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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
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ABSTRACT

DNA immunization is a rapidly developing vaccine platform for cancer, infectious disease, and allergies. The efficiency of DNA vaccination is largely determined by the efficiency of delivery and subsequent expression of the HIV-1 genes in the cells. 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. A number of studies have compared the effect of the delivery methods on the amount and quality of DNA-directed immunogen expression, as well as on the magnitude and specificity of the immune response they generate. Several studies were based on post mortem studies of the tissues, or on indirect expression monitored by techniques such as *in vivo* imaging of reporter genes co-delivered or fused to immunogens.

The aim of this work was to develop *in vivo* imaging applications for DNA immunization. The first aim was to optimize delivery techniques in order to increase the efficacy and immunogenicity of DNA vaccines. Furthermore we set out to use the differences in the strength and type of immune response induced by DNA immunogens administered by intradermal (ID) or intramuscular (IM) injection routes, each followed by electroporation. In particular, the task was to determine the extent to which the method of DNA delivery influences the immune response to T_h1 and T_h2 type immunogens, represented by the viral protease (PR) and reverse transcriptase (RT) of HIV-1, respectively. Our final objective was to use the acquired results in an attempt to model immune responses induced by DNA immunogens *in silico*.

BALB/c mice were immunized with DNA immunogens mixed with a gene encoding a bioluminescent reporter. We used bioluminescence imaging (BLI) as a tool to monitor the expression of delivered reporter genes *in vivo*. By combining the readouts form BLI and immunoassays we were able to produce a set of delivery parameters that result in the best immunization outcome in terms of expression and immunogenicity. Upon the optimization of delivery conditions we exploited different immunization routes to determine the one that is best suited and providing maximal immunogenicity for DNA vaccines. Here we show that ID administration of DNA immunogens results in a significant enhancement of both cellular and humoral immune responses in mice as compared to IM. The increase in the magnitude of immune responses was evident regardless of the nature of the immunogen (T_h1 vs. T_h2). 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 to characterize the strength of specific immune responses against the delivered immunogen. Thus, we were able to assess the immunogenicity of a DNA vaccine by non-invasive imaging of bioluminescence from the co-delivered reporters.

The use of bioluminescent reporters is a new strategy to assess the delivery of DNA immunogens and their expression from the start to the completion of the immunization

experiment. The level of reporter expression in the presence of the DNA immunogens reflects the *in vivo* immunogenicity of the construct, presenting anon-invasive method (technique) to assess the dynamics of the immune responses in individual DNA immunogen recipients useful for determination of the study end-points. The application of this technique allows us to significantly refine and reduce animal experimentation in gene vaccine development.

LIST OF SCIENTIFIC PAPERS

I. **Petkov S**, Heuts F, Krotova O, Kilpeläinen A, Engström G, Starodubova E, Isaguliants M. Evaluation of immunogen delivery by DNA immunization using non-invasive bioluminescence imaging. *Hum. Vaccin. Immunother 2013, Oct; 9(10): 2228-2236.*

II. Krotova O, Starodubova E, **Petkov S**, Kostic L, Agapkina J, Hallengärd D, Viklund A, Latyshev O, Gelius E, Dillenbeck T, Karpov V, Gottikh M, Belyakov I, Lukashov V, Isaguliants M. Consensus HIV-1 FSU-A integrase gene variants electroporated into mice induce polyfunctional antigen-specific CD4+ and CD8+ T cells. *PLoS ONE 201, 8(5): e62720.*

III. **Petkov S**, Latanova A, Starodubova E, Kilpeläinen A, Isaguliants M. Expression localization determines the level of expression and the strength but not the type of immune responses to DNA immunogens in mice. *Manuscript.*

CONTENTS

LIST OF ABBREVIATIONS

3D	three dimensional
Ad5	adenovirus 5
APC	antigen presenting cell
BLI	bioluminescence imaging
BLT	bioluminescence tomography
CCD	charge-coupled device
CpG	cytosine-phosphate-guanine oligonucleotide
CT	computed tomography
CTL	cytotoxic lymphocyte
DC	dendritic cell
DNA	deoxyribonucleic acid
EP	electroporation
FACS	fluorescence associated cell sorting
FSU	former soviet union
GSHV	ground squirrel hepatitis virus
HIV-1	human immunodeficiency virus 1
HPV	human papilloma virus
HRP	horseradish peroxidase
ID	intradermal
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IM	intramuscular
IN	integrase
IVIS	in vivo imaging system
LN	lymph node
MHC	major histocompatibility complex
MRI	magnetic resonance imaging
NPE	non-penetrating electrode

PE	penetrating electrode
PET	positron emission tomography
PR	protease
RT	reverse transcriptase
SPECT	single-photon emission computed tomography
TMB	3,3’,5,5’-Tetramethylbenzidine
TNF	tumor necrosis factor

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 a eukaryotic promoter, which, when delivered *in vivo* result in expression of the encoded protein (1). Although DNA vaccines are referred to as a relatively new vaccination vehicle 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 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 (GSHV) genomic DNA elicited the production of specific antibodies against a GSHV 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). Concurrently, 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 even cancer. DNA vaccines were safe and very well tolerated, but the overall results showed less immunogenicity in humans than had been expected from animals studies. Immunogenicity was disappointing, which was reflected by low CD8+ and CD4+ T-cell responses and low antibody titers. However, these studies served to show that DNA vaccines could safely be used to induce immune responses in humans (even though they were of low frequency).

1.2 BENEFITS OF DNA VACCINES

DNA vaccines feature several fundamental advantages that set them apart from conventional vaccination platforms, such as protein, viral inactivated, or live attenuated vaccines. DNA vaccines are much safer than attenuated and inactivated vaccines, which may hold the risk of triggering an infection by the vaccine. Plasmids, the backbone of this vaccine vehicle, are relatively easy to design and produce even in large scale. Additionally, they are rather stable, facilitating their production and distribution. Also, complete genes are readily incorporated in DNA constructs, which allows for intact conformation and assembly of the protein product, potentially providing a higher degree of native immunogenicity. 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(17).

Furthermore, DNA plasmids possess an inherent adjuvanticity because of the incorporation of cytosine-phosphate-guanine oligonucleotide sequences (CpG). Bacterial antibiotic resistance genes are a required component in most DNA plasmids, which means that these unmethylated stretches are found in most DNA vaccines. Toll-like receptor 9, a receptor found on the surface antigen presenting cells (APCs), recognizes CpGs (18) 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 (19). The presence of CpG motifs is not required for the induction of immune responses, however, they are however undoubtedly involved in the process (19).

1.3 IMMUNE RESPONSES INDUCED BY DNA VACCINES

Historically, one of the most significant hindrances in the development of DNA vaccines has been the inability to achieve results of similar protective immunity in larger animals as in small animal models (20–22). Numerous examples of this can be found in the literature, such as the study by Casimiro et al. aiming to compare cellular responses following an IM injection of either a viral vector (adenovirus serotype 5, Ad5) or a DNA plasmid-based vaccine. The results showed that the plasmid-based vaccine retained only about one-third of its immunogenicity compared to the Ad5 vaccine (23).

1.3.1 Cellular responses

Cellular responses following DNA vaccine delivery mimic the situation after infection by live virus. In either case the end result is synthesis of antigen within the host cell leading to processing, loading, and surface presentation via MHC I molecules. However, 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 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 (24, 25), however, maintained expression of vaccine antigen can still serve the function of providing a source of antigen and augmentation of response after DNA immunization (26). 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. There are also reports of endogenous antigen entering the exogenous processing pathway and being presented on MHC II molecules (1, 27). Those APCs possess co-stimulatory signals and can therefore prime naïve CD8+ T cells and induce CTLs (28, 29). They can also prime CD4+ T_h cells via MHC II presentation (27). Thirdly, APCs can acquire exogenous antigen that has been secreted by transfected somatic cells or from phagocytosing apoptotic cells. This results in 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 the endosome into the cytosol, where it goes through the endogenous antigen processing pathway and is finally presented on MHC I molecules (30, 31). 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 (32) or cross-dressed (33) and directly presented on the surface of the obtaining APC. In these ways exogenous antigens acquired by APCs can theoretically serve for priming both naïve CD4+ T_h cells and naïve CD8+ T cells or CTLs by utilizing the appropriate presentation pathway.

1.3.2 Antibody responses

The ability of DNA vaccines to induce antibody responses are usually less potent than the capacity of raising cellular responses (4). A possible explanation is the endogenous nature of some antigens. The intracellular localization of the antigen pushes its subsequent processing in the direction of the MHC I pathway. By definition induction of humoral responses requires antigen processing to go through the MHC II pathway or be recognized by the B cell receptor, 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 lower 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 (34–36). It has also been reported that the induction of vaccine-specific CTLs has resulted in enhancement of humoral responses (37) suggesting the existence of a synergistic generation activation of both compartments of adaptive immunity. Induction of antigen-specific T_h and CD8+ T-cells after DNA vaccination has also been observed in cases where protective antibody responses were also present (38). Antibody responses take between 4 and 12 weeks to reach maximum potency after DNA vaccine administration and the antibodies raised have a long duration (39), good neutralizing capacity, and good avidity (1). Live virus (40) and protein subunit (41) vaccines have been reported to induce a higher frequency of antibody responses compared to their DNA counterparts. The most frequently observed antibody subtypes after DNA immunization are IgA and IgG and the subclass is usually heavily influenced by the overall Th1 polarization caused by DNA vaccines may result in higher abundance of IgG2a/b than IgG1 (42). Typically, immunization with DNA constructs encoding secreted antigen results in the generation of IgG1 antibodies (36), which is also an effect observed after using delivery modalities, such as the gene gun or biojector (42).

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 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 (43).

1.4 DELIVERY OF DNA VACCINES

The new generations of DNA vaccines that are currently being developed have brought about significant improvements and have successfully turned the spotlight back to this vaccine modality. New DNA vaccines are capable of generating improved cellular and humoral responses even in large animal models. Significantly, they have been shown to possess the capacity of inducing effective CTL responses in large animal models (44).

One of the notorious attributes of DNA vaccines, low immunogenicity, is heavily attributed to inefficient delivery of plasmids and subsequent poor uptake by cells. Therefore, much effort has been dedicated to devising new methods of delivering DNA vaccines that can maximize the transfection efficiency *in vivo*. Some of the main focus of recent research has been the optimization of factors, such as immunogen design, vaccine formulation, and, importantly, the delivery of DNA into the targeted anatomical location (45).

1.5 ELECTROPORATION

Electroporation (EP) is a delivery method, which utilized pulses of electrical current to increase cell transfection rate and generally enhance the uptake and thus, the immunogenicity of a DNA vaccine. The exact mechanism by with this technique provides its benefits has not been elucidated, however, there are a few major theories. It has been proposed that the electrical pulses applied to the site of immunization create transient pores in the cell membranes thereby facilitating the process of DNA uptake into cells (46, 47). Additionally, the electric pulses might be a source of tissue damage, which causes local inflammation, serves as a danger signal and recruits macrophages, dendritic cells (DCs), and lymphocytes (48, 49). The technique relies on a fine balance between voltage and current in order to avoid inflicting excessive damage to the immunization site. Some devices used for EP operate by delivering constant voltage and disregard alterations in tissue resistance may deliver excessive current to the targeted tissue. If this process in not carefully controlled it might result in deterioration, rather than enhancement of vaccine uptake. Other types of devices use square wave pulses of constant current and do not vary the voltage delivered, which is beneficial as it serves to reduce tissue damage and possibly results in lower loss of plasmid expression that might occur during tissue repair (50).

The use of *in vitro* EP to facilitate DNA uptake in cells has been long utilized and is by no means novel. However, 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 trasngenes in rat livers (51) and rat brain tumors (52). Those early studies successfully demonstrated the ability of EP to mediate gene transfer and expression. Additional experiments continued to show that transfection was not only possible, but the rates of transgene expression were from 100-1000 fold higher in both muscle and skin as compared to the injection of DNA without EP (53–55). Furthermore, efficient electrotransfer of genes has been demonstrated in various tissue types with prophylactic and therapeutic applications targeting infectious diseases, cancer therapy, metabolic disorders and vaccines (56).

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 when these DNA constructs were EP-transfected in muscle a significant increase in humoral responses against HBV (57) and cellular immune responses against HIV-1 (58) was observed. Recently, many more pathogens have been added to the list success stories, which EP has contributed to. The use of EP has enhanced immune responses against infectious agents such as: Influenza (59–62), HIV (63), HCV, HPV and many others. Enhanced immunogenicity has also been demonstrated after delivery of DNA vaccines encoding antigens from numerous parasitic and bacterial agents. 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 an extensive panel of pathogens vaccines for which are in dire need.

Muscle has been the traditional target for vaccine delivery and therefore the entirety of 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 (64, 65). One of the undesirable effects associated with IM EP delivery was the high degree of pain experienced by the subjects (66). 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 (67–69). There also are a wealth of data demonstrating the superiority of skin in inducing cellular immune responses after DNA immunization (70).

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 *(Fig. 1)*. The epidermal layer is composed of keratinocytes, which form the bulk of it, however it also consists of dendritic cells known as Langerhans cells and a proportion of melanocytes. Cells of the epidermis are constantly sloughed off with the average turnover time being 27 days (71). The dermal layer of the skin mainly consists of fibroblasts and dermal DCs. This is the layer that targeted when ID immunization is administered. This is also where hair follicles form and blood vessels are found. 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 (72).

1.5.1 Electrodes for dermal electroporation

Currently there are several types of electrodes that have been developed to deliver electrical pulses in skin. They can be slip in three categories: penetrating (PE), non-penetrating (NPE), and microneedle electrodes. 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 (73, 74). 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 0.05-650 ms, and pulse number of 1-18. 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 assess 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 (75–77).

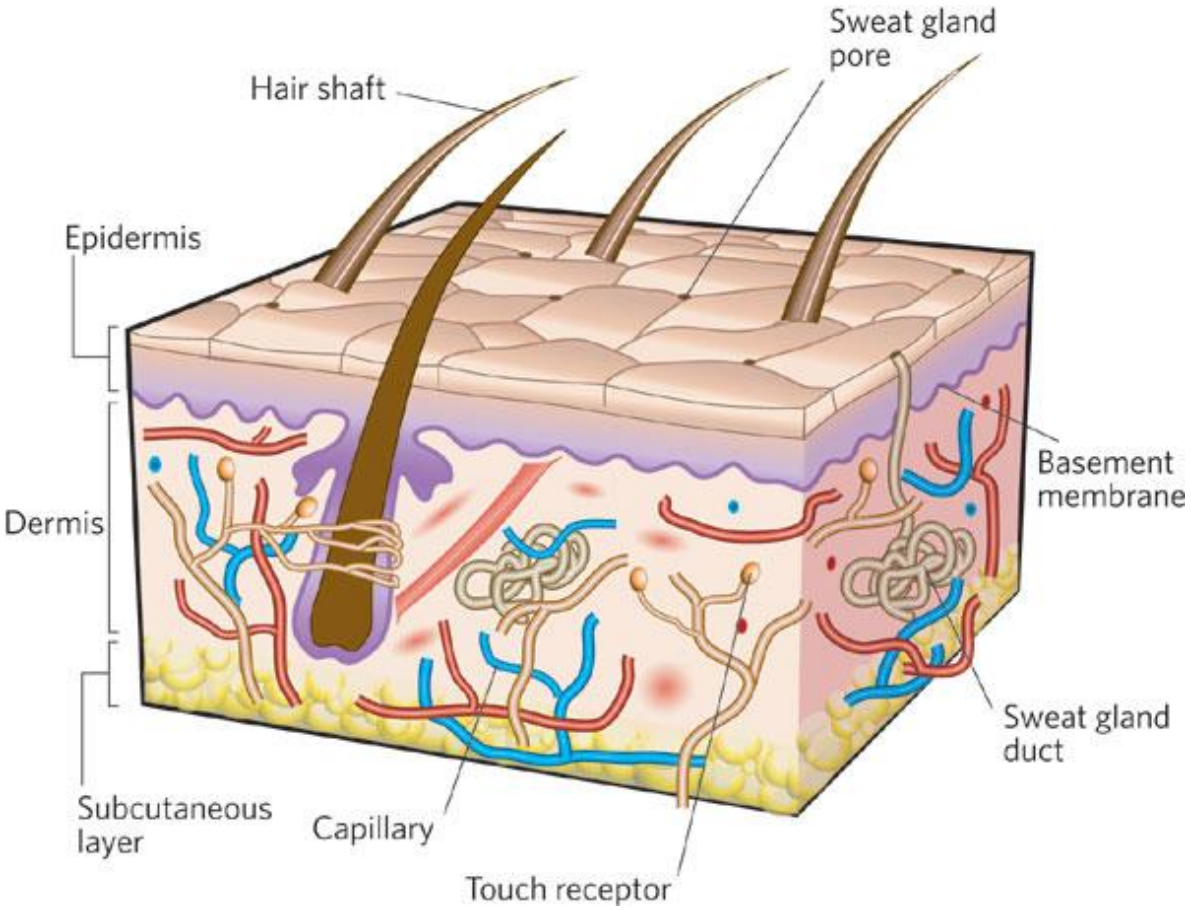

***Figure 1**. Structure of normal human skin. (Image: skininfo.org)*

1.6 OPTICAL IMAGING

A variety of imaging methods have been established to look beyond the physical barrier of skin *in vivo*. Based on 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 reverting back to their original state is recorded and quantified. These are techniques that help us understand the anatomical characteristics of different organisms, however, when combined with contrast agents and alternative imaging modalities such as single photon emission computed tomography (SPECT) and positron emission tomography (PET) they can serve to monitor molecular processes (78–80).

The advances in genetic engineering have enabled scientists to design proteins emitting luminescent or fluorescent light, which can be detected by various optical devices. As an

imaging modality, optical imaging possesses some key advantages over other existing 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 scientists. Optical imaging is very well suited for *in vivo* studies, where it can be used for monitoring processes like biodistribution (81), gene expression (82), enzyme activity (83), inflammation (84), and tumor spread (85) 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 only 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 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.

1.6.1 Bioluminescence imaging

Bioluminescence is a natural phenomenon exhibited by a range terrestrial and marine species for various behavioral reasons such as defense, camouflage, communication, etc. (86) 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 research.

In order to make bioluminescence imaging (BLI) possible *in vivo*, a gene encoding a luminescent reporter must be introduced into the tissue to be imaged. Currently there exist a variety of ways to transfer transgenes, such as using viral or bacterial vectors, injection of cells, electroporation-mediated transfer of plasmid DNA, and 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. 2)*. 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 in light scattering and attenuation at different wavelengths are taken into account in order to determine the depth of the bioluminescence signal. Due to the wide emission range of luciferase (560-660 nm) 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 (87).

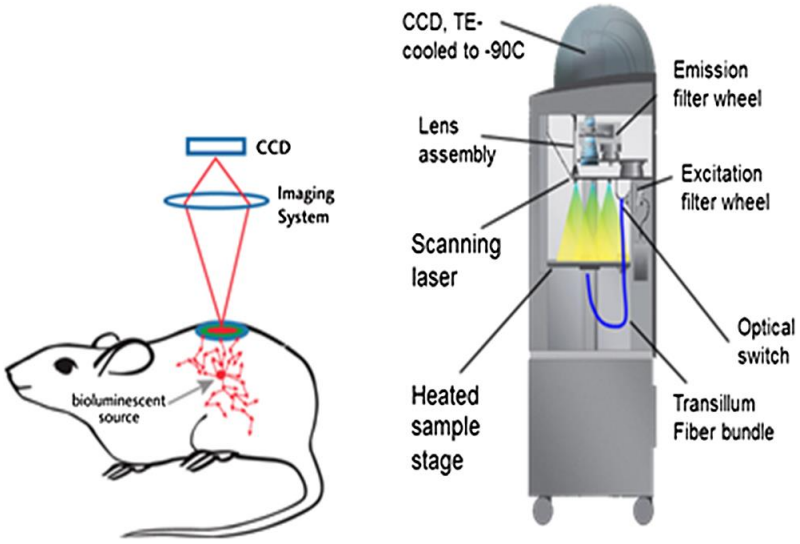

Figure 2. *Graphical representation of the IVIS Spectrum used for bioluminescence imaging (BLI)* (118).

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 (88), which uses luciferin as a substrate for an oxidation reaction in the presence of ATP and oxygen to generate light (89):

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

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

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

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 (90). In the presence of an excess of luciferin, magnesium, and ATP the photons released are proportional to the amount of luciferase (91). 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 (92).

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.

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 (93).

1.6.2 Bioluminescence optical imaging *in vivo*

The amount of detectable luminescence *in vivo* is largely dependent of 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 (94). 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 (82).

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 were expressing the bacterial luciferase

(95). 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 (96). However, bacteria are but 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 or deliver the luciferase gene (97, 98).

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 (99). A vivid example of this method are tumor studies, which used tumor cells engineered to express luciferase and then transferred into animal models (100, 101). This powerful approach has been demonstrated in many studies that have investigated the dynamics of tumor growth and regression *in vivo*. Using a similar methods gene expression has also been successfully imaged with transgenic animal models expressing luciferase under specific promoters (99).

2 AIMS

The present work will focus on the use of *in vivo* bioluminescence imaging (BLI) techniques to:

- Study the applicability of bioluminescent reporter expression suitable for monitoring of the delivery and expression (localization, level) of plasmid-based DNA immunogens.
- By usinng bioluminescent reporters, determine the way in which the route and site of DNA vaccine delivery influences its further expression and immunogenicity.
- Develop BLI applications for monitoring the development of immune response *in vivo*, to omit intermediate immune tests and create new end-points for terminating the trial of ineffective/non-immunogenic and promotion of effective DNA vaccine candidates.

3 MATERIALS AND METHODS

3.1 ANIMALS

The performed experiments involving animals were approved by the Northern Stockholm's Unit of the Ethics of Animal Research on 16-05-2013 with ethical permit N66/13, entitled "Evaluation of improved vaccines against infectious diseases and cancer". The series of experiments were aimed at improving vaccines and vaccination strategies to combat serious viral infections, such as HIV, and to advance existing clinical practices in current vaccination. The vaccine candidates allowed for testing in the context of this ethical permit included DNA immunogens, proteins, and peptides, administered with or without adjuvants. Vaccine administration was allowed by intramuscular, intradermal, or subcutaneous routes to be performed using needle injections, inoculations with auxiliary devices, such as Biojector with or without electroporation. All pain inflicting procedures including injections, electroporations, and biojections were to be delivered under inhalation anesthesia, consisting of mixture of air and 2.5% isofluorane. The methods in these experiments were deemed to result in low degree of pain reflected by the no to little effect on normal weight, food and water consumption or behavior of the mice involved. Additionally, any possible mouse discomfort was alleviated by the application anesthesia. The animals were sacrificed by cervical dislocation.

The animals used in the described experiments were 8 week-old, female BALB/c mice ordered from Charles River Laboratories (Sandhofer, Germany) or from the breeding facility of the Department of Microbiology, Tumor and Cell Biology (Karolinska Institute, Stockholm, Sweden). Depending of the origin, animals were housed in Astrid Fagraeus Laboratory or in the animal facility of the Department of Microbiology, Tumor and Cell Biology under a light-dark cycle of 12 h / 12 h. Five to eight mice were contained in environment-enriched cages with food and water available *ad libtum*. Mice were regularly inspected for irregularities in food or water intake, weight, fur and behavior changes by the staff of the animal facility. Immunizations were delivered with 29 G needles and never exceeded the volume of 20 microliters. In order to screen immune responses, mice were bled from the tail vein two and four weeks after the` administration of vaccines. Expression of DNA-immunogens was assessed by imaging co-delivered reporter gene encoding firefly luciferase using the IVIS Spectrum or Spectrum CT imaging devices (Perkin Elmer). In computer tomography, Spectrum CT delivered a total of 23 mGy of radiation per mouse, totally 115 mGy under the whole observation period. The radiation was not expected to cause any noticeable immune suppression, as myeloablation in mice requires the delivery of over 500 cGy (102). Bioluminescence imaging (BLI) was performed in light-tight, thermo-regulated chamber, supplied with inhalable anesthesia. Prior to immunization, electroporation or imaging mice were anesthetized with a mixture of air and 4% isofluorane during induction and 2.5% during maintenance.

3.2 PLASMID DNA

The luciferase-coding plasmid, pVax-luc 4663 bp (pVaxLuc) constructed by inserting the cDNA of firefly luciferase from pGL2-basic vector (Promega, #E1641) into vector pVAX1 (Invitrogen, #V260-20) under the control of a human cytomegalovirus immediate/early promoter and a polyadenylation signal from the bovine growth hormone gene (103), was kindly provided by Maltais AK (Eurocine Vaccines, Sweden).

The gene encoding HIV-1 reverse transcriptase with multiple mutations of drug resistance (Ref to sequence; Isaguliants M, Zuber B) was expression optimized by codon humanization. To ensure the proper protein expression, the gene was provided with an AAT-ATG-GGA sequence fused to its 5′-end, which resulted in the addition of Met-Gly to the N-terminus of the protein. The humanized gene of the expression-optimized multidrug-resistant RT (RT1.14opt) was synthesized (Evrogen, Moscow, Russia) and cloned into pVax1 vector to generate pVaxRT1.14opt (104). The enzymatic activity of RT1.14opt was abrogated by the point-mutations D187N, D188N, and E480Q, which were introduced into the RT1.14opt gene by site-directed mutagenesis (Evrogen). The latter yielded the expression-optimized gene for inactivated drug-resistant RT (RT1.14opt-in) in the pVax-1 backbone dubbed pVaxRT1.14opt-in (104).

The HIV protease (PR) plasmid was constructed by Hallengärd et al. by ligating a codon-optimized PR gene into a pKCMV vector. Mutations resulting in enzymatic inactivation (D25N) were introduced in the gene by site directed mutagenesis (105).

Expression-optimized genes encoding consensus integrase of HIV-1 clade A FSU-A strain (IN_A), and its variant containing mutations conferring resistance to raltegravir (IN_A_e3), alongside with their inactivated versions carrying mutation were synthesized and cloned into the pVax-1 vector (Evrogen) (106). The empty vector used as control in all immunization experiments was pVax-1 (Invitrogen Corporation).

3.3 GENE IMMUNIZATION AND *IN VIVO* ELECTROPORATION

Groups of BALB/c mice were immunized with DNA immunogen or empty vector control. In both case and control group plasmids were mixed with pVax-luc reporter in 1:1 (w/w) ratio. The total amount of DNA per injection never exceeded 20 micrograms delivered in a volume of 20 microliters saline solution. Plasmids were delivered by intradermal or intramuscular injection using a 29 G insulin-grade syringe (Micro- Fine U-100; BD Consumer Healthcare, Franklin Lakes, NJ). Mice were immunized on the lower back, on both sides of the base of the tail. Injections were immediately followed by electroporation of the immunization sites. Needle-array electrodes consisting of eight 2-mm pins arranged in 2 rows (BTX, #47- 0040) were used to deliver 2 pulses of 1125 V/cm (50 microsecond interval) and 8 pulses of 275 V/cm (10 microsecond interval) (103). Electrical pulses were generated by the DERMA

VAX Clinical DNA vaccine delivery system (Cellectis, Glen Burnie, USA). Electroporation was performed in a controlled (keeping pre-pulse skin resistance < 3000 Ω) fashion.

3.4 REAL TIME *IN VIVO* BIOLUMINESCENCE IMAGING AND SIGNAL QUANTIFICATION

To monitor luciferase expression *in vivo* imaging using a CCD camera, mounted in light-tight chamber was performed (IVIS200, or Spectrum CT, Perkin Elmer). On days 1, 3, 6, 9, 15 and 21 mice were injected intraperitoneally with a 150 mg/kg dose of D-luciferin (PerkinElmer, #122796) dissolved in 200 μl PBS. Five minutes after the injection animals were placed in a chamber with inhalable anesthesia consisting of air mixed with 4% isofluorane for induction and then reduced to 2.5% for the duration of the imaging process. After a total of 10 minutes following the injection of D-luciferin the mice were moved into the Spectrum CT, five at a time and two at a time for three-dimensional (3D) imaging. Planar bioluminescence imaging (BLI) was performed using automatic exposure times ranging from 1-60 seconds, depending on the intensity of the bioluminescence source. In the cases, when 3D signal acquisition (BLT) was needed a microCT scan was performed prior to BLI using the Spectrum CT. Luminescent sites where quantified as luminescence flux in photons/s using the Living Image software version 4.1 (Perkin Elmer). The same software product was also used to process the imaging data and generate signal intensity, expression localization (depth), and size of expression area (volume) values.

3.5 INTERFERON GAMMA AND INTERLEUKIN-2 FLUOROSPOT ASSAY

For the assessment of cellular immune responses, mice were sacrificed and spleens were harvested 23 days after DNA-immunization. Spleens were homogenized by smashing them through 70 μm cell strainers (Falcon) to obtain a single-cell suspension of splenocytes. The suspension was then treated with red cell lysing buffer (Beckton Dickenson, Franklin Lakes, NJ, US) and the cells were re-suspended in RPMI supplemented with 2 mM L-glutamine, 2 mM Penicillin-Streptomycin (all from Sigma-Aldrich, St. Louis, MO) and 10% FBS (Gibco, Invitrogen, Carlsbad, California) (complete media). The fluoroSpot assay was performed according to the manufacturer's instructions (MabTech AB, # FS-4142- 10) as previously described (107, 108). Polyvinylidene difluoride plates (MabTech AB, # FS-S5EJ-T) were treated with ethanol and coated with monoclonal antibodies for IFN-γ (AN18) and IL-2 (1A12) detection. A total of 2.5 × 105 splenocytes per well were plated and stimulated for 20 h at 37°C and 5% CO_2 with peptide and protein antigens in the presence of anti-CD28 antibody. Complete RPMI was used as a negative control and Concanavalin A (Con A, 5 μg/ml) as positive controls. Bound cytokines were detected with BAM-labeled antibody (R4-6A2-BAM) and biotynylated antibody (5H4) followed by anti-BAM-490 antibody conjugated to a green fluorochrome and streptavidin (SA-550) conjugated to a red fluorochrome. The plates were then treated with a Fluorescence enhancer II (Mabtech) and dried before detection. Spot-forming cells (SFC) secreting cytokines were analyzed using the

AID iSpot FluoroSpot Reader System (AID GmbH, Strassberg, Germany). Responses were presented as SFC per million splenocytes with the background spots in the negative control wells subtracted. Responses were considered to be significant only when the spots exceeded +3SD the number of those produced by splenocytes from vector-immunized mice in response to *in vitro* stimulation with the same antigen.

3.6 INTRACELLULAR CYTOKINE STAINING AND FACS ANALYSIS

All of the reagents used for these analyses were purchased from BD Biosciences (Franklin Lakes, NJ, US) unless stated otherwise. Splenocytes from immunized or control mice (3 x 10^6) were stimulated for 4-6 hr at 37°C and 5% CO_2 with recombinant proteins (10 μg/ml) or an equimolar mixture of peptides representing (10 μg/ml) T cell epitopes. Concanavalin A (5 μg/ml) was used as a positive control. The stimuli were diluted in complete culture media consisting of RPMI supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/mL streptomycin, and 0.3 mg/ml glutamine (Gibco, Life technologies Co.) and GolgiPlug.

To block unspecific binding of immunoglobulins to Fcγ receptors a CD16/CD32 (cat. # 553141) antibody was added to each well 10 minutes before the end of the incubation. Before proceeding to staining surface molecules, cells were stained for a viability using the Fixable Viability Stain 660 (FSV660) as recommended by the manufacturer. Surface staining was then performed by incubating the cells with a mixture of antibodies including: FITC-conjugated anti-mouse CD8a, APC-H7-conjugated anti-mouse CD4 and PerCP-conjugated anti-mouse CD3. Thereafter, the cells were fixed and permeabilized at room temperature for 20 minutes in 100 μl Cytofix/Cytoperm solution, washed with Perm/Wash buffer, and stained at 4°C for 30 minutes with PE-Cy7-conjugated anti-mouse IFN-γ, BV421-conjugated anti-mouse IL-2, BV510-conjugated anti-mouse TNFα and PE-conjugated anti-mouse FoxP3 antibodies. The samples where then analyzed on a FACSVerse cytometer (BD Biosciences) and the data was exported as FCS3.0 files using the FACSuite software. The FCS files were subsequently read using BioConductor's (109) package flowCore (110) in the R software language (111). Finally, the cytometry data were normalized using the flowStats package (112) and gated. First a general lymphocyte area was defined and viable cells were identified by the lack for FSV660 staining. From the viable population, single cells were defined by their expressing of surface markers, such as CD3, CD4, CD8 and their production of cytokines, such as IFN-γ, IL-2, and TNFα.

3.7 ASSESSMENT OF HUMORAL IMMUNE REPONSES

To assay immune responses, 96-well Maxisorb plates (Nunc Maxisorp, Denmark) were coated with antigen diluted in carbonate buffer pH 9.3 at a concentration of 0.3 μg/ml and incubated overnight at 4-6°C. The next day, the plates were washed 5 times with PBS containing 0.05% Tween-20. Mouse serum samples were then appropriately diluted in HIV-scan buffer (HSB; 2% normal goat serum, 0,5% BSA, 0, 05% Tween-20, 0,01% sodium

merthiolate), applied 100 µl/well on the microtiter plates and incubated overnight at 4-6°C. Following the incubation, plates werewashed 5 times as above, and treated for 1.5 hr at 37°C with anti-mouse IgG (Dako,A/S, Glostrup, Denmark) or IgG1, or IgG2a, or IgG2b, or IgA conjugated to HRP. The plates were then washed again as above and developed with 3,3', 5,5'-tetramethylbenzidine solution (TMB) diluted 1:10 in substrate buffer (both Medico-Diagnostic Laboratory, Moscow, Russia). The reaction was stopped with 50 µl of 2.5M sulfuric acid, and optical density (OD) was measured at a dual wavelength of 450–620 nm. The cut-off value for specific antibody response at each time-point was set to the mean OD values demonstrated by the sera of the vector-immunized mice at this time-point +3 SD. For positive sera showing OD values exceeding the cut-off values, end-point dilution titers were established from the titration curves.

4 RESULTS AND DISCUSSION

4.1 THE DELIVERY OF DNA IMMUNOGENS DEPENDS ON THE QUALITY OF ELECTROPORATION

The method of delivery of DNA vaccines in one of the crucial determinants of their subsequent immunogenicity. Other factors, such as the choice of target of gene delivery, play an instrumental role in the shaping of the induced immune response. Therefore, we have undertaken the task of thoroughly investigating the effect of injection site/expression localization on the efficacy of genetics vaccines. We also sought to study additional aspects of the process of DNA transfer such as electroporation. Our previous results had suggested an implicit relationship between the efficiency of DNA transfer and features affecting electroporation, such as the resistance of skin (113). By electrotransfer a DNA plasmid, pVax1-luc encoding the firefly luciferase into BALB/c mice we were able to follow the expression of the gene using non-invasive bioluminescence imaging (BLI) in BALB/c mice (114). 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. 3 A,C,D).*

Data showed that that efficient transgene expression after injection of DNA required electroporation 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 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. A controlled electroporation resulted in a significantly tighter variance range of luminescence values as well as higher overall intensity after delivery of the gene *(Fig. 3 B).*

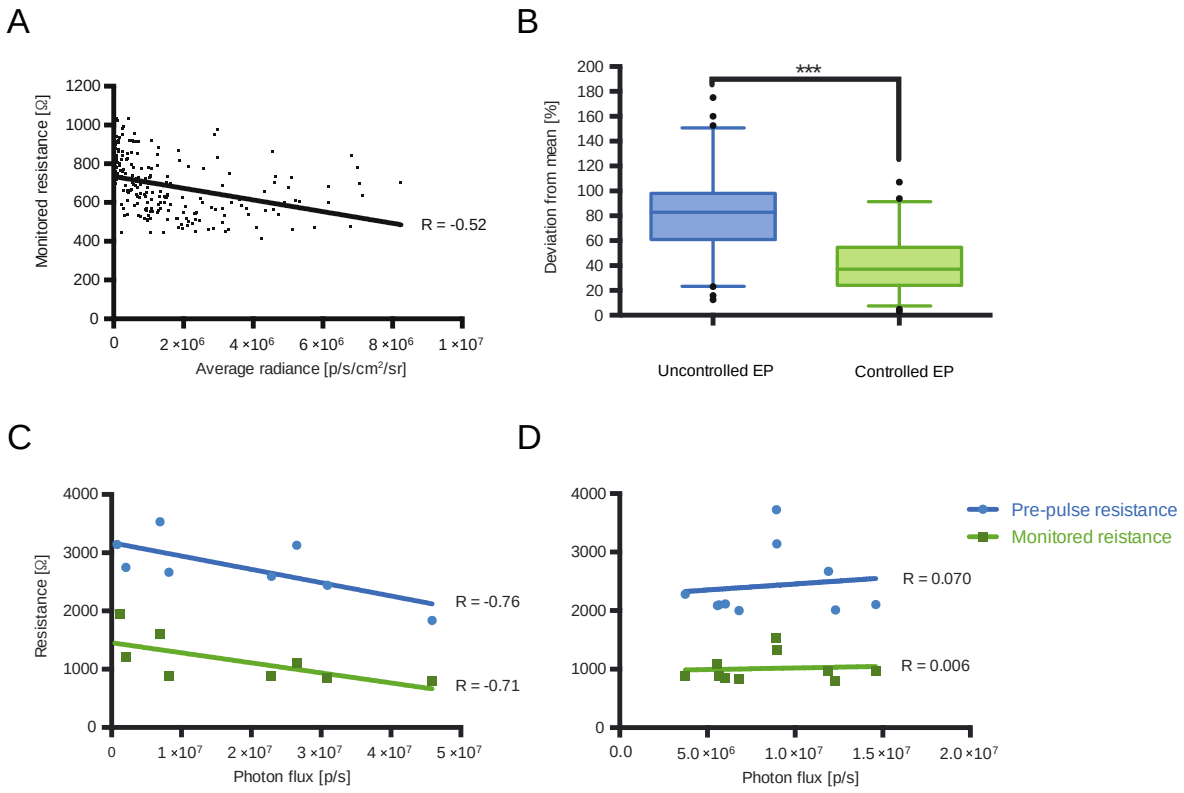

Figure 3*. Dependence of expression of luciferase gene assessed as the total photon flux to the estimated pre-pulse and monitored skin resistance during electroporation (Derma Vax). Analysis of the monitored skin resistance and average photon flux data from pervious Luc gene injection experiments involving 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.*

4.2 TRANSGENE EXPRESSION IS INFLUENCED BY THE ANATOMICAL TARGET OF DELIVERY

In the process of optimization of electroporation we observed a wide variation in the immunogen expression after DNA delivery. This posed a question of the capacity of different tissues to accept the electro-transferred 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 (115) of injection referred from here onwards as intradermal (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 intramuscular (IM). 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 3-dimensional (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. 4)*. 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 *(Fig. 5C)*. These data demonstrate that in comparison to the superficial skin-targeting injection (ID), plasmid immunogen delivered via deep injection (IM) results in the higher and more prolonged protein expression, and also gives an early peak in the expression of the immunogen (108) *(Fig. 5)*.

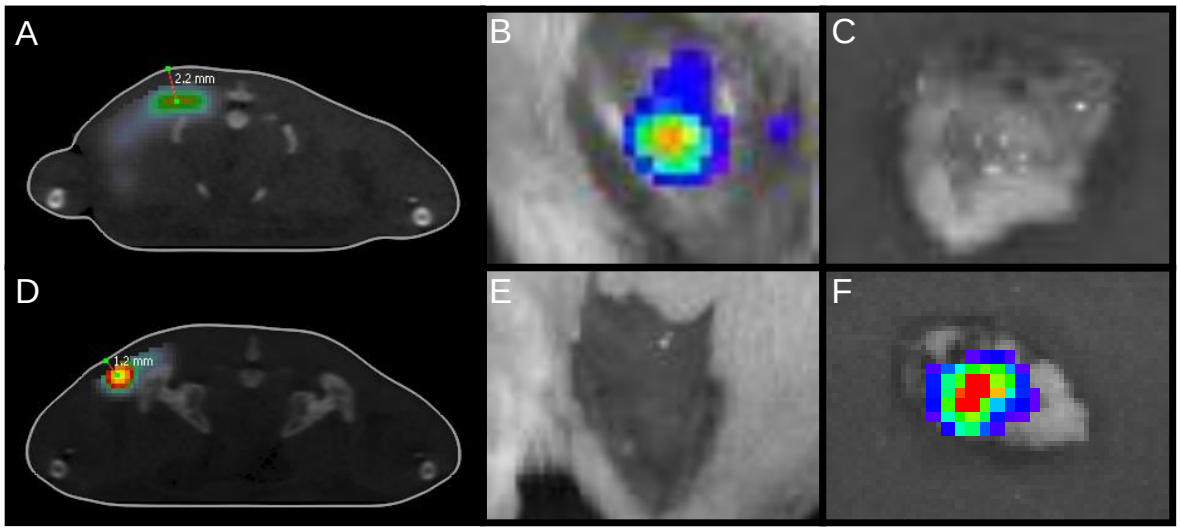

Figure 4. Expression of reporter gene after IM (A) and ID (D) injection. Tissue from injected mice was excised and monitored for reporter activity. Mice receiving IM injections did not have any luciferase present in excised skin but showed ample signal in muscle tissue (B,C). The opposite was true after ID administration of the reporter gene (E,F).

Further, we assessed the immune responses induced by ID and IM administration of the luciferase gene. IM immunization resulted in a 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) *(Fig. 6)*. These data show that the ID injection of a DNA–immunogen (116) in skin results in a weaker and less durable antigen

synthesis, 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 high and longer-lasting antigen production and, thus, a better humoral response to the encoded immunogen.

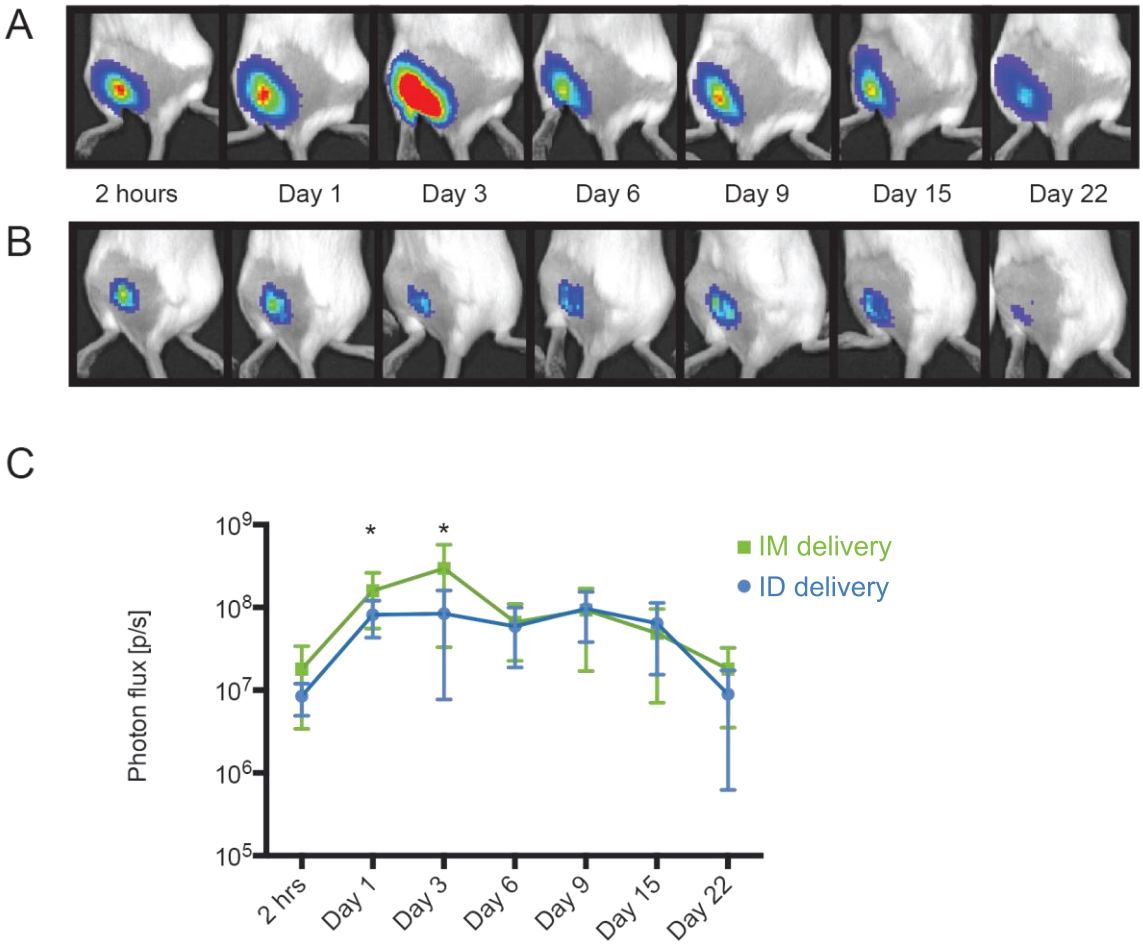

Figure 5*. Imaging of luminescence in mice receiving IM and ID injections. The activity of luciferase was monitored by in vivo bioluminescent imaging of mice receiving Luc gene by intramuscular (A) or intradermal (B) injections. The images represent a composite of luminescence data (photons/sec) overlaid with a photograph of the subjects. ID injections resulted in higher and longer-lasting expression of luciferase (C).*

4.3 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. For this, we conducted a series of experiments, which sought to uncover the effects of intradermal versus intramuscular delivery on the expression and immunogenic performance of polarized DNA-immunogens known to stimulate either T_h1-, or T_h2-type immunity. Specifically, we immunized mice with HIV-1 protease (PR) as a model T_h1-

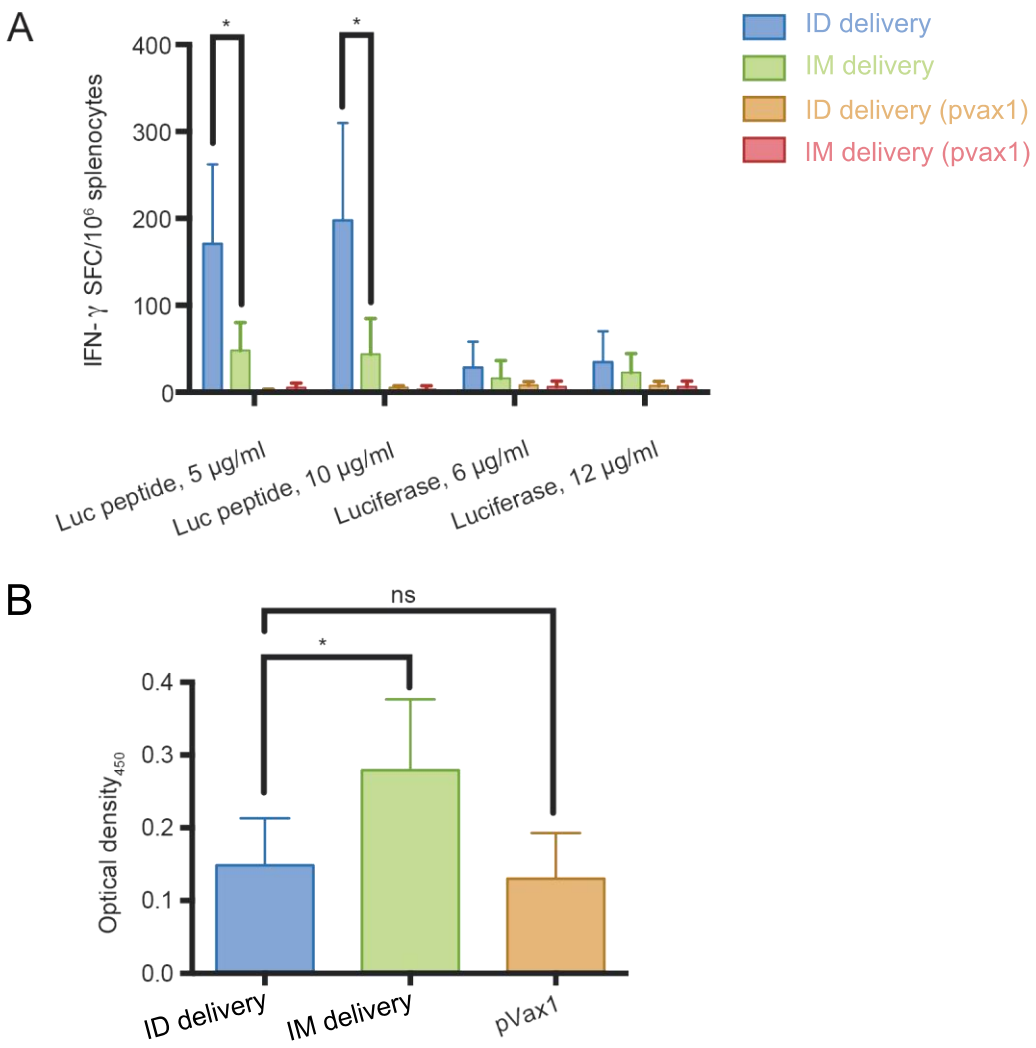

Figure 6. *Immunogenicity of luciferase DNA in BALB/c mice immunized by superficial or deep injections of luciferase gene followed by electroporation. On day 23-post immunization splenocytes were harvested from mice receiving superficial (ID) and deep (IM) injections of luciferase-coding pVax-Luc and vector-only controls (pVax1). Responses of splenocytes stimulated by a peptide representing CD8+ epitope of Luc (GFQSMYTFV) or recombinant luciferase measured by fluorospot: secretion of IFN-γ (A). Optical density of luciferase-specific antibodies measured in serum samples (diluted 1:50) by ELISA (B).*

immunogen (105) and HIV-1 reverse transcriptase (RT) as a model T_h2-type immunogen (114). The plasmids were delivered by either ID or IM injections. To determine how the route of plasmid delivery influenced the induction of cellular immune responses, splenocytes were harvested from the animals 21 days after immunization, and stimulated *in vitro* by peptides representing known CD8+ and CD4+ T cell epitopes of PR and RT recognized in BALB/c mice.

For mice DNA-immunized with RT there was little difference in cytokine production induced by ID and IM gene immunizations (n.s.; p>0.1). Interestingly, mice responding to RT have a

pronounced shift in the subsets of activated T cells towards CD4+ responses. Both ID and IM immunization induced responses against the same epitopes in the same proportion, and in both cases, skewed towards CD4+ T cell responses. Secretion of both IFN-γ and IL-2 was on the average 10-fold higher in response to peptides representing the immunodominant CD4+ T_h- as compared to CTL epitopes independently of the delivery route *(Fig. 7, Table 1)*. This performance falls in line with the T_h2-tilt in RT gene immunogenicity described by us previously (114). The T_h2-nature of anti-RT immune response was supported by the assessments of RT-specific antibodies; the titer of anti-RT IgG reached after single gene injection was as high as 80,000 in ID, and 20,000 in IM immunizations. Thus, for RT gene, ID immunization resulted in significantly higher levels of specific antibody production than IM, whereas the magnitude and specificity of T-cell responses was not influenced by the delivery route. These results indicated that a T_h2-polarized gene immunogen as HIV-1 RT promotes aT_h2 type of immune response irrespectively of the immunization route *(Fig. 7)*.

Cytokine	Total responses (spots/mln)		Responses to CTL epitopes		Responses to CD4+ T cell epitopes	
	ID	IM	ID	IM	ID	IM
IFN-γ	41.5	46.5	4.5	3	37	43.5
IL-2	138	144.5	23	35.5	115	109
IFN-γ /IL-2	40	28.5	3.5	1.5	36.5	27

Table 1. *Comparison of fine specificity of responses against RT induced by respective gene immunization via ID and IM routes. (total responses as sum of spots to peptides).*

This however, was not the case for a T_h1-polarized gene immunogen, such as HIV-1 PR. For the splenocytes from the PR-immunized animals, there was a clear divide in the readout.

Being a bona fide T_h1 antigen (105), PR immunization resulted in the potent stimulation of cellular immunity both when delivered ID and IM. However, the ID delivery generated much stronger cellular response as was reflected by approximately two-fold higher levels of total *in vitro* IFN-γ and IL-2 production in response to stimulation with a PR-peptide in splenocytes of mice receiving PR gene by ID route compared to IM *(Fig. 7 D, E; Table 2).*

Cytokine	Total responses (spots/mln)		Responses to CTL epitopes		Responses to CD4+ T cell epitopes	
	ID	IM	ID	IM	ID	IM
IFN-γ	1233.8	635.7	708.4	382.9	525.4	252.8
IL-2	544.1	275.7	257.9	131.6	286.2	144.1
IFN-γ /IL-2	426.6	223.3	230.2	117.2	196.4	106.1

Table 2. *Comparison of fine specificity of responses against PR induced by respective gene immunization via ID and IM routes. (total responses as sum of spots to peptides).*

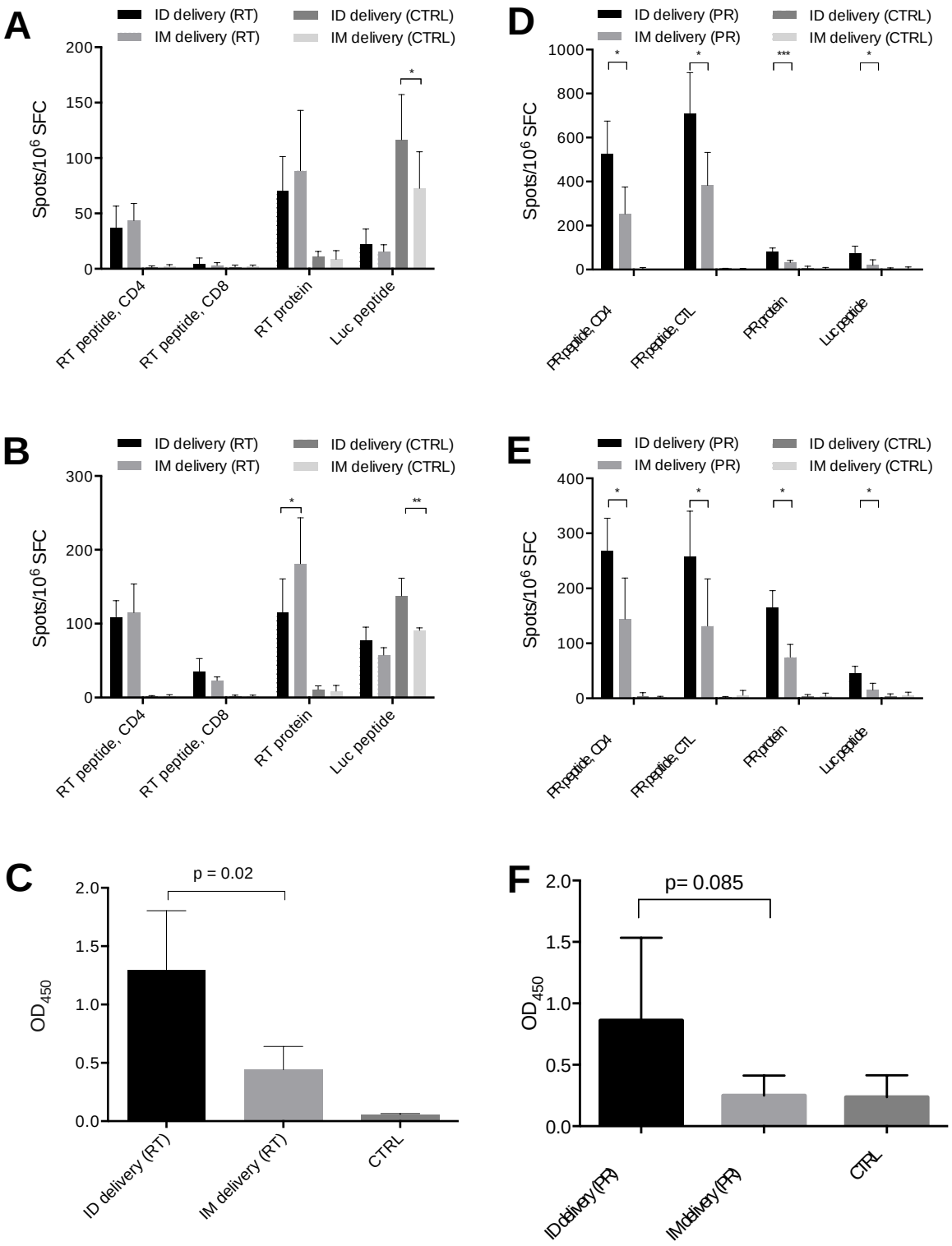

Figure 7. *Immune responses in mice 21 days post immunization of DNA encoding PT or RT. IFN-γ, IL-2 and IgG production after immunization with RT (A,B,C, respectively) and PR (D, E, F, respectively). Serum dilution in (C) is 1:4000 and in (F) is 1:50.*

To find out if the route of delivery influences the type of effector cells induced, we studied if immunization by ID or IM route results in a differential immune recognition of CD4+ and/or CD8+ T-cell epitopes. For this, we compared the magnitude of T cell responses to peptides representing known CD4+ and CD8+ T-cell epitopes of BALB/c mice raised by PR gene administered by ID and IM routes. ID route favored significantly stronger IFN-γ T-cell responses against both CD8+ and CD4+ T cell epitopes *(Fig. 7 D, E; Table 2)*, however, the route of immunization had no effect on the proportion of T cells responding to CTL or to CD4+ T cell epitopes by either IFN- γ, or IL-2 (or dual) production *(Table 2)*. For example, IFN-γ response to CTL epitopes in PR constituted 57% of all IFN-γ responses in ID, and 60% in IM delivery (n.s.); similarly, IL-2 response to CTL epitopes in PR constituted 47% of all IL-2 responses in ID, and 48% in IM PR immunization (*Table 2*; ns). Absence of change of epitope choice and/or hierarchy was evident from the ratio of total CD4+/CTL responses in ID and IM PR gene immunizations. Responses to individual CD4+ and CTL epitopes followed the same pattern *(Fig. 7)*.

As expected for a T_h1-immunogen, peptides representing CTL epitopes of PR tended to induce a stronger IFN-γ response than those representing epitopes of T-helper cells in both ID and IM PR-immunized animals (57% vs. 43% in ID, and 60% vs. 40% in IM immunization, Table 2; $p<0.01$). At the same time, no difference was observed in the magnitude of IL-2 responses to CTL as compared to CD4+ T cell epitopes, in either ID or IM PR gene immunizations. Thus, for T_h1-type immunogens as HIV-1 PR, ID route of immunization favored stronger IFN-γ responses against both CD4+ and CD8+ T cell epitopes than the IM route. IL-2 responses were not influenced by the route of gene delivery.

Being a T_h1 immunogen, PR induced a very weak antibody response with titers of 200 in ID, and no antibodies after IM immunization *(Fig. 7 F)* i.e. none of the immunization routes favored the formation of anti-PR antibodies.

Thus, in both DNA immunizations the results demonstrate an advantage of the intradermal route of DNA delivery as could be seen from the magnitude of cellular (IFN-γ/IL-2) and antibody assays. At the same time, the delivery route appeared to have no major influence on the T_h-type of response and the choice (CTL or CD4+) of the recognized epitopes (choice of CD4+ versus CTL epitopes, or epitope hierarchy; in-between categories, and within each category).

4.4 ANTIGEN-SPECIFIC IMMUNE RESPONSES CORRELATE WITH THE EXPRESSION OF REPORTER GENE

The presence of a single CD8+ T cell epitope on the luciferase protein (116) 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 analysis

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 and the level of emitted luminescence. This relationship was evident as early as 3 days post injection and reappeared after 21 days *(Fig. 8)*. No correlation was discovered when mice received IM injections due to a low magnitude of specific immune response (data not shown).

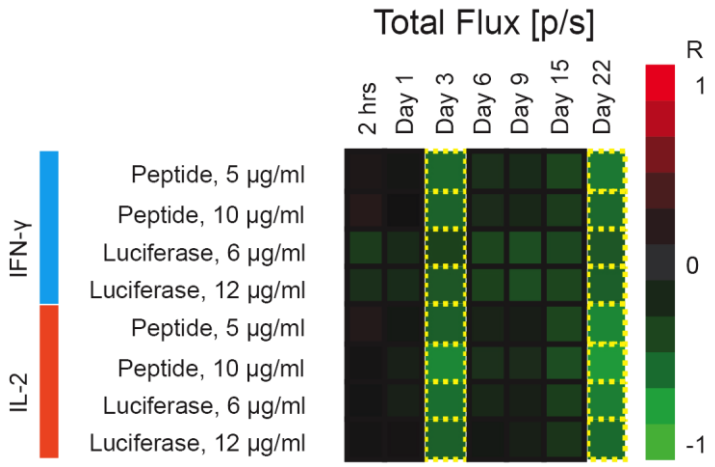

Figure 8*. Correlation between luminescence intensity due to luciferase expression and cytokine production after ID immunization with pVax-Luc.*

We further verified the potential of luciferase to serve as means to follow the kinetics of expression of other (heterologous) gene immunogens. As such, we used plasmids encoding variants of the consensus integrase of HIV-1 clade A FSU-A strain (IN_A). IN_A and Luc encoding plasmids were administered to mice in a 1:1 ratio (w/w). BLI was used to follow the levels of reporter gene expression from hour 2 to day 21 after immunization. By the end of the study the expression of luciferase in mice receiving ID injection of the plasmid mixture had decreased significantly. Similar to what we observed previously, luminescence on day 21 inversely correlated with the levels of *in vitro* IFN-γ and IL-2 production by splenocytes of IN-gene immunized mice stimulated with peptides representing main IN epitopes recognized by BALB/c mice. Equally strong inverse correlations were found between the end-point luminescence and the magnitude of IN-specific triple cytokine response of CD4+ and of CD8+ T cells (106) assessed by mutiparametric FACS *(Fig. 9)*. Luminescence intensity at an early time point (day 3) also correlated inversely with the attributes of immune response as observed previously. This demonstrates the magnitude of the immune response is predetermined by the efficacy of DNA transfer and its early expression. Overall, these data serves to show that the induction of antigen-specific multi-cytokine response of CD4+ and

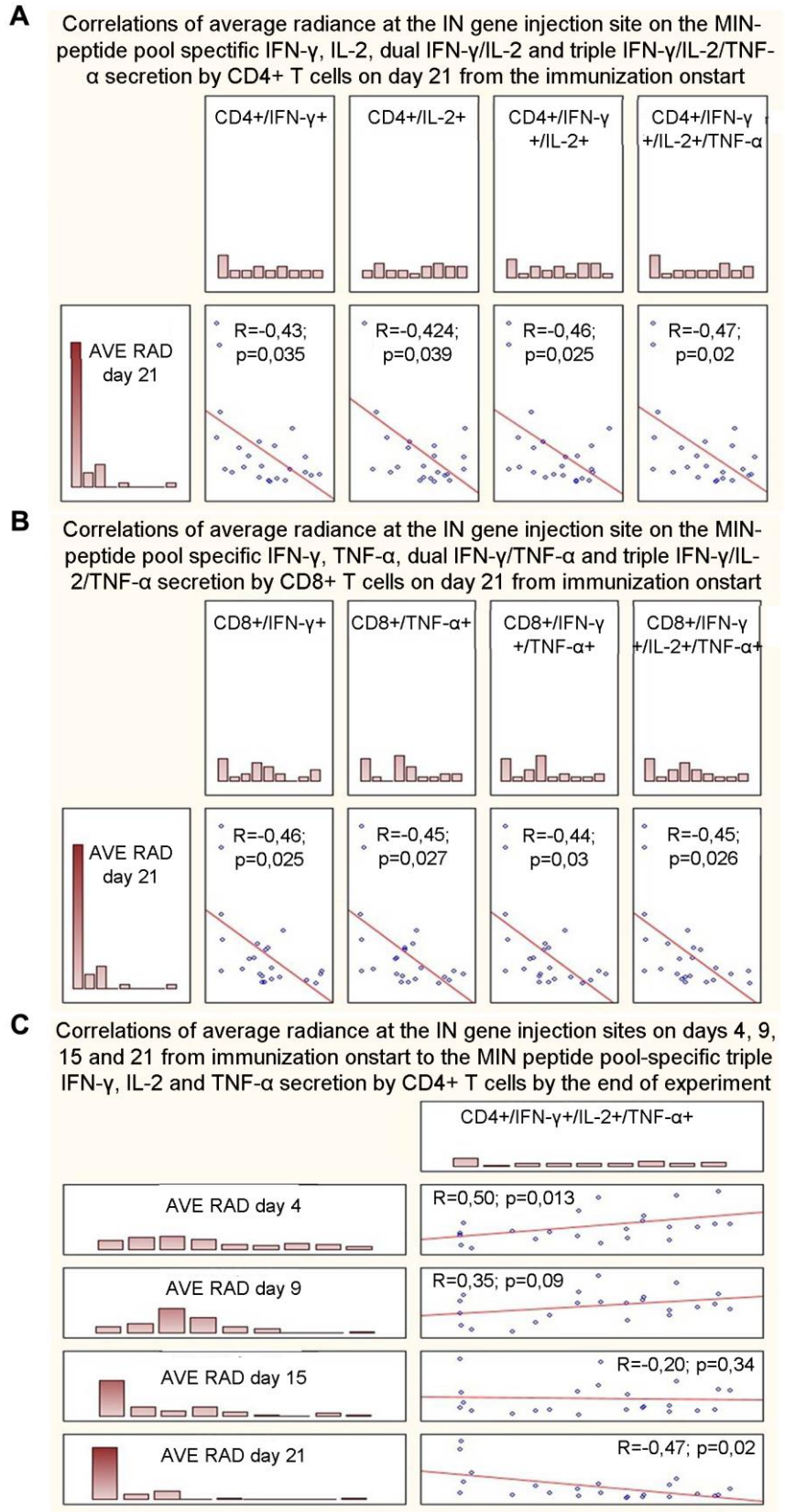

Figure 9. *Average radiance at the sites of the IN/Luc-reporter genes co-injection correlates to IN-specific cytokine response.*

CD8+ T cells cause the extermination of luciferase expressing cells at the sites of immunization.

Next, we attempted to use the data on the reporter gene expression and IFN-γ and IL-2 secretion to immunogen-derived epitopes, to build a model which would predict the level of specific T cell responses raised by the end of immunization from the kinetics of reporter gene expression. For this, we used the respective data obtained in a series of immunizations with DNA encoding expression-optimized gene for inactivated HIV-1 RT (RT). Cellular immune responses to RT epitopes were characterized by IFN-γ and IL-2 fluorospot *(Fig. 7 A, B)* and by mutliparametric FACS assessing IFN-γ, IL-2, TNF-α production by CD4+ and CD8+ T-cells.

RT gene immunization generated a set of correlates between the magnitude of specific IFN-γ and IL-2 responses and parameters of expression of co-delivered reporter gene. We analyzed the relationship between the production of IFN-γ, IL-2, and the simultaneous secretion of both by the same cell. When plasmid DNA was delivered via the IM route we observed a series of direct correlations between luminescence intensity and specific response against CD4+ T cell epitopes. However, most of them did not reach the level of statistical significance. There were fluctuating correlations between luminescence and cytokine production in response to stimulation with peptides encoding both CD4+ and CD8+ T cell epitopes. At the same time, the correlation between the response to the CTL epitope of luciferase and the detected luminescence was direct throughout the follow-up with an endpoint r of 0.59. This value was not significant ($p=0.150$), however it implies that to develop, the immune response against the reporter requires a strong and prolonged luciferase expression. Indeed, Podetz-Pedersen et al. have shown that there exists a threshold of luciferase expression, that when exceeded, results in the generation immune response against luciferase (117). This threshold is defined by the amount of protein expressed and the duration of its production.

The administration of RT via ID injection resulted in clear reversal of the correlations between luminescence and IFN- γ secretion. Almost all of these relationships were inverse on day 21, suggesting that lower level of luminescence corresponds to higher responses, which is consistent with the hypothesis proposed by us earlier (108). Stimulation with two peptides (aa 207-223 and aa 528-543) encoding CD4+ T cell epitopes resulted in statistically significant correlations as early as day 3 after injection *(Fig. 10)*. Contrary to IM delivery, the ID injection of the same mixture of RT and luciferase reporter resulted in an inverse correlation between luminescence and IFN-γ production already from the first day after injection. This is consistent with the luciferase expression kinetics that are exhibited after this type of administration, which are characterized by lower and shorter expression of the reporter.

IL-2 and dual IFN- γ/IL-2 production followed that same pattern of correlation that was observed in IFN-γ secretion. One exception to that was the relationship between luciferase expression and IL-2 production after ID delivery. In that case the correlation was entirely direct after day 1 post delivery. However, this correlation failed to pass the significance criteria and was not further analyzed.

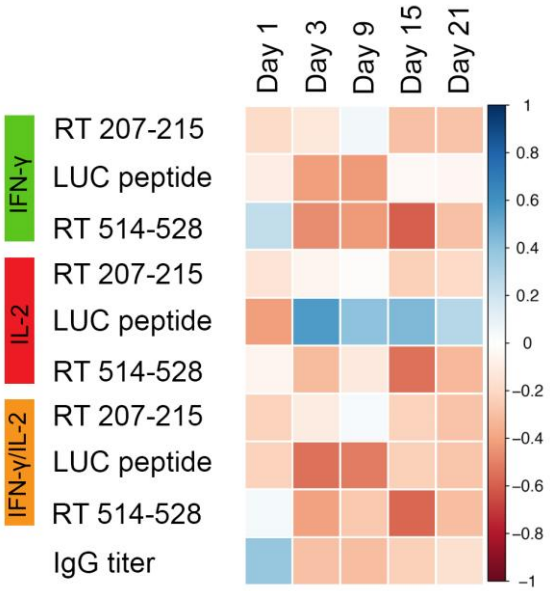

Figure 10. *Correlation analysis of BLI and immune response data for mice DNA immunized with RT.*

In RT immunized mice we also verified if a correlation existed between antigen-specific antibody responses and values of the signal from the reporter gene. Indeed, after ID administration of the immunogen characteristic and statistically significant correlations were detected. Emerging as early as day 1, the direct relationship between luminescence and IgG titers was strong with R=0.46. This correlation progressed to become inverse by day 3, when R equaled -0.30. This correlation pattern seemed to be valid only when mice were immunized via ID injection, because after IM delivery the only significant correlation that was identified was a weak one at the last day of the follow-up. Correlation analysis of the expression of reporter gene co-delivered with RT gene immunogen, and of the magnitude of IFN-γ and IL-2 secretion in response to RT epitopes induced at the end of immunization, allowed us to identify BLI parameters which could serve as surrogate markers of the developing immune response, as BLI by day 1 and 21 were indicative for the potency of CTL; and BLI by day 9, of T-helper cell responses manifested by the secretion of IFN-γ.

The existence of multiple correlations between cytokine production and luminescence level kinetics led us to attempt to build a model for this relationship, and later try to prove its validity on the data obtained in a new series of immunizations. To create such model, we constructed a dataset consisting of the luminescence intensity from each BLI time point and

the endpoint cytokine responses after stimulation with either a CD4+ or CD8+ T cell epitope encoding peptide. These data were used to perform stepwise multiple regression analyses to obtain a minimalistic model which would be able to predict the cytokine levels raised after completion of immunization, based on the levels of expression of co-delivered reporter gene quantified by to photon emission from the injected area (BLI). Bioluminescence data was presented as: a. raw bioluminescence intensity values from each mouse; b. transformed values, representing a fraction of the luminescence intensity present as compared to the levels of day 1; c. transformed values, representing the proportion of luminescence in percent to the maximum BLI value recorded during the complete follow-up. BLI and cytokine secretion data was obtained from series of DNA immunizations with RT altogether. BLI and immune response parameters were tested in the multiple regression analysis. The total photon flux made a significant input into the prediction of IFN-γ and/or IL-2 responses after RT gene immunization and was therefore used in the analyses.

These parameters were incorporated into a linear model, which was used to predict cytokine secretion in an independent run of DNA immunizations with RT. The regression analyses using RT data could with very high reliability model cytokine secretion by CD4+ T-cells after stimulation with peptides representing respective epitopes, such as RT amino acid regions 207-223, 528-543. The resulting model performed exceptionally well in predicting IFN-g production in response to (adj. $R=0.91$, $p<0.01$). IL-2 and dual IFN- γ /IL-2 responses were predictable, although with somewhat lower significance (adj. $R=0.64$, adj. $R=0.75$, $p<0.01$) *(Table 3)*. The model could not predict T cell responses to CD8+ T cell epitopes (adj. $R<0.5$) due to low level of such responses in RT gene immunized animals.

Responses against PR could not be modeled, although a set of correlations was discovered. We speculate that the reason for this might be in the nature of the luciferase reporter as another possibly competing T_h1 immunogen. Our data show that when administered as a mixture with a potent inducer of immune responses, luciferase might trigger cellular immune response of varying magnitude. Indeed, we have observed the differential luciferase-specific production of IFN- γ and IL-2 after immunization of mice with weakly and highly immunogenic RT DNA constructs. When the reporter was co-delivered with RT that generated immune response of low magnitude there was a profound boost in luciferase-specific cytokine production as compared with almost negligible amounts after immunization with the expression optimized and highly immunogenic version of RT. Similar artifacts may exist when using a mixture with luciferase and PR, both of which have been shown to induce T_h1 responses. Options to modify the reporter for further use it also for monitoring of T_h1 type of cellular responses need to be further elucidated.

Response to epitope	*Predicted parameter*		
	IFN-γ	**IL-2**	**IFN-γ/IL-2**
Luc peptide	0.93	0.59	0.72
RT protein	0.59	0.79	0.73
RT 528-543	0.62	0.53	0.55
RT 207-223	0.84	0.89	0.91

Table 3*. Characteristics of the models predicting in vitro T-cell responses by CD4+ and CTL of RT gene immunized mice by multiparametric (multiple regression) analysis of BLI of co-delivered reporter gene expression.*

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. Additional research is, however, needed to upgrade this vaccine modality to the levels acceptable for standard clinical applications. With the number of studies focused on the problem growing exponentially the solution slowly getting closer. Various strategies are implemented to improve plasmid design, such as consensus immunogens and codon optimization. Many molecular adjuvants are currently developed and show great potential of enhancing the efficiency of DNA vaccines. Future studies will determine whether this platform is suitable for combating major health problems of our time.

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