Provisional chapter

Plasmid-Based DNA Vaccines

Leonardo A. Gómez and Angel A. Oñate

Additional information is available at the end of the chapter

Abstract

Plasmids are circular deoxyribonucleic acid (DNA) vectors that can be used as vaccines to prevent various types of diseases. These plasmids are DNA platforms that are usually composed of a viral promoter gene, a gene coding resistance to antibiotics, a bacterial origin of replication gene and a multiple cloning site (MCS) for a transgenic region, where one or several genes of antigenic interest can be inserted. Immunization with these recombinant vectors allows intracellular expression of the encoded antigens by molecular and cellular machinery of transfected cells, stimulating an antigen-specific immune response. This process provides an effective protection against diverse types of pathogens, tumor cells and even allergy and autoimmune diseases. Protective efficacy is achieved by the induction of a strong humoral and cellular immune response dependent on B and T cells. The immunity induced by these DNA vaccines, added to the ease of production, administration, genetic stability, and safety, has transformed plasmid-based immunization into a safe strategy in prevention of various diseases.

Keywords: antigen, recombinant plasmids, vaccines, infectious diseases, immunotherapy

1. Introduction

Vaccination practices have made an enormous contribution to human and animal well-being, becoming one of the greatest cost-benefit achievements in global health. Since its implementation, vaccines have managed to eradicate two important diseases in humans and animals: smallpox and rinderpest, and have successfully prevented a wide variety of infectious diseases: polio, diphtheria, measles, and hepatitis, thus saving the lives of millions of people every year [1, 2].

The vaccination process consists of administering an infectious agent modified to a point where it cannot cause damage or disease but allows the induction of a specific immune response and the development of an immune memory to provide protection against agent inoculated. The same effect may be attained inoculating a part of this agent. Contact between the immune system and the infectious agent's antigens allows the stimulation of this system, activating a specific protective immune response which leads to the prevention of the disease in the vaccinated host. Successful results have been obtained using vaccines based on live or dead attenuated microorganisms (such as smallpox or yellow fever vaccines, or bacterial bacillus Calmette-Guérin strains), or vaccines composed by parts of pathogenic agents: toxoids (such as vaccines against diphtheria or tetanus), protein subunits, or polysaccharide conjugates (such as vaccines against pneumococcus, *Haemophilus influenzae* type B or meningococcus) [1–3]. Recently, it has been possible to develop DNA vaccines, also called genetic vaccines, through advances in genetics and molecular biology. This method of vaccination is based on the immunization with naked recombinant plasmids, coding one or more antigens derived from infectious agents or tumor cells, which are administered directly into the tissues, generating an antigen-specific antibody response and cell-mediated immunity, conferring protection against the antigens of interest. These recombinant plasmids can be intradermal or intramuscularly introduced or can be also nasally or orally administrated. In these tissues or anatomical regions, plasmids transfect resident cells and use the cellular machinery to express the encoded antigens, stimulating the host's immune response [4].

DNA vaccination offers a series of advantages, including their ability to stimulate the innate and adaptive immune responses. Innate immunity can be activated by recognition of the double-stranded DNA (dsDNA) of the plasmid backbone, while adaptive responses involve antigen processing and presentation in class I or class II major histocompatibility complex molecules (MHC-I or MHC-II) to $CD8^+$ and $CD4^+$ T cells, respectively. Another advantage of DNA vaccines is their safety because the plasmid DNA is stable in biological systems and avoids using whole infectious organisms. Additionally, the ease of manufacturing these vaccines on a large scale makes them more attractive vaccine candidates. These advantages make DNA vaccination an attractive and novel strategy to apply in human and veterinary medicine, capable of providing effective protection against various infectious agents of viral, bacterial, or parasitic origin. DNA immunization is also effective in eliminating tumor cells and protect against allergic and autoimmune diseases through immunotherapy [5, 6]. Furthermore, the optimization of their design, which increases immunogenicity and specificity of antigen delivery, has diversified its applications [7, 8]. Two DNA vaccines against viral diseases have been licensed for horses and fish, one against melanoma in dogs and one growth hormone releasing hormone (GHRH) product for swine [4]. Various clinical trials are being conducted for their application in humans. Promising results made DNA vaccines a biotechnological product that entered the veterinary market already, and it is hoped that soon there will be an effective and safe product for the prevention of human diseases.

2. Plasmid-based DNA vaccine design and construction

DNA vaccines are designed using expression plasmids that are safe for both humans and animals. Expression plasmids are also easily produced on a commercial scale. These vectors are characterized by containing an expression/transcription unit which allows expression of a transgene and a production unit or backbone of the plasmid (**Figure 1**) [4, 9]. Expression units are constituted by promoter/enhancer sequences which are usually of viral origin (cytomegalovirus (CMV), Rous sarcoma virus (RSV) or simian virus (SV) 40 promoters). These sequences regulate antigen expression in various target tissues (high diversity of mammalian cells). This sequence is followed by a MCS or polylinker, corresponding to a short segment containing many restriction sites (sequences that can be cut by restriction enzymes), where the transgene is inserted. Transgenes are found in regions capable of encoding multiple proteins in a single construct, an important advantage presented by DNA vaccines when compared to other platforms. Recombinant plasmids can incorporate several antigens, including sequences with adjuvant activity that increase DNA vaccine efficiency and the amplitude of induced immune responses. Finally, there is the termination sequence called poly-adenylation (poly-A), which is essential for gene expression because it stabilizes the translation of mRNA (alternatively, many vectors contain a bovine growth hormone (BGH) poly-adenylation signal). On the other hand, the production unit or backbone of the plasmid is composed of all bacterial sequences necessary for plasmid amplification and selection. That is, they have

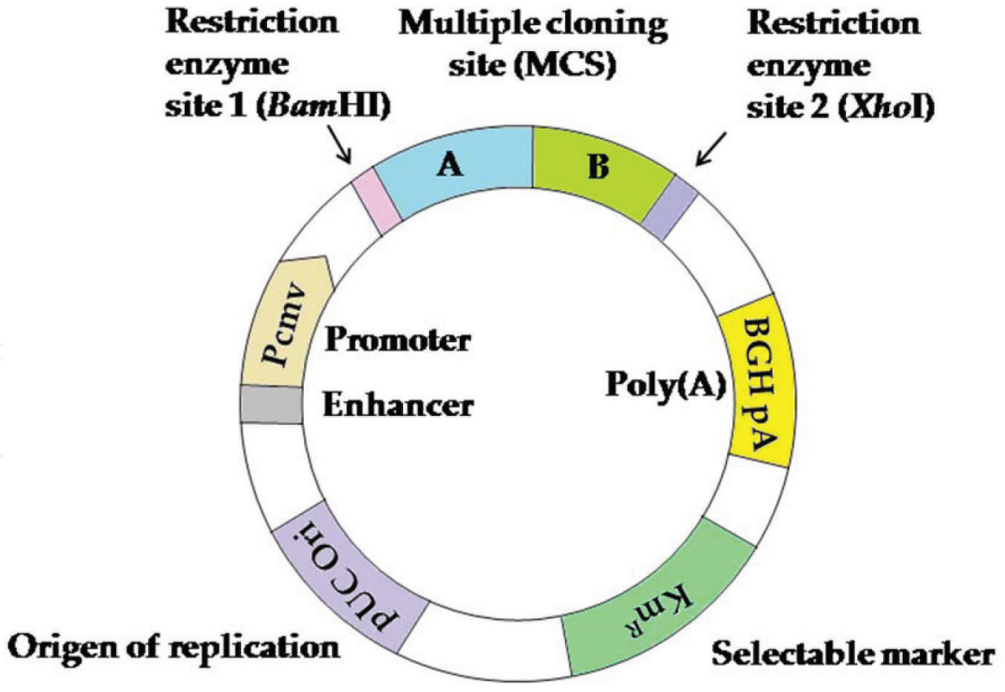

Figure 1. Hypothetical structure of a plasmid-based DNA vaccine encoding of A-B fusion protein. Design of this plasmid is based on Kutzler & Weiner [4] and the commercial pVAX1 vector (Invitrogen, Thermo fisher scientific).

a bacterial replication origin, which usually correspond to a replication origin of *Escherichia coli*, the main bacterial species used for plasmid amplification; antibiotic resistance genes (e.g., resistance to Kanamycin, Km^R) used for the selection of bacteria transformed with recombinant plasmids in culture media with antibiotic (**Figure 1**). In addition, recombinant plasmid replication may also have a replication origin of mammalian, which facilitates replication in animal cells, prolonging the antigen persistence and expression in host cells [9, 10]. Examples of available commercial plasmids approved for clinical use include pVAX1 and pcDNA3.1 vectors (Invitrogen, Thermo Fisher Scientific).

Design of antigenic gene is fundamental to optimize the expression and induction of protective immune response. This design usually incorporates codon optimization to minimize the presence of rare codons and to reduce the formation of secondary structures in the mRNA sequences, preventing translation process inhibition of antigenic proteins. In addition, expression of antigens in transfected eukaryotic cells can be optimized by adding a Kozak consensus sequence responsible for mRNA recognition by eukaryotic ribosomes. Another fundamental variable for cloning antigenic sequences in the plasmid requires that the 5′ and 3′ ends of these sequences possess sites for restriction enzymes (**Figures 1** and **2**). DNA vaccines versatility allows the incorporation of sequences encoding one or several antigens, as well as immunedominant epitopes for MHC-I and MHC-II molecules, which enhances antigen recognition and adaptive immunity activation. The efficiency of DNA vaccines can also be improved if

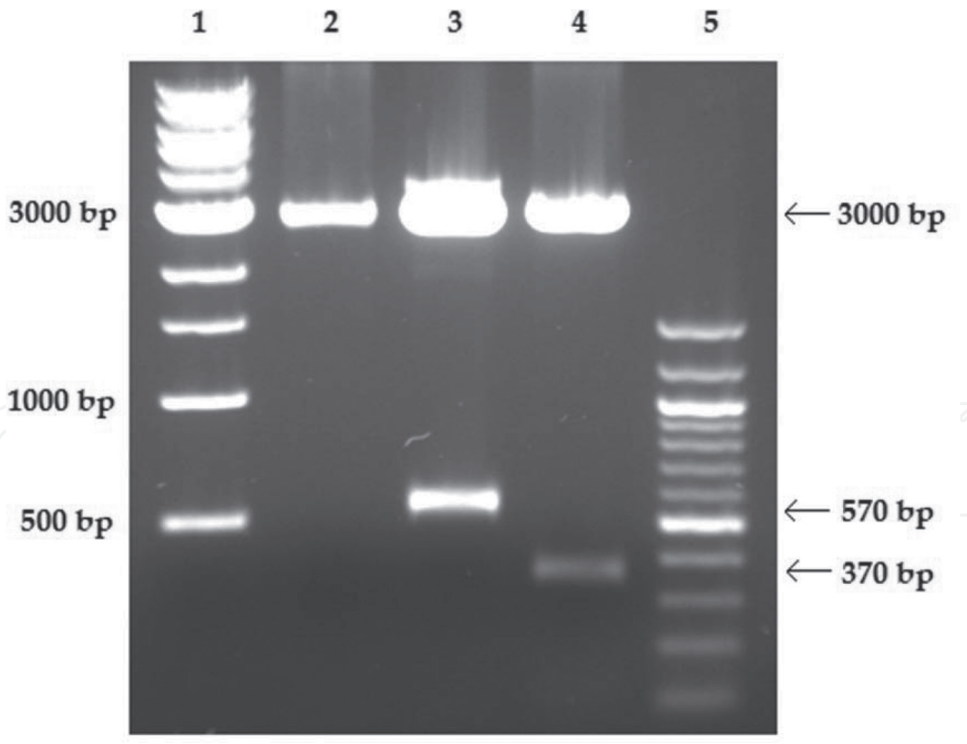

Figure 2. Gel electrophoresis of two DNA vaccines based on the pVAX1 commercial plasmid. These vaccines are digested with *Bam*HI and *Xho*I restriction enzymes. Lane 1: Molecular weight marker (1 kb); lane 2: pVAX1 (3000 bp); lane 3: pVAX1 encoding of a gene (570 bp); lane 4: pVAX1 encoding of B gene (370 bp); lane 5: molecular weight marker (100 bp) [17].

Qualities	Description
Immunogenicity	DNA vaccines have the ability to induce a specific humoral immune response associated to antibody production and a cellular immune response associated to CD4 and CD8 T cells against antigens encoded in the plasmids.
Administration	Intramuscular, electroporation, gene gun, ultrasound, transcutaneous micro-needle, skin abrasion, tattoo perforating needle, jet-injector, or topical patch.
Safety	DNA vaccines are safe since they can revert to virulent forms, due to the absence of pathogens. In addition, several early clinical trials have proved their safety, being well tolerated in humans. Adaptive immune responses against the plasmid do not occur.
Adjuvanticity	Double-stranded DNA is recognized by intracellular sensors such as TLR9, AIM2, STING and TBK1, which activate signaling cascades required for the activation of innate and adaptive immunity.
Stability	They are more resilient to temperature.
Economy	Rapid production and formulation, being highly cost-effective.
Adaptability	DNA vaccines can encode one or more antigens (fusion proteins) from one or more pathogens or tumor cells. In addition, they can code multi-epitopes.
Storage and mobility	Neither requires cold chains, nor special transport conditions.

Table 1. Advantages of plasmid-based DNA vaccines.

co-stimulatory molecules (cytokines, chemokines, or ligands for toll-like receptors (TLR), such as sequences rich in unmethylated cytosine-phosphate-guanine (CpG) [TLR9 ligand] or double-stranded RNA [TLR3 ligand]) are included in the vaccine plasmid [9–16].

After recombinant plasmids are designed and constructed, they are introduced into bacteria using electroporation (electric pulses) or chemical transformation (calcium chloride) methods. Transformed bacteria, usually *E. coli*, are cultured until reaching their logarithmic growth phase, allowing the production of multiple copies of the recombinant plasmid. Subsequently, the plasmids are extracted from these bacteria, avoiding contamination with lipopolysaccharide (LPS), a component of the *E. coli* outer membrane, which is pro-inflammatory and whose administration can produce adverse reactions in individuals vaccinated with this DNA [18]. DNA concentrations obtained are adjusted in physiological saline or phosphate buffered saline (PBS) and stored. Because DNA is a stable molecule, it does not require the use of cold chains, facilitating easier storage and distribution. These are additional advantages of DNA vaccines, which are described in **Table 1**.

3. Cellular mechanisms induced by plasmid immunization

Cellular mechanisms which generate protective immunity against antigenic proteins through immunization with DNA vaccines are being elucidated. Following intradermal, subcutaneous, intravenous, oral, intranasal, or intramuscular plasmid administration, the plasmids transfect resident cells in these tissues or anatomical regions, which are mainly professional

antigen presenting cells (APCs, which include dendritic cells, macrophages, and B cells) but also non-APCs. Antigens encoded in the recombinant plasmids are expressed by host cellular machinery, inducing an antigen-specific immune response. It has been demonstrated that plasmids administered orally are transfected by the intestinal epithelial cells (IECs), while in intradermal or subcutaneous administration, plasmids target are skin keratinocytes, fibroblasts and Langerhans cells. Langerhans cells are main APCs of skin, which participate in antigen internalization and migrate to lymph nodes, where they present the antigens to T and B cells. This dermal administration route usually produces a humoral immune response with the production of immunoglobulin A (IgA) and G1 (IgG1). On the other hand, in intramuscular administration, the main immunization routes with DNA are myocytes and APCs, which capture the recombinant plasmids. This route of administration usually induces a cellular response with the activation of cytotoxic $CD8^+$ T and $CD4^+$ T helper type 1 cells [19, 20].

Inside transfected cells, genes encoded in the plasmids are transcribed to mRNA and then translated into proteins. These proteins are processed as peptides by the ubiquitin/proteasomes system and transported by TAP molecules to the endoplasmic reticulum (ER), where they are assembled into MHC class I molecules. MHC-I/peptides complexes are presented on cell surfaces of APCs or non-APCs for recognition by $CD8^+$ T cells. In addition, many of these proteins can be released from transfected cells, being captured, endocytosed and presented by MHC class II molecules expressed by APCs to T $CD4^+$ cells. In parallel, antigen-loaded APCs travel to the lymph nodes where they present MHC/peptides complexes to T naive cells. Soon thereafter, they activate, expand and differentiate $CD4^+$ and $CD8^+$ T cells to various effector phenotypes. In this microenvironment, T cell activation promotes cytokine secretion, along with the release of soluble antigens, activating and differentiating B cells toward plasma cells that produce antigen-specific antibodies. Furthermore, the expression of antigens bound to MHC-I by transfected myocytes activates the cytotoxic functions of $CD8^+$ T cells, causing the release of more antigens [4, 7, 16].

Prior to the activation of the aforementioned adaptive immunity, immunizations with these recombinant plasmids induce the activation of innate immunity. This activation occurs because plasmids are elements of dsDNA of bacterial origin that acts as pathogen-associated molecular pattern molecules (PAMPs), which can be recognized by pattern recognition receptors (PRRs) such as Toll-9 type receptors (TLR9). TLR9 is a receptor which is highly expressed in APCs endosomes. Recognition of plasmids by TLR9 triggers signaling by myeloid differentiation factor 88 (MyD88). This factor, in turn, induces the activation of the interleukin-1 receptor-associated kinase (IRAK) and the tumor necrosis factor receptor-associated factor (TRAF), which activate mitogen-activated protein kinases (MAPKs) and the nuclear factor (NF)-κB (NF-κB). The latter are the elements responsible for the transcription of IFN type I and various pro-inflammatory cytokines which promote cell recruitment, giving way to adaptive immunity activation (activation of T and B cells). In addition, other intracellular sensors for the dsDNA have been reported: stimulator of IFN genes (STING), TANK binding kinase 1 (TBK1) and absent in melanoma 2 (AIM2) proteins. Signaling by STING/TBK1 directs the phosphorylation of interferon regulatory factors (IRF) 3 and 7, activating IFN type I production, while AIM2 receptor activates the inflammasome and the release of biologically active interleukin-1β (IL-1β) [9, 11–13]. These receptors recognize the plasmid backbone, which has

an adjuvant effect that induces the production of IFN type I, which is critical for the induction of an innate and adaptive immune response.

Therefore, understanding the intracellular recognition of plasmid DNA and the identification of its receptors has allowed for improving the effectiveness of immune response induced by DNA vaccines. Furthermore, the flexibility of these vaccines allows them to be administered in conjunction with co-stimulatory molecules, cytokines, chemokines, or ligands for intracellular receptors such as TLRs, for instance, the CpG (TLR9) and double-stranded RNA (TLR3) motifs, or the intracellular receptors AIM2, SINTG, or TBK1, whose signaling cascades promote the activation of innate immunity, giving way to adaptive immunity activation. This knowledge, together with the improvements in the targeting of the plasmids to the appropriate APCs, the strategies of 'Prime-Boost' (immunization of DNA followed by protein antigen), and the methods of administration (**Table 1**), will allow to improve the immunogenicity of these vaccines, protecting the host against the challenges represented by diseases caused by pathogens and tumor cells.

4. DNA vaccines used to prevent infectious diseases

Vaccination has helped control the spread of many infectious diseases: polio, diphtheria, measles, hepatitis B, mumps, whooping cough, pneumonia, rotavirus diarrhea, rubella, and tetanus [21]. Protection conferred by vaccination has managed to prevent diseases, disabilities, and the death of millions of people each year. Although the implementation of immunization plans has been very successful in various regions of the world, there are still enormous challenges in the field of vaccinology. Because, in each phylogenetic group (virus, bacteria, or parasites), there are numerous pathogens capable of producing high mortality rates, for example, human immunodeficiency virus (HIV/causal agent of acquired immune deficiency syndrome [AIDS]), *Mycobacterium tuberculosis* (tuberculosis) and the protozoan *Plasmodium* (malaria), alone are capable of causing the death of approximately 4 million people each year in the world [22]. Currently, there are no effective vaccines against many pathogenic microorganisms, and therefore, the diseases produced by them can be disseminated directly or indirectly from one individual to another, producing outbreaks and epidemics with high mortality rates in several regions in the world.

In the search for new strategies to prevent infectious diseases, immunization with plasmid-based DNA vaccines was introduced in the clinical field at the beginning of the nineties. Several DNA vaccines have been developed to fight against viral, bacterial, and parasitic diseases. The DNA vaccination against viruses, obligate intracellular pathogens and highly specialized in sequestering molecular mechanisms of their host cells in order to replicate themselves, has been evaluated. These vaccines were shown to be able to induce an antibody response against several pathogens: herpes simplex, hepatitis B, HIV, and influenza [23–26]. However, to successfully eliminate infection by many of these pathogens, coordination of multiple effector mechanisms of innate immunity and adaptive immunity is required. These defensive mechanisms involve viral neutralization by antibodies produced by plasma cells but also involve the cytolytic activity (perforin/granzyme, Fas ligand, and tumor necrosis factor α [TNF-α]) of

$CD8^+$ T cells for the elimination of infected cells and the production of IFN-γ to inhibit viral replication [27]. These DNA vaccines have also been tested as immune therapy for human papilloma virus, hepatitis C virus, Rabies virus, Filovirus, Flavivirus, and Bunyavirus [28–33]. These preclinical and clinical trials have shown the efficacy of DNA vaccines against various viral pathogens, being safe and well tolerated in humans. Success achieved through immunization with DNA vaccines has allowed the licensing of two vaccines to prevent diseases caused by viruses. These vaccines correspond to West Nile Innovator products developed by the Center for Disease Control and Prevention and the Fort Dodge Laboratories (USA, 2005), to protect horses from the West Nile virus and the Apex-IHN vaccine produced by Novartis (Canada, 2005) to protect salmon from the infectious hematopoietic necrosis virus [4].

With regard to the prevention of bacterial diseases, only a limited number of vaccines are available against a small number of pathogens. In addition, most of these vaccines do not confer complete protection against these pathogens. Vaccine designs depend on the bacterial pathogen lifestyle, which requires that immunization induce a specific type of immune response. Infections by intracellular bacteria are predominantly controlled by a cellular response dependent on macrophages, natural killer (NK) cells, Th1 type $CD4^+$ T cells, and cytotoxic $CD8^+$ T cells. Infection control of extracellular bacteria requires neutralization of these pathogens, activating a humoral response dependent on the complement system, B cells and plasma cells which produce antibodies [27]. Bacterial complexity requires the development of specific humoral and cellular immune response against different structural proteins, toxins, or capsular sugars. The relevance of microbial antigenic epitopes to obtain an effective response is the key to progress in the development of DNA vaccines. Therefore, DNA vaccines are good candidates for the prophylaxis of intracellular and extracellular pathogens due to their ability to induce humoral and cellular immune responses. Their efficacy has been evaluated against intra- and extracellular bacteria such as *Brucella abortus*, *Vibrio anguillarum*, *Edwardsiella tarda*, *Helicobacter pylori,* or *Mycobacterium tuberculosis* [34–39].

The development of DNA vaccines to fight against parasitic diseases is an emerging field. Nevertheless, numerous challenges are involved including identification of suitable antigens due to the complexity of parasite life cycles and their antigenic variability. Some parasites such as *Plasmodium* and *Giardia* have the ability to vary their antigens during certain stages of development, while others such as *Plasmodium*, *Leishmania,* or *Toxoplasma* have developed various mechanisms to escape surveillance of the host's immune system [40]. Nevertheless, to prevent disease by these pathogens, DNA vaccines are a platform that allows integrating various antigens present in different life-cycle stages, or antigens of different subspecies of the parasite, simultaneously. This property of DNA vaccines is essential for the design of effective vaccinations against diseases such as trypanosomiasis (variety of *Trypanosoma cruzi* subspecies), malaria (*Plasmodium spp.*), leishmaniasis (*Leishmania*), or schistosomiasis (*Schistosoma*), which present different life-cycle stages inside the host and kill millions of people every year [41–44].

5. DNA vaccines against tumor cells

Cancer is one of the leading causes of death in the world. Finding effective therapies to combat cancer has been one of the main objectives, since standard treatments such as surgery,

radiation, and chemotherapy have had limited success. These therapies are usually effective in early stages but rarely effective in the late stages. Tumor cells may lose the capacity to stimulate or be detected by immune system cells, since they acquire phenotypic modifications. These modifications include the loss of the expression of MHC class I and/or class II molecules, or their ability to process and present antigens due to modifications in the exogenous and endogenous pathways which activate $CD4^+$ and $CD8^+$ T cells, respectively [9]. In addition, tumor cells exhibit a great heterogeneity of mechanisms to evade immune responses, including the recruitment of regulatory cells (regulatory T cells, myeloid-derived suppression cells, and type 2 macrophages), production of suppressors [interleukin-10 (IL-10), and transforming growth factor-β (TGF-β)], and the expression of inhibitory molecules [cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), lymphocyte-activation gene (LAG-3), and programmed cell death-1 (PD-1)], which leads to T cell suppression [45–50]. This immune tolerance induced by tumor cells successfully manages to evade host immune responses, which represents a challenge, but at the same time, their understanding is a path to the development of effective immunotherapy against cancer.

The ability of the immune system to distinguish between normal and malignant cells is essential for the development of effective immunotherapy. The main cells that play a key role in the elimination of tumor cells are innate immunity cells such as NK cells, natural killer T (NKT) cells, macrophages, dendritic cells, and adaptive immunity cells such as helper type 1 $CD4^+$ T cells and $CD8^+$ T cells [27]. Tumor cells express a variety of antigens with potential to produce a tumor-specific immune response. Application of DNA vaccines as a new and novel therapeutic strategy to combat tumor cells has arisen from this property. These vaccines have been developed thanks to the identification of tumor-associated antigens (TAA). These TAAs are expressed in tumor tissues under the control of oncogenes or have been differentiated during cancer development. Many of these antigens are shared among tumors, while others are unique to each tumor [51]. Because some of the TAA are expressed in normal tissues, they hinder the direction of the immune response induced by vaccines, and can generate adverse side effects associated with autoimmune sequelae.

Since the effector responses of T cells against several of these TAAs can be diminished by central tolerance, which reduces the ability to kill tumor cells due to the preexisting tumor suppressor microenvironment, some of these DNA vaccines are designed to express tumor antigens which are fused to co-stimulator and/or cytokine [granulocyte macrophage colony stimulating factor (GM-CSF) or interleukin-2 (IL-2)] proteins, for the recruitment and activation of dendritic cells [14–16, 51]. Therefore, cancer DNA vaccines combine the best tumor antigens with the most effective immunotherapeutic agents. In addition, the antigen choice involves characteristics associated with therapeutic function, immunogenicity, antigen roles in tumors, specificity, expression level and percentage of antigen-positive cells, stem cell expression, number of patients with cancers with positive antigen, number of antigenic epitopes, and cellular localization of antigen expression [52]. These efforts have represented the logical steps for the development of DNA vaccines against cancer, and whose advances have allowed the development of numerous preclinical and clinical trials (phases I and II) against various types of cancer: lymphomas, melanomas, cervical, breast, kidney, and prostate [51, 52]. The success of these cancer DNA vaccines is reflected by the Canine Melanoma Vaccine, product developed by Merial, Memorial Sloan-Kettering Cancer Center and The New York Animal Medical Center (USA, 2007), a licensed DNA vaccine used to protect dogs from melanoma [4].

6. Conclusions

Plasmid-based DNA vaccines are a novel, economic, and effective strategy which induces antigen-specific immunity capable of conferring effective protection against various infectious diseases and tumor cells. Its applications are diverse because plasmids are versatile platforms in which one or several antigens can be incorporated, that allow inducing an innate and adaptive humoral and cellular-type immune response. In addition, their handling, design, and construction are relatively easy to perform. The success of these vaccines has been demonstrated by the number of clinical trials conducted in humans, and by DNA vaccines already licensed in the field of infectious diseases and cancer immunotherapy in the veterinary field. Although there are still many challenges in developing a vaccine for humans, the improvements in design, methods of DNA administration and delivery, associated with new technology, bring us closer to achieving this goal every day. Finally, although immunization with DNA is a successful strategy, its advantages must be evaluated case by case and its applicability depends on the nature of the agent to be immunized, the nature of the antigen and the type of immune response required to achieve effective protection.

Acknowledgements

This work was supported by grant 1180122 from the Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT), Santiago, Chile and grant VRID 217.036.046-1.0 Universidad de Concepción.

Conflicts of interest

Authors did not have any conflicts of interest.

Author details

Leonardo A. Gómez and Angel A. Oñate*

*Address all correspondence to: aonate@udec.cl

Department of Microbiology, Laboratory of Molecular Immunology, Faculty of Biological Sciences, University of Concepción, Concepción, Chile

References

[1] Greenwood B. The contribution of vaccination to global health: Past, present and future. Philosophical Transactions of the Royal Society of London, series B, Biological Sciences. 2014;**369**:1-9

[2] Nabel G. Designing tomorrow's vaccines. The New England Journal of Medicine. 2013; **368**:551-560

[3] Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nature Immunology. 2011;**12**:509-517

[4] Kutzler M, Weiner D. DNA vaccines: Ready for prime time? Nature Reviews Genetics. 2008;**9**:776-788

[5] Coban C, Koyama S, Takeshita F, Akira S, Ishii K. Molecular and cellular mechanisms of DNA vaccines. Human Vaccines. 2008;**4**:453-456

[6] Bona A, Bot A. Genetic Immunization. NY: Kluwer Academic/Plenum Publishers; 2000. p. 179

[7] Iurescia S, Fioretti D, Rinaldi M. Strategies for improving DNA vaccine performance. In: Rinaldi M, Fioretti D, Iurescia S, editors. DNA Vaccines: Methods and Protocols. Methods in Molecular Biology. 3rd ed. Vol. 1143. NY: Humana Press; 2014. pp. 21-31

[8] Coban C, Kobiyama K, Aoshi T, et al. Novel strategies to improve DNA vaccine immunogenicity. Current Gene Therapy. 2011;**11**:479-484

[9] Ingolotti M, Kawalekar O, Shedlock D, Muthumani K, Weiner D. DNA vaccines for targeting bacterial infections. Expert Review of Vaccines. 2010;**9**:747-763

[10] Montgomery D, Prather K. Design of plasmid DNA constructs for vaccines. In: Saltzman W, Shen H, Brandsma J, editors. DNA Vaccines: Methods and Protocols. Methods in Molecular Medicine, 2nd ed. NJ: Humana Press; 2006;**127**:11-22

[11] Desmet C, Ishii K. Nucleic acid sensing at the interface between innate and adaptive immunity in vaccination. Nature Reviews Immunology. 2012;**12**:479-491

[12] Kobiyama K, Jounai N, Aoshi T, Tozuka M, Takeshita F, Coban C, Ishii K. Innate immune signaling by, and genetic adjuvants for DNA vaccination. Vaccine. 2013;**1**:278-292

[13] Li L, Saade F, Petrovsky N. The future of human DNA vaccines. Journal of Biotechnology. 2012;**162**:171-182

[14] Melkebeek V, Van den Broeck W, Verdonck F, Goddeeris BM, Cox E. Effect of plasmid DNA encoding the porcine granulocyte-macrophage colony-stimulating factor on antigen-presenting cells in pigs. Veterinary Immunology and Immunopathology. 2008;**125**: 354-360

[15] Barouch D, McKay P, Sumida S, Santra S, Jackson S, Gorgone D, Lifton M, Chakrabarti B, Xu L, Nabel G, Letvin N. Plasmid chemokines and colony-stimulating factors enhance the immunogenicity of DNA priming-viral vector boosting human immunodeficiency virus type 1 vaccines. Journal of Virology. 2003;**77**:8729-8735

[16] Laddy D, Weiner D. From plasmids to protection: A review of DNA vaccines against infectious diseases. International Reviews of Immunology. 2006;**25**:99-123

[17] Gómez L. Evaluación de vacunas de ADN basadas en los marcos de lectura abiertos BAB1_0267 y BAB1_0270 de *Brucella abortus* 2308 en un modelo murino [thesis]. Concepción: University of Concepción; 2016

[18] Van Amersfoort E, Van Berkel T, Kuiper J. Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clinical Microbiology Reviews. 2003;**16**:379-414

[19] Akbari O, Panjwani N, Garcia S, Tascon R, Lowrie D, Stockinger B. DNA vaccination: Transfection and activation of dendritic cells as key events for immunity. Journal of Experimental Medicine. 1999;**189**:169-178

[20] Shedlock D, Weiner D. DNA vaccination: Antigen presentation and the induction of immunity. Journal of Leukocyte Biology. 2000;**68**:793-806

[21] Duclos P, Okwo-Bele JM, Gacic-Dobo M, Cherian T. Global immunization: Status, progress, challenges and future. BMC International Health and Human Rights. 2009;**9**:1-11

[22] Murray C, Ortblad K, Guinovart C, Lim S, Wolock T, et al. Global, regional, and national incidence and mortality for HIV, tuberculosis, and malaria during 1990-2013: A systematic analysis for the global burden of disease study 2013. The Lancet. 2014;**384**:1005-1070

[23] Strasser J, Arnold R, Pachuk C, Higgins T, Bernstein D. Herpes simplex virus DNA vaccine efficacy: Effect of glycoprotein D plasmid constructs. The Journal of Infectious Diseases. 2000;**182**:1304-1310

[24] Kwissa M et al. Efficient vaccination by intradermal or intramuscular inoculation of plasmid DNA expressing hepatitis B surface antigen under desmin promoter/enhancer control. Vaccine. 2000;**18**:2337-2344

[25] Ulmer J, Wahren B, Liu M. DNA vaccines for HIV/AIDS. Current Opinion in HIV and AIDS. 2006;**1**:309-313

[26] Laddy D, Yan J, Kutzler M, Kobasa D, Kobinger G, Khan A, Greenhouse J, Sardesai N, Draghia-Akli R, Weiner D. Heterosubtypic protection against pathogenic human and avian influenza viruses via in vivo electroporation of synthetic consensus DNA antigens. PLoS One. 2008;**3**:e2517

[27] Abbas A, Lichtman A, Pillai S. Cellular and Molecular Immunology. 8th ed. PA: Saunders/Elsevier; 2014. p. 544

[28] Cheng M, Farmer E, Huang C, Lin J, Hung C, Wu T. Therapeutic DNA vaccines for human papillomavirus and associated diseases. Human Gene Therapy. 2018:1-78

[29] Lee H, Jeong M, Oh J, Cho Y, Shen X, Stone J, Yan J, Rothkopf Z, Khan A, Cho B, Park Y, Weiner D, Son W, Maslow J. Preclinical evaluation of multi antigenic HCV DNA vaccine for the prevention of hepatitis C virus infection. Scientific Reports. 2017;**7**:43531

[30] Xiang Z, Spitalnik S, Tran M, Wunner W, Cheng J, Ertl H. Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus. Virology. 1994;**199**:132-140

[31] Grant-Klein R, Altamura L, Badger C, Bounds C, Van Deusen N, Kwilas S, Vu H, Warfield K, Hooper J, Hannaman D, Dupuy L, Schmaljohn C. Codon-optimized filovirus DNA vaccines delivered by intramuscular electroporation protect cynomolgus macaques from

lethal Ebola and Marburg virus challenges. Human Vaccines and Immunotherapeutics. 2015;**11**:1991-2004

[32] Chang G, Davis B, Hunt A, Holmes D, Kuno G. Flavivirus DNA vaccines: Current status and potential. Annals of the New York Academy of Sciences. 2001;**951**:272-285

[33] Spik K, Shurtleff A, McElroy A, Guttieri M, Hooper J, Schmaljohn C. Immunogenicity of combination DNA vaccines for Rift Valley fever virus, tick-borne encephalitis virus, Hantaan virus, and Crimean Congo hemorrhagic fever virus. Vaccine. 2006;**24**(21):4657-4666

[34] Oñate A, Céspedes S, Cabrera A, Rivers R, González A, Muñoz C, Folch H, Andrews E. A DNA vaccine encoding Cu, Zn superoxide dismutase of *Brucella abortus* induces protective immunity in BALB/c mice. Infection and Immunity. 2003;**71**:4857-4861

[35] Yang H, Chen J, Yang G, Zhang XH, Liu R, Xue X. Protection of Japanese flounder (*Paralichthys olivaceus*) against *Vibrio anguillarum* with a DNA vaccine containing the mutated zinc-metalloprotease gene. Vaccine. 2009;**27**:2150-2155

[36] Jiao X, Zhang M, Hu Y, Sun L. Construction and evaluation of DNA vaccines encoding *Edwardsiella tarda* antigens. Vaccine. 2009;**27**:5195-5202

[37] Hatzifoti C, Roussel Y, Harris A, Wren B, Morrow J, Bajaj-Elliott M. Mucosal immunization with a urease B DNA vaccine induces innate and cellular immune responses against *Helicobacter pylori*. Helicobacter. 2006;**11**:113-122

[38] Bruffaerts N, Huygen K, Romano M. DNA vaccines against tuberculosis. Expert Opinion on Biological Therapy. 2014;**14**:1801-1813

[39] Romano M, Rindi L, Korf H, Bonanni D, Adnet P, Jurion F, Garzelli C, Huygen K. Immunogenicity and protective efficacy of tuberculosis subunit vaccines expressing PPE44 (Rv2770c). Vaccine. 2008;**26**:6053-6063

[40] Carvalho J, Rodgers J, Atouguia J, Prazeres D, Monteiro G. DNA vaccines: A rational design against parasitic diseases. Expert Review of Vaccines. 2010;**9**:175-191

[41] Arce-Fonseca M, Rios-Castro M, Carrillo-Sánchez S, Martinez-Cruz M, Rodríguez-Morales O. Prophylactic and therapeutic DNA vaccines against Chagas disease. Parasites & Vectors. 2015;**8**:1-7

[42] Le T, Coonan K, Hedstrom R, Charoenvit Y, Sedegah M, Epstein J, Kumar S, Wang R, Doolan D, Maguire J, Parker S, Hobart P, Norman J, Hoffman S. Safety, tolerability and humoral immune responses after intramuscular administration of a malaria DNA vaccine to healthy adult volunteers. Vaccine. 2000;**18**:1893-1901

[43] Masih S, Arora S, Vasishta R. Efficacy of Leishmania donovani ribosomal P1 gene as DNA vaccine in experimental visceral leishmaniasis. Experimental Parasitology. 2011;**129**: 55-64

[44] Tebeje B, Harvie M, You H, Loukas A, McManus D. Schistosomiasis vaccines: where do we stand? Parasites & Vectors. 2016;**9**:1-15

[45] Yang B, Jeang J, Yang A, Wu T, Hung C. DNA vaccine for cancer immunotherapy. Human Vaccines and Immunotherapeutics. 2014;**10**:3153-3164

[46] Santarpia M, Karachaliou N. Tumor immune microenvironment characterization and response to anti-PD-1 therapy. Cancer Biology and Medicine. 2015;**12**:74-78

[47] Sukari A, Nagasaka M, Al-Hadidi A, Lum L. Cancer immunology and immunotherapy. Anticancer Research. 2016;**36**:5593-5606

[48] Vinay D, Ryan E, Pawelec G, Talib W, Stagg J, et al. Immune evasion in cancer: Mechanistic basis and therapeutic strategies. Seminars in Cancer Biology. 2015;**35**:185-198

[49] Beatty G, Gladney W. Immune escape mechanisms as a guide for cancer immunotherapy. Clinical Cancer Research. 2015;**21**(4):687-692

[50] Colluru V, Johnson L, Olson B, McNeel D. Preclinical and clinical development of DNA vaccines for prostate cancer. Urologic Oncology. 2016;**34**:193-204

[51] Fioretti D, Iurescia S, Fazio V, Rinaldi M. DNA vaccines: Developing new strategies against cancer. Journal of Biomedicine and Biotechnology. 2010;**174378**:1-16

[52] Rice J, Ottensmeier C, Stevenson F. DNA vaccines: Precision tools for activating effective immunity against cancer. Nature Reviews Cancer. 2008;**8**:108-120