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IN VIVO BIOLUMINESCENCE IMAGING IN PRECLINICAL TRIALS OF GENETIC VACCINES

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In vivo bioluminescence imaging in preclinical trials of genetic vaccines
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

DNA immunization is a rapidly developing vaccine platform for infectious diseases, cancer and allergies. The efficiency of DNA vaccination is largely determined by the efficiency of delivery and subsequent expression of genes encoding microbial and tumor antigens or allergens in the cells of vaccine recipients. DNA immunogens are generally administered by intramuscular or intradermal injections, followed by electroporation to enhance the DNA uptake into the cells. An intense debate on the pros and cons of different routes of DNA delivery is still ongoing.

The aim of this work was to develop *in vivo* imaging applications for improvement of DNA immunization. The first aim was to optimize delivery techniques in order to increase the efficacy of *in vivo* delivery of DNA vaccines and subsequent immune response. Using model DNA immunogens encoding luciferase, and HIV-derived immunogens encoding protease (PR) and reverse transcriptase (RT), we defined the differences in the strength and type of immune responses induced by them when administered by intradermal or intramuscular injection routes followed by electroporation. Furthermore, we determined the extent to which the method of DNA delivery influences the immune response to Th1 and Th2 type immunogens, represented by plasmids encoding PR and RT of HIV-1. Finally, we developed imaging applications for the *in vivo* assessment of the effector/lytic potential of the immune response in tumor and surrogate pathogen challenge models.

We immunized mice with DNA immunogens mixed with a gene encoding a bioluminescent reporter. Bioluminescence imaging (BLI) served as a tool to monitor the expression of delivered reporter genes *in vivo*. By combining the readouts form BLI and immunoassays we defined a set of delivery parameters that led to the best immunization outcome in terms of both immunogen expression and subsequent immune response. After optimizing the delivery conditions we tested different immunization routes to determine the one that ensures maximal immunogenicity of DNA immunogen. Here we show that intradermal administration resulted in a significant enhancement of both cellular and humoral immune responses as compared to intramuscular delivery. This was evident regardless of the nature of the immunogen (Th1 vs. Th2). The kinetics of the loss of co-delivered reporter gene expression was found to correlate with the antigen-specific production of IFN-γ and IL-2 and could thus be used as *in vivo* correlate of the strength of specific immune responses. Thus, non-invasive imaging allowed to assess the immunogenicity of DNA vaccines *in vivo*. Using the same parameters we developed a surrogate method that could assess effector memory responses. Finally, we applied BLI to study the growth of luciferase-labeled tumors in luciferase-immunized animals, which provided a functional measure of vaccine efficacy.

Overall, the use of BLI allowed us to establish a methodology to increase the efficacy of delivery, define optimal regimens and test the effector capacity of the immune response induced by DNA vaccination. The application of this technique made it possible to significantly refine and reduce animal experimentation in gene vaccine development.

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CONTENTS

LIST OF ABBREVIATIONS

3D	three dimensional
Ad5	adenovirus 5
ADCC	antibody-dependent cellular cytotoxicity
APC	antigen presenting cell
BLI	bioluminescence imaging
BLT	bioluminescence tomography
CCD	charge-coupled device
c-di-GMP	cyclic dinucleotide diguanylate monophosphate
CpG	cytosine-phosphate-guanine oligonucleotide
CT	computed tomography
CTL	cytotoxic lymphocyte
DAI	DNA-dependent activator of interferon
DC	dendritic cell
DNA	deoxyribonucleic acid
EP	electroporation
FACS	fluorescence-activated cell sorting
HBV	hepatitis B virus
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HPV	human papilloma virus
HRP	horseradish peroxidase
ID	intradermal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
IN	integrase
IRF	interferon regulatory factor
IVIS	in vivo imaging system

LN	lymph node
Luc	luciferase
NF-κB	nuclear factor kappaB
MAGE	melanoma antigen
MEA	multielectrode array
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
NK	natural killer
NPE	non-penetrating electrode
PE	penetrating electrode
PET	positron emission tomography
Pol	polymerase
PR	protease
RIG-I	retinoic acid inducible gene 1
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	reverse transcriptase
SPECT	single-photon emission computed tomography
STING	stimulator of interferon genes
TAA	tumor associated antigens
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
WT1	Wilm’s tumor gene 1

1 INTRODUCTION

1.1 DNA VACCINES

A DNA vaccine is described as a genetically engineered plasmid that codes for antigenic proteins under the control of an eukaryotic promoter, which when delivered *in vivo* directs expression of the encoded protein(s) (1). Although DNA vaccines are referred to as a relatively new vaccination vehicles, the inception of this strategy was commenced more than 50 years ago during the conduction of tumorigenesis studies. Independently, two groups were able to show that introduction of tumor DNA derived from mice resulted in the development of tumors in the mice, in which it was injected (2, 3). However, it was not until the 1980s when the studies of *in vivo* expression of injected plasmid DNA really exploded (4). Studies proved the concept of *in vivo* activity in animal models: it was demonstrated that Hepatitis B Virus (HBV) DNA could induce hepatitis in chimpanzees (5) and that the synthesis of growth hormone can be triggered by the injection of its gene in rats (6). Even at this early stage some studies were able to show the induction of immune responses after DNA injection. Seeger *et al.* demonstrated that an intrahepatic injection of Ground Squirrel Hepatitis Virus genomic DNA elicited the production of specific antibodies against its antigen, which confirmed the activation of humoral immunity in these animals (7).

Although many of these studies were able to validate the principle of *in vivo* expression of injected DNA, they frequently utilized special DNA preparations, including liposome encapsulation or calcium phosphate precipitation to improve cell transfection rates (8–10). Not long thereafter, researchers were able to show that the injection of a pure DNA plasmid was also capable of *in vivo* transfection and protein expression. Wolff *et al.* were among the first to manifest the phenomenon by administering a selection of reporter genes by intramuscular (IM) injection in mice and observing the gene products in transfected murine cells (11).

The demonstration of efficacy of *in vivo* DNA transfection led to the initiation of a plethora of studies exploring DNA vaccination. Groups reported production of antibodies against Human Growth Hormone in mice following a genetic immunization with genes derived from Human Growth Hormone (12). The immunological protection from disease by DNA immunization is attributed to Ulmer *et al.* (13) for cell mediated immunity and Fynan *et al.* for humoral immunity (14). The former demonstrated protection against H7N7 influenza after administration of two doses of an H7-expressing DNA construct. The latter further elaborated on the role of different delivery routes in protection against influenza challenge. In these early stages of technological breakthrough many renowned international vaccine meetings featured presentations on the use of DNA vaccines against infectious diseases (13–15).

Due to the promising results already acquired in small animal models, clinical trials were bound to soon ensue. Almost 20 years ago, the first phase I trial became a reality. Its purpose

was to evaluate the efficacy of a therapeutic/prophylactic DNA vaccine targeting human immunodeficiency virus type 1 (HIV-1) (16). The range of targets expanded rapidly as studies targeted other infectious agents such as influenza, hepatitis, human papillomavirus (HPV), and also cancer. DNA vaccines were safe and very well tolerated, but the overall results showed less immunogenicity in humans than had been expected from animal studies. Immunogenicity manifested by low $CD8^+$ and $CD4^+$ T-cell responses and low antibody titers was disappointing. Nevertheless, these studies served to show that DNA vaccines could safely be used to induce immune responses in humans (even though they were of suboptimal frequency).

1.2 BENEFITS OF DNA VACCINES

DNA vaccines feature several fundamental advantages that set them apart from the conventional vaccination platforms, such as protein, viral inactivated, or live attenuated viral vaccines. They are much safer than attenuated and inactivated vaccines, which may hold the risk of triggering an infection due to incomplete inactivation or poor attenuation of the virus. Existing reports have pointed out the risks associated with the latter, especially for debilitating diseases such as poliomyelitis, where the strain of poliovirus in the oral poliovirus vaccine had reverted to neurovirulence and caused vaccine-associated paralytic poliomyelitis in vaccinees or lead to emergence of vaccine-derived poliovirus strains (17, 18).

The backbone of DNA vaccines are bacterial plasmids, which are relatively easy to design and produce even on a large scale. Additionally, they are relatively stable (19), which facilitates their production and distribution. Full-length genes are readily incorporated in DNA constructs, which allows for correct subsequent maturation, glycosylation and processing, potentially providing immunogenicity close to that of the native protein. Importantly, DNA plasmid vectors can be designed to express only the antigen of interest, while the vectors are designed to be non-immunogenic. This offers the benefit of using prime-boost regimens and avoiding the development of vector-specific immune response, as opposed to the situation with carriers of viral or bacterial origin (20).

Furthermore, DNA plasmids possess an inherent adjuvanticity because of the incorporation of cytosine-phosphate-guanine oligonucleotide sequences (CpG), the so called CpG motives. Most DNA immunogens contain bacterial antibiotic resistance genes as well as bacterial regulatory sequences needed for plasmid propagation in *E. coli*. This creates multiple stretches of unmethylated DNA. Toll-like receptor 9 (TLR9), a receptor found on the surface antigen presenting cells (APCs), recognizes CpGs (21) and may drive the priming and differentiation of cytotoxic T lymphocytes (CTLs) by induction of pro-inflammatory cytokines, such as type I interferon and IL-12 (22). The presence of CpG motifs is not absolutely required for the induction of immune responses, however, they are undoubtedly involved in the induction of the immune response (22).

1.3 IMMUNE ACTIVATION BY DNA

The capacity of DNA vaccines to engage pattern recognition receptors (PRR) such as TLR9 has been shown to be significant in prime, but not in prime-boost immunization schedules (23). Although many studies have investigated and confirmed the connection between TLR9 activation and induction of DNA-specific responses, TLR9 knockout models have shown that the receptor is not essential for DNA vaccines to work (22, 24), which implies the involvement of other DNA sensors that contribute to the immunogenicity *(Fig. 1)*.

The first of what turned out to be a long array of cytosolic DNA receptors was identified in 2007 and called DNA-dependent activator of interferon regulatory factors (DAI) (25). Takaoka *et al.* showed that DAI was capable of upregulating the expression of type I IFNs via NF-κB and IRF3 and to bind to DNA. Two years later, in 2009 another pathway in response to DNA was discovered, which was unusual because of the involvement of RIG-I and MAVS (26, 27). RNA polymerase III (RNA Pol III) was shown to be able to transcribe AT-rich dsDNA in RNA, which subsequently triggers RIG-I leading to production of IFN-β. However, there were still a multitude of DNA responses that could not be accounted for, especially provided that RNA Pol III-RIG-I pathway could only explain the detection of AT-rich stretches of DNA and DAI was only known to act in a cell-specific manner (28). This suggested that additional mechanisms of DNA sensing existed that remained to be elucidated.

In 2008, an important event in the field occurred – several groups in parallel identified the existence of a signaling adaptor protein, STING (29–32). It was shown to be important in IFN-β response to DNA and in Sting-knockout mice responses to infection by DNA viruses were severely abrogated (33). Although all of the mechanisms by which STING activates NF-κB have not yet been determined, a lot is already known, such as its role in the response to viral and bacterial pathogens, self-DNA in autoimmune disorders and mediation of immune activation by DNA-based adjuvants (34). The function of STING in recognition of bacterial second messenger molecules such as cyclic dinucleotide diguanylate monophosphate (c-di-GMP) and recently of the mammalian second messenger cyclic-GMP-AMP (cGAMP) has been of high interest. In addition to its adaptor functions in IFN response to DNA, STING was shown to directly bind c-di-GMP serving as a direct sensor of cyclic dinucleotides (35). Cyclic dinucleotides have recently emerged as effective vaccine adjuvants and immunotherapeutics and the mechanisms by which they are sensed by the innate immune system has been a heavily researched topic (36). Recent work has established an association between STING and both protective and debilitating responses *in vivo*. Some studies exploring its role in cancer immunotherapy have shown that, in mouse tumor models, activation of STING in dendritic cells (DCs) by the recognition of tumor cell DNA can induce protective IFN-β responses that in turn enable DCs to present tumor associated antigen to $CD8^+$ T cells (37, 38). Inversely, STING has been implicated in exacerbation of a

condition where defective DNase activity causes excessive accumulation of self-DNA and STING-mediated inflammatory responses (39).

The discovery of STING stimulated intense research that led to a more detailed description of this signaling pathway. In 2013, Wu *et al.* identified a factor called cGAMP, which was shown to bind STING and activate IRF3 (40). This novel pathway, upstream of STING, involved cGAMP synthase (cGAS), which is activated upon DNA binding, causing its conformation to change in turn allowing access of nucleotide substrates to its active site, followed by cGAMP synthesis (41). DNA sensing by cGAS has been confirmed in cGAS knockout mice, which were not able to induce IFN responses to DNA or infection by DNA viruses (vaccinia, HSV-1) and were significantly more susceptible to lethal challenge by HSV-1 as compared to wild type mice (42, 43). cGAS has also been shown to play a role in sensing of HIV-1 as infected lymphocytes are known to produce cGAMP and the virus induced IFN response was cGAS-dependent (44).

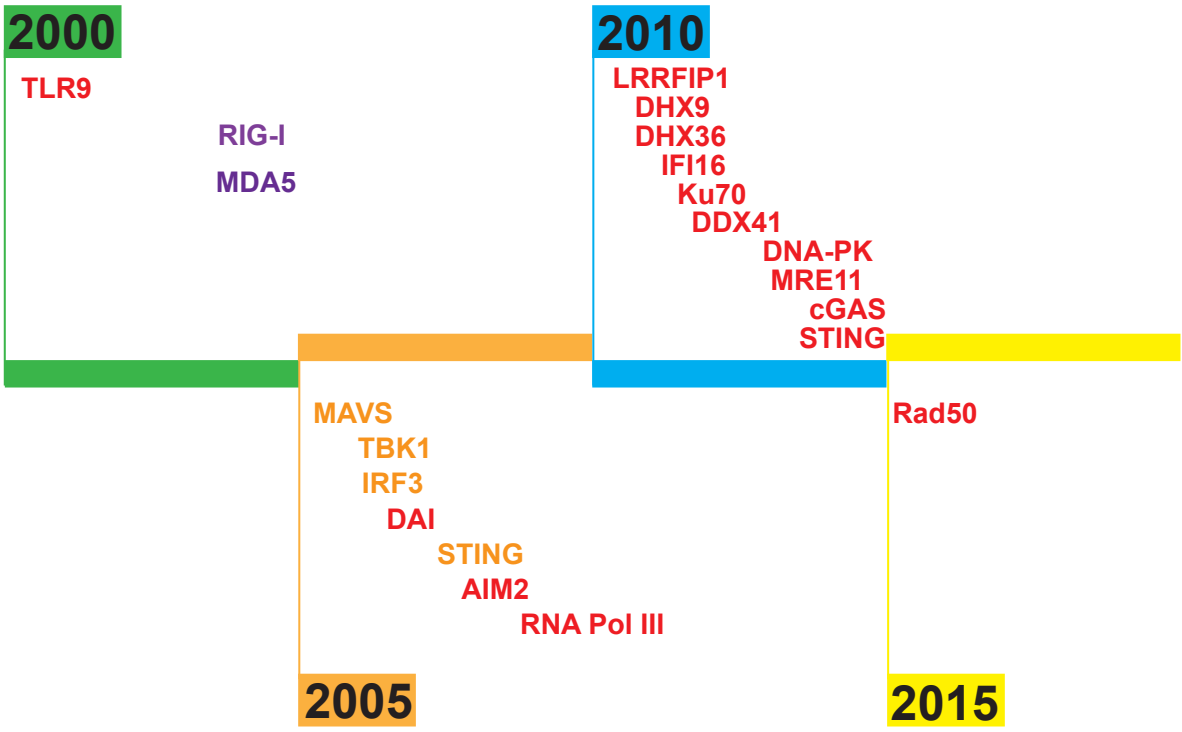

***Figure 1**. Timeline of discovery of cytosolic DNA sensors and signaling molecules. DNA sensors are colored in red, signaling molecules in orange and RNA sensors – in purple. Inspired by Dempsey et al.* (45)

1.4 IMMUNE RESPONSES INDUCED BY DNA VACCINES

Historically, one of the most significant hindrances in the development of DNA vaccines has been the inability to reproduce the results of successful protective immunity, demonstrated in small animal models, in larger animals (46–48). However, recent developments such as codon optimization, gene design and the use of adjuvants have brought DNA vaccines back

into the spotlight. When combined, optimization strategies have been shown to enhance both cellular (49) and antibody (50) responses. Importantly, this has been reported in rodents as well as in larger animal models. These results go to show that a better understanding of the way immune responses are elicited by DNA vaccines is key for our ability to enhance them.

1.4.1 Cellular responses

Cellular responses following DNA vaccine delivery mimic the sequence of events seen after infection by a live virus. In either case the end result is the synthesis of an antigen within the host cell followed by its processing, loading, and surface presentation of the processed antigen in complex with MHC molecules. There are a few distinct ways that the vaccine antigen can be acquired, processed and presented, which in turn determine the overall resulting immune response. Firstly, immune cells can be primed by somatic cells that have been transfected and made to express the vaccine-encoded antigen. Upon transfection, somatic cells process the antigen via the endogenous pathway and subsequently present it loaded on MHC I molecules to antigen-specific $CD8^+$ T cells. Lacking any means of co-stimulation, somatic cells are unable to prime naïve $CD8^+$ T cells (51, 52); however, maintained expression of vaccine antigen can still provide the antigen and augment the response after DNA immunization (53). APCs can acquire exogenous antigen that has been secreted by transfected somatic cells or from phagocytosing apoptotic cells. Secondly, APCs present at the site of immunization or in draining lymph node cells (LN) can be directly transfected by the vaccine immunogen, process and present it on MHC I molecules. Those APCs possess co-stimulatory signals and can therefore prime naïve CD8+ T cells and induce CTLs (54, 55). They can also prime $CD4^+$ T helper cells via MHC II presentation (56). There are also reports of endogenous antigen entering the exogenous processing pathway and being presented on MHC II molecules (1, 56). Another way of acquiring antigen is the recycling of antigen from dying APCs. During this process pre-loaded MHC I molecules can be processed and the antigen presented on MHC II molecules (57) or cross-dressed (58) and directly presented on the surface of other phagocytizing APCs. All of the latter pathways result in the antigen being normally processed and presented on MHC II molecules. However, APCs are special in their ability to cross-present, which translates into antigen escaping from the endosome into the cytosol, where it goes through the endogenous antigen processing pathway and is finally presented on MHC I molecules (59, 60). Due to these processes exogenous antigens acquired by APCs can theoretically serve for priming both naïve $CD4^+$ T helper (Th) cells and naïve $CD8^+$ T cells or CTLs by utilizing the appropriate presentation pathway.

1.4.2 Antibody-dependent cellular cytotoxicity

A hybrid way of cytolysis of antigen-expressing cells is mediated by effector cells in the presence of antigen-specific IgG. This phenomenon was first described in the mid-1960s by Erna Möller, who showed that incubation of mouse tumor cells with serum from rabbits previously immunized with these cells, followed by incubation with lymphocytes from non-

immunized mice resulted in elimination of the tumor cells (61). It was characterized as "serum-induced aggregation" between the tumor and effector cells, however, later on it became to be known as "antibody-dependent lymphocyte-mediated cytotoxicity" (62) and finally as antibody-dependent cell-mediated cytotoxicity (ADCC) that is the term most frequently used today. The main components in ADCC are target cells, antibodies and effector cells and this mechanism is known for its capacity to lyse tumor and pathogen-infected cells. A simplified summary of the process can be defined as the ability of effector cells expressing Fc receptors to lyse cells bearing surface antigens complexed with antibodies.

The understanding of ADCC depends on the elaborate knowledge of the key players involved. Although there have been diverse evidence of the participating effector cells, recent data show that their unifying characteristics can be summarized as granular leukocytes expressing FC receptors. It is important to state that both mononuclear (NK cells, macrophages, γδ T cells) and polymorphonuclear (neutrophils, basophils, eosinophils) leukocytes can carry out ADCC activity (63). A large proportion of studies classify it either NK- or PBMC-mediated, which leads to bias and an unintentional undermining of polymorphonuclear leukocytes in this immunological context.

IgG-dependent ADCC involves the engagement of three types of Fcγ receptors: FcγRIIIA (CD16), FcγRII (CD32) and FcγRI (CD64), with CD16 being the one that is most often mediating the binding process as it is expressed on NK cells (64, 65). IgA-dependent ADCC utilizing FcαR (CD89), which is most abundant on the surface of monocytes, has also been reported (66).

Initiation of ADCC requires interaction between the antibody-antigen complex and the Fc receptor on the effector cell. After binding to its cognate antigen, the Fc region antibody undergoes a conformational change that increases its affinity for a specific Fc receptor on effector cells. The affinity to different Fc receptor is heavily affected by the degree of glycosylation (67, 68). Since the main body of data describes ADDC activity after cross-linking between FcγRIIIA on NK cells and Fc part of IgGs, the best described downstream pathway is the one taking place in NK cells. The established model postulates that after cross-linking the gamma subunit of the FcγRIIIA receptor, containing tyrosine-based activation motifs (ITAMS), becomes phosphorylated by spleen tyrosine kinase (Syk). Binding of Syk activates the three main pathways involved in ADCC, which in turn can activate three mechanisms of killing: perforin/granzyme assisted pathway, FAS-ligand (FAS-L) pathway, and reactive oxygen intermediates/species (ROI/ROS) pathway (69). The perforin/granzyme pathway is the one having attracted the most attention and as a result is the best described. After FcγRIIIA cross-linking signaling pathways lead to increased calcium content, intracellular microtubule reorganization and polarization and release of cytotoxic perforin/granzyme granules (70). Killing of target cells occurs through the well-coordinated

actions of both perforin and granzyme B. Perforin by itself is capable of mediating cell lysis but not apoptosis, which requires granzyme B. It penetrates the cell, activating caspases that trigger DNA fragmentation or initiating a sequence of events that results in the release of mitochondrial contents that either enhance caspase activation or induce caspase-independent apoptosis (71). The FAS-L pathway is significantly less studied and evidence for it is also less substantial. It attributes killing of target cells to a combination of granule secretion and transcriptional activation of FAS-L, which enables NK cells to kill cells expressing FAS receptors (72). The last mechanism of killing, by ROI/ROS is still very controversial. It is based on the fact that phagocytic cells produce ROS in response to antigen opsonization. ROS such as hydrogen peroxide, superoxide and other free radicals are released and in turn damage the opsonized entity (73).

1.4.3 Antibody responses

The capacity of DNA vaccines to induce antibody responses are usually less potent than the capacity to elicit the cellular immune responses (4). A possible explanation for this is the endogenous nature of the encoded antigens. The intracellular localization of the antigen pushes its subsequent processing in the direction of the MHC I pathway. Live virus (74) and protein subunit (75) vaccines have been reported to induce a higher magnitude of antibody responses compared to their DNA counterparts. By definition, the induction of humoral responses requires antigen to be recognized by the B cell receptor, or be processed through the MHC II pathway, which is not possible unless the source of antigen is exogenous. Thus, a likely bottleneck effect might be created by the lack of extracellular antigen, which in turn leads to insufficient activation of this arm of the immune system. This explanation is supported by the fact that DNA vaccines encoding secreted immunogens result in much more potent humoral responses than those encoding intracellular ones (76–78). It has also been reported that the induction of vaccine-specific CTLs has resulted in enhancement of humoral responses (79) suggesting the existence of a synergistic activation of both compartments of the adaptive immune response. Induction of antigen-specific Th and $CD8^+$ T cells after DNA vaccination has also been observed in cases where protective antibody responses were involved (80). To reach maximum potency antibody responses take between 4 and 12 weeks starting from DNA vaccine administration; the antibodies raised are durable (81), have good neutralizing capacity and high avidity (1). The most frequently observed antibody subtypes after DNA immunization are IgA and IgG and the subclass, which is usually heavily influenced by the overall Th1 polarization caused by DNA vaccines, may result in higher abundance of IgG2a/b than IgG1 (82). Typically, immunization with DNA constructs encoding secreted antigen results in the generation of IgG1 antibodies (78), which is also an effect observed after using delivery modalities, such as the gene gun or biojector (82).

Importantly, the route of DNA administration and the way it is delivered can heavily influence the immune response, which may have to deal with the type and location of the cell

that is transfected and in turn expresses the antigen. In mice, the intramuscular (IM) route of DNA administration resulted in significant antigen-specific antibody responses, which were not directly depending on expression of the antigen at the site of immunization. In comparison, when DNA was administered via the intradermal (ID) route by gene gun, humoral responses were of lower magnitude and seemed to require antigen expression at the site of delivery. Thus, it appeared that in ID immunization skin has a vital role in the generation of antibody responses; however, in IM vaccination muscle cells did not provide essential input (83).

1.5 DNA VACCINES FOR CANCER IMMUNOTHERAPY

Cancer immunotherapy recruiting the immune system of the host to eliminate tumors and preventing their reoccurrence has been generating significant attention in recent years. Despite the advantages of DNA vaccines and their ability to induce potent cellular and humoral immune responses, they have had only limited success in fighting cancer. Intense optimization has managed to significantly enhance the immunological efficiency of DNA vaccines, contributing to the ultimate goal of precluding foreign agents such as viruses from causing disease (84). However, the oncogenic etiology of most tumors stems from uninfected, normal tissue expressing antigens, which are either recognized as self, resulting in tolerance and even if modified are still weak immunogens unable to drive effective immune responses. Additionally, such autoimmune reactivities are tightly controlled by $CD4^+CD25^+FoxP3^+$ regulatory T cells, which can suppress such lymphocyte functionality (85). Another hindrance for an effective response is the fact that cytotoxic lymphocytes that recognize an aberrant cancer antigen can become anergic due to the lack of expression of costimulatory molecules, again resulting in immunological tolerance. Finally, tumors are sites of increased mutagenesis, which often results in the loss of immunodominant epitopes that can prevent the immune system from mounting a tumor-specific response thus further impeding the effect of vaccines that target them (85). Despite this, recent clinical trials have been using a personalized approach of inducing T cell response against neoantigens unique for the patient undergoing treatment. These studies used computational methods to predict arising mutations and deliver a vaccine that is often combined with additional therapy such as PD-1 blocking. The results in vaccination against melanoma using this approach have shown significantly reduced metastatic events and tumor progression, which ultimately resulted in tumor control and sustained survival in these patients (86, 87).

One of the absolute prerequisites for developing a successful DNA vaccine is the identification of tumor-specific antigens tumor-associated antigens (TAAs) (88). The first human TAAs were discovered about 20 years ago; however, there was a series of preceding events that ultimately lead to this initial stage of discovery. For a period of 20 years starting from the 1940s the scientists worked on coining the basic idea that tumors induced by oncogenic viruses can be rejected in mice following recognition of viral antigens by the

immune system (89). As a consequence of this, it was shown that chemically-induced tumors, could also be recognized by the immune system and mice were able to reject the same tumor cells upon repeated challenge (90). Contrary to this, in the mid-1970s it was observed that when tumors developed spontaneously in mice there was no evidence of the immunological response being able to control their growth (91). During the same period Boon *et al.* laid the foundation of tumor-specific antigen discovery with their work in mice showing that immune tolerance to mouse teratocarcinoma could be broken when they induced mutations in a tumorigenic cell line producing so the called "tum$^-$" variants not capable of forming tumors. When syngeneic mice were injected with these cells, an immune response was mounted against them (92). Interestingly, injecting teratocarcinoma cells in mice which had earlier received "tum$^-$" cell line variants prevented any tumor development. This served as evidence that identification of the mechanism of tumor rejection in humans can translate into a vaccine, which could induce the immune response inhibit tumor formation in patients (93, 94).

In the early 1990s, van der Bruggen *et al.* used the approach that helped to discover the existence of tumor specific antigens in mice and described the first TAA to be recognized by T cells, called melanoma antigen family A, 1 (MAGEA1). Multiple classes of self TAAs have been identified since this discovery (88). Knowledge of TAAs has been successfully applied in the development of licensed veterinary DNA vaccines (43). However, low immunogenicity still remains the main barrier for the progress of such vaccines in humans. The few exceptions to that are antigens derived from oncogenic viruses such as HPV, which served as a basis for the prophylactic vaccine against cervical cancer (95). Very recent attempts have shown promising results by utilizing novel immunization platforms to induce potent responses against a germline tumor antigen, Wilm's tumor gene 1 (WT1), which is overexpressed in many human malignancies. Walters *et al*. have managed to significantly enhance anti-tumor immune responses by using synthetic micro-consensus DNA vaccine approach to break tolerance in non-human primates (96). The synthetic DNA encodes a consensus protein sequence that was generated by using amino acid sequences of the target protein from various species, but without altering protein structure. Immunization with electroporation (EP) elicited immune responses against native WT1 peptides and was capable of slowing tumor growth. This is just a singular example of a way in which antigen optimization can potentiate immune responses in an environment, where their induction is a significant issue. Similar attempts have been a continuous research trait in DNA vaccine targeting prevention and treatment of viral infection by highly variable pathogens such as HIV-1 and some other viral antigens. Although eliciting immune responses against foreign antigens is theoretically simpler than immune recruitment against self, it takes deep understanding of the functions of the cellular and humoral compartments of the immune system to successfully stimulate them.

1.6 DELIVERY OF DNA VACCINES

The new generation of DNA vaccines that are currently being developed have brought about significant improvements and have successfully turned the spotlight back to this vaccine modality. These new DNA vaccines are capable of generating enhanced cellular and humoral responses in small, as well large animal models including primates (97).

Low immunogenicity of DNA vaccines has been to large extent attributed to inefficient delivery of plasmids, their poor uptake by cells which led to low level of expression of encoded immunogens. Therefore, much effort has been dedicated to devising new methods of delivering DNA vaccines that can maximize the efficacy of *in vivo* transfection. The research was focused on optimization of a number of parameters, such as immunogen design, vaccine formulation, and, importantly, the delivery of DNA into the targeted anatomical location/target tissues (98).

1.7 ELECTROPORATION

EP is a method of delivery of charged (macro)molecules *(Fig. 2)*. Substantial improvements in EP have been achieved during the past three decades allowing for its clinical integration in life-saving procedures such as electrochemotherapy (99). In application to DNA, it utilizes pulses of electrical current to achieve transfection of the cells. The electrotransfer of DNA and genes in varying cell types or electrogenetherapy (100) is now widely researched with multiple ongoing clinical trials, but is yet to be established as a standard procedure in the clinic (101). Clinical studies have shown this modality of gene delivery to be safe in patients. A phase I clinical trial was completed confirming the safety of EP-assisted transfection of IL-12 in patients with metastatic melanoma (102). In case of DNA vaccination, it greatly enhances the rate of *in vivo* transfection of the cells at the site of vaccine administration, and hence, vaccine immunogenicity (103).

1.7.1 Electric field and DNA-membrane interactions

The exact mechanism by which this technique increases the efficacy of DNA vaccination has not been elucidated, however, there are several theories supported by experimental data. Early single-cell experiments have allowed us to look into the mechanics of molecule electrotransfer. Size is known to be a limiting factor for entry into cells and small molecules have almost unrestricted mobility under the conditions of electrotransfer (104). They can cross the plasma membrane of electropermeabilized cells during the application of electrical pulses or in the interval of a few minutes following it (105). However, when the electrotransfer of larger molecules such as DNA is considered, the process seems to follow a more elaborate sequence of events. DNA must first approach, insert itself into and translocate across the cell membrane, then migrate through the cytosol towards the nucleus and finally cross the nuclear membrane (106–109). The electrotransfer of DNA is only possible when DNA is present prior to the introduction of the electric field. Experiments have shown that if

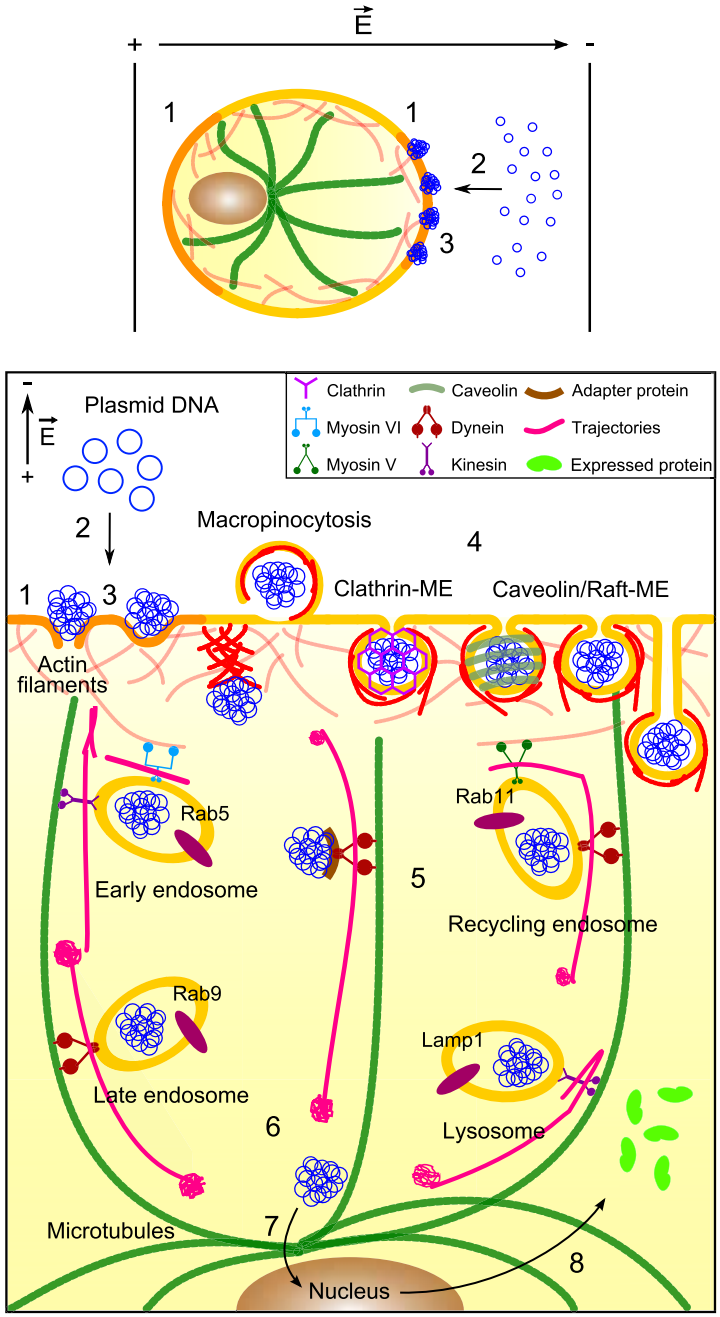

Figure 2. *Mechanism of DNA electrotransfer. When an electric field is applied (1) the plasma membrane is permeabilized and DNA is dragged into the cell membrane proximal to the cathode by electrophoretic forces (2). DNA-membrane interactions occur (3), which causes the formation of aggregates and transient accumulation. Following electric field application and membrane resealing (4) DNA is internalized mainly by endocytosis. During the intracellular transport DNA passes through different endosomal compartments (5). The successful migration of DNA depends on interactions with cellular motor or adapter proteins. In order to be expressed DNA must then escape the endosomal compartment in proximity to the nucleus (6). Finally, DNA must cross the nuclear envelope (7) and be processed yielding proteins that are released into the cytoplasm (8). The figure was adapted from* (110).

DNA is added even 2 seconds after EP, transfection either does not occur or is insignificant (111). Although there are contrasting hypotheses on the matter, some studies have suggested that the permeable membrane structures formed by the electric field are very short-lived and only exist while the electric field lasts (101, 109). Moreover, the interaction between DNA and the cell membrane requires electrophoresis to overcome the negative charge on both, which would otherwise cause a repulsion. Importantly (and confirming the importance of electrophoresis), adding agents that reduce electrophoretic mobility (cations) causes a sharp drop in the transfection efficacy (112). This observation has been experimentally confirmed for multiple tissue types both *in vitro* and *in vivo* (113, 114).

The importance of electrophoretic mobility for induction of DNA-cell membrane interaction is further emphasized by the asymmetric nature of DNA migration under in the electric field. Visualizations have shown that fluorescently labeled DNA interacts with the membrane only on the side facing the cathode. When an electric field is applied DNA moves to the anode permeabilizing any cells in its way and forms DNA-membrane complexes. These interactions are directly dependent on the polarity of the electric field (115). However, if a bipolar electric field (alternating current) is applied, the interactions described above will occur on both sides of any cell within the electric field, which will improve the uptake of DNA (116). Complexes between the cell membrane and DNA are formed in two distinct ways: the latter can either become anchored at one side or it can become inserted within the membrane (117). The EP parameters determine the nature of the complexes that are formed (115), however the biophysical structure and significance of the different DNA-membrane complexes remains to be elucidated.

1.7.2 DNA internalization

The actual process, by which internalization occurs, and the activities of the cell during and after the transfer are not fully understood (118). There are several hypothetic models, but they fail to explain the whole process in its complexity.

One of the prevailing theories relies on the formation of electropores. This was the first of the proposed mechanisms which suggested that plasmids enter the cell via stable macropores on the cell membrane (119, 120). Application of electric field was postulated to alter the membrane potential not resulting in the membrane rupture, but rather in the generation of hundreds of pores of size varying between 1 and 400 nm. The model predicted that this process creates a sufficient number pores large enough to allow plasmids to enter cells even in their circular conformation. The pores were proposed to maintain an open state for the entire duration of the EP procedure (121).

Another model also revolves around the existence of electropores, however, the entry of DNA into the cell is attributed mainly to the electrophoretic forces, which aggregate the plasmids on the cell surface and then push them through the pores (112, 122). The electric field is proposed not to be able to penetrate the initially intact membrane. However, upon pore formation it is able to cross the membrane through them (123). In fact, the electric field is concentrated on the pores and even if their size of about 1 nm is insufficient for the plasmid to pass, the DNA enters, driven by the electrophoretic pressure on the permeabilized membrane (124). This mechanical interaction is suggested to be able to adjust the size of the pores and also prevent their closure if the plasmid is partially through them, even after the electric field has been discontinued (125).

Even though electropermeabilization of the cell membrane remains the stepping stone for successful gene electrotransfer, internalization through pores has become more and more difficult to accept due to the fundamental differences between small molecules and DNA, which tends to exist as large complexes before entering the cell. A third model proposes an entirely different pathway for DNA internalization. At its foundation is the phenomenon that application of an electric field could encapsulate DNA inside giant unilamellar vesicles (126). The model suggests an endocytosis-like internalization of DNA via the formation of vesicles. This endocytic uptake of DNA was first theorized in the early 1990s (112, 127), however it received little attention because the main focus at the time was on the electropore theories, which were seen as much more plausible considering the well described mobility of small molecules across the membrane. This is why endocytic pathways for internalization have been recently gaining more and more attention.

1.7.3 Intracellular DNA transport

The cell cytoplasm consists of an intricate and dense network of microfilaments, microtubules and intermediate filaments (forming the cytoskeleton) in addition to various organelles and proteins. The tightness of this molecular mesh makes diffusion of large DNA complexes a very unlikely event. While the mobility of molecules of size about 700 Da is only 4 times lower in the cytoplasm as compared to water, increased size quickly renders larger molecules immobile (128). When plasmid DNA is microinjected into the cytoplasm or nucleus its mobility is shown to be negligible (129, 130). DNA fragments larger than 2 kb are unable to diffuse through the cytoplasm (131). In the post-EP cellular environment, DNA takes much longer to reach the nucleus compared to small molecules (few hours vs few minutes) (117). Expression of the transgene is already observed 3 h after EP and if ATP is depleted 2 h after EP, gene expression is significantly reduced with no effect on cell viability (132). Very similar transgene expression kinetics have been observed after transfection, which was not assisted by EP (133). These data suggest that intracellular trafficking of DNA depends on the ATP levels, i.e. is not purely mechanical, but is driven by the cellular machinery.

According to the existing theories of DNA internalization, it can either enter the cell through electropores propelled by electrophoresis, or is shuttled in endosomal vesicles. In the first case, DNA would rapidly form complexes with DNA-binding proteins and intracellular polycations (134). This will neutralize its negative charge, make DNA more compact and also protect it from degradation (135). DNA is not known to bind to any motor proteins, so the only scenario of active transport of these complexes is if some of the DNA-binding proteins or polycations serve as adaptors and anchor it to the cellular motors. However, if DNA entered the cell in vesicle, it would already be equipped with the necessary proteins to attach to these motors (136) as endosomes can take advantage of the cellular machinery and be transported freely through the cytoplasm and reach the nucleus.

Colocalization studies in electroporated CHO cells have shown that DNA can be found in significant amounts in vesicles expressing Rab5 (early endosomes), Rab11 (recycling endosomes), Rab9 (late endosomes) and Lamp1 (lysosomes) (137). Rosazza *et al*. also found that in the early stages after EP most of the DNA was within Rab11 and Rab9 vesicles, whereas 2 h after that it was encapsulated by Lamp1 lysosomes (138). This shows that DNA trafficking essentially follows classical intracellular transport pathways. Understanding intracellular DNA trafficking is crucial for optimization of its delivery and subsequent expression. The knowledge that DNA complexes undergo lysosomal degradation prompted efforts to inhibit this process, which has actually been shown to improve its expression (139, 140). A different approach could consist of using nanosecond electric pulses which would only permeabilize the internal membranes, and not the cell or nuclear membranes (141). This would help to release DNA at the endosomal stage, delivering it closer to the nucleus. These and similar strategies and their combinations can tremendously improve the process of *in vivo* transfection.

1.7.4 Crossing the nuclear membrane

The nuclear membrane is the last hurdle that internalized DNA needs to overcome before gene expression can be initiated. Localization studies have shown that even as late as 24 hours after EP, when transgenes are already being translated, most of the electrotransferred plasmid is still in the perinuclear region (117). Only a very small fraction of the plasmid is able to cross the nuclear membrane. This hindrance is due to the large size of the DNA molecule. Molecules up to 40 kDa can easily diffuse through the nuclear envelope; however, transport of the plasmid, which a molecular mass of 1 MDa or more (1 kb = 0.66 MDa) requires a DNA nuclear targeting signal (142). In the absence of a targeting signal, DNA can also be imported into the nucleus during mitotic destabilization. Increased EP-assisted transfection rates have been documented in dividing as opposed to quiescent cells (143); however in some of those reports the plasmids did contain targeting sequences, which could drive the nuclear import (144). Thus, it still remains unclear how plasmids cross the nuclear membrane.

1.7.5 Post-EP tissue traumatization

Electropermeabilization of cells *in vivo* can be a source of significant disturbance of tissue homeostasis. Several direct consequences of applying electric fields are currently known. EP has been shown to result in the induction of high levels of ROS, which could be due to mitochondrial membrane instability and might negatively affect overall cell viability (145, 146). To remedy this deleterious effect, antioxidants have been administered simultaneously with or before EP. In a study that investigated this phenomenon plasmid DNA was mixed with the antioxidant tempol and then electroporated into skeletal muscle. The authors reported a 40% increase in the transfection rate compared to the tempol untreated controls (147). Thus, diminishing of traumatization enhanced the expression, but not necessarily the subsequent immune response.

Another side-effect of the electric pulses traveling through tissue is the generation of heat. Some EP protocols can significantly raise the local temperature and damage or even denaturate tissue (148). Lackovic *et al.* performed an in depth analysis of how different parameters of EP affect heat generation and distribution during pulse delivery. They found that the negative effects of the procedure can be minimized by using shorter pulses with small amplitude (148).

The effect of electrical pulses on the blood flow is also a concern, even more so, for intravenous DNA delivery (149). A study investigated the effects of varying pH values in the post-EP tissue environment and discovered that when hyaluronidase, used to increase DNA uptake (150), is added to plasmid DNA, the electric current increases causing a strong shift in pH and significant tissue damage (151).

The effect of electrical pulses on the blood flow is also a concern especially if DNA is delivered intravenously, as is required for systemic expression of the immunogen(s). A study done in skeletal muscles showed that EP results in a short-term reduction of perfusion (152). Another experiment indicated that electric pulses affect the subcutaneous vasculature. The authors showed that DNA was sensitive to vascular lock (i.e. the ability of blood to move through the vasculature) and constriction of the blood vessels significantly perturbed the movement of DNA through the vascular walls (153).

Importantly, tissue damage inferred by the electric pulses leads to local inflammation, serving as a danger signal and recruiting macrophages, DCs, and lymphocytes (154, 155). This APC-attracting effect has been observed in multiple animal models and is known to occur independently of plasmid delivery (156). A detailed study of skeletal muscle EP in mice showed that after application of ten 20-ms-long, 175 V/cm pulses morphological changes were readily observable after 3 h and lasted for up to 5 days. Infiltration of inflammatory cells coincided with observable tissue traumatization and mainly consisted of $CD11c^+$ DCs. The study showed that 3 h post-EP there was no migration of lymphocytes to the site, however, 4

days after EP $CD4^+$ T cells were detectable in moderate amounts, but CD8+ T cells presence was modest. APC infiltration also overlapped with a significant increase in the levels of TNF-α and IL-1β, which are pro-inflammatory cytokines recognized as danger signals (156). A very similar adjuvant effect of electrical pulses has been demonstrated after intradermal EP-assisted delivery of plasmids in macaques (157). The response to this sterile inflammation was driven by M2 macrophages and promoted tissue repair, which involved $CD4^+$ T cells (133).

1.7.6 In vivo application of electroporation

The first studies that applied this method *in vivo* were conducted in the late 1990s. They evaluated the use of EP for *in vivo* delivery of transgenes in rat livers (158) and rat brain tumors (159). Those early studies successfully demonstrated the ability of EP to mediate gene transfer and expression. Additional experiments demonstrated that a huge enhancement of transfection efficiency: the rates of transgene expression were from 100 to 1000 fold higher in both muscle and skin as compared to the injection of DNA without EP (160–162). The efficacy of gene electrotransfer has been demonstrated in various tissue types with prophylactic and therapeutic applications targeting infectious diseases, cancer therapy, metabolic disorders and vaccines (163).

In vivo plasmid DNA electrotransfer in humans is currently one of the most efficient non-viral methods of gene delivery. Studies have shown it to be superior to the gene gun (164), liposomes (165), sonoporation (166) and the use of cationic lipids (167). The resulting gene expression is transient but can vary from a few weeks (168) to several months (169) with a possibility for repeated transfection capable of reproducing similar levels of expression. Adaptation of the EP procedure now allows the delivery of a gene(s) of interest in various target tissues such as skin, skeletal muscle, liver, kidneys, brain, heart, tumor and eyes without inflicting significant damage (170, 171).

Some of the initial EP mediated vaccination studies aimed at assessing the expression of DNA-encoded antigens and their immunogenic potential. Primary targets of these studies were various HBV proteins and HIV-1 gag. Results showed that electrotransfer of these DNA constructs into muscle induced a significant increase in humoral response against HBV (172) and cellular immune responses against HIV-1 (173) proteins. Recently, many more pathogens have been added to the list of success stories, which EP has contributed to. The use of EP has enhanced immune responses against infectious agents such as: influenza (174–177), HIV (178), HCV, HPV and many others. Enhanced immunogenicity has also been demonstrated after delivery of DNA vaccines encoding antigens from numerous parasitic and bacterial agents (179). This data clearly shows that EP can be utilized not only to improve the delivery and expression of transgenes, but also as a reliable means of increasing immune responses against a broad panel of pathogens for which vaccines are in dire need.

Muscle has been the traditional target for vaccine delivery and therefore the early device production was aimed at manufacturing invasive EP electrodes that inserted deep into muscle tissue. The rationale for using it as a target was that it is highly vascularized, multinucleated and it has the ability to express transgenes at a high rate for extended periods of time (180, 181). Skeletal muscle is also unique in immunological terms. It fits the definition of all immuno-privileged organs by having a slow turnover of cells which is instrumental for the survival of the individual and of the species (182, 183). Similar to other immuno-privileged sites, skeletal muscle in physiological conditions lacks APCs and has low levels of expression of MHC I and II molecules. However, unlike the classical immunologically privileged sites, it is rich in lymphatic vasculature and presents no physical barriers to the immune system. Inflammation in the skeletal muscles is often followed by activation of macrophages and accumulation of regulatory T cells, which favors tissue regeneration and limits autoimmunity (184). Accumulation of $CD4^+$ and $CD8^+$ lymphocytes has also been reported, mostly due to the persistent inflammation or immune-mediated damage (185). Altogether, this suggests that immune intervention at this site would prioritize tissue repair and timely termination of the inflammation process. One of the undesirable effects associated with IM EP delivery was the high degree of pain experienced by the recipients (186). Subsequently, alternative sites for delivery have been explored with skin emerging as a prime competitor. It is a very attractive target for vaccine delivery because of the fact that skin is rich in APCs and is very accessible. Recent studies have shown that expression of transgenes in skin benefits greatly from EP mediated delivery (187–189). There also are a wealth of data demonstrating the superiority of skin in inducing cellular immune responses after DNA immunization (190).

Skin is the largest organ in the human body possessing a high degree on immunological complexity. It serves as a physical barrier, which deters the entry of external agents and also performs various regulatory functions such as temperature control, fluid balance and many others. The thickness of human skin ranges between 0.5mm at its thinnest (eyelids) to around 4.0 mm on the soles of the feet and hands. Structurally is can be divided into epidermis, dermis, and a subcutaneous layer. The epidermal layer is composed of keratinocytes, which form the bulk of it, however, it also consists of dendritic cells known as Langerhans cells, resident $CD8^+$ T cells and a proportion of melanocytes (191). Langerhans cells are the prevailing APCs of the epidermal layer and as such are very competent at taking up vaccine antigens, processing and presenting them to lymphocytes (192). Although they are not classified as APCs, keratinocytes are also highly important in the induction of immune responses since they can be a major source of immune modulators (193). The cells of the epidermis are constantly sloughed off with the average turnover time being 27 days (194). The dermis has a more complicated structure with a heterogenous cellular composition. This compartment is populated by multiple types of specialized immune cells such as DCs, NK cells and $CD4^+$ Th cells (195). Thus, by administering an immunogen into the skin one could target multiple immune cell types and efficiently induce a potent multi-facetted immune

response. The innermost layer of the skin is the subcutaneous layer. It is composed of fatty and connective tissue with the main cell types being adipocytes, fibroblasts, and macrophages (196). The skin is another very popular target for gene electrotransfer. This is largely due to its cell composition, easy accessibility and similar to muscle tissue, its ability to release gene products into the blood stream. The therapeutic potential of gene electrotransfer in skin has been employed in a human trial focusing on treatment of skin cancer (102). Other studies have shown conflicting results – some providing strong evidence of its immunological superiority over skeletal muscle and others demonstrating no benefits or even disadvantages of using skin as a delivery target tissue for DNA immunogens (197, 198).

1.7.7 Electrodes

Electric field in the tissues is administered through application of the electrodes, a conductive device that translates the electric current from the generator (electroporator) into the tissues. In the dawn of EP, electrodes were manufactured for transfection of cell cultures, and represented two parallel plates being inserted in a suspension of cells. Later, cuvettes were developed which are still widely used. However, due to the inconvenience of fitting parts of animals into chambers, the first electrode designs for in vivo use featured metal plates (tweezers or calipers) or needles/needle arrays, which penetrated the tissues (199). Later, numerous variations in the design of electrodes were adapted to the type of transfected tissues and requested expression profile. This was valid also for the needle electrodes which come in multiple configurations (number and length of needles) depending on the target tissues. Such electrodes can be inserted to variable depths and often have insulated shafts to prevent the spread of electrical pulses in the neighboring tissues. The maximal electric field of these electrodes is at the tips of the needles and decreases in a distance-dependent manner (200). Uninsulated needles can deliver pulses over a wider area and are thus also suitable for surface electroporation.

Several types of electrodes have been developed to deliver electrical pulses into the skin. They can be split in two categories: penetrating (PE) and non-penetrating (NPE). NPEs are available as plate, tweezers, and caliper electrodes. All of these modalities are available in both single and multiple conformations and are designed to improve the delivery and expression of DNA plasmids in skin (201, 202). PEs are typically available as needle array electrodes in different configurations. They can provide a range of electric fields between 50-1800 V/cm, pulse length of 0.05 to 650 ms, and pulse number of 1 to 18. Many reports have recently shown the great efficiency with which PEs facilitate gene electrotransfer resulting in a high rate of expression. A number of PEs have been used to carry out immunization trials against various pathogens with data showing that they were able to enhance both humoral and cellular responses as compared to immunization with DNA without EP (203–205). However, recent efforts have switched focus to development of NPE due to the increased pain associated with PEs. Heller and colleagues have been successful in developing minimally

invasive multielectrode array (MEA) for skin EP and optimizing the EP protocol as to reduce the pain caused by strong electric fields (206). They achieved that by reducing the distance between the electrodes so that lower voltages can create the same electric attained by using higher voltage pulses. The resulting decrease of area was compensated for by the introduction of additional electrode pairs on the surface of the device. The MEA features 16 electrodes spaced out at a distance of 2mm in a 4 x 4 array configuration. To achieve an electric field of 250 V/cm it used 50 V pulses as opposed to the 200 V pulses required by typical electrode with a 8 mm gap (206). Similar alternations of this approach have managed to further reduce the voltage required for efficient gene electrotransfer to as little as 15 V (199).

1.8 OPTICAL IMAGING

A variety of imaging methods has been established to look beyond the physical barrier of skin *in vivo*. Based on the classical X-ray imaging, computer X-ray computed tomography (CT) has been developed for the identification of anatomical features, where an image is acquired based on the capacity of different tissues to absorb X-rays. Magnetic resonance imaging (MRI) represents a different approach that exploits the magnetic properties of hydrogen atoms. In that scenario hydrogen atoms are being excited by radio waves and then the radio waves that they emit when reverting back to their original state are recorded and quantified. These are techniques that help us understand the anatomical characteristics of different organisms. If combined with contrast agents and alternative imaging modalities such as single photon emission computed tomography and positron emission tomography they can serve to monitor processes at the molecular level (207–209).

The advances in genetic engineering have enabled scientists to design proteins emitting luminescent or fluorescent light, detectable by various optical devices. Optical imaging possesses some key advantages over other imaging methods. It has been developed to have a relatively high throughput, where multiple animals can be imaged simultaneously over a short period of time. Image acquisition, which is performed using a CCD camera is usually quite straightforward and does not require the attendance of a specialist thereby unlocking the technique for use by a wide variety of researchers. Optical imaging is very well suited for *in vivo* studies, where it can be applied for monitoring at the cellular level of the different processes such as biodistribution (210), gene expression (211), enzyme activity (212), inflammation (213), and tumor spread (214) at the cellular level. In the field of optical imaging bioluminescence imaging (BLI) holds several distinct advantages over modalities utilizing fluorescence. A key difference between these methods is the virtual lack of background luminescence signal in animal tissues. Luminescent light is produced in detectable levels only when the enzyme reacts with an exogenously provided substrate. Unlike luminescence, fluorescence works by excitation from a source different than the emitting subject. Hence, the excitation light can also impact other fluorescent molecules present in tissues and result in a high degree of auto fluorescence background, which would

obstruct the detection of reporters. Furthermore, the requirement of florescent proteins to be illuminated by an excitation source doubles the travel distance of fluorescent light in tissues, which increases its scattering and results in a lower signal/noise ratio.

1.8.1 Bioluminescence imaging of luciferases

Bioluminescence is a natural phenomenon exhibited by a range terrestrial and marine species for various behavioral reasons such as defense, camouflage, communication, etc. (215) The process of emitting bioluminescent light has been thoroughly studied and reproduced by researchers *in vitro*. It is a result of the reaction between luciferase and a substrate know as luciferin. When this interaction occurs in the presence of oxygen and ATP the outcome is the oxidation of the substrate with release of a byproduct – luminescent light. The extensive understanding of this process has facilitated its integration as an essential tool in biomedical research.

In order to make BLI possible *in vivo*, a gene encoding a luminescent reporter protein must be introduced into the tissue to be imaged. Currently there exists a variety of ways to transfer transgenes in animal models, such as using viral or bacterial vectors, injection of cells, electroporation-mediated transfer of plasmid DNA, as well as inducible expression in animals transgenic for the gene of interest. To perform BLI in live animals the subjects are anesthetized and placed in a light-tight chamber equipped with a CCD camera. Before detection of luminescent photons begins a reference picture is taken under low illumination, after which the CCD captures photons in complete darkness and during various exposure times. The data are then analyzed on a computer running the quantification software. The anatomical location of the signal source can be pinpointed by producing an overlay from the greyscale reference picture and the pseudocolor intensity picture that results from detection of luminescent photons *(Fig. 3)*.

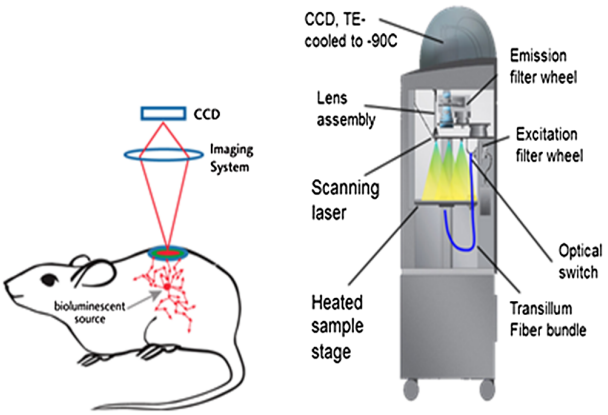

***Figure 3**. Graphical representation of the IVIS Spectrum used for bioluminescence imaging (BLI)* (259).

The localization of bioluminescence signal can be further improved by the use of complementary methods such as bioluminescence tomography (BLT). In that imaging modality the difference in light scattering and attenuation at different wavelengths are taken into account in order to determine the depth of origin of the bioluminescence signal. Due to

the wide emission range of the bioluminescence emitting enzymes (560-660 nm for luciferase) a series of planar acquisitions can be performed allowing the calculation of the depth of the source, based on the signal intensity and adjusted with the known tissue attenuation at the respective wavelength. This information is then combined with computed tomography data to form a complete three-dimensional (3D) model of the subject (216).

Luciferases are at the core of the phenomenon of bioluminescence, making the process possible by their enzymatic properties. The luciferase from the firefly (*Photinus pyralis*) consists of a single polypeptide (217), which uses luciferin as a substrate for an oxidation reaction in the presence of ATP and oxygen to generate light (218):

$$\text{Luciferase} + \text{Luciferin} + \text{ATP} + O_2 \xrightarrow{Mg^{++}} \text{Luciferase-Luciferin} + \text{AMP} + \text{PPi} \quad (1)$$

$$\text{Luciferase-Luciferin} + \text{AMP} + O_2 \longrightarrow \text{Oxyluciferin} + CO_2 + \text{AMP} \quad (2)$$

$$\text{Oxyluciferin} \longrightarrow \text{Oxyluciferin} + \text{light} \quad (3)$$

The reaction between luciferase and its substrate results in the production of oxyluciferin. It is an electrically excited form of luciferin, which upon the return to its ground state releases a single photon (219). In the presence of an excess of luciferin, magnesium, and ATP the photons released are proportional to the amount of luciferase (220). Providing luciferin to cells expressing luciferase will result in emission of luminescence peaking at around 560 nm. The gene for luciferase has been cloned and optimized for eukaryotic expression making it convenient to use in various animal models. Alternative forms of luciferase are also available from organisms such as the yellow click beetle (*Pyrophorus plagiophthalamus*). These luciferases have different emission spectra that can either be green- or red-shifted with light peaking at 543 nm and 618 nm, respectively.

Another variation of luciferase can be isolated from the marine species *Renilla*. Unlike firefly luciferase, it uses a coelenterazine as a substrate. The reaction between luciferase and its substrate is independent of cellular sources of energy, so ATP is not required for the generation of photons (221).

$$\text{Coentelerazine} \longrightarrow \text{Coentelerazine} + CO_2 + \text{light}$$

The process of oxidative decarboxylation of coelentaerazine by the luciferase results in the production of coelenteramide, carbon dioxide, and light peaking at 450 nm.

Bacterial luciferase is yet another form of the enzyme available in nature. In luminescent bacteria the enzyme uses reduced flavin mononucleotide ($FMNH_2$), fatty aldehydes, and oxygen to produce light that peaks at 490 nm.

$$FMH2 + O_2 \longrightarrow FMN + RCOOH + H_2O + light$$

Bacterial luciferases are encoded by the *lux* gene, which also codes for an enzyme responsible for the synthesis of the substrate. The lux operon consists of 5 genes (*luxA-luxE*) and has been expression-optimized in mammals. Its use is further facilitated by the fact that it retains significant enzymatic activity at 37°C (222).

1.8.2 Bioluminescence optical imaging in vivo

The amount of detectable luminescence *in vivo* is largely dependent on the optical properties of the tissues, through which it has to pass in order to reach the CCD. Absorption and scattering are the main parameters that affect passing light. Emission of light with wavelength less than 600 nm is heavily affected by mammalian tissues, whereas red light (wavelengths longer than 600 nm) is not influenced as much. The main factors responsible for absorption of light in tissues are hemoglobin and melanin with both of them absorbing blue and green wavelengths. It is therefore advisable to utilize luciferases producing red-shifted light when targeting deep tissues, so that signal loss is minimized (223). In such cases enzymes like the *Renilla* luciferase should be avoided as they produce little light over 600 nm. Firefly luciferase, however, has an emission spectrum with more than 30% of light that has wavelength longer than 600 nm (211).

Up to date BLI has been successfully implemented in the study of the animal models of many human diseases. One of the first instances of the imaging of luminescent reporters was conducted using *Salmonella typhimurium*, which expressed the bacterial luciferase (224). The luminescent signal from expressing bacteria was detected in many organs of infected animals. After these pioneering studies showed significant success many other bacterial strains have been modified to allow the expression of the lux operon and their application in disease models (225). However, bacteria are only a fraction of the organisms that are amenable to similar engineering. Other types of infectious agents such as viruses and fungi were also successfully modified to carry, deliver and express the luciferase gene (226, 227). Very recent studies have demonstrated how respiratory syncytial virus expressing luciferase can be utilized in studying virus replication and burden *in vivo*. The authors reported that performing whole body BLI in mice provided data of virus titers that correlated well with *ex vivo* plaque assays and qRT-PCR. Moreover, the technique opened up possibilities to monitor in real time the reduction of viral titers and replication after vaccination (228).

Another tremendous advance in the study of *in vivo* processes was the use of BLI to monitor cells labeled with luciferase and assess their population kinetics and gene expression (229). A vivid example of this method are tumor studies, which used tumor cells engineered to express luciferase and then transferred into animal models (230, 231). This powerful approach has been applied in many *in vivo* studies that have investigated the dynamics of tumor growth and

regression aimed to assess the efficacy of various treatments including chemotherapy and prophylactic and therapeutic vaccination (232, 233). Using a similar methods gene expression has also been successfully imaged with transgenic animal models expressing luciferase under specific promoters (229).

2 AIMS

The present work focused on the development of *in vivo* bioluminescence imaging (BLI) techniques to:

- Improve the delivery and expression (localization, level) of plasmid-based DNA immunogens in order to enhance their immunogenic performance.
- Determine how the route and site of DNA vaccine delivery influences its further expression and immunogenicity and if these effect(s) are similar for immunogens polarized to induce cellular versus humoral immune response.
- Follow the development of immune response *in vivo* replacing the intermediate immune tests and defining new end-points to terminate the trials of ineffective/non-immunogenic and promote the effective DNA vaccine candidates.
- Evaluate the *in vivo* effector/lytic potential of immune responses to eliminate the reporter/immunogen co-expressing cells, and create surrogate challenge models for infections in small laboratory animals.

3 RESULTS AND DISCUSSION

In this section of my thesis I will summarize and discuss the main findings of its four constituent papers. The data will not follow the chronological order of the papers, but will rather be presented in a logical progression consisting of four sections. The basic experimental setup used to acquire each set of data is summarized in Table 1. Please refer to the papers for any fine details that might be missing in this section.

Experiment	Immunogen	nn mice	Route	Immunization days				Dose/immunogen (µg)	Dose / reporter (µg)
				1	5	21	28		
Paper I	pVaxLuc	5	im	•				2 x 10	NA
	pVaxLuc	5	id	•				2 x 10	NA
	pVax1	5	im	•				2 x 10	2 x 10
	pVax1	5	id	•				2 x 10	2 x 10
	pVaxLuc	10	im	•				1 x 20	NA
	pVaxLuc	10	id	•				1 x 20	NA
	pVax1	10	im	•				1 x 20	NA
	pVax1	10	id	•				1 x 20	NA
Paper II	pVaxLuc	12	id	•			•	2 x 20	NA
	pVaxLuc	12	id	•			•	2 x 20	NA
	pVaxLuc	12	id	•			•	2 x 20	NA
	pVaxLuc	12	id	•			•	2 x 20	NA
	pVax1	12	id	•			•	2 x 20	NA
	pVax1	12	id	•			•	2 x 20	NA
	pVax1	12	id	•			•	2 x 20	NA
	pVax1	12	id	•			•	2 x 20	NA
Paper III	RTwt-opt-in	5	id	•				2 x 10	2 x 10
	RTwt-opt-in	5	im	•				2 x 10	2 x 10
	PR_Bin	5	id	•				2 x 10	2 x 10
	PR_Bin	5	im	•				2 x 10	2 x 10
	pVax1	5	id	•				2 x 10	2 x 10
	pVax1	5	im	•				2 x 10	2 x 10
	RTwt-opt-in	5	id	•		•		2 x 10	2 x 10
	PR_Ain	5	id	•		•		2 x 10	2 x 10
Paper IV	RTwt-opt-in	5	id	•				2 x 10	2 x 10
	pVax1	5	id	•				2 x 10	2 x 10
	RTwt-opt-in	5	id	•	•			2 x 10	2 x 10
	pVax1	5	id	•	•			2 x 10	2 x 10
	RTwt-opt-in	8	id	•			•	2 x 10	2 x 10
	pVax1	8	id	•			•	2 x 10	2 x 10

Table 1. *Summary of mouse DNA immunization experiments*

3.1 THE DELIVERY AND EXPRESSION OF DNA IMMUNOGENS DEPEND ON THE PARAMETERS OF ELECTROPORATION

The method of delivery of DNA vaccines is one of the crucial determinants of their subsequent immunogenicity. Other factors, such as the choice of target of gene delivery, also play an instrumental role in shaping of the subsequent immune responses. To address these, we undertook the task to thoroughly investigate the effect of injection site and expression localization on the efficacy of genetic vaccines. We also sought to study additional aspects of the process of DNA transfer such as electroporation. EP greatly benefits transgene expression and largely predefines the outcome of DNA immunization. An optimal gene electrotransfer depends on the combination of four main parameters: voltage, current, pulse duration and pulse polarity. These intrinsic properties of the electrical pulses are relayed to an important EP instrument – the electrode.

We evaluated the importance of the electrotransfer parameters by transfecting mouse skin or muscle with the luciferase (Luc) reporter gene and performing a longitudinal follow up of Luc expression by non-invasive BLI. Luminescence intensity is known to be directly proportional to the amount of expressed luciferase (234). Thus, by assessing bioluminescence, we can directly measure the level of protein expression. A plasmid, pVaxLuc encoding the firefly luciferase was introduced into BALB/c mice using different EP regimens.

Most of the early work on delivery and imaging of Luc reporter genes was performed with a CELLECTIS DermaVax device, which is unfortunately limited in the selection of electrodes that it could utilize. Due to this we switched to the CUY21EditII pulse generator (BEX Ltd., Tokyo, Japan). Before proceeding with further testing, we compared the performance of the two devices equipped with different electrodes. Mice immunized with a single dose of expression optimized gene encoding HIV-1 reverse transcriptase (RT)/Luc, using either of devices resulted in similar level of production of IFN-γ/IL-2 response (**Paper IV**). These data confirmed that the CUY21EditII was as good as the DermaVax and therefore suitable further testing of the remaining variables in the EP process.

A wide range of parameters had to be tested due to the lack of established protocols with specific recommendations for each electrode in different animal models. The process of optimization began with comparing electrodes, to choose ones serving for the highest *in vivo* transfection/gene expression levels. Most of our preclinical experience was founded on the use of the multineedle array electrode mounted on the DermaVax device. This electrode was adapted for small animal models by reducing the number of pins of the clinically approved version of the same electrode. However, there were alternative options available including some that were less and some that were more invasive. We tested plate (tweezer) electrodes, which do not penetrate the skin. These types of electrodes require the injection site on the skin to be pinched. Another prerequisite is a stronger electric field since the DNA suspension

is under the skin and the electrodes do not directly come into contact with it. This often results in considerable tissue damage and burns (190). We found this type of electrodes to be inferior to multineedle arrays. We also tested two-needle and fork-plate electrodes *(Fig. 4A)*. The former are penetrating electrodes with insulated needles. It can be inserted to a depth up to 1 cm and delivers the electric pulses at the tips of the needles. Although EP administration was easier using this electrode, its performance was suboptimal compared to multineedle arrays. The last alternative we assessed was the fork-plate electrode. It provided reasonable expression levels that were comparable to those obtained by multineedle array EP, however, its application significantly increased the time required to administer the electric pulses.

Next we addressed the effect of voltage *(Fig. 4B)*. Earlier studies have suggested that voltages between 10 and 100 V are optimal for gene delivery in skin. Exceeding this limit could result in excessive tissue trauma and hamper the overall transfection rate (235). Particular emphasis has been placed on the utilization of low voltage pulses due to the decreased sensation of pain associated with the procedure. Some studies have shown that skin electroporation with <60 V pulses results in improved transgene expression (236). By delivery of either pVaxLuc (Luc DNA) or a mixture of Luc DNA with an optimized DNA immunogen based on HIV-1 RT (RT/Luc DNA) we studied how these parameters interact and contribute to cellular and humoral immune responses after EP-assisted immunization.

Using a multineedle array electrode we compared early expression in mice injected with an RT/Luc mix at 15, 30, 50 and 100 V. Mice immunized with unipolar 15 V and 30 V pulses exhibited no or very poor reporter gene expression and 100 V EP resulted in higher luminescence levels as compared to 50 V (**Paper IV**). Contrary to previous studies we found that higher voltages generated better transfection without any adverse effects as the immunization sites did not show any permanent trauma.

One of the features of the CUY21EditII device that set it apart from the DermaVax is the ability to generate bipolar electrical pulses. As a next step of the optimization process we included alternating polarity pulses in the testing conditions and compared these with the unipolar 50 V and 100 V pulses. The results confirmed previous data suggesting an enhanced level of expression when a higher electric field is applied (115). We observed an increased reporter expression on both day 1 and day 3 after EP *(Fig. 4 C)*.

Changing the duration of the electrical pulses also contributed significantly to increasing the expression. Efficient electrotransfer of genes requires two key events to occur – permeabilization and electrophoretic drag of DNA into the cell. In order for these events to take place there are certain conditions to be met concerning the duration of both permeabilizing and driving (electrophoretic) pulses. The poration pulses must be of high voltage and in the μs range. We have empirically established a good permeablizing protocol that consists of 400 V 50 μs pulses. The voltage of the driving pulses was also established to

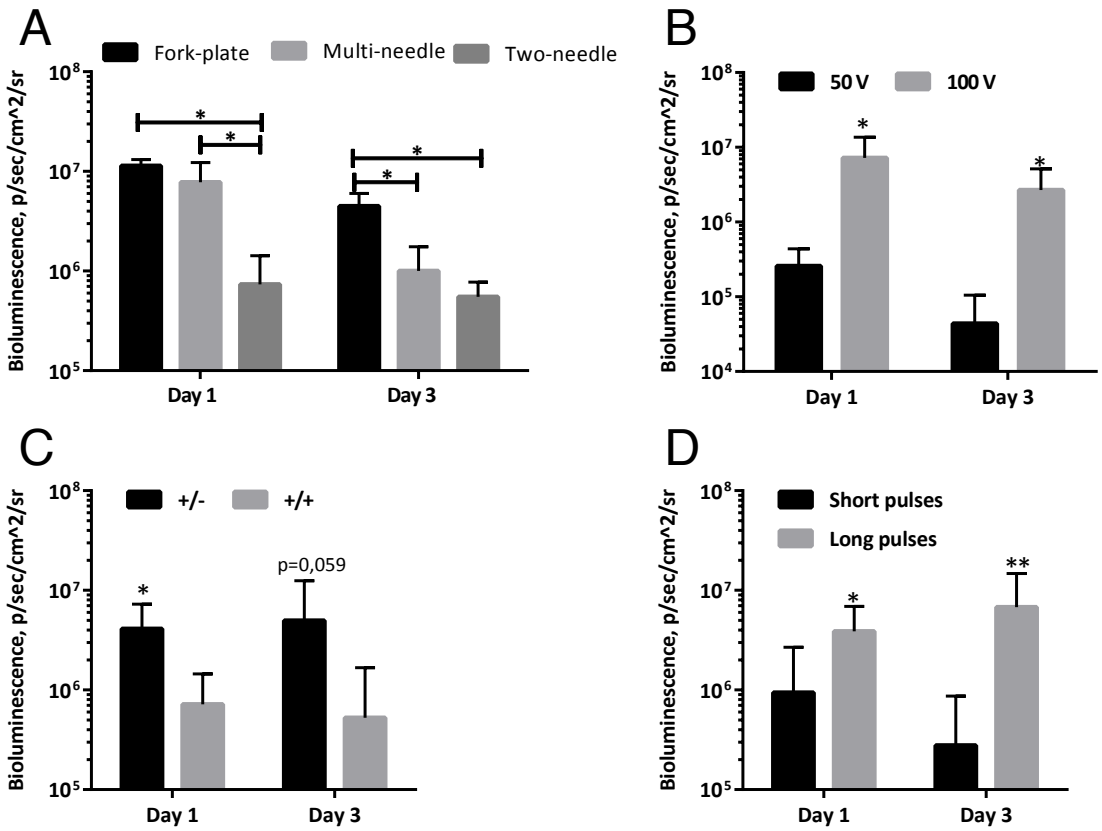

Figure 4. *(A) Comparison of fork-plate, multi-needle and two-needle electrodes in the capacity to promote the expression of luciferase reporter shortly after gene delivery. Levels of bioluminescence at the sites of injections on day 1 and 3. BALB/c mice were injected with 20 μg Luc administered ID to the left and to the right sites from the back of the tail, and immediately after electroporated with CUY21EditII (BEX Ltd, Japan) device equipped with fork-plate, multi-needle or two-needle electrodes. Electroporation was started with a poration pulse of 400 V, followed by a train of eight 100 V pulses, each 10 ms long administered with 20 ms intervals. Parameters of driving pulses optimal for gene delivery by intradermal injections followed by electroporation, defined on the example of luciferase reporter; the effects of pulse: (B) voltage; (C) polarity, (D) duration. Electroporation was initiated with a poration pulse of 400 V of 0,05 ms, followed by a train of eight 50 V (B, C, D) or 100 V (B) pulses of the same (+/+; B, C, D) or opposing/alternating polarity (+/-; C, D) administered for 10 ms (Short pulses; B, C, D) or 50 ms (Long pulses; C, D) with 20 ms (Short pulses) or 950 ms (Long pulses) intervals. Data represent an average photon flux from all injections sites in the group (photons/sec/cm^2/sr) + SD. *p<0,05, **p<0,01 (Mann-Whitney test).*

be 100 V, however the pulse generator manufacturer's recommendation of duration seemed to conflict with the voltage chosen by us. This is why we conducted a trial of optimization and compared 50 ms (recommended) and 10 ms pulses. The 50 ms pulses inflicted significant tissue damage leading to low expression rates and a ultimately premature experimental end-point. By reducing the pulse duration to 10 ms we were able to increase reporter expression while simultaneously eliminating any visible tissue damage *(Fig. 4D)*.

Our optimal EP-assisted immunization protocol consisted of ID injection with immediate application of 400 V poration pulses and 10 ms driving pulses with alternating polarity. By using the CUY21EditII we were also able to carefully control pre-pulse skin resistance and thus minimize tissue damage all while maintaining an adequate current. Importantly, non-EP assisted delivery was ineffective – it resulted in 100 to 1000 times lower Luc expression levels (10^4 – 10^5 without compared to 10^7 to 10^8 p/s with EP) (data not shown).

Our previous results had suggested an implicit relationship between the efficiency of DNA transfer and other parameters affecting electroporation, such as the resistance of skin (237). We investigated how skin resistance influenced transgene expression by electroporating pVaxLuc into the skin of BALB/c mice and monitoring the expression of the gene using BLI (238).

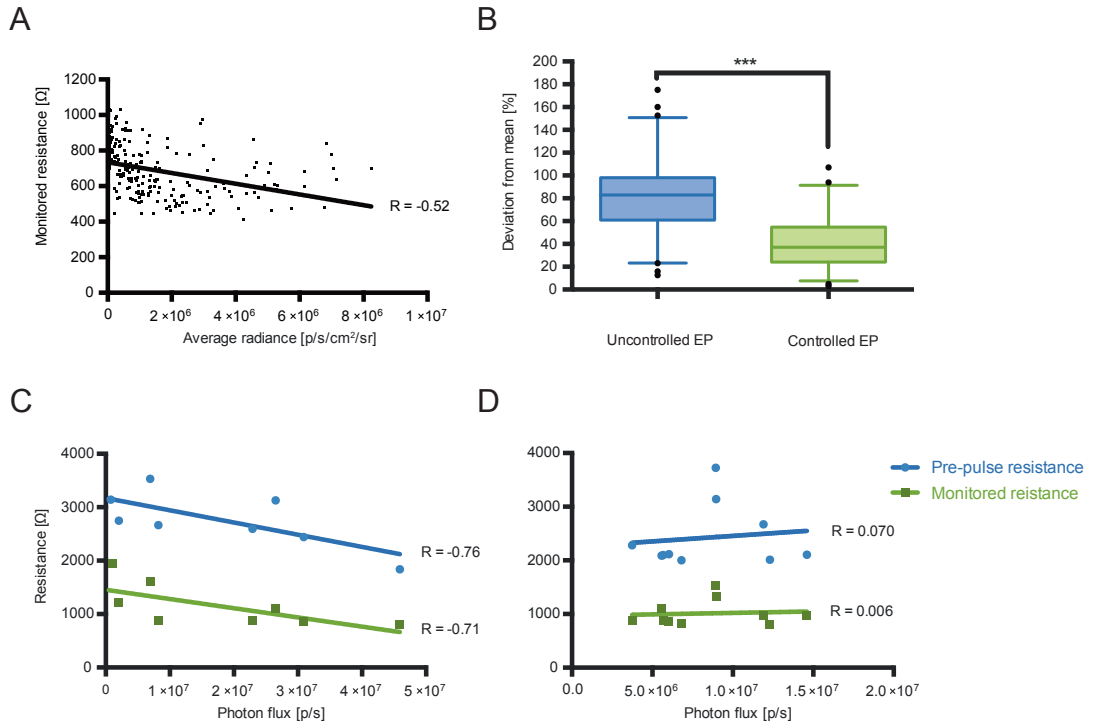

Figure 5. *Dependence of expression of luciferase gene assessed as the total photon flux to the estimated pre-pulse and monitored skin resistance during electroporation (Derma Vax). Analysis of the monitored skin resistance and average photon flux data from pervious Luc gene injection experiments involving 232 injections (A); Variance of average flux from the injection sites four days after Luc gene injection followed by pre-pulse resistance controlled vs. uncontrolled electroporation (B); Correlation between total photon flux (photons/sec) and electroporation parameters 2 h after injection in mice receiving intramuscular (C) and intradermal (D) Luc gene injections.*

Our analyses of the detected luminescence intensity, known to be directly proportional to the amount of expressed luciferase present, showed that skin resistance inversely correlated with the efficiency of *in vivo* transfection and subsequent protein expression *(Fig. 5 A, C, D)*.

In **Paper I** we showed that efficient transgene expression after injection of DNA required electroporation (DermaVax, multineedle electrodes) delivered in a controlled fashion with pre-pulse resistance value maintained below 3000 Ω and monitored resistance values not exceeding 1000 Ω. The validity of this approach was clearly demonstrated by an experiment we performed comparing the outcome of DNA electrotransfer of a luciferase reporter in terms of emitted luminescence after a controlled versus an uncontrolled delivery of electroporation *(Fig. 5B)*. By using BLI we were able to acquire quick and reliable feedback of how different parameters of the EP process affected the delivery and expression of the Luc gene. A controlled electroporation resulted in a significantly tighter variance range of luminescence values as well as higher overall intensity after ID delivery of the gene.

3.2 IMMUNOGEN EXPRESSION IS INFLUENCED BY THE ANATOMICAL TARGET OF DELIVERY

In the process of optimization of EP we observed a wide variation in the immunogen expression after DNA delivery. This posed a question for the capacity of different tissues cell types to internalize and express the electrotransferred DNA immunogens. To address this, we transfected the luciferase gene into the skin or muscle tissues and followed the expression by BLI for 21 days. The reporter gene was delivered to the skin using the standard Mantoux method (239) from here on referred to as ID injection. The target site for delivery into muscle was the caudal thigh muscles proximal the base of the tail. These injections are referred to as IM. All injections were followed by electroporation (DermaVax, multineedle electrodes) in conditions earlier found to promote high gene expression and good immune response (240). Luc expression of injected/electroporated sites was followed by 2D and 3D imaging started 1 hour after the delivery.

Two hours after injection no differences were observed in the intensities of luminescence from the sites of injection in muscle or skin. To ascertain the precision of delivery we performed 3D bioluminescence tomography (BLT) and indeed, saw a difference in the depth at which luciferase was expressed. ID injections resulted in superficial localization of luciferase (~1mm), whereas the highest intensity of bioluminescence after IM injection came from deeper layers of tissue (>2mm) *(Fig. 6)*. Twenty hours post injection the relative luminescence emitted by the muscle was significantly higher than that of the skin. Notably, muscle and intradermal injections were found to result in different luminescence kinetics. DNA transfected in the skin yielded a maximum antigen production by day 1, whereas in muscle the maximum was reached by day 3 post injection. There were no statistically significant differences in the levels of luminescence from day 6 to day 21 of the follow-up, however, the tendency of signal reduction was evident and much clearly pronounced in skin

than in muscle. These data demonstrate that in comparison to the superficial skin-targeting injection (ID), plasmid immunogen delivered via deep injection (IM) results in a more sustained protein expression, and also gives a later peak in the expression of the immunogen (241) *(Fig. 5)*.

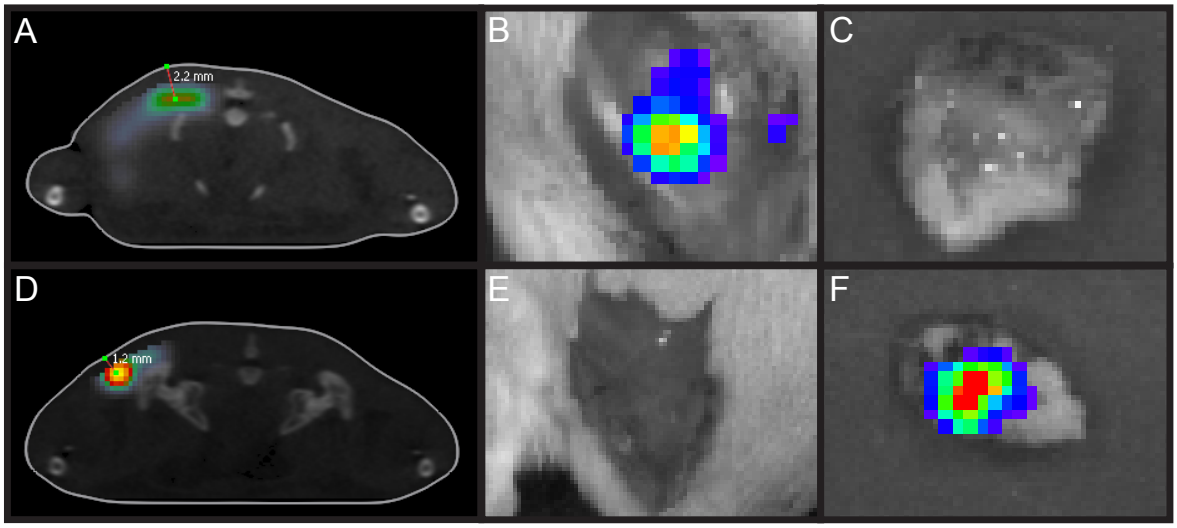

Figure 6. *Expression of reporter gene after IM (A) and ID (D) injection. Tissue from injected mice was excised and monitored for reporter activity. Mice receiving IM injections did not have any luciferase present in excised skin but showed ample signal in muscle tissue (B, C). The opposite was true after ID administration of the reporter gene (E, F).*

Further, we assessed the immune responses induced by ID and IM administration of the luciferase gene. ID immunization resulted in 3-times higher secretion of IFN-γ compared to IM. IL-2 secretion in response to stimulation with peptides encoding $CD8^+$ T cell epitope was also found to be higher in splenocytes from mice receiving ID injections. However, no luciferase-specific antibodies were detected after ID administration of luciferase, while IM injection induced a weak anti-Luc IgG response (titer 50). These data show that the ID injection of a DNA immunogen (242) in skin results in a less durable antigen production, but yields more potent cellular immune responses as compared to the DNA being delivered into the muscle tissues. On the other hand, IM administration supports longer-lasting antigen production, which might play a role in the induction of humoral response to the encoded immunogen.

We performed a similar experiment comparing the expression kinetics of luciferase co-delivered with plasmids encoding model Th1 and Th2 immunogens. We choose two HIV-1 clade B-based DNA immunogens of opposing Th-polarity: HIV-1 protease (PR), which induces potent cellular immune responses of Th1-type while generating no or very poor humoral immune response (243), and reverse transcriptase (RT), which as DNA immunogen induces a Th2-type of immune response characterized by high titer of IgG, induction of anti-RT IgA and production of IL-4 and IL-10 (244). Plasmids encoding PR and RT were

delivered by ID and IM in a mixture with the Luc reporter and then the luminescence levels were monitored for 21 days (**Paper III**).

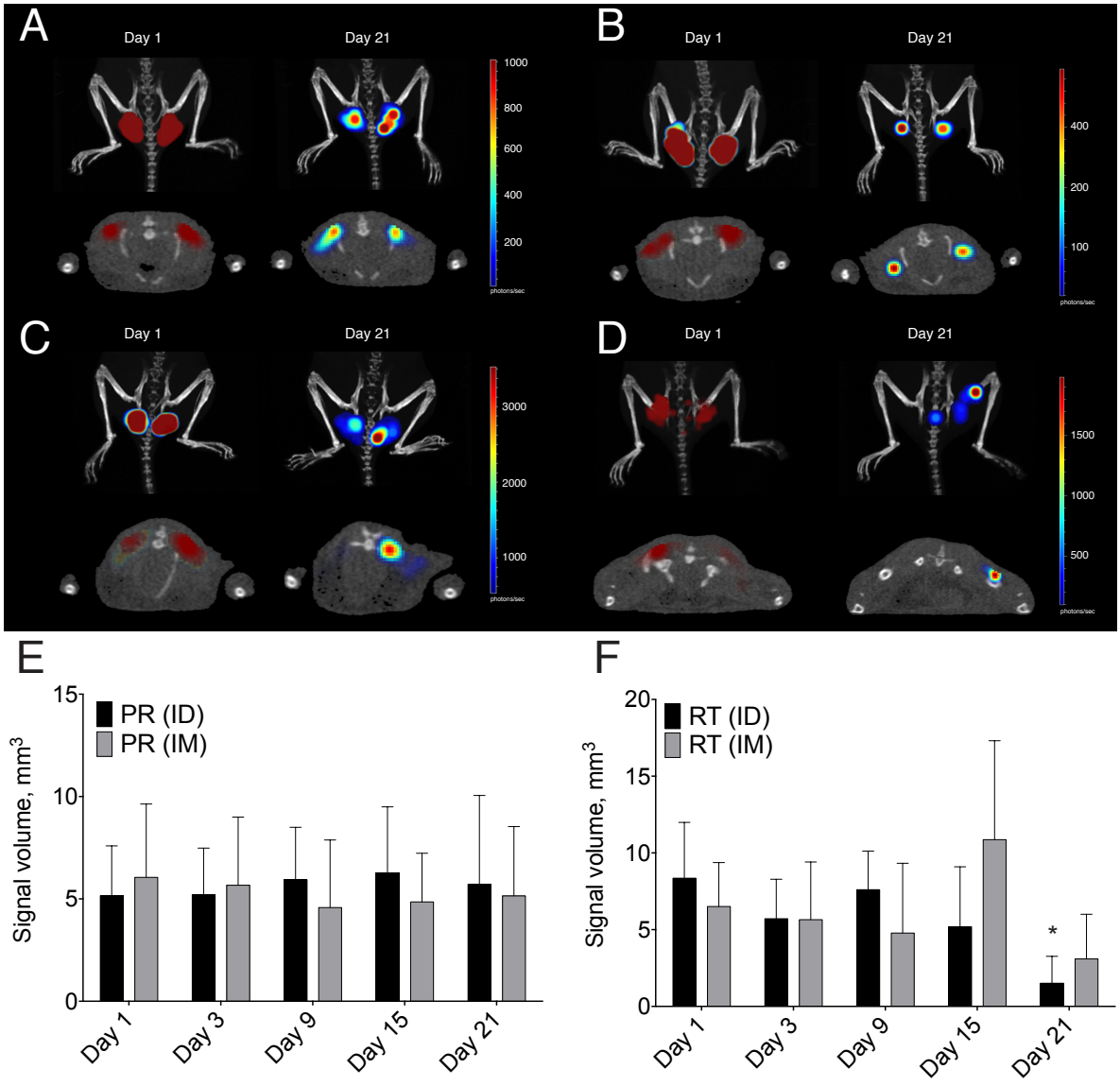

Figure 7. *In vivo volume registration by BLT. The volume of tissue expressing luciferase in mice immunized intradermally (A, B) or intramuscularly (C, D) with PR/Luc DNA (A, C) or RT/Luc DNA (B, D) with sequential imaging done 20-24 hours post injection (day 1) and by the experimental end-point (day 21) (as indicated by text boxed over the panels). Luminescence kinetics demonstrate no reduction in the volume of expressing tissues in either ID or IM PR/Luc DNA immunizations (E). On the contrary, a significant reduction in volume is registered by day 21 for RT/Luc expressing tissues, after ID, and to smaller extent, IM immunization (F). Images were acquired by combined 3D BLI and micro-CT on Spectrum CT with data analysis using Living Image 4.5 software . Statistical comparison was done using Mann Whitney U-test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

At the early time points, skin and muscle supported similar levels of reporter expression. Patterns of the reporter expression for PR/Luc and RT/Luc delivered IM and ID were similar up to day 3. Interestingly, we revealed that the delivery of Th2-polarized DNA immunogen HIV-1 RT, both via ID and IM routes, resulted in a much lower end-point luminescence levels and volume of expressing tissues than the delivery of the Th1-polarized one, such as HIV-1 PR. Also, the bioluminescent signal from the sites of RT expression decayed at a lower initial, but higher overall rate than the signal from the sites of expression of PR. This indicated that the kinetics and the magnitude of reporter expression depended not only on the site of gene immunogen/reporter delivery, but also on the nature of the delivered gene. Overall, the Th2-polarizing immunogens generated a more pronounced loss of reporter expression with "contraction" of expressing tissues, after both ID and IM administrations than the Th1-polarized ones *(Fig. 7)*.

3.3 THE SITE OF DNA VACCINE DELIVERY AND EXPRESSION INFLUENCES THE POTENCY BUT NOT THE TYPE OF IMMUNE RESPONSE

Next we tested if monitoring of the reporter gene activity could be applied to follow not only the delivery, but also the subsequent immunogenic performance of different types of DNA immunogens (**Paper III**). For this, we conducted a series of experiments, which sought to uncover the effects of ID versus IM delivery on the expression and immunogenic performance of polarized DNA-immunogens known to stimulate either Th1-, or Th2-type immunity. Specifically, we immunized mice with HIV-1 PR as a model Th1- immunogen (243) and HIV-1 RT as a model Th2-type immunogen (238). The plasmids were delivered by either ID or IM injections with EP (DermaVax) and the accuracy of delivery was confirmed and followed by both planar and 3D *in vivo* BLI. Tissue targeting was confirmed by the differences we observed in the kinetics of expression, which was monitored via co-delivery of the target DNA immunogens with a plasmid encoding Luc. In ID deliveries of DNA immunogen/Luc, the reporter expression peaked early and steadily declined thereafter. On the contrary, IM delivery of DNA immunogen/Luc followed by EP led to a strong sustained reporter expression. These profiles corroborate our previous findings on the kinetics of Luc expression after ID and IM delivery of DNA encoding Luc alone (**Paper I**) and are consistent with the expression kinetics described earlier for intradermal and intramuscular DNA immunizations.

Murine splenocytes were stimulated *in vitro* with peptides that we have shown to induce a multi-cytokine response, namely aa 1-15 and 75-84 of PR, and aa 465-476 and 528-543 of RT *(Fig. 8 A-D)*. Splenocytes from the PR-immunized animals exhibited a different readout after ID and IM immunization. Being a Th1 antigen, PR immunization resulted in the potent stimulation of cellular immunity both when delivered ID and IM. However, ID delivery generated significantly stronger cellular responses manifested by the production of IFN-γ and

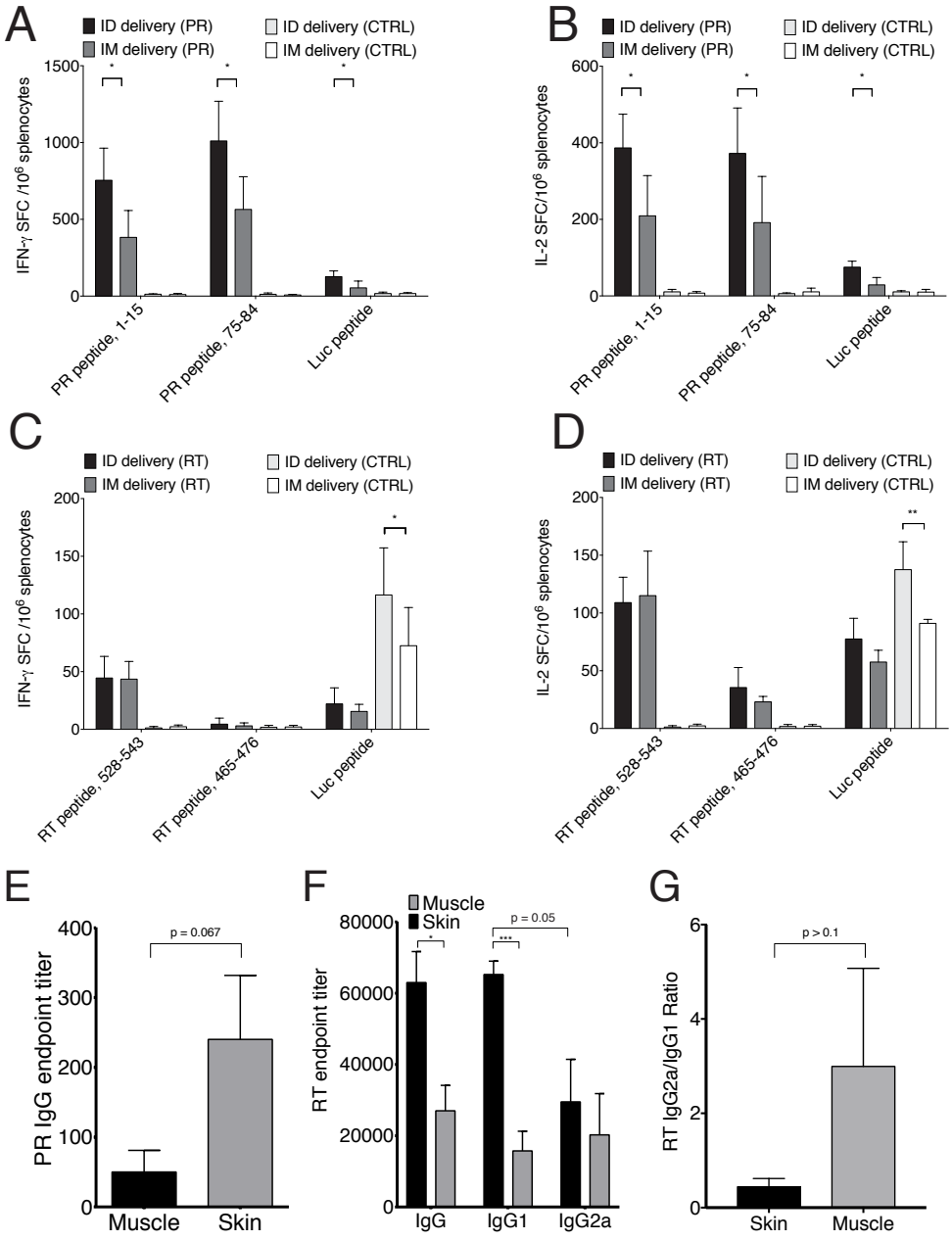

Figure 8. *Immune recognition of the peptides representing $CD4^+$ and $CD8^+$ epitopes of PR (A, B) and RT (C, D) by FluoroSpot test assessing in vitro production of IFN-γ (A, C) and IL-2 (B, D) in mice immunized with the plasmid encoding inactivated PR of HIV-1 HXB2 in pVax1 vector mixed with pVaxLuc (A, B); plasmid encoding inactivated RT of HIV-1 HXB2 in pVax1 vector mixed with pVaxLuc (C, D). Cytokine response to the immunodominant CTL epitope of Luc in PR/Luc, RT/Luc and control empty vector/Luc DNA immunized mice (CTRL) is presented everywhere for comparison. Antibody response was assessed in mice receiving PR and RT DNA by ID (skin) and IM (muscle) delivery. Titer of IgG against PR (E), IgG, IgG1 and IgG2a against RT (F), and the ratio of anti-RT IgG2a/IgG1 (G). Mice were immunized and their responses were assessed on experimental end-point at day 21.*

IL-2. This difference was observed when cells were stimulated both with peptides representing PR aa 1-15, and aa 75-84. Interestingly, for mice immunized with RT/Luc DNA there was little difference in the cytokine production after ID and IM immunizations. Both ID and IM immunization induced IFN-γ and IL-2 response against the peptide representing RT aa 528-543, preferentially recognized by the murine $CD4^+$ T cells. Cellular response to the peptide representing RT aa 465-476 and 528-543 were low and mainly constituted of $CD4^+$ T cells. This reflected the nature of RT as Th2-type immunogen with preferential induction of T helper over CTL response. The data may also reflect the peculiarity of FluoroSpot tests to capture IL-2 in the cell culture fluid giving preference to the registration of reactivities of Th2-polarized $CD4^+$ T cells, which depend not (exclusively) on IL-2, but also on retained IL-4 (245). Apart from this, anti-RT and anti-PR immune responses induced by ID and IM administrations targeted the same $CD4^+$ and $CD8^+$ T cell epitopes. Cellular response to the immunodominant epitope of Luc was weak to insignificant after ID, as well as after IM delivery.

As a Th1-polarized immunogen, PR induced a very weak antibody response with IgG titers less than 200 after ID, and no antibodies after IM immunization. In contrast, the Th2-polarized RT induced a strong antibody response. After single DNA-immunization, the titer of anti-RT IgG reached $2.7\pm0.7 \times 10^4$ in IM, and three-times higher levels $6.3\pm0.8 \times 10^4$ in ID immunization *(Fig. 8 E-G)*. To assess if the Th-bias of RT would be affected by the route of delivery, we assayed the abundance of anti-RT antibodies of IgG1 and IgG2a subclasses, and calculated the IgG2a/IgG1 ratio, considering IgG2a/IgG1 <1 as a tell-tale sign of an antigen inducing a Th2-type immune response (246, 247). ID immunization tended to give a stronger IgG1 compared to IgG2a response, whereas the levels of anti-RT IgG1 and IgG2a in mice receiving IM injections of RT/Luc DNA did not differ. Due to this, IM administration yielded an average IgG2a/IgG1 ratio of more than 1, higher than that after ID administrations, although the difference did not reach the level of significance.

Thus, for HIV-1 PR, immunization by the ID route enhanced cellular response and induced a weak antibody response, and via the IM route, induced only cellular response. For HIV-1 RT, immunization via the ID route enhanced the antibody response and the degree of polarization, while the IM route gave the opposite effect.

In conclusion, by both *in vitro* and *in vivo* bioluminescence-based assays we have demonstrated that ID delivery strongly increases the magnitude of the immune response induced by Th1-, and to lesser extent, by Th2-polarized DNA immunogens. ID delivery amplified the response against Th1 and Th2 immunogens in a similar way, enhancing the responses characteristic to each of the immunogen types, cellular for Th1-type immunogens as HIV-1 PR, and antibody for Th2-polarized ones such as HIV-1 RT, while not altering the response polarization, or epitope hierarchy/dominance predefined by the protein structure. Using ID DNA immunization followed by EP we raised potent cellular and antibody

responses against HIV-1 enzymes capable of clearing the expressing cells from the sites of immunization. The magnitude of these responses and their effector capacity turn these immunogens into attractive components of therapeutic DNA vaccines against drug resistant HIV-1.

3.4 REPORTER EXPRESSION AS A SURROGATE MARKER FOR ANTIGEN-SPECIFIC IMMUNE RESPONSE

The presence of only a single $CD8^+$ T cell epitope on the luciferase protein (242) makes it perfectly suited for the assessment of immune responses with a method utilizing its bioluminescent nature such as BLI. To do this we performed correlation analyses incorporating the luminescence intensity values at different time points and attributes of immune response including levels of IFN-γ and IL-2 production by stimulated splenocytes in *in vitro* tests. Mice which received ID injections demonstrated a strong inverse correlation between the amount IFN-γ, IL-2 and the level of emitted luminescence. This relationship was evident as early as 3 days post injection and reappeared after 21 days. No correlation was discovered when mice received IM injections due to a low magnitude of specific immune response (data not shown).

We further verified the potential of luciferase to serve as means to follow the kinetics of expression of co-delivered gene immunogens. We were able to correlate the decrease of luminescence with the immune response against the DNA immunogen of lytic $CD8^+$ and/or lytic $CD4^+$ T cells (243, 248, 249). However, it remained unclear how this would relate to the Th2-polarized immunogens, which induce only limited cellular responses. To find out, we examined the bioluminescence/immune response correlations for mice immunized with Luc reporter DNA together with a Th2-polarized (RT) in comparison to a Th1 immunogen (PR). We correlated bioluminescence loss with the cellular and antibody responses of mice DNA-immunized with RT or PR via the ID route since it promoted a stronger overall immune response. Cellular responses were represented by the number of splenocytes producing IFN-γ, IL-2 and both cytokines in response to *in vitro* stimulation with peptides capable of inducing secretion of multiple cytokines, and antibody responses, by the end-point titer of specific IgG. These responses were correlated to the loss of bioluminescent signal calculated as the fraction of maximum signal retained at the sites of RT/Luc and PR/Luc delivery starting from when we began to register the loss of the signal to the experimental end-point.

The analysis revealed significant correlations between the loss of bioluminescence signal and IFN-γ and IL-2 secretion in PR-immunized mice in response to stimulation with peptides representing PR aa 1-15 and 71-84/75-84. A highly significant inverse correlation of the magnitude of luminescence with the frequency of T cells secreting IFN-γ in response to stimulation with peptides representing CTL epitope of PR aa 1-15 and 75-84 was observed starting from day 15. Similar and even stronger correlations were identified for IL-2 and dual IFN-γ/IL-2 response. On the contrary, in RT DNA-immunized mice, correlations of

bioluminescent signal with the number of cells secreting IFN-γ and IL-2 *in vitro* in response to stimulation with RT aa 465-476 and 528-543 (which induce a multi-cytokine response of $CD4^+$ and $CD8^+$ T cells) were not significant. Interestingly, however, a strong inverse correlation of bioluminescence was observed for IFN-γ response induced by the peptide representing aa 207-223, which contains a cluster of $CD4^+$ and $CD8^+$ T cell epitopes. In one of the experiments, mice received an RT DNA boost immunization one month after the first administration of RT/Luc DNA. These mice demonstrated a strong inverse correlation of bioluminescence with IL-2 response to RT aa 207-223 already on day 1 after the boost. Apart from this, for RT/Luc DNA immunized mice, we observed a strong inverse correlation of the reporter signal with the magnitude of the end-point RT-specific antibody response ($r = -0.7$; $p = 0.0006$). Interestingly, a direct relationship ($r = 0.46$) was found when antibody titers were correlated with luminescence signal levels on day 1, indicating that a high level of antigen expression early after gene delivery has a positive impact on the development of antibody responses. Of note, all correlations of anti-RT immune response with photon emission after day 15 were non-significant due to the extremely low residual level of bioluminescence observed at late time points. The loss of luminescence and antibody response against PR were not correlated (as the levels of antibodies were negligible).

Altogether, we demonstrated that the loss of bioluminescence from the sites of co-delivery of the reporter with Th1-polarized DNA immunogen (as PR) is correlated to CTLs, and of the Th2-polarized DNA immunogen (as RT), to the antibody response and boostable RT-specific response of effector $CD8^+$ and lytic $CD4^+$ T cells, indicating that these responses are involved in the clearance of the immunogen/reporter co-expressing cells. Recently, there has been an increasing interest in a systems biology approach for modelling the immune system and the way it responds to various stimuli such as infection or vaccination (250, 251). These efforts have a significant impact on the development of therapies and vaccines for different diseases. As the kinetics of reporter clearance correlate extremely well with antibody responses for Th2, and with cellular responses for Th1 immunogens, they can be advanced as a surrogate *in vivo* biomarker of the induction of the effector immune response in gene immunization. Furthermore, correlations of bioluminescence loss with the cytokine production in response to immunodominant T helper cell and CTL epitopes can be useful for predicting the immune responses in DNA-immunizations done with a mixture of immunogen/reporter expressing plasmids and for the immunogens and/or in the species, for which the immunodominant epitopes to use in the assessment of immune response are not known.

Our data demonstrates that the clearance of RT expressing cells is antibody dependent, indicating involvement of pathways, alternative to $CD4^+$/$CD8^+$-driven cellular cytotoxicity, possibly ADCC. Series of studies stress the role of ADCC activity in controlling experimental HIV/SIV infections (252, 253). Peptides derived from Pol polyprotein are common ADCC targets in HIV infection (254). In rhesus macaques the induction of ADCC-promoting antibodies specific for $CD4^+$-induced epitopes on the background of a balanced

$CD4^+$ T cell response correlated with protection from SIV infection (252). Corroborating these findings, we show that the intradermal DNA immunization with HIV-1 RT induces a strong antibody response associated with close to complete clearance of RT/reporter co-expressing cells from the sites of immunization. Furthermore, the antibody production is accompanied by a $CD4^+$ T cell response against multiple epitopes within HIV RT, with the dominant epitope correlated to the *in vivo* clearance of RT/reporter expressing cells. The precise mechanism of immune clearance of RT-expressing cells needs to be further elucidated, but already at this stage, the lytic potential of the response points at the utility of this enzyme as a component of therapeutic HIV DNA vaccines.

3.5 IN VIVO IMAGING IN ASSESSMENT OF THE EFFECTOR CAPACITY OF THE IMMUNE RESPONSE

To evaluate the *in vivo* effector capacity of immune responses, we immunized mice with RT encoding plasmids, mixed 1:1 (w/w) with the plasmid encoding Luc. Bioluminescence from the injection sites was monitored by *in vivo* imaging on days 1, 3, 9, 15 and 21 post immunization. A statistically significant decrease of the bioluminescence levels was observed in mice receiving Luc mixed with expression optimized RT compared to empty vector. The loss became dramatic by day 15. When Luc DNA was administered with the expression-optimized RT genes, bioluminescence signals decreased by 99% within two weeks, and 99.9%, within three weeks of DNA delivery. The loss of luminescent signal coincided with the development of cellular and antibody response against RT confirming the correlation between the levels of luminescence and lytic/effector potential of the specific immune response.

In **paper III** we provided evidence that the development of cellular and antibody immune response could be monitored by co-delivery of a Luc reporter gene and *in vivo* imaging of the bioluminescence kinetics. The study was based on a single injection with EP by multineedle array (DermaVax) of an optimized PR or RT immunogens. In **paper IV** we went on to further optimize the EP parameters thus improving immunogen expression. The next unaddressed question was whether we can achieve an enhancement of immune response by applying different immunization schemes, and if so, whether this could be observed by *in vivo* imaging.

The application of prime-boost vaccination strategies has been shown induce substantially higher magnitude of cellular and humoral responses than those obtained after a single immunization. DNA priming is believed to provide multiple advantages such as (a) efficient generation of memory T cells that can later be boosted in a homologous or heterologous manner (255, 256), (b) induction of effector T cells with enhanced IFN-γ production profile (257) and (c) induction of broad T cells responses against multiple epitopes including sub-dominant ones (257). The timing of the booster immunization plays an essential role in the enhancement of memory immune responses (258). In **paper IV** we used a set of expression-

optimized inactivated RT-based DNA immunogens to prime mice and then deliver a homologous DNA boost four weeks later. Assessment of the immune responses of these animals three weeks after the boost showed a higher overall titer of RT specific IgG.

In order to obtain *in vivo* evidence of boosting of the effector immune response and well as the proof that it can be monitored by *in vivo* imaging, we designed the experiment in which animals were first primed with RT DNA and electroporated using protocol optimized for BEX device with fork-plate electrodes. Priming was done with RT DNA immunogen alone (without adding Luc DNA). Booster immunization consisted of a mixture of RT DNA and Luc DNA in a 1:1 w/w ratio. This allowed us to monitor only the RT-specific recall responses as the animals had no prior exposure to Luc immunogen that has the capacity to contribute to the immune-mediated clearance of cell in which it is expressed (**Paper I**). Shortly after delivery of the plasmid mixture luminescence levels were indistinguishable from those observed in previous priming immunizations. However, by day nine we observed almost complete clearance of luminescence - an event normally registered two weeks after the single RT/Luc DNA co-injections. Both antibody and cellular RT-specific immune responses were found to significantly correlate with the drop of luminescence levels (**Paper III** and **Paper IV**).

The experimental setup we developed, in which the co-delivered reporter gene serves as a surrogate marker gauging the functionality of the immune response can be applied to other microbial vaccine tests that lack a challenge model in small laboratory animals. Moreover, the "antigen challenge" approach can be applied to practically any vaccination scenario and allows to significantly minimize assessment risks linked to the challenge with pathogens, as well as to reduce the cost and time spent in assessing the vaccine efficacy.

We used this technique to obtain the *in vivo* proof of boosting of anti-RT effector immune response. If functional, the boosted immune response should clear the immunogen/reporter-co-expressing cells in mice boosted with the RT gene faster than in mice immunized for the first time. To find out if this was the case, mice were primed with the expression-optimized inactivated RT gene variants as described above, and four weeks later boosted with a 1:1 (w/w) mixture of Luc with the RT gene variant used in priming. Loss of bioluminescence in mice primed with an RT gene, and boosted with a mixture of this RT and Luc genes was compared to that in mice receiving this mixture for the first time. A dramatic loss of bioluminescence was observed by day 9 after the boost, i.e. one week earlier than in mice immunized with RT/Luc mixture once *(Fig. 9)*. This shift indicated a pre-existence of the RT-specific effector immune response induced by priming and furthered by booster injections (**Paper IV**).

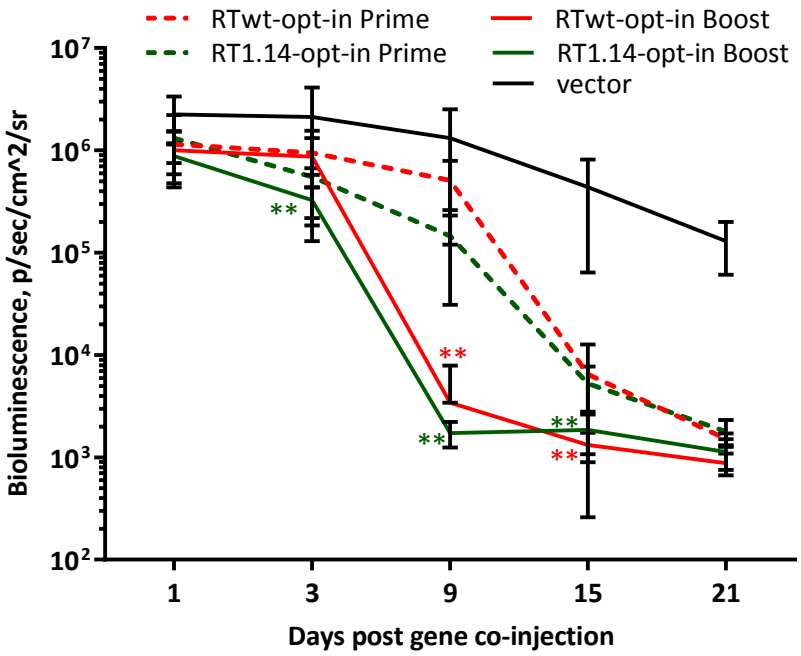

Figure 9. *Antigen challenge model for testing effector immune responses. Mice were immunized with DNA encoding two variants of RT in a mixture with Luc or alone. Animals receiving the mix of genes were monitored by BLI until day 21. Three weeks after the prime all mice were boosted with a mix of RT/Luc DNA. Dotted lines represent luminescence kinetics after prime and solid lines – after boost immunization. Days after each immunization are shown on the x axis. Luminescence levels decreased significantly faster after mice were administered a DNA boost. Statistical significant difference between values after prime and boost immunizations are indicated by asterisks; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 (Mann-Whitney test).*

We also performed an *in vivo* evaluation of the effector/lytic potential of the immune response induced by DNA immunization in the settings of tumor challenge. For this, we used a syngeneic breast adenocarcinoma cell line engineered to express firefly luciferase (4T1luc2, PerkinElmer) and tested if by immunization with Luc DNA we can protect BALB/c mice from developing solid tumors and metastatic lesions (**Paper II**). The animals were primed and boosted by ID injection of plasmids encoding Luc and then electroporated with multineedle electrodes using either DermaVax (100 V pulses) or the CUY21EditII (50 V or 100 V pulses) EP devices. Two weeks after the boost, mice were challenged with a subcutaneous injection of 5.0 x 10^3 4T1luc2 cells. We had previously established that the ectopic implantation of these cells does not affect their growth rate of metastatic capacity, so we continued to monitor luminescence produced by the injected cells for 23 days as well as the residual luminescence from the sites of injection of Luc DNA. All mice that were immunized using 100 V pulses showed no increase of luminescence from the sites of implantation of tumor cells, and developed no solid tumors (which normally develop during

6-7 days post implantation). Nine days after implantation less than 15% of the initial signal was detectable from the implantation site in these animals. As we previously observed, electroporation using 50 V pulses yielded weaker Luc expression in the immunized animals. Overall, the resistance to tumor growth coincided with the IFN-γ response against the $CD8^+$ T cell epitope of Luc. All animals immunized with non-coding vector DNA showed no production of IFN-γ and developed palpable tumors by day nine.

5. CONCLUDING REMARKS

DNA vaccines represent an attractive vaccination platform for many infectious diseases because of their safety, stability, and ease of manufacture. However, they fall behind in their immunogenic performance, especially when compared to that of live attenuated, recombinant protein or viral vector vaccines. Recent developments in techniques such as *in vivo* electroporation have improved the immunogenicity of DNA vaccines considerably. Further improvements in gene immunogen delivery are needed to increase the ability of DNA vaccines to induce potent immune responses. In this work we showed that the efficacy of DNA immunogens could be enhanced by improving the delivery including optimization of electroporation procedure and selection of an appropriate delivery route best fitting a given immunogen. Furthermore, we have developed a functional method relying on non-invasive bioluminescence imaging to assess the immunogenicity of prototype DNA vaccines *in vivo*. We described correlations of bioluminescence loss with the cytokine production in response to immunodominant T helper cell and CTL epitopes of viral proteins encoded by DNA immunogens. These observations can be useful to predict the immune responses in DNA immunizations done with novel DNA immunogens and/or in the species, where the immunodominant epitopes are yet unknown. Further, an experimental setup was developed, in which the co-delivered reporter gene serves as a surrogate marker gauging the functionality of the immune response. This approach can be applied to test vaccine candidates for microbes which lack a challenge model in small laboratory animals. The “antigen challenge” approach, applicable to practically any vaccination scenario, allows to significantly minimize assessment risks linked to the challenge with pathogens, as well as to reduce the cost and time spent in assessing the vaccine efficacy. These findings would help to upgrade the DNA vaccine modality to the levels acceptable for standard clinical applications and make it suitable for combating the major health problems of our time.

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