to the expression and processing of antigens, which directly enter the presentation pathway. Langerhans cells, which serve as professional antigen presenting cells (APCs), then migrate to the lymph nodes and present antigens to naïve T-cells.[55,56] These endogenously produced antigens are presented by major histocompatibility complex (MHC) class I molecules on Langerhans cells to activate CD8+ cytotoxic lymphocytes (CTLs).[53]

Intramuscular delivery of DNA vaccines results in the transfection of myocytes. Although myocytes are efficient in expressing the transfected antigens, they are incapable of activating strong specific immune responses since they are not professional APCs.[57] Instead, APCs, like dendritic cells, are attracted to the site of transfection, where inflammation and cytokines are generated by vaccination. APCs then capture antigens produced by transfected cells through means like phagocytosis.[58] These exogenous antigens are then presented by dendritic cells through MHC class II molecules and interact with CD4+ helper T cells, resulting in the activation of a humoral response.[59] Alternatively, the captured exogenous antigens can be presented in the context of MHC class I molecules through cross presentation to CD8+ cytotoxic T cells, leading to activation of a cellular immune response.[60] Importantly, Type I IFNs promote this process.[61] Direct transfection of DNA may also occur in APCs where direct antigen presentation through MHC class I can prime CD8+ T cells.[47,56]

The activation of cellular immune responses is important in eliciting antitumor immunity. Specifically, a potent CD8+ cytotoxic T cell response has been shown to correlate strongly with a positive prognosis of tumor control and clearance. DNA vaccines are particularly suited to induce CD8+ T cell responses, as they can generate antigens intracellularly, which triggers the MHC class I antigen presentation pathway.[62] Though in principle
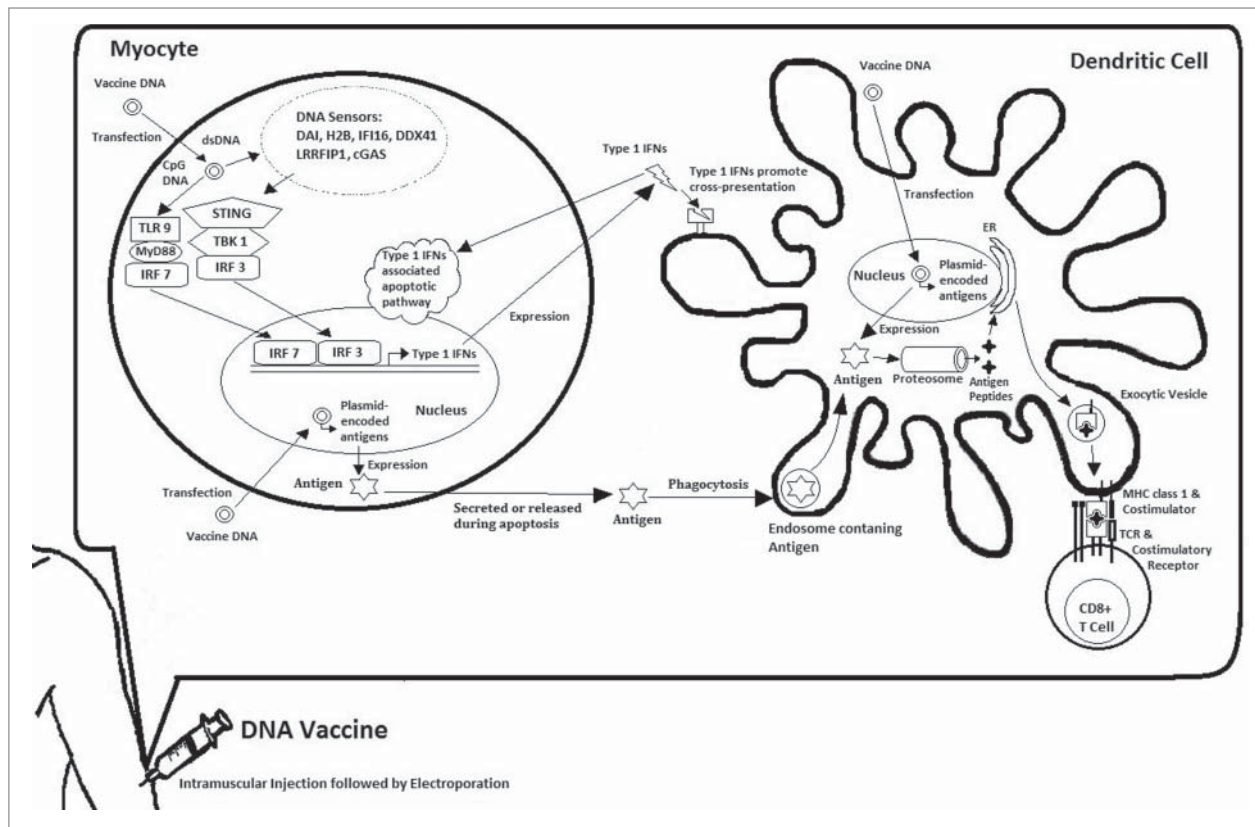
Figure 1. Immune activation following DNA Vaccination. Intramuscular administration of DNA vaccine leads to transfection of DNA plasmids encoding antigens mainly in myocytes with some transfection in dendritic cells. DNA sensors such as DAI, H2B, IFI16, DDX41, LRRFIP1, and cGAS are able to detect the presence of dsDNA in the cytosol and induce the activation of STING-TBK1 signaling cascade leading to activation of IRF3 and resulting in expression of Type I IFNs. TLR9 can recognize the unmethylated CpG DNA, which through the signaling of MyD88 activates IRF7 also resulting in expression of Type I IFNs. Dendritic cells can pick up the myocyte-expressed antigens through phagocytosis as they get secreted or released following apoptosis. The antigens are then processed and presented through MHC class I to CD8+ T cells in cross-presentation. Interestingly, this process is promoted by Type I IFNs. Alternatively, dendritic cells can be directly transfected and express the antigens, which then can be presented through MHC class I to CD8+ T cells.

DNA vaccines are capable of inducing therapeutic antitumor immune responses, some limitations have prevented easy and direct applications of DNA vaccines in the clinics. The designs of DNA vaccines have to be strategically optimized to achieve the desired translational efficacy.

Advantages and Disadvantages of DNA Vaccine

The use of DNA plasmids to generate antigens in vivo for cancer immunotherapy offers several advantages and practical benefits (**Table 1**). Molecular recombinant DNA technology allows for flexible design of DNA vectors to encode a wide range of antigens and immunomodulatory molecules.[63] The DNA plasmid also possesses intrinsic abilities to induce innate immune responses.[45] By utilizing different routes of administration and manipulating the antigen processing pathways, DNA vaccines can preferentially trigger the activation of either Th1 Helper T cells or Th2 Helper T cells and polarize the resulting immune response into being either humoral or cellular based. DNA vaccines also have the advantage of eliciting CD8+ CTL-mediated immune responses more important for tumor killing.[62] Since the antigens are expressed intracellularly by the plasmid-transfected host's cells, they can be presented by MHC class I and prime

Table 1. Advantages of DNA Vaccine

Design	Allows for simple and flexible design, can encode a wide range of antigens and immunomodulatory molecules
Immunology	Trigger both innate and adaptive immune responses, induce both antibody and cytotoxic mediated cellular immunity, long term antigen production
Safety	No risk of pathogenic infection, no clinical adverse effect or toxicity, no production of anti-DNA antibody allowing for repeated administration
Stability	Heat stable, easy to store and transport without the need of a cold chain
Cost effectiveness	Rapid production, easily engineered, very reproducible, perfect for large scale production and administration

antigen-specific CD8+ T cells. In addition to the antigens, the vector can also encode various immunomodulatory molecules like immunostimulatory cytokines[64], or agents that can target the antigens to specific processing and presentation pathways, leading to selected antibody or T-cell-mediated effects.[65] DNA plasmid transfection into host cells also allows for steady expression and supply of antigens.

DNA vaccines represent a platform for cancer immunotherapy with the potential for mass application. They can be easily engineered and produced rapidly in large quantities. DNA vectors are very stable and can be easily stored and transported. Their safety has also been well demonstrated in both animal models and human clinical trials.[66-68] Unlike live attenuated bacterial or viral vaccines, there is no risk of pathogenic infection. Furthermore, the DNA vectors do not elicit anti-vector neutralizing antibody production, so multiple doses of the vaccine can be administered.[7] Various methods of DNA vaccine delivery have resulted in only minor, tolerable discomfort with no significant adverse effects. Practical features such as safety, ease of manufacture, and low cost make DNA vaccines an appealing option.

Despite all the positive characteristics, DNA cancer vaccines still suffer from relatively low immunogenicity, which hampers desired clinical success. Naked DNA does not easily spread from cell to cell in vivo. APCs do not readily take up expressed antigens and activate satisfactory immune responses. Thus, effective strategies that help enhance DNA vaccine potency need to be developed.

DNA Vaccine Delivery for Optimal Priming

Delivery of the encoded DNA vector is the first step of the immune activating cascade of DNA vaccines. Due to the inherently low immunogenic nature of DNA vaccines, it is imperative to adopt the route of administration that can trigger the strongest immune priming.

DNA plasmids are usually introduced intradermally or intramuscularly with the majority of the vectors ending up in the extracellular space. However, most cells at the injection site are inefficient in uptaking the injected DNA vectors resulting in low transfection efficiency. Large amounts of DNA would need to be administered for sufficient DNA uptake, reducing the cost efficiency of DNA vaccines. Strategies that directly target DNA into APCs have been explored. Within a DNA vaccine delivery device called Gene Gun, plasmid DNA is coated onto heavy metal nanoparticles, usually made of gold, and bombarded into the keratinocytes with the help of compressed helium as an acceleration force. This results in direct introduction of the antigen into the immature dendritic cells, which can process the antigen and migrate to lymph nodes where they present the antigens and activate lymphocytes. This system is able to induce substantial CD8+ T cell responses and is shown to be very efficient, requiring only an amount of DNA in the nanogram range.[53,69] The ease of administration also makes Gene Gun an attractive approach.

The intramuscular injection route has been another focus of DNA vaccine delivery. It has been shown that skeletal muscles are capable of generating long-term expression of transfected plasmid DNA and triggering strong immune responses in mouse models. However, scaling up from mice to human subjects, the low transfection efficiency means that a large amount of DNA would need to be applied, making such an approach impractical.[58] A potential solution to this problem is electroporation (EP). EP greatly increases the uptake of plasmid DNA in muscle cells by applying brief electric pulses that transiently permeabilize the cell membrane.[70,71] The result is a one thousand fold increase in antigen delivery compared to naked DNA injection alone.[72] EP itself also serves as a form of adjuvant by damaging the application site, leading to inflammation and cytokine release and ultimately recruiting APCs, such as dendritic cells and macrophages.[73,74] Furthermore, EP greatly reduces the amount of DNA required and greatly enhances both cellular and humoral immune responses.[75-77] In addition, EP does not induce an immune response against the delivery mechanism, allowing for repeated administration, and is also very well tolerated by patients showing no apparent long-term adverse effects. It appears that EP technology may be the key for successful DNA vaccine translation and is being tested in several clinical trials.[78,79]

DNA Vaccine Design: Circumventing Immune Tolerance

Numerous strategies to improve the immunogenicity of DNA vaccines have been developed and studied, including plasmid designs that produce enhanced epitopes, which more effectively activate lymphocytes, coadministration of DNA encoding immunomodulatory and immunostimulatory molecules, the use of prime boost strategies, and approaches to break the immunosuppressive networks in the tumor microenvironment (reviewed in [63]). The problem of immune tolerance still remains a major hurdle for DNA vaccination, as most tumor antigens are self-antigens that cannot trigger potent immune responses. Attempts have been made to engineer DNA vaccines that can break immune tolerance. Previously, a study showed that DNA vaccine encoding alphavirus replicon can activate the innate immune pathway and elicit antitumor immune responses against self-TAA tyrosinase related protein 1, effectively overcoming immune tolerance.[80]

Recently, more innovative DNA vaccine designs aiming to circumvent central tolerance have been investigated in which antigen-specific antitumor immune responses were observed. One approach to circumvent central tolerance against self antigens is by creating a DNA construct encoding a secreted chimeric protein consisting of a single-chain trimer (SCT) of MHC class I heavy chain, β2-microglobulin, and peptide antigen linked to immunoglobulin G.[81] This chimeric protein is shown to form a dimer that can strongly bind to antigen-specific CD8+ T cells with high efficiency and directly induce T cell activation and proliferation. The SCT region of the construct possesses the property of directly and stably displaying an antigenic peptide to CD8+ cytotoxic T cells with high affinity. This effect provides an

advantage of bypassing the antigen-processing pathway that may result in suboptimal efficiency of antigen presentation. Furthermore, the IgG domain of the construct causes the chimeric protein to form a dimer and bind to the Fc receptors on APCs, such as dendritic cells, which migrate to the lymph nodes where the antigen on the chimeric protein is displayed to prime antigen-specific T cells. Importantly, intradermal vaccination of DNA encoding the SCT chimeric protein linked to a melanoma antigen tyrosinase related protein 2 (Trp2), a self-antigen also expressed in tumors, is able to elicit strong Trp2-specific CD8+ T cell mediated immune responses and demonstrates therapeutic antitumor effects in B16 melanoma tumor models in mice.[81] This innovative DNA construct demonstrates translational value by inducing strong immune responses against a self-antigen. Further studies need to be performed to examine the effect of DNA vaccination using this approach against other self-antigens in tumors.

The potential of using xenogeneic versions of antigens in DNA vaccines to bypass central immune tolerance has been investigated, mostly in melanoma. Interestingly, a recent study reported the use xenogeneic p53 in colon cancer. The tumor suppressor gene p53 is mutated and overexpressed in various cancers. P53 represents another self-antigen in which only low affinity CD8+ T cells against p53 may be generated resulting in weak antigen-specific immune responses. A study shows that a xenogeneic version of the p53 gene is able to induce potent p53-specific immune responses.[82] Interestingly, intramuscular vaccination of DNA encoding the human p53 gene followed by electroporation elicits a strong CD8+ T cell response against mouse p53 in mice. Furthermore, these effects are shown to induce both prophylactic and therapeutic antitumor effects against murine colon cancer MC38 expressing mouse p53.[82] It is likely that the xenogeneic version of p53 is recognized by the immune system as foreign due to its origin from a different species. It is important to note that this particular strategy utilizes DNA sequences encoding genes homologous between 2 species. Therefore, the expressed antigen has to be similar to be recognized as the same host antigen, yet different to bypass tolerance against the self-antigen. Future studies are needed to evaluate the effectiveness of the xenogeneic p53 DNA vaccine on other p53-expressing cancers.

The DNA vaccine strategy of encoding a xenogeneic version of an antigen was examined in another pre-clinical study. Human telomerase reverse transcriptase (hTERT) is another self-antigen highly expressed in a wide range of human cancers. A DNA vaccine encoding a synthetic, highly optimized full-length hTERT (phTERT) has been engineered. Intramuscular injection of the DNA followed by electroporation has elicited a strong CD8+ T cell response against hTERT and has generated activated T cells expressing CD107a, IFNγ, and TNFα in mice.[83] More importantly, vaccination with phTERT is able to induce robust CD8+ T cell-mediated antitumor immune responses in a non-human primate (NHP) model. Immune studies in NHP models carry significant translational potential as the immune systems of NHPs closely match that of humans. In addition, in this particular DNA vaccine study, sequence homology analysis reveals that hTERT shares 96% identity with NHP TERT. Vaccination with phTERT in NHPs leads to improved tumor control and survival in both prophylactic and therapeutic studies in HPV-16 associated tumor models.[83] A cytotoxicity assay also showed that phTERT-induced T cells can eliminate hTERT target cells. phTERT is shown to be able to break immune tolerance in the human-related NHP model, and warrants further investigation.

A heterologous prime boost vaccine system using DNA and an adenoviral vector has been reported to induce antigen-specific immune responses and antitumor effects against self-TAAs. Evidence has shown that DNA plasmids are most efficient in priming the immune system, while boosting with a viral vector leads to superior immune responses compared to boosting with additional DNA plasmids. Intramuscular injection of 2 DNA plasmids encoding TAA constructs consisting of CEA fused to the B subunit of Escherichia coli heat-labile toxin and the extracellular and transmembrane domain Her2 followed by electroporation, followed by boosting with an adenoviral subtype 6 dicistronic vector carrying the same genes was able to generate both T cell-mediated and antibody responses against both TAAs in mice.[84] Furthermore, the vaccine system was able to induce therapeutic antitumor immunity in both Her2+ mammary tumors and CEA+ colon tumors. The heterologous prime boost vaccine demonstrated immune activation against immune tolerant self-TAAs, and its safety and immunogenicity in humans are currently being evaluated in a phase I clinical trial.[79]

A DNA vaccine encoding wild type c-Myb cDNA flanked by 2 helper T cell epitopes of tetanus toxin(TT) has been shown to trigger an immune response against the self- antigen c-Myb by utilizing associative recognition. The epitopes of tetanus toxin are highly immunogenic to helper T cells, which help induce CD8+ cytotoxic T cell and CD4+ helper T cell responses against the weakly immunogenic c-Myb.[85] Interestingly, the DNA vaccine is administered intravenously as opposed to the common intramuscular or intradermal delivery routes. Nevertheless, the vaccine is able to generate prophylactic antitumor immunity mediated by CD8+ and CD4+ T cells against murine colon cancer model MC83 in the absence of adjuvant or immunostimulatory molecules.[85] The use of TT derived T cell epitopes in DNA vaccines have been further investigated in recent studies. A fusion DNA vaccine coupling fragment C domain (DOM) of TT with PASD1, a CT antigen, was able to induce CTL response against human multiple myeloma in HLA transgenic mice.[86] In another study, DNA encoding the TT domain fused with S9C, an immunodomant HLA-A2-binding peptide in NY-ESO-1, generated T cells capable of killing tumor cells expressing endogenous NY-ESO-1.[87] These studies demonstrate the potential of DNA vaccines incorporating TT epitopes in enhancing immunogenicity. The effect of utilizing potent tetanus toxin epitopes to help elicit immune responses against weak self-antigens should be further studied using other TAAs.

It has been shown that administration of molecules to modulate immune cells can enhance the potency of DNA vaccines against self-antigens. A previous study showed that blockade of CTLA-4 with anti-CTLA-4 antibody after vaccination with DNA encoding melanoma differentiation antigens Trp2 and

gp100 resulted in enhanced B16 tumor rejection.[88] In another study, an anti-glucocorticoid-induced tumor necrosis factor receptor family-related gene (GITR) monoclonal antibody was also shown to enhance CD8+ T cell immune responses against the same melanoma antigens.[89] These earlier studies demonstrate the adjuvant effect of immunostimulatory agents. In a recent study, plasmid DNA encoding p53 or gp100 along with DNA encoding CD40L, a costimulatory molecule that activates APCs, were injected intramuscularly and followed by electroporation. Significant increase in antigen specific CTL cellular immune responses was observed in both p53 and gp100 tumor models.[90] Furthermore, the immune responses were shown to be CD8+ T cell mediated and result in potent antitumor response. The results show that immunostimulatory molecules can be incorporated into DNA vaccines to enhance the vaccines' potency. Future DNA vaccine strategies should aim to incorporate immunomodulating molecules either as adjuvants or encoded in DNA plasmids to further enhance the immunogenicity against self-antigens.

Importantly, these DNA vaccine strategies demonstrating effectiveness in inducing antigen-specific immune responses against self-antigens have not been shown to trigger damaging autoimmune attacks against normal tissues. The first DNA vaccine that has broken immune tolerance and has been translated into the market was actually implemented in dogs. A DNA vaccine encoding xenogeneic tyrosinase has been shown to elicit antitumor response against oral melanoma in dogs and demonstrated safety and therapeutic efficacy in a phase I trial.[91,92] These promising results have led to the USDA licensure of the DNA

vaccine, Oncept, as an immunotherapy for oral melanoma in dogs.[93] Although no DNA vaccines for humans have been standardized for cancer treatment, numerous clinical trials are currently underway to assess their translational potential.

Human Clinical Trials

Clinical trials provide opportunities to evaluate whether DNA vaccines will be able to fulfill their ultimate goal of demonstrating efficacy in treating humans. Since the first DNA vaccine clinical trial on HIV-1, numerous clinical trials have demonstrated the safety and tolerability of various DNA vaccine platforms. Injection of DNA plasmids is well tolerated by patients with minimal to no systemic toxicities being reported. Integration of DNA vectors into the host genomes is not a concern, and no detectable increase in antibodies against DNA is observed.[94] The widely accepted safety profile of DNA vaccines has led to relaxed requirements for FDA approval and the frequent combination of first and second phase trials into one trial. Since the safety of DNA vaccines is well established, the main interest in clinical trials has become demonstrating efficacy. Here, we report a brief summary of results from a number of recent clinical trials on DNA vaccines against cancer (**Table 2**).

Melanoma

Malignant melanoma expresses a number of TAAs that can be used as targets for DNA vaccination. Intranodal delivery of DNA

Table 2. Human Clinical Trials

Disease	Antigens	Design	Phase	Outcome	Refs
Melanoma	Gp100	Xenogeneic mouse gp100 or human gp100	I	Increase in IFNγ+ production in CD8+ T cells against gp100, absence of toxicity	66
		Xenogeneic gp100 delivered by PMED	I	High IFNγ+ CD8+ T cells production, absence of toxicity	96
Breast cancer	HER2	Full length signaling-deficient HER2 gene with low doses of IL-2 and GM-CSF	I	Long-term antibody response, absence of toxicity	97
		Chimeric rat/human HER2 targeted to dendritic cells	I	IFNγ+ production from both CD4+ and CD8+ T cells	105
	Mam-A	Vaccine encoding Mam-A cDNA	I	Expansion of CD4+ helper T cells expressing IFNγ+, decreased number of regulatory T cells	99
Colorectal cancer	CEA	Modified human CEA fused to promiscuous T helper epitope of the tetanus toxoid with cyclophosphamide and GM-CSF	I	Absence of toxicity	67
Prostate cancer	PAP	PAP with GM-CSF adjuvant	I/II	PAP-specific IFNγ+ CD8+ T cell development, PAP-specific CD4+ and CD8+ T cell proliferation	100
				Multiple boosting enhance the immune responses	101
	PSMA	DNA encoding a tumor derived epitope from PSMA fused to a domain of fragment C of tetanus toxin	I/II	Induction of CD4+ T cell help and antibody production, development of PSMA-specific CD8+ T cells	102
Cervical cancer	HPV E6/E7	DNA encoding modified HPV E7 incapable of binding retinoblastoma protein fused to HSP70 and a secretion signal sequence	I	Absence of toxicity	68
		Highly optimized HPV16 and 18 E6/E7	I	HPV-specific CD8+ T cell responses with cytolytic functionality, absence of toxicity	103
		HPV16 E6 and E7	I	Local CD8+ T cell responses in the tumor microenvrionment	104

plasmids encoding Melan-A (MART-1) and tyrosinase have been shown to elicit both cellular and humoral-mediated immune responses in stage IV melanoma patients in previous trials.[95] A phase I trial has been conducted using DNA encoding xenogeneic mouse gp100 or human gp100.[66] Mouse or human gp100 plasmid DNA were injected with 3 dosages (100, 500, or 1500 ug) intramuscularly every 3 weeks, and then with the gp100 of the other species 3 times. Only mild toxicity was observed at the injection site in 12 out of 19 enrolled patients. Furthermore, CD8+ T cells binding gp100 HLA-A2 restricted tetramers were elicited in 5 patients while one patient showed an increase in IFNγ+ CD8+ T cells. However, no difference in progression-free survival was found between patients with or without immune responses.

Another pilot clinical trial was conducted to compare the immunological responses of intramuscular delivery and particle mediated epidermal delivery (PMED) of the xenogeneic gp100 DNA.[96] 27 stage IIB-IV melanoma patients received 8 vaccinations with either PMED or IM over 4 months with either 4ug or 2000ug of mouse gp100 DNA respectively. The safety profile of PMED was found to be comparable to that of the intramuscular administration route. Furthermore, PMED seemed to induce higher IFNγ+ CD8+ T cell production while requiring a significantly lower dose of DNA. Although 30% of the vaccinated patients displayed immune responses, no significant clinical outcomes were observed.

Breast cancer

Her2/neu (HER2) is an oncoprotein overexpressed in breast cancer, and is used as a target antigen for DNA vaccines in clinical trials. In a pilot clinical trial, a vaccine using DNA plasmid encoding full-length signaling-deficient version of HER2 was administered along with low doses of IL-2 and GM-CSF in metastatic HER2-expressing breast cancer patients. The vaccine was well tolerated with no clinical toxicity or autoimmunity observed. Although no improved T cell responses were observed, the vaccine was able to generate long-term antibody responses, and 2 out of the 6 patients who completed all 3 cycles of vaccination survived for more than 4 years after the vaccinations.[97] Another vaccine targeting dendritic cells with DNA encoding chimeric rat/human HER2 was tested in 28 HER2 breast and 16 HER2 pancreatic cancer patients.[98] The chimeric plasmid was able to induce T cell responses and significantly hinder HER2+ tumor growth, with the ability to circumvent suppressor effects of regulatory T cells, IL10, and TGF-β. No therapeutic outcome was reported. Thus, further testing should be conducted.

Mammaglobin-A (Mam-A) is another protein overexpressed in breast cancer. Preclinical studies have shown that a vaccine encoding Mam-A cDNA can generate Mam-A-specific CD8+ T cell immune responses. This led to a phase I trial of the Mam-A DNA vaccine in stage IV metastatic breast cancer patients. Patients were vaccinated with Mam-A cDNA on days 0, 28, and 56. Interestingly, an increase in Th1 CD4+ T cells was observed. Furthermore, the activated CD4+ helper T cells shifted from expressing IL-10 to expressing IFNγ and induced preferential lysis of Mam-A-expressing breast cancer cells.[99] This result shows that Mam-A cDNA vaccine is able to elicit antitumor immunity against breast cancer. Further studies should be conducted to evaluate long-term therapeutic outcomes.

Colorectal cancer

Application of a DNA vaccine against colorectal cancer has also been studied in a phase I clinical trial. The vaccine plasmid encodes a modified version of the human carcinoembryonic antigen (CEA) gene fused to a promiscuous T helper epitope of the tetanus toxoid, and has been shown to be immunogenic in mice. In the trial, 10 patients were treated with cyclophosphamide intravenously before the first vaccination. CEA66 DNA plasmids were injected either intradermally (2mg) or intramuscularly (8mg) on week 0, 2, and 6, along with subcutaneous injection of GM-CSF(150ug). Only minor adverse effects, such as fatigue, headache, arthralgia, chest tightness, and myalgia, were observed at the vaccination site. During the follow-up period, one patient had a recurrence, and interestingly, another patient was diagnosed with urinary bladder cancer, which was unrelated to the DNA vaccination treatment.[67]

Prostate cancer

Preclinical studies have demonstrated that a DNA vaccine encoding Prostatic acid phosphatase (PAP), a prostate associated antigen, can elicit PAP-specific CD8+ T cell immune responses. A phase I/II trial was conducted using a DNA vaccine encoding human PAP to treat 22 stage D0 prostate cancer patients. In this dose escalation study with 100 mg, 500 mg, or 1500 mg of plasmids, DNA was injected intradermally along with the adjuvant GM-CSF (200mg) 6 times at 14-day intervals. During the one-year observation after treatment, 3 of 22 patients developed PAP-specific IFNγ+ CD8+ T cells and 9 of 22 patients showed proliferation of PAP-specific CD4+ and CD8+ T cells.[100] Although no PSA values in patients declined by more than 50%, several patients were reported with a decrease in the rate of serum PSA rise after treatment. A subsequent study was conducted on this vaccine and found that multiple boosting can enhance the elicited immune responses.[101] Future trials evaluating the vaccine's clinical therapeutic effect are warranted.

In a phase I/II trial, a DNA vaccine encoding a tumor derived epitope from prostate-specific membrane antigen (PSMA) fused to a domain of fragment C of tetanus toxin was administered either intramuscularly alone or intramuscularly followed by electroporation. The tetanus toxin fragment C domain was shown to induce CD4+ T cell help and elicit antibody responses. Furthermore, intramuscular injection of the vaccine followed by EP generated a greater amount of antibodies. The results showed that intramuscular injection followed by EP is safe and can generate strong humoral responses. Moreover, PSMA-specific CD8+ T cells were detected in patients after administration of the DNA vaccine.[102] However, no effect of DNA dose on outcome was detected. Nevertheless, a reduction on the rate of disease progression was observed.

Cervical cancer

DNA vaccines theoretically should generate the strongest immune responses against cervical cancer due its etiological factor being human papillomavirus infection. HPV E6 and E7 are foreign antigens and are only expressed in transformed cancer cells, making them ideal targets. Various DNA vaccines encoding the viral oncoproteins HPV E6 and E7 have shown to generate potent cellular and humoral immune responses in mice. Numerous fusion DNA vaccines encoding HPV E7 and other manipulative molecules have been developed. A phase I trial was conducted in patients with grade 2/3 cervical intraepithelial neoplasia (CIN). This DNA vaccine encodes a modified version of HPV E7 incapable of binding retinoblastoma protein and fused to heat shock protein 70 (HSP70) as well as a secretion signal sequence. The patients received 3 IM vaccinations (0.5, 1, or 3mg) on days 0, 28, and 56. Histologic outcomes based on resection were evaluated at week 15, and histologic regressions were observed in 33% of the patients in the highest-dose cohort.[68]

In another phase I trial, the effect of a highly optimized DNA vaccine, VGX-3100, encoding HPV 16 and 18 E6/E7 antigens was evaluated in 18 patients with grade 2/3 CIN. The DNA vaccine was injected intramuscularly followed by electroporation 3 times in a dose escalating manner (0.3, 1, and 3 mg). The vaccine was able to induce HPV specific CD8+ T cells that loaded granzyme B and perforin.[103] Higher levels of interferon gamma were also observed in the 3 mg cohort compared to the other 2 doses. The immunization was also well tolerated with no dose limiting toxicity. It would be of interest to evaluate whether this treatment strategy can lead to regression or clearance of lesion.

In another study conducted on grade 2/3 CIN patients, intramuscular injection of DNA vaccine targeting HPV16 E6/E7 was shown to increase local CD8+ T cell responses in the tumor microenvironment. Particularly, histologic and molecular changes, including an average of threefold increase in intensity of CD8+ T cell infiltrates in both the stromal and epithelial compartments, were observed. Increased expression of genes associated with immune activation and effector functions were also detected in CD8+ T cell infiltrates.[104] Specifically, the histological changes in the stroma were characterized by increases in the expressions of immune activation gene CXCR3 and effector function genes Tbet and IFN β. The result from this study shows that HPV DNA vaccine can induce robust localized effector immune responses in cervical cancer patients.

Conclusion

DNA vaccine has demonstrated promising potential as an effective immunotherapeutic strategy against cancer. DNA plasmids can be easily designed and manipulated to induce potent cell-mediated immune responses. Furthermore, their ease of transportation and storage makes mass production and administration easily achievable. In addition, increasing numbers of clinical trials on various DNA vaccines against different cancers have shown promising results.

Although numerous strategies to enhance the immunogenicity and potency of DNA vaccines have already been developed and studied, future DNA vaccines should aim to further enhance antitumor immunity by circumventing immune tolerance, breaking the immunosuppressive networks in the tumor microenvironment, and inducing long lasting memory. DNA plasmids should be optimally designed to induce the strongest priming of immune responses. The activities of immunosuppressive agents in the tumor microenvironment, such as regulatory T cells and myeloid derived suppressor cells, need to be controlled. Furthermore, DNA vaccines can be used in conjunction with other cancer treatments to further control and eliminate tumors. As efforts to optimize DNA vaccines continue, we will come closer to alleviating the burden of cancer.

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