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DNA Vaccination by Electroporation

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1. Introduction

Vaccination: Traditional and new generation vaccines

Vaccination is historically one of the most important methods for the prevention of infectious diseases in humans and animals. When Edward Jenner inoculated James Phipps with a bovine poxvirus to induce protection against the closely related human pathogen smallpox virus in 1796 and then, almost a century later, Pasteur developed a live attenuated vaccine against rabies, the basic principles for vaccine development were established (Fraser and Rappuoli 2005). Traditionally, a vaccine is known as a preparation of attenuated or killed microorganisms or of subunit vaccines (purified components of a pathogen including the protein-conjugated capsular polysaccharides, toxoids, cell-free extracts, recombinant proteins and stand-alone capsular polysaccharides) administered for inducing active immunity to a specific disease.

Two types of immunization exist with intrinsic differences between them: prophylactic vaccination initiates a response against an antigen to which the immune response is naïve, leading to a long-term memory cell maintenance and protective efficacy; therapeutic vaccination stimulates the immune system to a chronically displayed antigen, leading to a clearance of an established infection.

Several infectious diseases can be prevented by vaccines produced with conventional approaches. These methods are based on the cultivation in laboratory conditions of the microorganism from which single components are isolated individually by using biochemical, microbiological and serological techniques. Each antigen is produced in pure form either directly from the bacterium or using the DNA recombinant technology, and finally tested for its ability to induce an immune response (Serruto and Rappuoli 2006).

Conventional approaches provided the basis of vaccinology and led to great achievements such as the eradication of smallpox and the virtual disappearance of diseases like diphtheria, tetanus, poliomyelitis, pertussis, measles, mumps, rubella and invasive *Haemophilus influenzae* B, increasing the life quality and expectancy (Andre 2003). Nevertheless, they present major disadvantages such as to be time-consuming and, more important, to be impractical in some circumstances due to the difficulty in cultivating some microorganisms *in vitro* and to the fact that even attenuation may result in detrimental or unwanted immune responses (Purcell et al.

2007). Moreover, in many cases the antigens expressed during infection are not produced in laboratory conditions, as well as the proteins that are most abundant and easily purified are not necessarily protective antigens and, in any case, only few molecules can be isolated and tested simultaneously (Serruto and Rappuoli 2006).

The last decade has witnessed a revolution in the approach to vaccine design and development. These advances include new delivery technologies aimed at improving the safety and immunogenicity of traditional vaccines, new strategies to identify protective antigens, generation of improved adjuvants.

Considering that new diseases are sure to emerge through evolution by mutation and gene exchange, interspecies transfer or human exposure to novel environments, more reliable approaches must be available to promptly respond to those threats (Plotkin 2005). Thanks to sophisticated technologies such as genomics, proteomics, functional genomics and synthetic chemistry, the rational identification of antigens, the synthesis of complex glycans, the generation of engineered carrier proteins are possible. This leads to identify, generate and test new vaccines, to use not only against infectious diseases but also in the treatment of autoimmune disorders, allergies, chronic inflammatory diseases and cancer. There are several vaccine modalities currently under investigation, including subunit vaccines, syntetic peptide vaccines, ex-vivo loaded dendritic cells (DCs) and genetic vaccination, that will be here discussed.

1.1 Subunit vaccines

Subunit vaccines have improved conventional attenuated or killed vaccines in many aspects, including safety and production. The systems mostly used to produce these vaccines are based on bacteria, yeast, insect or mammalian cells. However, production of recombinant vaccine proteins in these expression systems is expensive in many cases, requiring large scale fermenters and stringent purification protocols. Worldwide, only a small number of facilities exists with capabilities to produce kilograms of a specific protein to be used as immunogen, and the construction, validation, and final approval of new production facilities take many years implying important investments in capital and human resources. Additionally, some antigens require post-translational modifications that cannot be achieved using all expression systems. In the last decade, non-fermentative alternatives based on living organisms have been developed to solve such problems and provide low-cost technologies for vaccine production. Insects and plants have been adapted for subunit vaccine production with clear advantages to conventional fermentative systems, especially in terms of time of development, scaling-up production and cost-efficiency (Brun et al. 2011). Despite the improvements in the recombinant technology, these vaccines remain hard to produce due to their inherent toxicity for the bacterial/viral expression system (e.g., Human Papilloma Virus type 16-E2, wild-type p53) (van der Burg et al. 2006).

1.2 Syntetic peptide vaccines

Identification of individual epitopes within protective proteins allows the development of peptide vaccines as alternative approach respect to using a whole protein as a vaccine. Selected peptide epitopes represent the minimal immunogenic region of a protein antigen and allow for precise direction of immune responses aiming at the induction of T-cell immunity. A peptide vaccine should ideally include epitopes recognized both by B and T cells, and take into account the MHC restriction of the T-cell response. In some cases B and

T-cell epitopes can overlap substantially within the sequence of an antigen and, in others, they might be present in separate discrete regions of the antigen or present in different antigens from the targeted pathogen. The simplicity of producing clinical grade peptides allows swift changes in the design of peptide vaccines and, therefore, rapid translation of new immunological concepts, which represent a great advantage for the development of vaccines against rapidly changing viruses such as influenza (Brun et al. 2011). Despite the potential advantages of this approach, the development of successful peptide vaccines has been limited mainly by difficulties associated with stability, poor immunogenicity of simple peptides and by the MHC polymorphism of the host species (Tam 1996).

1.3 Ex-vivo loaded dendritic cells

Antigen Presenting Cells-based vaccines represent another explored field in vaccine research. With this approach, DC are harvested from the patient, pulsed with antigens or transfected with genes encoding these antigens, and readministered to the patient. This vaccine strategy has the potential to augment presentation through the MHC-class I pathway and subsequently drive the expansion of tumour-specific CTLs. In translational studies with melanoma patients, DC vaccines have demonstrated a keen ability to elicit detectable immune responses. However, such responses often fail to elicit substantial clinical responses. As it is often difficult to discern the relative contributions of DCs and effector T cells in these situations, a thorough investigation of the *in vivo* interactions between these immune cell populations may be required before a complete understanding of DC role (Palucka et al. 2007).

1.4 Genetic vaccination

Recently, new methods of vaccination such as those based on gene transfer have emerged. Genetic vaccination originates from gene therapy. The objective of genetic vaccination is to transfer in the host a gene encoding for the disease target antigen with the aim to induce a specific immune response, whereas the goal of gene therapy is to ensure production of a protein which is lacking or defective in the host. To date, the vast majority of gene therapy clinical trials have addressed cancer (66.5%), cardiovascular diseases (9.1%) and infectious diseases (6.5%). For infectious diseases, a total of 85 gene therapy trials have been carried out, the majority of these trials being performed on human immunodeficiency virus infection, tetanus, cytomegalovirus and adenovirus infections (Chiarella et al. 2008a).

Current techniques of gene transfer in mammals include packaging the DNA into carriers for gene delivery. The ideal carriers for gene delivery should be safe and yet ensure that the DNA survives the extra and intracellular environment, efficiently transfer to the appropriate cellular compartments assuring good and long-lasting expression levels.

Presently, viral vectors are more efficient than non-viral systems, achieving high levels of efficiency, estimated around 90%, for both gene delivery and expression. However, immunogenicity, inflammatory reactions, problems associated with scale-up costs and, more important, the risk of integration in the host genome, are limiting their clinical use in preclinical and clinical protocols respect to the past: i.e. during 2000 year, around 75% of clinical protocols involving gene therapy used recombinant virus-based vectors for DNA delivery (Chiarella et al. 2008a). In the last years, lot of clinical trials pointed out that the use of viral vectors as antigen delivery systems has numerous other drawbacks such as toxicity, recombination, precedent host immunity, higher immunogenicity in comparison to the

target antigen and limited DNA carrying capacity (Harrington et al. 2002; Ramirez et al. 2000). The recent advances made on the knowledge of the immune system biology have led to consider non-viral systems as naked DNA vaccination an alternative, safer and promising approach for introducing foreign antigens into the host to induce an immune response. At the moment, non-viral systems, especially those based on plasmid DNA delivery, have become increasingly desirable in both basic research laboratories and clinical settings.

2. DNA vaccination

2.1 DNA vaccines: An emerging field

In 1990, Wolff and collaborators found that bacterial plasmid DNA encoding a reporter gene could result in *in vivo* expression of the encoded protein after simple intramuscular injection without the need for more complex vectors (Wolff et al. 1990). Following Wolff's findings a new era of vaccination started.

Naked-DNA vaccines are for definition vectors based on bacterial plasmids engineered to express the disease-specific antigen using promoter elements active in mammalian cells, without the addition of surrounding chemicals or a viral coat. The main advantages of naked DNA vaccines are safety and production in large amount, as well as stability at different temperatures and, more important, flexibility in design, since multiple antigenic targets or multiple independent cytokines or co-stimulatory sequences can be incorporated into a single DNA vector. They are also likely to be attractive from a health economics perspective: they are relatively easy to manufacture in large quantities in contrast to the complicated processes requested for attenuated virus vaccines, and do not require any special transportation or storage conditions that could hinder their widespread distribution as it happens for live pathogens-based vaccines which need to be distributed and stored in cold conditions. The genes encoding the antigens can be chemically constructed without deriving them from live virulent organisms so avoiding for operators and patients the risks of exposure to dangerous pathogens. They are commonly delivered by a simple intramuscular injection. In mammals the skeletal muscle represents approximately the 30% of the body mass, and muscle fibres are ideal targets for DNA transfection. These are stable and large syncytial cells containing several nuclei that can actively take part in immune reactions. For the easy accessibility of the skeletal muscle and good vasculature, the delivery of DNA vaccine into this organ is highly preferable (Wiendl et al. 2005). Immunisation with DNA induces all three arms of adaptive immunity (antibodies, helper T cells, CTLs), and even innate immune responses can be easily and rapidly made while maintaining fidelity to the immunological aspects necessary for a pathogen, yet excluding other undesirable proteins or immune responses (Liu 2011).

On the other hand, a major disadvantage of plasmid DNA vaccines is their poor immunogenicity when administered as an unformulated intramuscular injection. Large quantities of DNA are required to induce only modest immunogenicity and many efforts have focussed on the development of new technologies aimed at increasing the DNA vaccine potency (Chiarella et al. 2008a). That said, better strategies are needed in designing more effective vectors and combined protocols so as to induce a strong immune response to weakly immunogenic antigens. These strategies comprise new insights in studying the mechanism of action and induction of the immune response in a host injected with a DNA-based vaccine. Recently, most relevant patented strategies have been developed to enhance the plasmid DNA vaccine immunogenicity taking into account DNA plasmid construction,

epitope and antigen choice, selection and use of new adjuvants and different delivery methods.

In table 1 the advantages and disadvantages of DNA vaccines are listed.

Characteristic	Advantage/disadvantage of plasmid DNA vaccines
Antigen	In vivo antigen synthesis with native conformation
Antigen presentation	MHC-I, MHC-II, cross-priming
Immune Response	Humoural and cytotoxic
Manufacture	Easy and fast
Stability	Stable at various temperature (RT)
Risk	Does not induce the disease related to the encoded antigen
Applicability	Prophylaxis and therapy of disease
Indication of use	Infectious disease, allergy, cancer, autoimmune disease
Safety	Low risk of recombination and inflammation
Immunogenicity	Weak

Table 1. Advantages and disadvantages of plasmid DNA vaccines.

2.2 Mechanism of action and induction of the immune response

The crucial event responsible for the initiation of an immune response against a foreign antigen is recognition by specialized cells namely the antigen presenting cells (APCs), uptake and presentation of the antigen to naïve lymphocytes and induction of effector T helper (Th), cytotoxic (CTL) and B lymphocytes.

In this context the mechanism of action of DNA vaccines looks very simple. Once the DNA vaccine is delivered into the skeletal muscle, the plasmid DNA is taken up by the resident DCs and by the muscle fibres. While transfected muscle cells behave as permanent antigen reservoir as well as target of immune effector cells (Payette et al. 2001), resident DCs have the property to leave the muscle tissue and move to the closest draining lymph nodes in order to process and present the antigen to T lymphocytes (Fig. 1). DCs are specialized in capturing extracellular antigens by receptor-mediated endocytosis and pinocytosis mechanisms and following antigen uptake they undergo a complex multi-step maturation process. DC maturation depends also on the microbial and pathogens-derived signals which increase their capacity to migrate towards the draining lymph node. While DCs move to the lymphoid organs, they interact with various chemokines which contribute further to their maturation process (Palucka et al. 2010). Once in the lymph nodes, DCs shift from an antigen-capturing cell to a T sensitizing cell, being capable to present antigen in association with the class I and class II MHC molecules to CTLs and Th lymphocytes. Interaction between the DC and the T lymphocyte induces formation of the immunological synapse (IS) via complex MHC-antigen- T cell receptor (TCR) resulting in the clonal expansion of the T lymphocyte and differentiation in T memory cell. Professional DCs can also capture antigens released in the interstitial space by skeletal muscle fibres or in form of apoptotic bodies activating the cross-presentation pathway (Fig. 1) (Russo et al. 2000). This route allows presentation of extracellular/exogenous antigens through the MHC-I restriction pathways (Kurts et al. 2010). Therefore, extracellular antigens which normally induce a humoral immune response can also access to the MHC-I compartment through endoplasmic reticulum, leading to simultaneous stimulation of the CTL immune response. Antigen synthesized

by DC or skeletal muscle cell can also be released in the extracellular environment and activate directly the B lymphocytes through antigen-antibody interaction (**Fig.1**).

Considering the mechanism described above, plasmid DNA vaccines are able to stimulate all the principal effector cells of the adaptive immune system but due to the presence of CpG islands intrinsic to the DNA structure they can also can mimic some aspects of live infection, activating important signals of the innate immune system (Matzinger 2007). Theoretically genetic immunisation by plasmid DNA vaccines seems to confer the same broad immunological advantages as immunisation with live, attenuated vaccines does, without the accompanying safety concerns associated with live infection, such as reversion to the virulent form and/or incomplete inactivation of live vaccines. However there are some obstacles that make naked DNA vaccination less potent than traditional vaccines.

Fig. 1. Mechanism of action of plasmid DNA vaccination.

The antigen sequence is cloned into a bacterial plasmid vector specific for vaccination. The DNA vaccine is administered by intramuscular injection. After plasmid uptake by muscle cells, the gene coding for the antigen is translated into a protein. Transfected skeletal muscle cells can present the antigen to CTL through MHC-I molecules (A) as well as through DCs cross presentation (B). Plasmid is also uptaken by resident DCs which synthesize the antigen and present it in association with MHC-I molecules and MHC-II molecules to CD8+ T and CD4+ T-helper lymphocytes (C). Furthermore, antigen is released in the extracellular environment inducing the production of antibodies by B lymphocytes whose activation is mediated by the CD4+ T-helper cells (D). SMC, skeletal muscle cell; DC, dendritic cell; CTL, cytotoxic lymphocyte. CD8 T cell, CD8 T lymphocyte; CD4 Th cell, CD4 T helper lymphocyte; B cell, B lymphocyte.

2.3 DNA plasmid construction

DNA plasmid vaccines offer several advantages when compared to viral vectors or live attenuated vaccines. First of all they are easy to construct and to manipulate which is an important characteristic required to vaccines against pandemic diseases. They are also very stable at room temperature, do not require particular storage conditions which makes them ideal candidates for long-term delivery in other countries. The antigen can be chemically synthesized and cloned directly into the plasmid vaccine simplifying the operations of amplification by molecular techniques and avoiding to work with potentially dangerous live antigen source. The DNA constructs can be easily made to encode for modified proteins deprived of regions that might be dangerous or toxic to the recipient cell or that might suppress the immune response. DNA is a highly flexible molecule, the basic construct can be manipulated in several ways by genetic engineering in order to increase antigen expression, immunogenicity and uptake by recipient cells. All the modifications can affect both the vector backbone and the gene sequence incorporated into the plasmid, which can include adjuvant-like sequences with stimulating activity on the immune system (Abdulhaqq and Weiner 2008). By using this approach, enhanced antigen specific immune responses were observed, suggesting that this could be a general method for targeting antigen to selected cell types. Different strategies can be used for enhancing the plasmid DNA vaccine potency. A first strategy allows to improve the vector construct *i.e.* by working on the plasmid backbone design and construction; a second strategy allows to improve the codon usage in order to maximize the antigen synthesis.

An expression vector for genetic vaccination generally consists of the following elements: 1) a promoter/enhancer sequence; 2) the gene of interest encoding the target antigen; 3) a polyadenylation/transcriptional terminator sequence; 4) a resistance gene for plasmid selection and an origin of replication (Ori) in order to allow production of high numbers plasmid copies. The capacity of a plasmid DNA vector to drive gene expression is obtained by an optimal combination of all these requisites, and by the possibility of introducing various modifications into the plasmid backbone. The promoters mostly used for gene expression vectors are the cytomegalovirus immediately-early promoter (CMV) the simian virus SV40 early promoter (SV40) and the Rous sarcoma virus promoter (RSV). The CMV promoter is the most popular, as it drives gene expression in a wide range of cells and tissues (Lundquist et al. 1999). Plasmid DNA vectors can also contain tissue specific, synthetic and controllable promoters, whose sequences are designed for a specific use (Papadakis et al. 2004). When the expression of a gene is desired in certain tissues, promoters that control expression in a cell- tissue-specific manner are used. For instance the alpha skeletal muscle actin promoter is specifically used for selective expression in skeletal muscle cells whereas endothelial cell-specific promoters are used to drive expression in the tumour-associated endothelial cells (Dong and Nor 2009). The flexibility of DNA vector permits investigators to exploit the concept of gene expression optimisation by creating synthetic promoters, such as enhancer/promoters composed of numerous combinations of various regulatory sequences (Edelman et al. 2000; Li et al. 1999). One of these regulatory synthetic sequence, the hybrid CMV-Ub promoter, was found to have higher expression than the natural muscle promoters (Yew et al. 2001). In order to display gene expression kinetics, naturally regulated systems were developed by incorporating sequence elements that respond to the local environment of the given cell or tissue or that are regulated by small molecule drugs (Yew 2005). Thus transgene expression can be regulated by modulating expression of these transcriptional factors or by altering their activity through drug administration. However, regulation of *in vivo*

transgene expression by such approaches is unreliable, mainly due to the low levels of control associated with the complexity of these systems. Appropriate choice of regulatory elements and vector backbone can lead the gene expression kinetics from a few days to several months. Nuclear localization of plasmid DNA is another prerequisite for the effective antigen expression. To this purpose our laboratory developed a series of plasmids with a functional nuclear translocation sequence (NTS) (Ciafre et al. 1998). All the characteristics of plasmid DNA vaccines mentioned above are summarized in **Table 1**.

The codon usage is an important issue to consider in the DNA vaccine manufacture. Plasmid DNA are totally dependent on the host cell machinery for protein transcription and translation. Since codon usage of bacterial organisms is different from the codon usage of mammalian, it is mandatory to optimize codon usage in DNA vaccine to allow maximum antigen expression (Bojak et al. 2002). Various codon usage approaches are now commonly exploited in both non-human primate studies and clinical trials. This strategy has been successfully used to optimize the sequence of mycobacterial antigens (e.g., Ag85B) improving protein expression and thereby enhancing the immunogenicity of DNA vaccines against *M. tuberculosis* (Ko et al. 2005).

2.4 Epitope and antigen selection

The first requisite for a DNA vaccine to induce effective immune response is related to the choice of the target antigen. Sometimes the immunogenic determinant of a certain pathogen is unknown hence selection of antigen sequences has to be included as first step in the design of an epitope-based vaccine. The goal is to identify relevant T cell epitopes, able to bind to MHC class I and II molecules that are both effective and sufficient in vaccine protection against pathogen challenge or, in the case of cancer vaccines, T cell epitopes of malignant antigens that are not ignored by the immune system. In particular for tumours, highly immunogenic antigen determinants remain to be identified for most cancers types. The “direct immunological approach” which consists of deriving tumour cell lines from malignant biopsies, isolating the cancer antigens and expanding the human CTLs specific for that given antigen is now substituted by the “reverse immunological approach” (Sette and Rappuoli 2010). With this new method a candidate cancer antigen expressed on a tumour is selected by *in silico* studies. First tumour antigens are identified by exploiting immune assays based on the availability of specific polyclonal and monoclonal antibodies. Otherwise the serological analysis of recombinantly expressed clones technology (SEREX) is also supportive to identify novel tumour antigens where the blood serum of patients affected by neoplastic diseases is screened against tumour antigen cDNA expression libraries (Jager et al. 2004). Techniques employed in the molecular biology field are also helpful. The analysis of the human transcriptome based on DNA and RNA microarrays allows identification of cancer antigens in a high-throughput system. As following step, putative antigenic determinants are predicted with the aid of bioinformatics. This makes it possible to identify a variety of epitopes within an antigen sequence and to choose the best candidates for the binding to specific MHC molecules. This system works well for epitope discovery, and predictions of the MHC class I pathway is being further improved by integration with prediction tools for proteasomal cleavage and Transporter associated with Antigen Processing (TAP) binding (Larsen et al. 2007). Furthermore, native epitopes that do not fit perfectly into the MHC groove can be modified at specific sites to increase their affinity to the MHC molecule of interest, leading to the generation of what are called “heteroclitic” epitopes (Dyall et al. 1998). The antigen determinant can be modified either by

increasing the affinity of the binding to the MHC molecules or by augmenting the binding ability of the peptide-MHC complex for the TCR. The first approach is the most widely used (Dudek NL et al. 2010). This strategy is commonly exploited in the design of cancer vaccines, allowing to convert a subdominant into a dominant epitope by making it more competitive in the binding to specific MHC alleles, and thereby enhancing the potency of the vaccine. The primary and/or secondary anchor residues of an epitope can be replaced with specific aminoacids that provide much of the specificity of binding to the MHC molecule. Epitope “enhancement” is possible for both classes of human MHC resulting in priming of the CD8⁺ cytotoxic T-lymphocytes (CTL) which can recognise the target epitope on tumour or infected cells and in activation of CD4⁺ T helper cells whose role is crucial for promoting humoral and cytolytic responses, regardless the CTL epitope enhancement. The selection of biologically relevant epitopes within an antigen sequence is performed with different bioinformatic softwares. Several databases of MHC binding peptides now exist and a number of programs performing such predictions are available on the web. We experienced the use of SYFPEITHI, BIMAS and PROPPED I-II programs (www.syfpeithi.de; www.bimas.cit.nih.gov; www.imtech.res.in/raghava/propred) (Parker et al. 1994; Rammensee et al. 1999; Singh and Raghava 2001). They are based on different algorithms that provide estimation of the binding of a certain peptide sequence to a wide spectrum of human MHC molecules. Unfortunately, a major drawback of these programs is their intrinsic feature of being ‘predictive’ in estimating the binding affinity between the MHC molecule and the antigenic epitope, with approximately 70% reliability. Another limit is they cannot calculate the binding of the MHC-peptide complex to the T cell receptor, which is a crucial point to verify the immunogenicity of the tumour antigenic determinant.

Once immunogenic peptides are predicted *in silico* they need to be verified experimentally. This goal is achieved by performing *in vitro* assays to confirm their stability and binding capacity. After that, human CTL isolated from patients are assayed *in vitro* to verify their capability to recognize specifically the selected epitope on the tumour cells. This result represents the validation of the tumour antigens. Once the antigenic epitope has been validated, it can be taken in consideration for DNA vaccines manufacture and for initiating vaccination trials.

2.5 Adjuvants

It is widely accepted immunogenicity of DNA vaccines is generally weak in comparison to that of traditional vaccines. Although plasmid vaccines are capable to induce a complete immune response involving activation of CTLs, Th and B lymphocytes and a certain activation of the innate compartment of the immune system, these vaccines show low potency and efficacy when administered as unformulated injection. DNA vaccines like all the subunit vaccines, which are made of purified or recombinant antigens, require additional components to help stimulating a “comprehensive” immune reaction. Such “help” is provided by substances and components termed adjuvants.

Adjuvants of current use, either in man or in animals, have for the most part been developed empirically, without a clear understanding of their cellular and molecular mechanisms of action. Indeed adjuvants were historically defined as “the dirty little secret of the immunologist” (Janeway 1989).

The main function of an adjuvant is to create a depot of antigen at the injection site, resulting in a gradual release of small quantities of antigen over a long period of time. The adjuvant also serves as a vehicle for delivering the antigen to the lymph organs, where antigenic

epitopes can be presented to T cells by professional APCs. However, recent advances in the immunological research suggest that most, if not all, adjuvants enhance T and B cell responses by engaging components of the innate immune system, rather than by exerting direct effects on the lymphocytes. In DNA vaccinations, adjuvants are used to achieve qualitative alteration of the immune response. Adjuvants confer to DNA or to subunit vaccines the ability to promote an immune response which might not occur in their absence. Here we describe certain classes of adjuvants most widely used in DNA vaccinations. A summary of the adjuvants described bellow is presented in **Table 2**.

Class of adjuvants	Adjuvant name	Nature of the adjuvant
Genetic adjuvants	Interleukin-2	Cytokine
	Interleukin-12	Cytokine
	Granulocyte Monocyte-Colony Stimulating Factor	Cytokine
	T-helper epitopes of toxins	Peptides
Adjuvants targeting Pattern Recognition Receptors	Monophosphoryl Lipid A AS02 AS01 QS-21 CpG-DNA	Lipid derivative Oil in water emulsion Liposomal formulation Saponin Oligodeoxynucleotides
Aluminium-based compounds	Aluminium Phosphate Aluminium Hydroxide	Mineral salt Mineral salt

Table 2. Adjuvants used in vaccination with naked DNA.

2.5.1 Genetic adjuvants

Genetic adjuvants are molecules such as cytokines, chemokines and co-stimulatory factors that may be cloned into the DNA plasmid vaccine and expressed *in vivo*. These adjuvants can be encoded on the same vector expressing the antigen or inserted into a separate vector and co-injected with the vaccine. This method provides adjuvant activity at the site of antigen production, with lasting effect from transfected cells. Among various factors, cytokines are highly preferred as genetic adjuvants because they act on cells involved in the host defense and can be used to modulate immune responses. Co-delivery of cytokines in DNA vaccine formulations has been used extensively for a wide range of infectious diseases such as malaria, leishmania, schistosoma to enhance the T cell mediated responses (Ivory and Chadee 2004). One of the earliest cytokines to be incorporated into a DNA vaccine was IL-2, a well known T-cell growth factor included in several immunotherapy protocols. Addition of this cytokine to a plasmid vaccination vector resulted in enhanced antibody responses in low responder mice against malaria (Good et al. 1988) and increased production of antibodies directed against the complementary determining hypervariable region 3 of the Ig heavy chain in human B-cell lymphoma (Rinaldi et al. 2001). IL-2

contributed to increase the efficacy of a DNA vaccine against a simian immunodeficiency virus when it was fused with the immunoglobulin Fc fragment, resulting in augmentation of the cytokine half-life (Barouch et al. 2000). However, the use of IL-2 is now being limited by the emerging evidence that this cytokine can play a major role in maintaining self-tolerance and in supporting survival of CD25⁺ CD4⁺ regulatory T cells (T-regs) (Bayer et al. 2005; Setoguchi et al. 2005).

IL-12 is another cytokine used in DNA vaccination. It acts on T and NK cells by inducing the generation of CTLs through T-helper 1 cell activation and IFN- γ production. The beneficial effect of IL-12 in pre-clinical experimental tumour models suggested the possibility of using IL-12 as an anti-tumour agent in clinical trials. Despite some toxicity associated with certain doses of IL-12 when administered as a drug in patients affected by melanoma and colon cancer, some clinical responses were observed; this indicates that IL-12 can be used in clinical protocols of cancer therapy where a toxic effect of the cytokine could be acceptable (Atkins et al. 1997; Gollob et al. 2003). Granulocyte/macrophage colony-stimulatory factor (GM-CSF) is probably the most attractive adjuvant for DNA vaccines for its ability to recruit antigen-presenting cells to the site where antigen synthesis occurs as well as for its capacity to stimulate DC maturation. Plasmid DNA vaccines were constructed fusing GM-CSF to the S antigen of Hepatitis B Virus (HBV) to vaccinate HBV-transgenic mice. This fusion construct worked well in conferring protection from the HBV to both normal and transgenic mice (Qing et al. 2010). In another study the utility of GM-CSF as a DNA vaccine adjuvant for glycoprotein B (gB) of pseudorabies virus (PrV) was evaluated in the vaccination of a murine model. Mouse co-inoculation with a vector expressing GM-CSF enhanced the protective immunity against PrV infection. This immunity was caused by the induction of increased humoral and cellular immunity in response to PrV antigen (Yoon et al. 2006). A DNA vaccine encoding the GM-CSF gene and a DNA vaccine encoding the H1N1 influenza (A/New Caledonia/20/99) HA antigen were co-administered by particle-mediated epidermal delivery in Rhesus Macaques. After three immunizations the DNA vaccines were shown to significantly enhance both the systemic and mucosal immunogenicity of the HA influenza vaccine (Loudon et al. 2010).

Among genetic adjuvants the pathogen-derived immune-enhancing proteins are noteworthy for their ability to stimulate the immune system when they are fused with target antigens. Modified bacterial toxins, such as anthrax, diphtheria and pertussis toxins, are being used in vaccination as effective carriers to deliver foreign epitopes which stimulate protective CTL responses in mammalian cells (Ballard et al. 1996; Carbonetti et al. 1999). However, the ability of modified toxins to activate the host immune system does not reside only in the delivery effect exerted on the fused antigen (Stevenson et al. 2004).

The tetanus toxin Fragment C (FrC) is one of the widely used genetic adjuvant as a fusion partner for foreign antigens. This protein was found to increase the immunogenicity of the *Schistosoma mansoni* glutathione S-transferase antigen when administered as genetic fusion in a live Aro-attenuated vaccine strain of *Salmonella* (Khan et al. 1994b) and similar results were obtained when a vaccine construct consisting of a portion of P28 glutathione S-transferase was administering intravenously as C-terminal fusion to tetanus toxin FrC in a live Aro-attenuated vaccine strain of *Salmonella* (Khan et al. 1994a). In cancer vaccination, a domain of the tetanus toxin FrC fused to a single antigenic determinant was demonstrated able to induce an anti-tumoural CTL mediated response in vaccinated mice (Rice et al. 2002). Likewise, in vaccination against B cell lymphoma, DNA vaccines containing the idiotypic determinants of the Ig variable region provided protective immunity against the tumour

when expressed as single-chain variable fragment (sc-Fv) fused to tetanus toxin FrC (King et al. 1998). Reproducible data from several published papers, show that the high immunogenicity of the tetanus toxin FrC depends on two main attributes; 1) a conformational sequence-dependent effect ; 2) the presence of promiscuous T-helper epitopes within the protein (Umland et al. 1997). Our group has analysed the sequence of the tetanus toxin FrC, and has identified numerous T-helper epitopes in the protein domain: 1) the universal p30 T-helper epitope (FNNFTVSFWLRVPKVSASHLE aa 947-967), a strong promiscuous immunogenic T-helper epitope consisting of at least three distinct overlapping helper peptides, each of which is presented in association with multiple HLA class II alleles [42]; 2) the p21TT helper epitope (IREDDNITLKLDRCNN aa 1064-1079); 3) the p23TT epitope (VSIDKFRIFCKALNPK aa 1084-1099), 4) the pGINGKA epitope (PGINGKAIHLVNNESE aa 916-932) [47]; 5) the p32TT epitope (LKFIKRYTPNNEID aa 1173-1188); 6) the pGQI epitope (GQIGNDPNRDIL aa 1273-1284 (Chiarella et al. 2007). Since CD4⁺ T-helper cells support both cell and humoral immunity, it seems that the antigen fusion to promiscuous T-helper peptides contributes to the activation of these lymphocytes. The result is enhancement of the immune response mediated by T-helper cells and this might explain the strong potency of the tetanus toxin FrC domain as vaccine adjuvant. Specific T-cell epitopes of FrC that are universally immunogenic, have also been widely exploited in peptide vaccination as they have been demonstrated to enhance the humoral immune response. In particular, T-helper epitopes were successfully used as vaccine carriers to induce humoral response against polysaccharide antigens when used in form of string-of-beads (Baraldo et al. 2005). This approach is based on the concept that response to the subset of antigens and epitopes, and not to the whole organism, can be sufficient for host protection. Furthermore, the availability of bioinformatic tools and softwares for prediction of the antigen binding to the human MHC molecules helps in the design of DNA vaccines. In multi-epitope vaccination, more than one CTL epitope belonging to a certain antigen of a specific disease can be linked to a series of promiscuous MHC-II binding T-helper epitope, to generate a string-of-beads vaccine, with or without intervening spacers. In several reports, vaccination with DNA constructs consisting of a single promiscuous T-helper epitope fused to antigen determinants has proved to be effective in stimulating a strong immune response when weakly immunogenic CTL epitopes are either co-injected or chemically linked to the T-helper sequence (Tymciu et al. 2004). Synthetic universal pan HLA-DR-binding T helper epitopes such as the PADRE were conceived and they were successfully used in making DNA vaccines against infectious diseases. PADRE is a synthetic universal peptide that binds to the more common HLA-DR molecules of the human population. Its efficacy in increasing immunogenicity of CTL and B epitopes was demonstrated to be higher (Alexander et al. 1994). The sequence of PADRE has been deduced from the core sequence of the ovalbumin master T-helper peptide (aa 323-339) and adapted for binding to the more representative human MHC class II molecules (del Guercio et al. 1997).

2.5.2 Adjuvants targeting pattern recognition receptors

Traditional vaccines based on live attenuated pathogens and inactivated whole pathogens have been extremely successful in preventing many common infectious diseases. The potent immunogenicity of such vaccines depends on the presence of “endogenous adjuvants” which are simply molecular portions of the pathogenic agent. *i.e.* now defined with the name of Pathogen Associated Molecular Pattern (PAMPs). These are

lipopolysaccharide (LPS), CpG-containing oligonucleotides (CpG), and peptidoglycans. LPS is a Gram-negative membrane molecule consisting of a hydrophilic polysaccharide and a lipophilic phospholipid (lipid A). The lipophilic portion of LPS is such a potent stimulus on the pro-inflammatory cytokine production that it can lead to septic shock (Heine et al. 2001) whereas monophosphoryl lipid A (MPL) is a lipid A derivative included in many adjuvant formulations with good adjuvanticity and lower toxicity (Ismaili et al. 2002). MPL was the first TLR ligand approved for human use in the hepatitis B vaccine, Fendrix (GlaxoSmithKline Biologicals, Rixensart, Belgium) (Baldridge and Crane 1999). The adjuvanticity of these adjuvant molecules depends on their ability to bind and activate the TLR4. As a result, many TLR agonists or lipid A mimetics displaying TLR4-dependent immunostimulating functions, have been synthesized and proposed as new adjuvants (Johnson et al. 1999). AS02 is an oil-in-water emulsion containing MPL and QS-21, a saponin-derived immunostimulator, induces strong antibody and Th1 responses. AS02 is being evaluated in clinical trials in vaccines against malaria, human papillomavirus (HPV), HBV, tuberculosis, and HIV (Vandepapeliere et al. 2007). Similarly to AS02, AS01 is a liposomal formulation containing MPL that induces potent humoral and cell-mediated responses, including cytotoxic T lymphocyte responses, and is being evaluated in clinical trials of vaccine against malaria.

In bacterial DNA there is a high frequency of unmethylated CpG dinucleotide sequences in comparison to the human genome which makes perception of plasmids as “foreign” elements by the human host. This is the only characteristic that confers a certain level of immunogenicity to plasmid DNA vaccines since the CpG unmethylated sequence is recognized by the Toll Like Receptor 9 preferentially expressed on the host APCs (Liu and Ulmer 2005). CpG-DNA is used as immunostimulatory potentiator in both pre-clinical peptide and DNA vaccination trials leading to activation of innate immunity and cytokine-dependent promotion of the Th-1 response. The CpG motifs, present in bacterial DNA, consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines. During an infection, the release of unmethylated CpG-DNA from bacteria serves as a danger signal stimulating the immune system of the host (Krieg 2002). Bacterial DNA and synthetic unmethylated CpG oligodeoxynucleotides trigger an immunostimulatory cascade that culminates in maturation, differentiation and proliferation of several immune cells creating a pro-inflammatory and Th1-biased environment. The immunostimulatory activity of this CpG-DNA is species-specific. As a result, sequences specific for the host are designed and optimized for selective TLR9 binding. In particular, *in vivo* CpG-DNA half-life has been improved by replacing phosphodiester CpG-DNA with a nuclease-resistant phosphothioate oligodeoxynucleotide although it may induce immune reaction leading to an anti-DNA immune response (Ciafre et al. 1995). Addition of the immunostimulatory CpG-DNA to peptide vaccines, tilts the balance towards a Th-1 immune response, which is often accompanied by a significant increase in IgG2a production in comparison to IgG1. The general effect of CpG-DNA addition is augmentation of the antibody serum titer against antigens and production of Th-1 cytokines such as IFN- γ (Klinman et al. 1999). By contrast, in DNA plasmid vaccination, the immunomodulating effect of bacterial CpG oligodeoxynucleotides on T-helper cell balance, shows great variability. Such variability often depends on the route of immunisation.

2.5.3 Aluminium-based compounds

Aluminium salt adjuvants (aluminium hydroxide, aluminium phosphate) are generally used in combination with protein antigens as they form a precipitated or adsorbed vaccine. An efficient, safe and well tolerated adjuvant in humans is aluminium hydroxide (Alum). It has been approved for clinical use. Although traditionally thought to function primarily by forming a long-lasting depot for antigen and by promoting their uptake by APCs, it is now clear that innate immune stimulation plays a primary role in the adjuvant activity of alum (Lambrecht et al. 2009). Aluminium hydroxide is used primarily to enhance antibody production and does not utilize TLR for its function *in vivo* (Gavin et al. 2006). This adjuvant induces a Th-2 biased immune response in mice whereas in humans it stimulates also a Th-1 type immunity. *In vitro* studies demonstrated that Alum can activate the inflammasome pathway to produce IL1- β (Li et al. 2007).

Recently, aluminium hydroxide has been also shown to work well in DNA vaccination in pre-clinical models (Kenney and Edelman 2003). However, the enhancing effect of aluminium hydroxide on the immune response elicited by DNA vaccines, is not related to the levels of antigen expression. Rather, it seems to affect antigen after *in vivo* expression, suggesting the adjuvanticity of this substance is strictly related to the antigen delivery mechanism (Ulmer et al. 1999).

2.6 Delivery methods

Different methods for enhancing naked DNA vaccine delivery into host cells have been studied. The best investigated strategies are based on chemical or physical devices aimed at facilitating the DNA entry into recipient cells.

2.6.1 Microparticles

Microparticle-based methods operate a DNA condensation and complexation in particles (O'Hagan et al. 2004). The encapsulation of plasmid DNA into micro- or nanospheres can provide protection from the environment prior to delivery and aid in targeting to a specific cell type for efficient delivery.

The major advantage of particulate delivery is that synthetic microparticles have excellent potential for targeting cells of the immune system stimulating antigen uptake. It has been demonstrated that particles of about 1-10 μm in diameter are preferred for their size that is readily phagocytosed by dendritic cells and other antigen-presenting cells. They are readily internalised by phagocytic cells of the immune system, leading to an enhanced antigen presentation to the immune effector cells. Furthermore, microparticulates appear to improve delivery of DNA to APCs by facilitating trafficking to the local lymphoid tissue via the afferent lymph and antigen uptake by dendritic cells (Denis-Mize et al. 2000; Denis-Mize et al. 2003; Dupuis et al. 2000). Moreover, antigen and adjuvant molecules can be delivered to the same cell at the same time being entrapped together in biodegradable microparticles such as poly-lactide-co-glycolide (PLG) or chitosan, or complexed with non-ionic block copolymers or polycations such as polyethyleneimine. Microparticulate adjuvants are currently tested in some clinical trials against human immunodeficiency virus (HIV), hepatitis B virus (HBV) and influenza (Fuller et al. 2006). DNA entrapment or encapsulation into biodegradable microspheres for DNA vaccine delivery has been illustrated in patent WO0203961 (Johnson 2003).

2.6.2 Cationic lipids/liposomes

Cationic Solid Lipid Nanoparticles (SLNs) have been recently proposed as alternative carriers for DNA delivery, due to many technological advantages such as large-scale production from substances generally recognized as safe, good storage stability and possibility of steam sterilization and lyophilisation. Cationic lipids are amphiphilic molecules composed of one or two fatty acid side chains (acyl) or alkyl, a linker and a hydrophilic amino group. The hydrophobic part can be cholesterol-derived moieties. In aqueous media, cationic lipids are assembled into a bilayer vesicular-like structure (liposomes). Liposomes/DNA complex is usually termed a lipoplex (Bolhassani et al. 2011). The future success of cationic SLNs for administration of genetic material will depend on their ability to efficiently cross the physiological barriers, selectively targeting a specific cell type in vivo and expressing therapeutic genes (Bondi and Craparo 2010).

2.6.3 Biolistic particle delivery

In order to address accelerating micro-projectiles into intact cells or tissues, is generally used a biolistic apparatus described in patent US6004287 (Loomis 1999). Application of this strategy to DNA vaccines resulted in the invention of a new DNA delivery technology that made it possible to move naked DNA plasmid into target cells on an accelerated particle carrier. This specific delivery system is based on the use of the gene gun device that, under pressurized helium, is capable of delivering plasmid DNA-coated gold beads to the epidermal layer of skin as described in patent US6436709 (Lin 2002). Because the DNA carrier is introduced directly into the skin cells, delivery of plasmid DNA vaccines using this strategy reduces the amount of DNA needed to induce immune responses. Robust immunogenicity has been shown in many different preclinical models and in clinical trials predominantly for infectious diseases (Fuller et al. 2006). In contrast to intramuscular or intradermal injection by needle, the gene gun delivery system releases plasmid DNA directly into the cells of the epidermis (Yang et al. 1990). Intradermal injection is becoming increasingly popular, as the dense network of antigen-presenting cells in the skin, absent in muscle, provides a favourable environment for induction of antigen uptake. This network of Langerhans cells (LCs) can help in the priming of both cellular and humoral immune responses. Importantly, direct transfection of Langerhans cells is carried out with very small doses of plasmid DNA (i.e. 1-10 µg), suggesting that minimum amounts of vector are required to induce the immune response. The advantage of using low doses of plasmid DNA is particularly attractive for prophylactic vaccines against infectious diseases, where a simple and rapid delivery is the main pre-requisite. Gene gun delivery has recently been used with success in a trial against the influenza virus, inducing sero-protective levels of antibody and it has been used in trials against HBV and HIV infections (Fuller et al. 2006). A further implementation of the biolistic delivery was obtained also by creating improved injection device suitable for application in human tissues. Patent US6730663 describes a flexible multi-needle injector device with a wide surface area as well as a modified injector device to be used for injection through an endoscopic device. Such a method leads to a deep injection of DNA within tissues (Hennighausen 2004).

2.6.4 Electroporation

The use of electric pulses as a safe tool to deliver therapeutic molecules to tissues and organs has been rapidly developed over the last decade. This technology leads to a transient increase in the permeability of cell membranes when exposed to electric field pulses. This

process is commonly known as electroporabilization or electroporation (EP) (Chiarella et al 2010; Favard et al. 2007; Mir et al. 1999). The simultaneous publications in 1998 by Aihara and Miyazaki and Harrison and co-workers, demonstrated EP as being a more efficient method for gene transfer into muscle than the simple i.m. injection of DNA (Aihara and Miyazaki 1998; Harrison et al. 1998). The strategy is not only promising for enhancing the gene delivery of therapeutic proteins and drugs. Infectious disease, cancer gene therapy and chemotherapy are other fields of application, making electrochemogenetherapy relevant in a variety of research branches and promising in the gene therapy field (Mir 2008; Wells 2004). The advantage of DNA electrotransfer is dual. On the one hand, a high number of muscle cells are transfected with the DNA vaccine; on the other hand the damaged muscle cells release danger signals that favour antigen presenting cell recruitment, thus enhancing the immune response (Chiarella et al. 2008b). For this reason we consider the electroporabilization such an important and very promising tool in the future of DNA vaccination therapy that it deserves a dedicated section of this book chapter.

3. Electrogene transfer

3.1 Mechanisms and application of gene transfer by electric fields

Naked DNA vaccination emerged as a promising approach for introducing foreign antigens into the host to induce protective immunity. The delivery of DNA vaccine into skeletal muscle is highly preferable as this organ is not only a passive site but can actively take part in immune reactions. However, one important limitation of intramuscular (i.m.) genetic vaccines is their weak performance in large animals as regards the low DNA transfection efficiency of the tissue (Escoffre et al. 2010). For this reason, novel and safe delivery systems have been developed to further improve the vaccine efficiency and immunogenicity. In several reports, electroporation-mediated DNA delivery was described as an effective tool in eliciting immune response in small and large animal models (Babiuk et al. 2002; Otten et al. 2004; Peng et al. 2007), with numerous studies proving that this technique is effective in the stimulation of humoral and cellular immunity (Dupuis et al. 2000; Tollefsen et al. 2002; Widera et al. 2000). Interest in the application of EP to DNA vaccination protocols is greatly growing in these last years for several considerations. It has been demonstrated that EP allows an augmented uptake of DNA in tissue cells especially if used in combination with hyaluronidase (McMahon et al. 2001). A higher DNA uptake *in vivo* is possible thanks to the enhancement of cell membrane permeabilization and electrophoretic movement of DNA molecules into the target cells. Moreover, if EP is applied in muscle cells, these work as a platform for antigen production within the skeletal muscle (Shirota et al. 2007). A combination of both these events facilitates target cell transfection, this resulting in a higher synthesis of the gene of interest and in an intensification of the immune response to the encoded protein.

Many studies have reported the beneficial effect of EP on the activated response by the immune system against the transferred antigen in several animal models (Tsang et al. 2007). Respect to a simple administration of DNA vaccines through i.m. injection, EP is responsible for a significant increase in antibody titre (Buchan et al. 2005), antigen-specific T-cell frequency and induction of several T-cell effector functions (Bachy et al. 2001; Capone et al. 2006). In particular, a study performed on DNA vaccination mediated by EP demonstrated that the concomitant injection of plasmid DNA and EP is crucial for the adjuvant effect exerted by EP, which is responsible for eliciting antigen-specific IgG2a antibody production

and Th-1 biased immune responses (Gronevik et al. 2005). Another study demonstrated that a single i.m. DNA vaccination in combination with EP enhanced significantly the onset and the duration of the primary antibody response affecting immune memory (Tsang et al. 2007). The use of EP for delivering a DNA vaccine encoding anthrax toxin protective agent has been demonstrated able in a rapid induction of antibodies against the antigen in 2 weeks following a single immunization in several experimental animals (Luxembourg et al. 2008).

Despite these evidences, details on possible mechanisms responsible for the positive effect of EP on the immune response to DNA vaccines were not completely characterised (Escoffre et al. 2009).

Recently EP has been reported as crucial event through which, inducing transient morphological changes and a local moderate damage in the treated muscle, is possible to generate an early production of endogenous cytokines responsible for signalling danger at the local level. The activation of a danger pro-inflammatory pathway and the recruitment of inflammatory cells result in T lymphocyte migration, indicating electroporation *per se* is able to recruit and trigger cells involved in antigen presentation (Chiarella et al. 2008b). Due to these immunostimulating effects, EP is now recognised as a good adjuvant (Chiarella et al. 2007), helpful in DNA vaccination for increasing the potency and safety of this therapeutic approach due to its property to induce a higher DNA uptake and its ability to stimulate both humoral and cellular immunity. At present, numerous findings are clarifying EP mechanism (Golzio et al. 2010), also showing the easy applicability of EP to large animals. In this view, many studies are concentrated to find the most appropriate and tolerable parameters that will make EP suitable for humans (Tjelle et al. 2008; Tjelle et al. 2006). To this purpose, various electroporating devices have been developed for animal and human use (Fig. 2).

Because these initial results seem promising, several clinical trials based on DNA vaccination assisted by electroporation are under investigation and the efficacy and tolerability of EP will be deeply studied in the near future both in preclinical and clinical gene therapy protocols.

3.2 Application of electrogene transfer in DNA vaccination protocols

The use of EP technology for DNA vaccination in the clinical settings takes advantage from the development of new protocols, which are effective in administering the vaccine with the minimum discomfort and maximum tolerability for the patient.

The type of EP device, intensity of electrical stimulations, number of electrical pulses and administrations, and choice of the target organ, are important parameters taken into consideration for the design of clinical protocols.

Because the need to develop non-invasive or minimally invasive genetic vaccination methods has become an important issue, several studies have been focussed on the needle-free injection of DNA vaccines. A recent patent, issued in 2007, reports the combination of needle-free injection and electroporation, demonstrating that this non-invasive strategy is sufficient to introduce the DNA vaccine in a form suitable for electrotransfer into a region of the host tissue. This needle-free injection may be used in combination with suitable non invasive electrode configurations (Hofmann 2007). Safe EP protocols for human disease therapies are under investigation and many preclinical studies are described in numerous works investigating the potential of EP in both infectious and tumour diseases.

The DNA vaccine is injected into the skeletal muscle of the mouse limb and penetration into the target tissue is achieved by electroporation of the muscle after DNA is injected with a syringe. The electrical impulse is applied by electrodes in contact with the skeletal muscle. Different electroporator devices are shown in the figure. A) BTX ECM 830 Harvard Apparatus; B) IGEA Cliniporator; C) Inovio MedPulser® EPT; D) Ichor TriGrid™.

Fig. 2. Administration of DNA vaccine by electroporation of the muscle

HIV vaccine administration has been conducted in several animal models. Electrically-mediated delivery technology has been applied to DNA vaccines against HIV virus, and substantially higher immune responses have been achieved in mice and rabbits following vaccination with DNA encoding HIV genes. Vaccines were administered with constant electric current or constant electric voltage, causing up to 20-fold higher immune responses in comparison to the application of DNA vaccines alone (Selby 2000). In another study in mice, *in vivo* EP amplified cellular and humoral immune responses to a HIV type 1 Env DNA vaccine, enabled a 10- fold reduction in vaccine dose, and resulted in increased recruitment of inflammatory cells (Liu et al. 2008). Another study on the development of plasmid DNA vaccine able in eliciting robust cell-mediated immune response to multiple HIV type 1 (HIV-1)-derived antigens has been conducted in Rhesus macaques. Vaccination in combination with *in vivo* EP led to a more rapid onset and enhanced vaccine-specific immune responses (Luckay et al. 2007). Also the hepatitis C virus disease is object of investigation. It was demonstrated that gene electrotransfer of a novel candidate DNA vaccine encoding an optimised version of the non

structural region of HCV (from NS3 to NS5B) induces substantially more potent, broad, and long-lasting CD4⁺ and CD8⁺ cellular immunity than a simple naked DNA injection in mice and in Rhesus macaques. As already discussed, the T-cell responses elicited by the DNA-based electroporation strategy can be useful in prophylactic vaccine approaches against HCV and this work supports this hypothesis (Capone et al. 2006). Because the administration of a plasmid cocktail, encoding antigen and adjuvants in combination with EP, is proposed as an efficient genetic immunisation strategy, the same group designed a protocol in which Hepatitis C virus (HCV) E2 and cytokine encoding plasmids have been co-injected in the mouse quadriceps with or without EP. The vaccination outcome has been evaluated by analysis of antigen-specific cellular- mediated or antibody-mediated immunity. The co-injection of cytokine and HCV E2-encoding plasmids followed by EP, strongly enhanced T- or B-cell responses to various levels, depending on the particular combination used (Arcuri et al. 2008).

Respect to cancer, strong cellular immune responses can be induced in both mice and non-human primates, following the administration with EP of a novel HPV18 DNA vaccine encoding an E6/E7 fusion consensus protein (Yan et al. 2008).

Improvement in the efficacy of a cancer vaccine administered by electroporation, could increase its chances for clinical success. A demonstration of the inhibition of tumour growth has been reported by Curcio and collaborators. They demonstrated that a vaccination protocol using a plasmid encoding the extracellular and transmembrane domains of the Neu oncogene delivered by electroporation, prevents longterm tumour formation in cancer-prone transgenic mice (Curcio et al. 2008).

Since electroporeabilization is considered a promising delivery system for plasmid DNA vaccination, several clinical trials are now experimenting EP as a medical technology in human patients affected by infectious as well as cancer diseases (Bodles-Brakhop et al. 2009).

A summary of the clinical trials performed by DNA vaccination and EP are shown in the following tables.

Clinical trial	Condition	Pathogen	Intervention	Phase
NCT01169077	Malaria	Plasmodium falciparum	EP-1300	I
NCT00685412	Human Papillomavirus infection	HPV (E6-E7)	VGX-3100 CELLECTRA	I
NCT00563173	Chronic Hepatitis C Virus Infection	HCV	CHRONVAC-C®	I/IIa
NCT00545987	AIDS (prophylactic)	HIV	ADVAX TriGrid™	I
NCT01082692	AIDS (therapeutic)	HIV-1	PENNVAX-B CELLECTRA	I

Table 3. Clinical trials in infectious diseases.

Clinical trial	Condition	Antigen	Intervention	Phase
NCT01064375	Colorectal Cancer	CEA	tetwtCEA DNA	I/II
			wt CEA with tetanus toxoid Th epitope Derma Vax (electroporation device)	
NCT00471133	Intraocular Melanoma	Tyrosinase	TriGrid	I
NCT01138410	Melanoma	Antibody (SCIB-1)	EP device	I/II
NCT00859729	Prostate cancer	PSA	pVAXrcPSAv53l DERMA VAX™	I/II
GTAC No 89	Prostate cancer	PSMA	EP device (Tjelle 2006)	I/II (Closed 1.4.2008)
NCT00753415	Colon cancer Breast Cancer Melanoma	V934/V935 hTERT	V934/V935 DNA	I

Table 4. Clinical trials in cancer diseases.

The results seem to be promising and applicable to a large cohort of diseases in the next future. Therefore electroporation would appear to be the more efficient technology for local injection of plasmid DNA vaccine into the tissue (Kato and Nakamua 1965; Wells 2010).

4. Conclusions

The last decade has witnessed a revolution in the approach to vaccine design and development. Despite considerable success in the prevention field, vaccinations against intracellular organisms, which require a cell-mediated immunity, are not yet available, while infectious diseases such as tuberculosis and malaria remain a serious problem in the Third World. Following the studies of Wolff and colleagues, in recent years immunisation with naked plasmid DNA encoding antigens has revealed a number of advantages, making DNA vaccination a promising therapeutic approach against infectious diseases and cancer. Of course improvement of vaccine efficacy has become a goal in the development of DNA vaccination protocols. Electroporpermabilization has been shown to increase both the number of transfected cells and also the number of plasmids that permeate into each cell, therefore, electroporpermabilization is now regarded as a promising delivery system for plasmid DNA vaccination. Intramuscular DNA vaccination combined with electroporpermabilization has been described as effective in activating both humoural and cellular immune response in the host as well as in enhancing expression of the encoded antigen. Several reports showed that EP has adjuvant-like properties when combined with plasmid DNA injection. This approach is currently used not only in preclinical protocols in animals but also in humans, and studies for evaluating pain and stress induced by the treatment are currently under investigation indicating this approach as applicable and promising. Because this procedure is used safely

without serious adverse effects related to the administration procedure, we strongly support improvements addressed to the efficacy of DNA vaccines administered by electroporation in clinical protocols. This new approach could successfully increase chances for clinical success in humans.

5. References

- Abdulhaqq, S. A., and Weiner, D. B. (2008). "DNA vaccines: developing new strategies to enhance immune responses." *Immunol Res*, 42(1-3), 219-32.
- Aihara, H., and Miyazaki, J. (1998). "Gene transfer into muscle by electroporation in vivo." *Nat Biotechnol*, 16(9), 867-70.
- Alexander, J., Sidney, J., Southwood, S., Ruppert, J., Oseroff, C., Maewal, A., Snoke, K., Serra, H. M., Kubo, R. T., Sette, A., and et al. (1994). "Development of high potency universal DR-restricted helper epitopes by modification of high affinity DR-blocking peptides." *Immunity*, 1(9), 751-61.
- Andre, F. E. (2003). "Vaccinology: past achievements, present roadblocks and future promises." *Vaccine*, 21(7-8), 593-5.
- Arcuri, M., Cappelletti, M., Zampaglione, I., Aurisicchio, L., Nicosia, A., Ciliberto, G., and Fattori, E. (2008). "Synergistic effect of gene-electro transfer and adjuvant cytokines in increasing the potency of hepatitis C virus genetic vaccination." *J Gene Med*, 10(9), 1048-54.
- Atkins, M. B., Robertson, M. J., Gordon, M., Lotze, M. T., DeCoste, M., DuBois, J. S., Ritz, J., Sandler, A. B., Edington, H. D., Garzone, P. D., Mier, J. W., Canning, C. M., Battiato, L., Tahara, H., and Sherman, M. L. (1997). "Phase I evaluation of intravenous recombinant human interleukin 12 in patients with advanced malignancies." *Clin Cancer Res*, 3(3), 409-17.
- Babiuk, S., Baca-Estrada, M. E., Foldvari, M., Storms, M., Rabussay, D., Widera, G., and Babiuk, L. A. (2002). "Electroporation improves the efficacy of DNA vaccines in large animals." *Vaccine*, 20(27-28), 3399-3408.
- Bachy, M., Boudet, F., Bureau, M., Girerd-Chambaz, Y., Wils, P., Scherman, D., and Meric, C. (2001). "Electric pulses increase the immunogenicity of an influenza DNA vaccine injected intramuscularly in the mouse." *Vaccine*, 19(13-14), 1688-93.
- Baldridge, J. R., and Crane, R. T. (1999). "Monophosphoryl lipid A (MPL) formulations for the next generation of vaccines." *Methods*, 19(1), 103-7.
- Ballard, J. D., Collier, R. J., and Starnbach, M. N. (1996). "Anthrax toxin-mediated delivery of a cytotoxic T-cell epitope in vivo." *Proc Natl Acad Sci U S A*, 93(22), 12531-4.
- Baraldo, K., Mori, E., Bartoloni, A., Norelli, F., Grandi, G., Rappuoli, R., Finco, O., and Del Giudice, G. (2005). "Combined conjugate vaccines: enhanced immunogenicity with the N19 polyepitope as a carrier protein." *Infect Immun*, 73(9), 5835-41.
- Barouch, D. H., Craiu, A., Kuroda, M. J., Schmitz, J. E., Zheng, X. X., Santra, S., Frost, J. D., Krivulka, G. R., Lifton, M. A., Crabbs, C. L., Heidecker, G., Perry, H. C., Davies, M. E., Xie, H., Nickerson, C. E., Steenbeke, T. D., Lord, C. I., Montefiori, D. C., Strom, T. B., Shiver, J. W., Lewis, M. G., and Letvin, N. L. (2000). "Augmentation of immune responses to HIV-1 and simian immunodeficiency virus DNA vaccines by IL-2/Ig plasmid administration in rhesus monkeys." *Proc Natl Acad Sci U S A*, 97(8), 4192-7.

- Bayer, A. L., Yu, A., Adeegbe, D., and Malek, T. R. (2005). "Essential role for interleukin-2 for CD4(+)CD25(+) T regulatory cell development during the neonatal period." *J Exp Med*, 201(5), 769-77.
- Bodles-Brakhop, A. M., Heller, R., and Draghia-Akli, R. (2009). "Electroporation for the delivery of DNA-based vaccines and immunotherapeutics: current clinical developments." *Mol Ther*, 17(4), 585-92.
- Bojak, A., Wild, J., Deml, L., and Wagner, R. (2002). "Impact of codon usage modification on T cell immunogenicity and longevity of HIV-1 gag-specific DNA vaccines." *Intervirology*, 45(4-6), 275-86.
- Bolhassani, A., Safaiyan, S., and Rafati, S. (2011). "Improvement of different vaccine delivery systems for cancer therapy." *Mol Cancer*, 10, 3.
- Bondi, M. L., and Craparo, E. F. (2010). "Solid lipid nanoparticles for applications in gene therapy: a review of the state of the art." *Expert Opin Drug Deliv*, 7(1), 7-18.
- Brun, A., Barcena, J., Blanco, E., Borrego, B., Dory, D., Escribano, J. M., Le Gall-Recule, G., Ortego, J., and Dixon, L. K. (2011). "Current strategies for subunit and genetic viral veterinary vaccine development." *Virus Res*, 157(1), 1-12.
- Buchan, S., Gronevik, E., Mathiesen, I., King, C. A., Stevenson, F. K., and Rice, J. (2005). "Electroporation as a "prime/boost" strategy for naked DNA vaccination against a tumor antigen." *J Immunol*, 174(10), 6292-8.
- Capone, S., Zampaglione, I., Vitelli, A., Pezzanera, M., Kierstead, L., Burns, J., Ruggeri, L., Arcuri, M., Cappelletti, M., Meola, A., Ercole, B. B., Tafi, R., Santini, C., Luzzago, A., Fu, T. M., Colloca, S., Ciliberto, G., Cortese, R., Nicosia, A., Fattori, E., and Folgori, A. (2006). "Modulation of the immune response induced by gene electrotransfer of a hepatitis C virus DNA vaccine in nonhuman primates." *J Immunol*, 177(10), 7462-71.
- Carbonetti, N. H., Irish, T. J., Chen, C. H., O'Connell, C. B., Hadley, G. A., McNamara, U., Tuskan, R. G., and Lewis, G. K. (1999). "Intracellular delivery of a cytolytic T-lymphocyte epitope peptide by pertussis toxin to major histocompatibility complex class I without involvement of the cytosolic class I antigen processing pathway." *Infect Immun*, 67(2), 602-7.
- Chiarella, P., Fazio, V.M., and Signori, E. (2010). "Application of electroporation in DNA vaccination protocols." *Curr Gene Ther*, 10 (4), 281-6.
- Chiarella, P., Massi, E., De Robertis, M., Fazio, V. M., and Signori, E. (2008a). "Strategies for effective naked-DNA vaccination against infectious diseases." *Recent Pat Antiinfect Drug Discov*, 3(2), 93-101.
- Chiarella, P., Massi, E., De Robertis, M., Sibilio, A., Parrella, P., Fazio, V. M., and Signori, E. (2008b). "Electroporation of skeletal muscle induces danger signal release and antigen-presenting cell recruitment independently of DNA vaccine administration." *Expert Opin Biol Ther*, 8(11), 1645-57.
- Chiarella, P., Massi, E., De Robertis, M., Signori, E., and Fazio, V. M. (2007). "Adjuvants in vaccines and for immunisation: current trends." *Expert Opin Biol Ther*, 7(10), 1551-62.
- Chu, C., Parker, R., Highlands, L., and Morales J. (2010). "Phase I of Human Papillomavirus (HPV) DNA Plasmid (VGX-3100) + Electroporation for CIN 2 or 3." *Date of access: 04/04/2011. Available from: Clinical Trials.gov.*

- Ciafre, S. A., Rinaldi, M., Gasparini, P., Seripa, D., Bisceglia, L., Zelante, L., Farace, M. G., and Fazio, V. M. (1995). "Stability and functional effectiveness of phosphorothioate modified duplex DNA and synthetic 'mini-genes'." *Nucleic Acids Res*, 23(20), 4134-42.
- Ciafre, S. A., Rinaldi, M., Vespignani, I., Parrella, P., Seripa, D., Signori, E., Ria, F., Farace, M. G., and Fazio, V. M. (1998). "A plasmid family containing two different expression cassettes suitable for immunomodulation and genetic immunization." *Plasmid*, 40(1), 84-9.
- Curcio, C., Khan, A. S., Amici, A., Spadaro, M., Quaglini, E., Cavallo, F., Forni, G., and Draghia-Akli, R. (2008). "DNA immunization using constant-current electroporation affords long-term protection from autochthonous mammary carcinomas in cancer-prone transgenic mice." *Cancer Gene Ther*, 15(2), 108-14.
- del Guercio, M. F., Alexander, J., Kubo, R. T., Arrhenius, T., Maewal, A., Appella, E., Hoffman, S. L., Jones, T., Valmori, D., Sakaguchi, K., Grey, H. M., and Sette, A. (1997). "Potent immunogenic short linear peptide constructs composed of B cell epitopes and Pan DR T helper epitopes (PADRE) for antibody responses in vivo." *Vaccine*, 15(4), 441-8.
- Denis-Mize, K. S., Dupuis, M., MacKichan, M. L., Singh, M., Doe, B., O'Hagan, D., Ulmer, J. B., Donnelly, J. J., McDonald, D. M., and Ott, G. (2000). "Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells." *Gene Ther*, 7(24), 2105-12.
- Denis-Mize, K. S., Dupuis, M., Singh, M., Woo, C., Ugozzoli, M., O'Hagan, D. T., Donnelly, J. J., 3rd, Ott, G., and McDonald, D. M. (2003). "Mechanisms of increased immunogenicity for DNA-based vaccines adsorbed onto cationic microparticles." *Cell Immunol*, 225(1), 12-20.
- Dong, Z., and Nor, J. E. (2009). "Transcriptional targeting of tumor endothelial cells for gene therapy." *Adv Drug Deliv Rev*, 61(7-8), 542-53.
- Dudek, N.L., Perlmutter, P., Aguilar, M.L., Croft, N.P., and Purcell, A.W. (2010). "Epitope discovery and their use in peptide based vaccines." *Curr Pharm Des*, 16(28), 3149-57.
- Dupuis, M., Denis-Mize, K., Woo, C., Goldbeck, C., Selby, M. J., Chen, M., Otten, G. R., Ulmer, J. B., Donnelly, J. J., Ott, G., and McDonald, D. M. (2000). "Distribution of DNA vaccines determines their immunogenicity after intramuscular injection in mice." *J Immunol*, 165(5), 2850-8.
- Dyall, R., Bowne, W. B., Weber, L. W., LeMaout, J., Szabo, P., Moroi, Y., Piskun, G., Lewis, J. J., Houghton, A. N., and Nikolic-Zugic, J. (1998). "Heteroclitic immunization induces tumor immunity." *J Exp Med*, 188(9), 1553-61.
- Edelman, G. M., Meech, R., Owens, G. C., and Jones, F. S. (2000). "Synthetic promoter elements obtained by nucleotide sequence variation and selection for activity." *Proc Natl Acad Sci U S A*, 97(7), 3038-43.
- Ertl, P. F. (2007). *Patent No. US2007248679*.
- Escoffre, J. M., Portet, T., Wasungu, L., Teissie, J., Dean, D., and Rols, M. P. (2009). "What is (still not) known of the mechanism by which electroporation mediates gene transfer and expression in cells and tissues." *Mol Biotechnol*, 41(3), 286-95.
- Escoffre, J. M., Teissie, J., and Rols, M. P. (2010). "Gene transfer: how can the biological barriers be overcome?" *J Membr Biol*, 236(1), 61-74.

- Favard, C., Dean, D. S., and Rols, M. P. (2007). "Electrotransfer as a non viral method of gene delivery." *Curr Gene Ther*, 7(1), 67-77.
- Fraser, C. M., and Rappuoli, R. (2005). "Application of microbial genomic science to advanced therapeutics." *Annu Rev Med*, 56, 459-74.
- Fuller, D. H., Loudon, P., and Schmaljohn, C. (2006). "Preclinical and clinical progress of particle-mediated DNA vaccines for infectious diseases." *Methods*, 40(1), 86-97.
- Gavin, A. L., Hoebe, K., Duong, B., Ota, T., Martin, C., Beutler, B., and Nemazee, D. (2006). "Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling." *Science*, 314(5807), 1936-8.
- Gollob, J. A., Veenstra, K. G., Parker, R. A., Mier, J. W., McDermott, D. F., Clancy, D., Tutin, L., Koon, H., and Atkins, M. B. (2003). "Phase I trial of concurrent twice-weekly recombinant human interleukin-12 plus low-dose IL-2 in patients with melanoma or renal cell carcinoma." *J Clin Oncol*, 21(13), 2564-73.
- Golzio, M., Escoffre, J. M., Portet, T., Mauroy, C., Teissie, J., Dean, D. S., and Rols, M. P. (2010). "Observations of the mechanisms of electromediated DNA uptake--from vesicles to tissues." *Curr Gene Ther*, 10(4), 256-66.
- Good, M. F., Pombo, D., Lunde, M. N., Maloy, W. L., Halenbeck, R., Kothe, K., Miller, L. H., and Berzofsky, J. A. (1988). "Recombinant human IL-2 overcomes genetic nonresponsiveness to malaria sporozoite peptides. Correlation of effect with biologic activity of IL-2." *J Immunol*, 141(3), 972-7.
- Gothelf, A., Mir, L. M., and Gehl, J. (2003). "Electrochemotherapy: results of cancer treatment using enhanced delivery of bleomycin by electroporation." *Cancer Treat Rev*, 29(5), 371-87.
- Gronevik, E., Mathiesen, I., and Lomo, T. (2005). "Early events of electroporation-mediated intramuscular DNA vaccination potentiate Th1-directed immune responses." *J Gene Med*, 7(9), 1246-54.
- Harrington, L. E., Most Rv, R., Whitton, J. L., and Ahmed, R. (2002). "Recombinant vaccinia virus-induced T-cell immunity: quantitation of the response to the virus vector and the foreign epitope." *J Virol*, 76(7), 3329-37.
- Harrison, R. L., Byrne, B. J., and Tung, L. (1998). "Electroporation-mediated gene transfer in cardiac tissue." *FEBS Lett*, 435(1), 1-5.
- Heine, H., Rietschel, E. T., and Ulmer, A. J. (2001). "The biology of endotoxin." *Mol Biotechnol*, 19(3), 279-96.
- Hennighausen, L., and Furth, P.A. (2004). "Targeting gene expression to living tissue using jet injection." *Patent No. US 6730663*.
- Hofmann, G. A., Rabussay, D.P., and Zhang L. (2007). "Intradermal delivery of active agents by needle-free injection and electroporation." *Patent No. US 7171264*.
- Ismaili, J., Rennesson, J., Aksoy, E., Vekemans, J., Vincart, B., Amraoui, Z., Van Laethem, F., Goldman, M., and Dubois, P. M. (2002). "Monophosphoryl lipid A activates both human dendritic cells and T cells." *J Immunol*, 168(2), 926-32.
- Ivory, C., and Chadee, K. (2004). "DNA vaccines: designing strategies against parasitic infections." *Genet Vaccines Ther*, 2(1), 17.
- Jager, D., Taverna, C., Zippelius, A., and Knuth, A. (2004). "Identification of tumor antigens as potential target antigens for immunotherapy by serological expression cloning." *Cancer Immunol Immunother*, 53(3), 144-7.

- Janeway, C. A., Jr. (1989). "Approaching the asymptote? Evolution and revolution in immunology." *Cold Spring Harb Symp Quant Biol*, 54 Pt 1, 1-13.
- Johnson, D. A., Keegan, D. S., Sowell, C. G., Livesay, M. T., Johnson, C. L., Taubner, L. M., Harris, A., Myers, K. R., Thompson, J. D., Gustafson, G. L., Rhodes, M. J., Ulrich, J. T., Ward, J. R., Yorgensen, Y. M., Cantrell, J. L., and Brookshire, V. G. (1999). "3-O-Desacyl monophosphoryl lipid A derivatives: synthesis and immunostimulant activities." *J Med Chem*, 42(22), 4640-9.
- Johnson, M. E., Mossman, S., Evans, L., and Cecil T. (2003). "Microspheres and adjuvants for DNA vaccine delivery." *Patent No. WO 0203961*.
- Kenney, R. T., and Edelman, R. (2003). "Survey of human-use adjuvants." *Expert Rev Vaccines*, 2(2), 167-88.
- Khan, C. M., Villarreal-Ramos, B., Pierce, R. J., Demarco de Hormaeche, R., McNeill, H., Ali, T., Chatfield, S., Capron, A., Dougan, G., and Hormaeche, C. E. (1994a). "Construction, expression, and immunogenicity of multiple tandem copies of the *Schistosoma mansoni* peptide 115-131 of the P28 glutathione S-transferase expressed as C-terminal fusions to tetanus toxin fragment C in a live aro-attenuated vaccine strain of *Salmonella*." *J Immunol*, 153(12), 5634-42.
- Khan, C. M., Villarreal-Ramos, B., Pierce, R. J., Riveau, G., Demarco de Hormaeche, R., McNeill, H., Ali, T., Fairweather, N., Chatfield, S., Capron, A., and et al. (1994b). "Construction, expression, and immunogenicity of the *Schistosoma mansoni* P28 glutathione S-transferase as a genetic fusion to tetanus toxin fragment C in a live Aro attenuated vaccine strain of *Salmonella*." *Proc Natl Acad Sci U S A*, 91(23), 11261-5.
- King, C. A., Spellerberg, M. B., Zhu, D., Rice, J., Sahota, S. S., Thompson, A. R., Hamblin, T. J., Radl, J., and Stevenson, F. K. (1998). "DNA vaccines with single-chain Fv fused to fragment C of tetanus toxin induce protective immunity against lymphoma and myeloma." *Nat Med*, 4(11), 1281-6.
- Klinman, D. M., Barnhart, K. M., and Conover, J. (1999). "CpG motifs as immune adjuvants." *Vaccine*, 17(1), 19-25.
- Ko, H. J., Ko, S. Y., Kim, Y. J., Lee, E. G., Cho, S. N., and Kang, C. Y. (2005). "Optimization of codon usage enhances the immunogenicity of a DNA vaccine encoding mycobacterial antigen Ag85B." *Infect Immun*, 73(9), 5666-74.
- Krieg, A. M. (2002). "CpG motifs in bacterial DNA and their immune effects." *Annu Rev Immunol*, 20, 709-60.
- Kurts, C., Robinson, B. W., and Knolle, P. A. (2010). "Cross-priming in health and disease." *Nat Rev Immunol*, 10(6), 403-14.
- Lambrecht, B. N., Kool, M., Willart, M. A., and Hammad, H. (2009). "Mechanism of action of clinically approved adjuvants." *Curr Opin Immunol*, 21(1), 23-9.
- Larsen, M. V., Lundegaard, C., Lamberth, K., Buus, S., Lund, O., and Nielsen, M. (2007). "Large-scale validation of methods for cytotoxic T-lymphocyte epitope prediction." *BMC Bioinformatics*, 8, 424.
- Li, H., Nookala, S., and Re, F. (2007). "Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1 β and IL-18 release." *J Immunol*, 178(8), 5271-6.
- Li, X., Eastman, E. M., Schwartz, R. J., and Draghia-Akli, R. (1999). "Synthetic muscle promoters: activities exceeding naturally occurring regulatory sequences." *Nat Biotechnol*, 17(3), 241-5.

- Lin, H. J., Huang, K.J., Yang, H.T., and Chen W.C. (2002). "Low-pressure accelerated particle gene gun." *Patent No US 6436709*.
- Liu, J., Kjekken, R., Mathiesen, I., and Barouch, D. H. (2008). "Recruitment of antigen-presenting cells to the site of inoculation and augmentation of human immunodeficiency virus type 1 DNA vaccine immunogenicity by in vivo electroporation." *J Virol*, 82(11), 5643-9.
- Liu, M. A. (2011). "DNA vaccines: an historical perspective and view to the future." *Immunol Rev*, 239(1), 62-84.
- Liu, M. A., and Ulmer, J. B. (2005). "Human clinical trials of plasmid DNA vaccines." *Adv Genet*, 55, 25-40.
- Loomis, D. J., Celeste, J., and Sanford, J. (1999). "Biolistic apparatus for delivering substances into cells and tissues." *Patent of USA, No US 6004287*.
- Loudon, P. T., Yager, E. J., Lynch, D. T., Narendran, A., Stagnar, C., Franchini, A. M., Fuller, J. T., White, P. A., Nyuandi, J., Wiley, C. A., Murphey-Corb, M., and Fuller, D. H. (2010). "GM-CSF increases mucosal and systemic immunogenicity of an H1N1 influenza DNA vaccine administered into the epidermis of non-human primates." *PLoS One*, 5(6), e11021.
- Luckay, A., Sidhu, M. K., Kjekken, R., Megati, S., Chong, S. Y., Roopchand, V., Garcia-Hand, D., Abdullah, R., Braun, R., Montefiori, D. C., Rosati, M., Felber, B. K., Pavlakis, G. N., Mathiesen, I., Israel, Z. R., Eldridge, J. H., and Egan, M. A. (2007). "Effect of plasmid DNA vaccine design and in vivo electroporation on the resulting vaccine-specific immune responses in rhesus macaques." *J Virol*, 81(10), 5257-69.
- Lundquist, C. A., Meier, J. L., and Stinski, M. F. (1999). "A strong negative transcriptional regulatory region between the human cytomegalovirus UL127 gene and the major immediate-early enhancer." *J Virol*, 73(11), 9039-52.
- Luxembourg, A., Hannaman, D., Nolan, E., Ellefsen, B., Nakamura, G., Chau, L., Tellez, O., Little, S., and Bernard, R. (2008). "Potentiation of an anthrax DNA vaccine with electroporation." *Vaccine*, 26(40), 5216-22.
- Matzinger, P. (2007). "Friendly and dangerous signals: is the tissue in control?" *Nat Immunol*, 8(1), 11-3.
- McMahon, J. M., Signori, E., Wells, K. E., Fazio, V. M., and Wells, D. J. (2001). "Optimisation of electrotransfer of plasmid into skeletal muscle by pretreatment with hyaluronidase -- increased expression with reduced muscle damage." *Gene Ther*, 8(16), 1264-70.
- Miller, A. M., Ozenci, V., Kiessling, R., and Pisa, P. (2005). "Immune monitoring in a phase 1 trial of a PSA DNA vaccine in patients with hormone-refractory prostate cancer." *J Immunother*, 28(4), 389-95.
- Mir, L. M. (2008). "Application of electroporation gene therapy: past, current, and future." *Methods Mol Biol*, 423, 3-17.
- Mir, L. M., Bureau, M. F., Gehl, J., Rangara, R., Rouy, D., Caillaud, J. M., Delaere, P., Branellec, D., Schwartz, B., and Scherman, D. (1999). "High-efficiency gene transfer into skeletal muscle mediated by electric pulses." *Proc Natl Acad Sci U S A*, 96(8), 4262-7.
- O'Hagan, D. T., Singh, M., and Ulmer, J. B. (2004). "Microparticles for the delivery of DNA vaccines." *Immunol Rev*, 199, 191-200.

- Otten, G., Schaefer, M., Doe, B., Liu, H., Srivastava, I., zur Megede, J., O'Hagan, D., Donnelly, J., Widera, G., Rabussay, D., Lewis, M. G., Barnett, S., and Ulmer, J. B. (2004). "Enhancement of DNA vaccine potency in rhesus macaques by electroporation." *Vaccine*, 22(19), 2489-93.
- Palucka, A. K., Ueno, H., Fay, J. W., and Banchereau, J. (2007). "Taming cancer by inducing immunity via dendritic cells." *Immunol Rev*, 220, 129-50.
- Palucka, K., Banchereau, J., and Mellman, I. (2010). "Designing vaccines based on biology of human dendritic cell subsets." *Immunity*, 33(4), 464-78.
- Papadakis, E. D., Nicklin, S. A., Baker, A. H., and White, S. J. (2004). "Promoters and control elements: designing expression cassettes for gene therapy." *Curr Gene Ther*, 4(1), 89-113.
- Parker, K. C., Bednarek, M. A., and Coligan, J. E. (1994). "Scheme for ranking potential HLA-A2 binding peptides based on independent binding of individual peptide side-chains." *J Immunol*, 152(1), 163-75.
- Pavlenko, M., Roos, A. K., Lundqvist, A., Palmborg, A., Miller, A. M., Ozenci, V., Bergman, B., Egevad, L., Hellstrom, M., Kiessling, R., Masucci, G., Wersall, P., Nilsson, S., and Pisa, P. (2004). "A phase I trial of DNA vaccination with a plasmid expressing prostate-specific antigen in patients with hormone-refractory prostate cancer." *Br J Cancer*, 91(4), 688-94.
- Payette, P. J., Weeratna, R. D., McCluskie, M. J., and Davis, H. L. (2001). "Immune-mediated destruction of transfected myocytes following DNA vaccination occurs via multiple mechanisms." *Gene Ther*, 8(18), 1395-400.
- Peng, B., Zhao, Y., Xu, L., and Xu, Y. (2007). "Electric pulses applied prior to intramuscular DNA vaccination greatly improve the vaccine immunogenicity." *Vaccine*, 25(11), 2064-73.
- Plotkin, S. A. (2005). "Vaccines: past, present and future." *Nat Med*, 11(4 Suppl), S5-11.
- Purcell, A. W., McCluskey, J., and Rossjohn, J. (2007). "More than one reason to rethink the use of peptides in vaccine design." *Nat Rev Drug Discov*, 6(5), 404-14.
- Qing, Y., Chen, M., Zhao, J., Hu, H., Xu, H., Ling, N., Peng, M., and Ren, H. (2010). "Construction of an HBV DNA vaccine by fusion of the GM-CSF gene to the HBV-S gene and examination of its immune effects in normal and HBV-transgenic mice." *Vaccine*, 28(26), 4301-7.
- Ramirez, J. C., Gherardi, M. M., Rodriguez, D., and Esteban, M. (2000). "Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector." *J Virol*, 74(16), 7651-5.
- Rammensee, H., Bachmann, J., Emmerich, N. P., Bachor, O. A., and Stevanovic, S. (1999). "SYFPEITHI: database for MHC ligands and peptide motifs." *Immunogenetics*, 50(3-4), 213-9.
- Rice, J., Buchan, S., and Stevenson, F. K. (2002). "Critical components of a DNA fusion vaccine able to induce protective cytotoxic T cells against a single epitope of a tumor antigen." *J Immunol*, 169(7), 3908-13.
- Rinaldi, M., Ria, F., Parrella, P., Signori, E., Serra, A., Ciafre, S. A., Vespignani, I., Lazzari, M., Farace, M. G., Saglio, G., and Fazio, V. M. (2001). "Antibodies elicited by naked DNA vaccination against the complementary-determining region 3 hypervariable region of immunoglobulin heavy chain idiotypic determinants of B-

- lymphoproliferative disorders specifically react with patients' tumor cells." *Cancer Res*, 61(4), 1555-62.
- Roos, A. K., King, A., and Pisa, P. (2008). "DNA vaccination for prostate cancer." *Methods Mol Biol*, 423, 463-72.
- Russo, V., Tanzarella, S., Dalerba, P., Rigatti, D., Rovere, P., Villa, A., Bordignon, C., and Traversari, C. (2000). "Dendritic cells acquire the MAGE-3 human tumor antigen from apoptotic cells and induce a class I-restricted T cell response." *Proc Natl Acad Sci U S A*, 97(5), 2185-90.
- Selby, M., Walsh, R., Pertile, T., Goldbeck, C., Ulmer, J., and Liu MA. (2000). "Electrically-mediated enhancement of DNA vaccine immunity and efficacy *in vivo*." *Patent No. WO/2000/045823*.
- Seo, S. H., Jin, H. T., Park, S. H., Youn, J. I., and Sung, Y. C. (2009). "Optimal induction of HPV DNA vaccine-induced CD8+ T cell responses and therapeutic antitumor effect by antigen engineering and electroporation." *Vaccine*, 27(42), 5906-12.
- Serruto, D., and Rappuoli, R. (2006). "Post-genomic vaccine development." *FEBS Lett*, 580(12), 2985-92.
- Setoguchi, R., Hori, S., Takahashi, T., and Sakaguchi, S. (2005). "Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization." *J Exp Med*, 201(5), 723-35.
- Sette, A., and Rappuoli, R. (2010). "Reverse vaccinology: developing vaccines in the era of genomics." *Immunity*, 33(4), 530-41.
- Shirota, H., Petrenko, L., Hong, C., and Klinman, D. M. (2007). "Potential of transfected muscle cells to contribute to DNA vaccine immunogenicity." *J Immunol*, 179(1), 329-36.
- Singh, H., and Raghava, G. P. (2001). "ProPred: prediction of HLA-DR binding sites." *Bioinformatics*, 17(12), 1236-7.
- Stevenson, F. K., Rice, J., Ottensmeier, C. H., Thirdborough, S. M., and Zhu, D. (2004). "DNA fusion gene vaccines against cancer: from the laboratory to the clinic." *Immunol Rev*, 199, 156-80.
- Tam, J. P. (1996). "Recent advances in multiple antigen peptides." *J Immunol Methods*, 196(1), 17-32.
- Tjelle, T. E., Rabussay, D., Ottensmeier, C., Mathiesen, I., and Kjekken, R. (2008). "Taking electroporation-based delivery of DNA vaccination into humans: a generic clinical protocol." *Methods Mol Biol*, 423, 497-507.
- Tjelle, T. E., Salte, R., Mathiesen, I., and Kjekken, R. (2006). "A novel electroporation device for gene delivery in large animals and humans." *Vaccine*, 24(21), 4667-70.
- Tollefsen, S., Tjelle, T., Schneider, J., Harboe, M., Wiker, H., Hewinson, G., Huygen, K., and Mathiesen, I. (2002). "Improved cellular and humoral immune responses against Mycobacterium tuberculosis antigens after intramuscular DNA immunisation combined with muscle electroporation." *Vaccine*, 20(27-28), 3370-8.
- Tsang, C., Babiuk, S., van Drunen Littel-van den Hurk, S., Babiuk, L. A., and Griebel, P. (2007). "A single DNA immunization in combination with electroporation prolongs the primary immune response and maintains immune memory for six months." *Vaccine*, 25(30), 5485-94.

- Tymciuk, S., Durieux-Alexandrenne, C., Wijkhuisen, A., Creminon, C., Frobert, Y., Grassi, J., Couraud, J. Y., and Boquet, D. (2004). "Enhancement of antibody responses in DNA vaccination using a vector encoding a universal T-helper cell epitope." *DNA Cell Biol*, 23(6), 395-402.
- Ulmer, J. B., DeWitt, C. M., Chastain, M., Friedman, A., Donnelly, J. J., McClements, W. L., Caulfield, M. J., Bohannon, K. E., Volkin, D. B., and Evans, R. K. (1999). "Enhancement of DNA vaccine potency using conventional aluminum adjuvants." *Vaccine*, 18(1-2), 18-28.
- Umland, T. C., Wingert, L. M., Swaminathan, S., Furey, W. F., Schmidt, J. J., and Sax, M. (1997). "Structure of the receptor binding fragment HC of tetanus neurotoxin." *Nat Struct Biol*, 4(10), 788-92.
- van der Burg, S. H., Bijker, M. S., Welters, M. J., Offringa, R., and Melief, C. J. (2006). "Improved peptide vaccine strategies, creating synthetic artificial infections to maximize immune efficacy." *Adv Drug Deliv Rev*, 58(8), 916-30.
- Vandepapeliere, P., Lau, G. K., Leroux-Roels, G., Horsmans, Y., Gane, E., Tawandee, T., Merican, M. I., Win, K. M., Treppe, C., Cooksley, G., Wettendorff, M., and Ferrari, C. (2007). "Therapeutic vaccination of chronic hepatitis B patients with virus suppression by antiviral therapy: a randomized, controlled study of co-administration of HBsAg/AS02 candidate vaccine and lamivudine." *Vaccine*, 25(51), 8585-97.
- Wells, D. J. (2004). "Gene therapy progress and prospects: electroporation and other physical methods." *Gene Ther*, 11(18), 1363-9.
- Wells, D. J. (2010). "Electroporation and ultrasound enhanced non-viral gene delivery in vitro and in vivo." *Cell Biol Toxicol*, 26(1), 21-8.
- Widera, G., Austin, M., Rabussay, D., Goldbeck, C., Barnett, S. W., Chen, M., Leung, L., Otten, G. R., Thudium, K., Selby, M. J., and Ulmer, J. B. (2000). "Increased DNA vaccine delivery and immunogenicity by electroporation in vivo." *J Immunol*, 164(9), 4635-40.
- Wiendl, H., Hohlfeld, R., and Kieseier, B. C. (2005). "Immunobiology of muscle: advances in understanding an immunological microenvironment." *Trends Immunol*, 26(7), 373-80.
- Wolff, J. A., Malone, R. W., Williams, P., Chong, W., Acsadi, G., Jani, A., and Felgner, P. L. (1990). "Direct gene transfer into mouse muscle in vivo." *Science*, 247(4949 Pt 1), 1465-8.
- Yan, J., Harris, K., Khan, A. S., Draghia-Akli, R., Sewell, D., and Weiner, D. B. (2008). "Cellular immunity induced by a novel HPV18 DNA vaccine encoding an E6/E7 fusion consensus protein in mice and rhesus macaques." *Vaccine*, 26(40), 5210-5.
- Yang, N. S., Burkholder, J., Roberts, B., Martinell, B., and McCabe, D. (1990). "In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment." *Proc Natl Acad Sci U S A*, 87(24), 9568-72.
- Yew, N. S. (2005). "Controlling the kinetics of transgene expression by plasmid design." *Adv Drug Deliv Rev*, 57(5), 769-80.
- Yew, N. S., Przybylska, M., Ziegler, R. J., Liu, D., and Cheng, S. H. (2001). "High and sustained transgene expression in vivo from plasmid vectors containing a hybrid ubiquitin promoter." *Mol Ther*, 4(1), 75-82.

Yoon, H. A., Aleyas, A. G., George, J. A., Park, S. O., Han, Y. W., Lee, J. H., Cho, J. G., and Eo, S. K. (2006). "Cytokine GM-CSF genetic adjuvant facilitates prophylactic DNA vaccine against pseudorabies virus through enhanced immune responses." *Microbiol Immunol*, 50(2), 83-92.

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This book focuses on recent advancement of gene delivery systems research. With the multidisciplinary contribution in gene delivery, the book covers several aspects in the gene therapy development: various gene delivery systems, methods to enhance delivery, materials with modification and multifunction for the tumor or tissue targeting. This book will help molecular biologists gain a basic knowledge of gene delivery vehicles, while drug delivery scientist will better understand DNA, molecular biology, and DNA manipulation.

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