FORMULATION AND DELIVERY OF DERMAL DNA VACCINES

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FORMULATION AND DELIVERY OF DERMAL DNA VACCINES

Formelering en toediening van dermale DNA vaccins
(met een samenvatting in het Nederlands)

PROEFSCHRIFT

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Joost Huibert van den Berg

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Promotoren:

Prof. dr. J.H. Beijnen
Prof. dr. ir. W.E. Hennink
Prof. dr. G. Storm

Co-promotoren:

Dr. B. Nuijen
Dr. J.B.A.G. Haanen

The research described in this thesis was performed at the:

The Department of Pharmacy & Pharmacology, Slotervaart Hospital/ The Netherlands Cancer Institute, Amsterdam, The Netherlands

&

The Department of Pharmaceutics, Utrecht University, Utrecht, The Netherlands

&

The Division of Immunology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

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Chapter 1

General introduction and outline

Vaccination

Since the discovery of smallpox vaccines by Edward Jenner in the late 18th century, active immunization has become a successful strategy in the prevention of infections by pathogens. Vaccines are pharmaceutical formulations of inactivated pathogens (such as viruses or bacteria) or the purified immunogenic proteins (antigens) of these pathogens. Injection of a vaccine to an individual leads to the activation of the immune system, our defence mechanism against foreign entities and tumors. This activation process is called immunization or vaccination. Successful vaccination starts with the uptake of the antigen by so-called Antigen Presenting Cells (APCs), which are abundantly present in the body, especially at natural entry sites of pathogens (such as skin and mucosa). APCs process the antigen into smaller peptides. Some of these peptides are subsequently presented, via intracellular processing, on the surface of the APC (bound into the groove of a major histocompatibility complex (MHC)). In the presence of sufficient "danger signals", APCs simultaneously display co-stimulatory molecules on their surface. After uptake, processing and presentation of the antigen, APCs migrate from the tissue to the lymphatic system, where the MHC-peptide complex is presented to naïve (in resting state) T lymphocytes. The body has a large repertoire of naïve T lymphocytes that each have a different T cell receptor (TCR) on their surface. Only naïve T lymphocytes that "see" both the MHC-peptide complex and co-stimulatory molecules, with a TCR with affinity for the MHC-peptide complex become activated (1). Presentation of vaccine derived peptides by a MHC class I molecule can lead to the activation of CD8 positive cytotoxic T lymphocytes (CTLs), which can directly kill virally infected cells or tumor cells (cellular immune response). Presentation of a peptide on a MHC class II molecule activates CD4 positive T helper lymphocytes, which can assist the development of CTLs, macrophages or B lymphocytes. B lymphocytes are responsible for the production of antigen specific antibodies (humoral immune response), which can neutralize pathogens that are present extracellularly. Vaccination strategies are very efficient and have led to the extinction or control of a variety of infection diseases in the past century (2,3).

DNA vaccination

Instead of the antigen itself, a DNA vaccine consists of a circular piece of bacterial DNA (called a plasmid), genetically encoding the antigen used for vaccination. Upon injection, both non-APCs and APCs can take up this plasmid and produce the encoding antigen under the control of an eukaryotic promoter, present in the plasmid (this process is called transfection). Similar to conventional vaccines, the production (called expression) of this antigen can initiate a vaccine specific immune response. Since the antigen production occurs intracellularly, DNA vaccination can be efficient in activating the cellular arm (CTL activation) of the immune system by antigen presentation via MHC class I (as further

discussed in **Chapter 2**) (4). This strong cellular immune response makes DNA vaccines attractive for therapeutic vaccination. Therapeutic vaccination rely on the induction of high numbers of CTLs (5). As such, DNA vaccination is most often evaluated for the treatment of malaria, HIV and cancer. In case of cancer, DNA vaccination aims to induce T cell responses against tumor associated antigens (TAAs).

Because the expression cassette of a plasmid can be easily modified by conventional molecular cloning, the characteristics of the produced protein can be simply tailored and optimized.

Dermal delivery and DNA tattooing

As discussed, successful vaccine specific immune activation requires antigen processing and presentation by APCs. Since the skin is a natural entry site of pathogens, a high number of APCs is present in the upper layers of the skin, functioning as the "gatekeepers" of the immune system, guarding the environment for foreign invaders (see Figure 1). The most abundant APCs in the skin are the epidermal Langerhans cells (LCs) and the dermal dendritic cells (dermal DCs) (6). In addition, macrophages can infiltrate into infected skin and can also function as APC (7).

Because of the high frequency of APCs and the good accessibility, the skin has gained interest as delivery site for both protein and DNA vaccines. For DNA vaccination, several techniques for the dermal administration of plasmids are currently in use or in development, such as gene gun, jet injectors, micro-needle systems and electroporation (8). The technique that is used throughout this thesis is called DNA tattooing. This strategy uses a permanent make-up device, currently applied in cosmetic tattooing, that delivers a DNA solution into the skin by thousands of small punctures. Tattooing leads to uptake of the DNA by skin cells (keratinocytes) and local production of the encoding antigen. Expression of the antigen induces a strong, cellular immune response specific against the antigen (9).

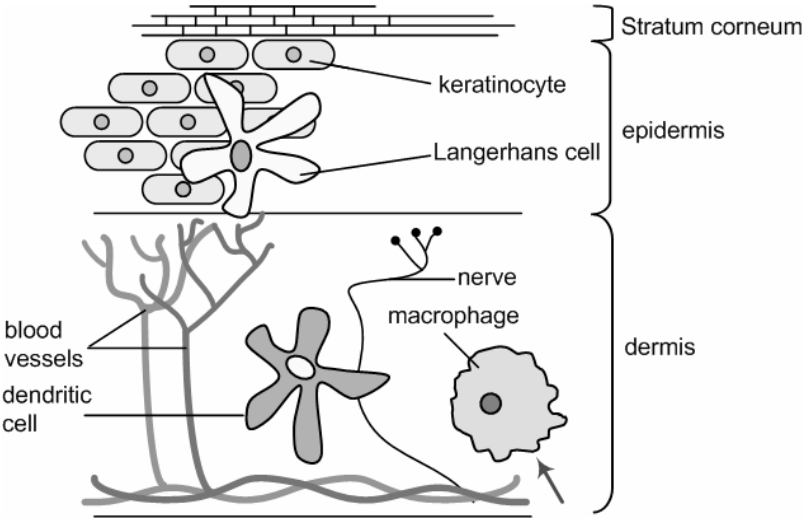

Figure 1: Schematic cross-section of skin with the major Antigen Presenting Cells.

Formulation and delivery aspects

Besides the ease of tailoring the expression cassette of a plasmid, DNA vaccination has also pharmaceutical advantages: the production process of a plasmid is cheaper and easier when compared to the production of proteins, which demand expensive and sensitive mammalian cell cultures as production cell (10). In addition, plasmid DNA is easy to formulate (with of without the use of a synthetic carrier (**Chapter 2**)) into a pharmaceutical dosage form and shows a more favourable long term stability upon storage (11). This thesis focused on optimizing 1) the pharmaceutical production and formulation and 2) the delivery of DNA vaccines administered by DNA tattooing.

Chapter 3 of this thesis describes a simple, scalable, RNAse and solvent free method for the production and formulation of plasmid DNA vaccines, complying to current Good Manufacturing Practice (GMP) guidelines. This method can be readily used in academic settings for the in-house production of relative small amounts of plasmid necessary for phase I and II trials.

Chapter 4 is focussing on the effect of lipopolysaccharides (LPS), a common contamination in plasmid DNA products, derived from the cell membrane of the bacterial production cells. The effect of LPS on toxicity, antigen expression and immunogenicity of DNA vaccines was studied in an *in vivo* model.

In the literature, mice are most frequently used to study the activity of DNA vaccines applied via the skin. Since mice skin is much thinner and has a high density of hair follicles compared to human skin (12), this is likely not the most optimal experimental model to study the performance of dermal DNA vaccines. In search for a model with a higher clinical predictive value, **Chapter 5** describes the development of an *ex vivo* human skin model, in which the expression properties of DNA vaccines can be studied. After development, this model was used throughout this thesis.

So far, pharmaceutical formulations of DNA vaccines used for the delivery by tattooing were solutions of plasmid DNA (called naked DNA), in the presence of a suitable lyoprotector when freeze drying was required. It is widely known that non-viral vehicles, such as cationic lipids and cationic polymers can potentially increase the transfection efficiency of naked DNA *in vitro* and *in vivo* (see **Chapter 2** for a review). Therefore, improving the formulation of plasmid DNA might be an useful approach to increase the efficiency of DNA vaccination by tattooing and therefore is an important aim in this thesis. In general, these carrier molecules complex plasmid DNA via electrostatic interaction leading to the formation of small, positively charged particles with a size between 50-300 nm (13). This small particle size

increases cellular uptake and the complexation with the carrier inhibits degradation by nucleases, increasing the half life of the plasmid DNA. **Chapter 6** describes the development of non-viral carrier systems for the delivery of DNA vaccines via dermal tattooing.

Plasmid DNA is a large molecule (more than 2500kD in size) that can form different three dimensional folding patterns, known as topoisoforms. It is widely accepted that the completely folded supercoiled (SC) form, with the smallest hydrodynamic radius, has the highest transfection efficiency *in vitro* and *in vivo*. However, during storage or administration, a part of this SC DNA can change into other topoisoforms. In **Chapter 7** the effect of the tattooing process on DNA topology and the biological performance of pharmaceutical formulations containing different topoisoforms was assessed.

Chapter 5 describes the optimization of the tattooing technique for naked DNA in the *ex vivo* skin model. The most optimal settings were defined as the settings that resulted in the highest level of gene expression, since the positive correlation between local antigen expression and systemic immunity was previously demonstrated in murine model (9). In search of another non-viral delivery technique than the discussed carriers, to further improve the delivery of dermal DNA vaccines, **Chapter 8** explores the possibilities of combining DNA tattooing of naked DNA or DNA nanoparticles with another commonly used dermal administration technique: electroporation. Electroporation leads to the formation of reversible pores in the cell membrane, formed by electrical pulses (14). This process allows the influx of macromolecules like plasmid DNA, which results in increased transfection levels in the electroporated tissue.

Chapter 9 summarizes the main results of this thesis and gives directions and suggestion for future investigations.

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Chapter 2

Synthetic vehicles for DNA vaccination

Joost H. van den Berg
Bastiaan Nuijen
Ton N. Schumacher
John B. A. G. Haanen
Gert Storm
Jos H. Beijnen
Wim E. Hennink

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Abstract

DNA vaccination is an attractive immunization method able to induce robust cellular immune responses in pre-clinical models. However, clinical DNA vaccination trials performed thus far have resulted in marginal responses. Consequently, strategies are currently under development to improve the efficacy of DNA vaccines. A promising strategy is the use of synthetic particle formulations as carrier systems for DNA vaccines. This review discusses commonly used synthetic carriers for DNA vaccination and provides an overview of in vivo studies that use this strategy. Future recommendations on particle characteristics, target cell types and evaluation models are suggested for the potential improvement of current and novel particle delivery systems. Finally, hurdles which need to be tackled for clinical evaluation of these systems are discussed.

Introduction

DNA vaccination is a very attractive addition to the current vaccine toolbox. Upon administration, circular plasmid DNA (pDNA) is taken up by cells that can subsequently produce (express) the encoded protein. In DNA vaccination strategies, this protein is an immunogenic antigen to which the injected individual can initiate an immune response. The main advantages of DNA vaccination above direct injection of the antigen include the simplicity for adjustment of the DNA sequence, the low production costs (1) and high pharmaceutical stability, and the ability to induce both a strong cellular and humoral immune response (2,3).

DNA vaccination is a particularly appealing strategy for vaccination against viral infection or tumors. As antigens are produced intracellularly upon gene administration, immunogenic epitopes of the antigen can be presented in the context of MHC class I molecules (see Figure 1 for an overview of the mechanisms involved in antigen expression and presentation upon DNA vaccination). In order to efficiently activate naïve T lymphocytes in the lymphatic system, those antigens are generally presented by professional Antigen Presenting Cells (APCs). Antigens can be produced directly in APCs that are directly transfected or can be produced in a non-APC and reach MHC Class I machinery of APC via a process called cross-presentation. This MHC class I routing makes DNA vaccination efficient in activating strong cytotoxic T lymphocyte (CTL) responses. High titers of CTL responses are essential in eliminating virally infected cells and tumors that express so-called tumor associated antigens (TAAs) (4).

Despite the robust cellular immune responses observed in animals (5,6), the immune responses and clinical outcome of DNA vaccination trials have been disappointing so far (7-9) and stress the need for improvement of current vaccine formulations and protocols.

Several strategies to improve the immunogenicity of current naked DNA vaccines are described in the literature: 1) optimization of the expression cassette, 2) adjuvants, 3) delivery techniques, 4) chemical carrier systems.

Optimization expression cassette

The first and most widely used method for enhanced immunogenicity is the optimization of the expression cassette of the DNA vaccine plasmid. This can be greatly enhanced by increasing the stability of the produced antigen, or by the introduction of strong $CD4^+$ helper epitopes, necessary in priming a strong CTL response (6,10,11). Additionally, optimization of promoter, open reading frame or polyadenylation signal can increase the expression properties of the DNA, which will result in higher concentrations of expressed antigen (12).

Adjuvants

Several studies have been published in which chemical or biological adjuvants are added to DNA vaccine formulations in order to increase immunogenicity. Adjuvants aim to enhance APC activation or attract APCs to the vaccination site. Cytokines (biological adjuvants) used are for example granulocyte-macrophage colony stimulating factor (GM-CSF), interferon (IFN)-γ, tumor necrosis factor (TNF)-α and several interleukins (IL) (13). The majority of chemical adjuvants are in the group of the Toll-like receptor (TLR) ligands, such as monophosphoryl lipid A (TLR-4) (14), imiquimod (TLR-7) (15,16), or CpG motifs (TLR-9) (17). These molecules can be directly added to the DNA vaccine formulation or can be administered before or after vaccine administration. In the case of proteins, incorporation of the encoding gene can lead to simultaneous expression of the adjuvant together with the antigen (18). Adjuvants have been incorporated in sustained release systems to improve their adjuvant properties (19,20).

Mechanical and physical techniques

In the past years, major progress has been made in developing new mechanical and physical techniques that are able to increase cellular uptake and immunogenicity of genetic vaccines. This include strategies such as electroporation (21-24), gene gun (25,26), jet injectors (27,28) or tattooing (29-32). Recently, the main focus in the development of these strategies has been on dermal delivery. This is a rational development since the skin is the natural infiltration site of pathogens, easy accessible and rich in APCs, including both epidermal Langerhans Cells (LCs) and dermal Dendritic Cells (DCs) necessary in presenting antigens to- and activation of naïve T lymphocytes (33).

Synthetic particles

In this review, we will focus on the development of synthetic particle carrier systems that aim to improve the performance of current DNA vaccines. In this approach, DNA is encapsulated into, or complexed with a synthetic carrier, resulting in particles with a size ranging from 50 nm to a few micrometer. These different types of particle systems will be discussed together with an overview of *in vivo* vaccination studies using these carriers. In addition, recommendations for the further development of new particle systems, or the improvement of current ones, will be given.

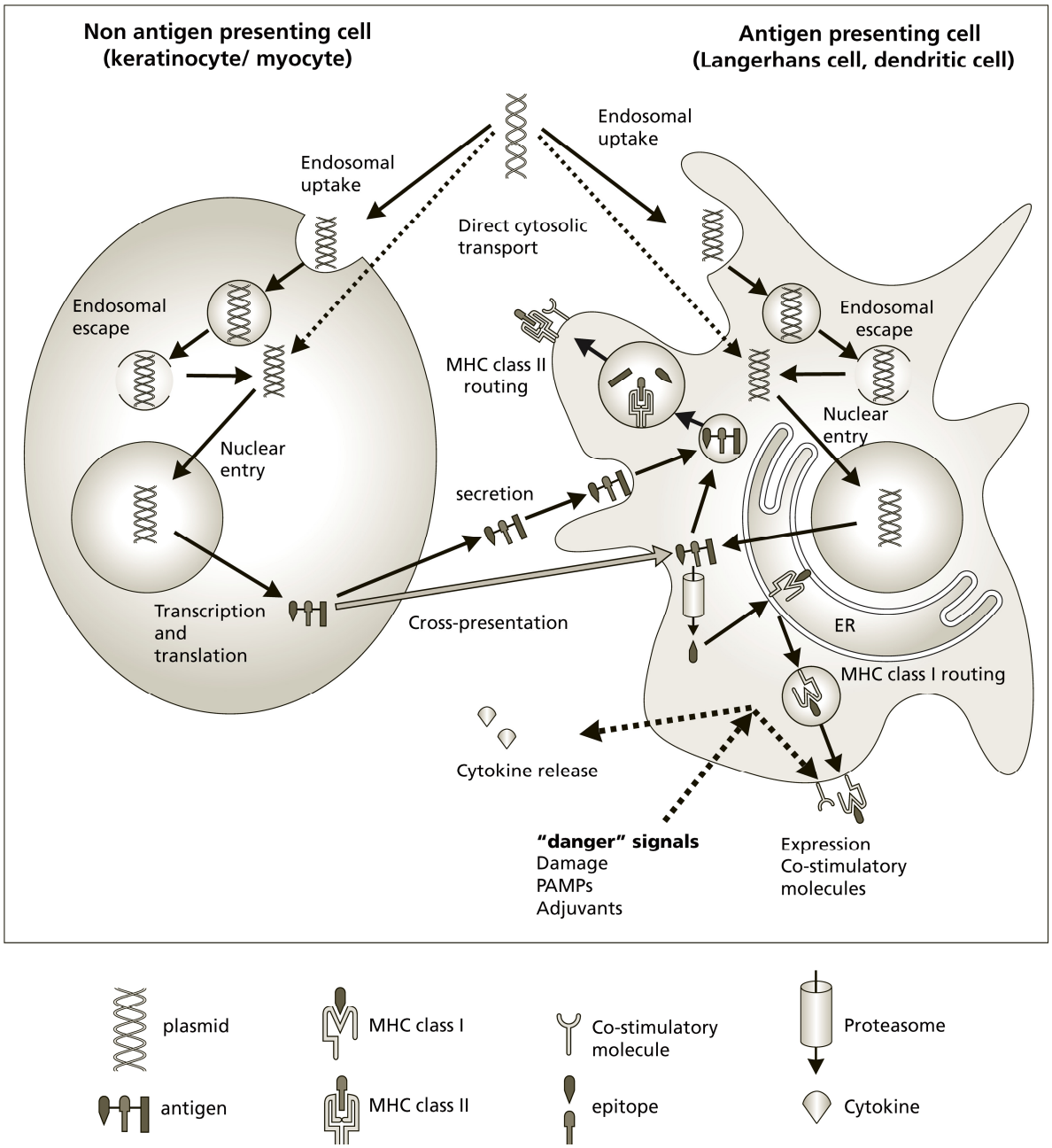

Figure 1: Schematic diagram of antigen expression and presentation upon DNA vaccination. DNA is taken up by cells via endocytosis or via direct cytosolic uptake. After endosomal escape, cytosolic trafficking and nuclear entry, the pDNA can be transcribed into mRNA, followed by intracellular translation of the antigen.
For T lymphocyte activation, antigens have to be presented in the context of MHC class I or MHC class II molecules in the presence of co-stimulatory molecules (such as CD80/86 and CD40). Since professional antigen presenting cells (APCs) are the only cell type that have both classes of MHC, can express co-stimulatory molecules and can migrate to the lymphatic system, their role in antigen presentation and T lymphocyte activation is crucial. Via extracellular release by non-APCs or cell death, antigen can enter the MHC class II pathway. Antigens produce by direct transfection of APCs are presented by MHC class I. In addition, antigen intracellularly produced by non APCs can enter the MHC class I pathway in APCs by a process called cross-presentation.

Advantages of particles

Condensation and encapsulation of DNA into micro- and nanoparticles is widely used for the delivery of nucleic acid for both *in vitro* as well as *in vivo* applications (34-37). The majority of particle formulations use electrostatic interaction between anionic phosphate groups in DNA with a cationic carrier, usually composed of building blocks with positively charged nitrogen atoms. In this complex formation process, an excess of carrier DNA is condensed into positively charged, nano-sized particles. Formulation into a particle has several advantages above the usage of naked DNA for vaccination:

Protection against endonucleases

Once complexed with a cationic carrier, the nucleic acids are better protected against endonuclease-mediated degradation upon injection. It is known that the administration of naked pDNA leads to rapid enzymatic degradation upon intramuscular, intradermal or intravenous injection (38-41). Since these routes are all important in vaccination strategies, prolonging of the half life of the administered genetic vaccines is a rational strategy to potentially increase transfection efficiency upon injection, thereby enhancing immunogenicity due to the increased expression of antigen. For several pDNA-containing nano- and microparticles an enhanced stability to nucleases *in vitro* (42,43) and an increased half-life when injected *in vivo* (44,45), compared with naked DNA, has been described. These studies emphasize the potential benefit of pDNA protection in genetic vaccines.

Increase cellular uptake

It has previously been shown that the transfection efficiency of dermally applied naked DNA vaccines is extremely low (31). This observation may also have relevance for other delivery routes. Besides protection against nuclease-induced degradation, encapsulation into particles has the potential to increase cellular uptake of nucleic acids, which can lead to higher transfection efficiencies. It has been shown that both a small nano-scale particle size and a positive surface charge are factors that increase cellular uptake *in vitro*.

Several studies showed that nano-sized DNA/carrier complexes are actively taken up by non phagocytotic cells via clathrin-mediated endocytosis or by caveolae mediated uptake, depending on the size of the complex used (46,47). Studies with fractionated particles of different sizes showed that smaller particles (<100 nm) have a much higher transfection activity than larger particles, and that the upper size limit of particles that can be taken up by non-phagocytotic cells is approximately 500 nm (48,49). In addition to a small particles size, ionic interactions between positively charged particles and negatively charged polyanionic glycosaminoglycans (GAGs) on cell surfaces generally lead to increased cell binding, followed by cellular uptake (50). Nevertheless, it has also been shown that these GAGs can inhibit transfection, depending on the type and quantity of the GAGs present (51,52). This

means that the optimal charge for transfection depends on cell type and on the matrix surrounding the target cell.

To further increase cellular uptake of particles, cell- penetrating peptides (CPPs) can be coupled to the particle surface (reviewed in (53)). It is important to stress that most particle systems increase the passage over the cell membrane (see Figure 1) as compared to naked DNA. This passage is the first obstacle towards protein expression. After that, the DNA has to escape from the endosome (when taken up via the endosomal route), be "unpacked" from the carrier and travel via the cytosol to the nucleus. To reach the nucleus, the DNA has to overcome probably the biggest hurdle towards protein synthesis, which in non-dividing cells is the nuclear membrane. Nuclear transport can potentially be increased by the coupling of nuclear localization signals (NLS) to the particle to allow interaction with the nuclear transport system (54). It has been shown that this strategy is more successful if the NLS is coupled to the carrier, rather than if coupled to the pDNA (55).

Uptake by Antigen Presenting Cells

Formulation of pDNA into a synthetic carrier can lead to specific uptake and activation of Antigen Presenting Cells (APCs). Larger particles, with a size between 0.5 and 5 µm can be specifically taken up by APCs via phagocytosis. This uptake is strongly dependent on both particle size and surface charge of the particles. In particular, uptake of particles >1 µm can be significantly enhanced *in vitro* by a cationic surface charge (56). Importantly, DNA-containing particles can also activate immature APCs, which leads to an increased expression of co-stimulatory molecules and release of cytokines. This up-regulation can be beneficial for vaccine formulations and depends on the characteristics of the particles used. In *in vitro* DC cultures, it was demonstrated that addition of anionic particles resulted in CD83 up-regulation and IL-12 release, whereas cationic particles up-regulated CD86 with no IL-12 release (57). As discussed later, APCs can also be targeted by specific ligands. Examples of APC ligands are mannose and other ligands for the C-type lectin receptor family like DC-SIGN. These molecules are frequently used *in vitro* to enhance uptake of particles by *in vitro* cultured DCs (58,59). Their performance *in vivo* will be discussed later.

A guidance in particle systems

In the next section we will briefly discuss the current types of delivery vehicles used in animals studies. Several extensive overviews of non-viral carriers for the delivery of plasmid DNA have previously been published (2,34,35,37,60-66). We will not discuss studies where DNA was coated onto gold microparticles in this review. Although this strategy is widely used in animals and patients (67,68), inert gold particles are exclusively restricted to gene gun application, one of the physical methods to administer genetic vaccines but which are beyond the further scope of this review.

Lipids

Cationic lipids are the oldest (69) and most commonly used delivery vehicles for genetic vaccines. In general, these cationic lipids with so-called helper lipids are formulated into unilamellar liposomes before they are complexed with DNA. Upon electrostatic interaction of a cationic liposome with negatively charged DNA, nano-sized complexes are formed, called lipoplexes. Lipid carrier systems have been used in several gene therapy trials in patients (70,71). After more than 20 years of developing these vectors, a wide variety of cationic lipids are currently available, some of them in (c)GMP grade. Commonly used lipids differ from each other in terms of cationic head groups, linkers and hydrophobic chains - the three structures that are generally present in lipids used for DNA transfection. From this variety of cationic lipids, 1,2-dioleoyl-3-trimethylammonium (DOTAP) is used most frequently and can be considered as the gold standard in lipid DNA carriers (61). Cationic lipids are frequently used in combination with a neutral helper lipid such as L-alpha-dioleoyl phosphatidylethanolamine (DOPE) or cholesterol to increase the transfection properties of the DNA/lipid complexes (61,72). A major obstacle for the wider use of these carriers in gene therapy is their toxicity, which is due to the cationic nitrogen group in these molecules (73). A quaternary amine group (such as present in N- [1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride (DOTMA) or DOTAP) is generally more toxic than a tertiary amine (present in 3β-[N-(N',N'- dimethyl-aminoethane)-carbamoyl] cholesterol (DC-Chol). Recently, successful attempts have been made to spread this cationic charge into a heterocyclic ring structure, which can lower toxicity with a retention of transfection properties as compared with DOTAP (74).

The observed toxicity of these molecules might also be an advantage in vaccination applications, since toxicity can also lead to immune activation (by release of "danger signals" by tissue damage). It is known that cationic lipids can up-regulate CD80/86 in DCs upon *in vitro* incubation (75,76), which can potentially increase the immunogenicity of the vaccination.

Cationic polymers

Cationic polymers are widely used as delivery vehicles for genes in both *in vitro* and *in vivo* applications (reviewed in (34,35,37)). Electrostatic complexation of a cationic polymer with negatively DNA results in a nanoparticle called a polyplex. The major advantages of polymers above lipids upon complexation with genes are that particle sizes are generally smaller and the polymer structure can be easily modified to tailor the characteristics of the formed particles. The two classical polymers used for gene transfer applications are poly(ethylenimine) (PEI) and poly(l-lysine) (PLL). In parallel with lipids, cationic polymers also show toxicity *in vitro* and *in vivo* (77), which is generally higher than the toxicity of lipids. The toxicity of polymers is positively correlated to the molecular weight of the polymer. Another

disadvantage of polymers is their lower biodegradability compared to lipids. In the past years, polymer development has focused on the development of biodegradable polymers that have similar or better transfection properties, a lower toxicity and decreased intracellular accumulation compared to PEI and PLL (65,78,79) Degradation of the polymeric carrier can also be used to increase the release of DNA from the polyplex in the cytosol (80).

PL(G)A polymers

Although cationic compounds are most commonly used for DNA complexation, non-ionic polymers are also useful as a vehicle for DNA vaccine delivery. Poly(lactide) (PLA) or poly(lactide-co-glycolide) (PLGA) are most commonly used since these polymers are biodegradable and non toxic (36). PL(G)A particles can be produced in the micro to nanoscale size range. DNA can be encapsulated inside these particles using different techniques such as spray-drying or solvent evaporation (36). Another strategy is to adsorb DNA onto the surface of these particles after coating with a cationic compound such as PEI or cetyltrimethylammonium bromide (CTAB). The advantage of adsorption is that the plasmid is not exposed to the acid microenvironment that is formed inside the particles upon degradation of PL(G)A particles. A disadvantage of adsorption is that the loading efficiency upon adsorption is lower than the already low efficiency upon encapsulation (max ~1% of particle mass). Remarkably, for both encapsulated and adsorbed DNA, protection against nuclease degradation has been demonstrated (81,82). In general, the surface charge of PL(G)A particles is negative, due to free carboxylic end groups of the polymer. This negative charge can be switched towards positive by coating these particles with a cationic compound. This makes it possible to study a wide variety of charges. It has been shown that PL(G)A particles can be useful in generating a sustained transgene expression depot upon intramuscular and intranasal administration, but the peak levels of expression are generally lower when compared with naked DNA (83,84). Another possible advantage of microsized carrier systems is that due to their size, they are preferentially taken up by APCs upon *in vivo* administration (85). Nevertheless, a major hurdle is that uptake should be followed by expression of the antigen-encoding DNA. Several *in vitro* studies showed that the transfection of DCs is relatively low for these systems (0-10%) (reviewed in (36)), especially without the help of physical techniques like electroporation. Although never determined, it is expected that the DC transfection efficiency upon *in vivo* administration is even lower for these systems.

In vivo performance of DNA particles

Table I shows an overview of recent *in vivo* DNA vaccination studies in which synthetic particle formulations were successfully tested to generate a cellular immune and/or a anti-tumor response. This overview illustrates that a variety of particle compositions, particle characteristics, antigens and administration routes have been studied *in vivo* thus far. Despite the successes that have been made in increasing T cell responses and tumor rejection, the mode of action of these systems is often marginally studied. Apart from the measurement of immune responses, it is also important to assess the mechanism of action of the particle formulations. This could at the level of increased antigen expression levels in non-APCs (due to increased DNA stability or uptake) or increased antigen presentation by APCs or activation of APCs. Measuring the level of antigen expression or antigen mRNA in specific cell types, in combination with the assessment of activation status should provide a better understanding of the mode of action of particle formulations and should be incorporated in future experimental set ups. Table I also shows that the cellular immunogenicity of these systems is rather low in pre-clinical testing thus far. Most vaccination studies require an *in vitro* re-stimulation of lymphocytes in order to detect the apparently low number of antigen specific effector T cells. Since tumor vaccination requires a high frequency of cytotoxic T lymphocytes to efficiently kill large tumor burdens, increasing the immunogenicity of particle DNA vaccine carriers is critical.

Table I: Overview of DNA vaccination studies in animals, using a synthetic DNA vehicles.

Type	Formulation	Size	Charge	Route of administration	Encoding antigen	Performance* (method used)			Reference
						Antigen expression	Cellular response	Tumor Survival	
Lipid	PEGylated lipoplexes	150 nm	neutral	Intradermal tattoo	Luciferase and influenza $NP_{366-374}$ epitope fusion	↑ (intravital)	Preserved or ↑ CD8+ (direct *ex vivo* tetramer stain)	n.d.	(98)
	Lipoplexes	n.d.	cationic	Intravenous injection	Ovalbumine (OVA)	n.d.	↑ (in vitro restimulation + ^{51}Cr release assay)	n.d.	(106)
	Lipoplexes Several composition	nano	cationic	Intramuscular injection	Hepatitis C non-structural protein 3	n.d.	↑ (ELISpot IL-2 and INFγ) Th1/Th2 ratio depends on lipid composition	n.d.	(107)
	Mannosylated lipoplexes	120 nm	cationic	Intraperitoneal injection	Ovalbumin	More OVA mRNA in CD11c+ cells	↑ (in vitro restimulation + ^{51}Cr release assay)	↑	(93,108)
	Lipid nanoparticles (+ or - CpG motifs)	150 nm	neutral	Intravenous injection	B galactosidase	in CD11b+ cells in spleen	↑ (in vitro restimulation + ^{51}Cr release assay or intracellular INFγ assay CD8+)	n.d.	(109)
Polymer	Methylated BSA polyplexes	200-400 nm	cationic	Intradermal injection	Ovalbumin	↓	↓ (in vitro restimulation + ^{51}Cr release assay)	n.d.	(110)
	PEGylated PAA polyplex	100	neutral	Intradermal tattoo	Luciferase and influenza $NP_{366-374}$ epitope fusion	↑ (intravital)	Preserved or ↑ CD8+ (direct *ex vivo* tetramer stain)	n.d.	(98)
	Poly-l-lysine coated nanoparticles	20,50, 1000 nm	cationic	Intradermal injection	Ovalbumin	n.d.	↑ (ELISpot INFγ) Only particles 50nm	n.d.	(111)
	PEI polyplex	Nano	cationic	Intravenous injection	Envelope glycoprotein 120	n.d.	↑ (ELISpot INFγ)	n.d.	(112)
	Mannosylated PEI polyplexes	100 nm	cationic	Topically (after tape-stripping)	HIV antigen (without integrase)	Increase in migrating DCs	↑ (*In vitro* restimulation + ELISpot INFγ or Intracellular INFγ staining)	n.d.	(95,113)
	Poly (methl methactylate) particles	460 nm	cationic	Intradermal gene gun	Calreticulin-HPV E7 fusion	n.d.	n.d.	↑compare to naked. ↓compare to gold particle	(114)

PLG(A) micro particle	PLG microparticle coated with cationic compound	1-2 µm	cationic	Intramuscular injection	Luciferase or HIV-1 p55 gag	↑	↑ (*in vitro* restimulation + ^{51}Cr release assay)	n.d.	(115)
	PLG microparticle coated with CTAB	micro	cationic	Intramusclar injection	Human carcinoembryonic antigen (CEA) + GM-CSF	n.d.	↑ (*in vitro* restimulation + ^{51}Cr release assay or intracellular INFγ/TNFα/IL2 assay CD3+)	n.d.	(116)
	PLGA microparticle coated with PEI	<10 µm	cationic	Intradermal and intramuscular injection	B cell antigen fused monocyte chemotactic protein	n.d.	n.d.	↑	(117)
	PLGA microparticle	7 µm	n.d.	Intraperatoneal or subcutaneous injection	Vescular stomatitis virus N52-59 eptipoe	n.d.	↑ (*in vitro* restimulation + ^{51}Cr release assay)	n.d.	(118)
	PLGA microparticle	2-3 µm	n.d.	Intramuscular injection	CYP1B1	n.d.	↑ (CD8 enrichment + ELISPOT INFγ)	n.d.	(119)
	Poly-β amino ester containig PLGA microparticle	4-6 µm	anionic to neutral	Intradermal injection	MHC 1 epitope (Kb)	n.d.	n.d.	↑	(120)

**= compared with naked DNA control*

n.d.= not determined, CTL= cytotoxic T lymphocyte, PEG = poly (ethylenglycol), PAA= poly (amino amide), PEI= poly (ethylenimine)

PLGA= Poly(lactide-co-glycolide), PLA = Poly(lactid), PLG = poly (lactide-co-glycolide, PEO-PPO-PEO= Poly(ethyleneoxide)-poly(propyleneoxide)-poly(ethyleneoxide)

CTAB= cetyl trimethyl ammonium bromide, NP= nucleoprotein, HIV= Human Immunodeficiency Virus, HPV= human papilloma virus ,

GM-CSF= granulocyte monocyte colony stimulating factor, TNFα= tumor necrosis factor-α, INFγ= interferon-γ, IL2= interleukin 2.

Future recommendations:

Cells to be transfected

APCs are the only cells that can express co-stimulatory molecules like CD80/86 and CD40 and can present $CD4^+$ helper epitopes on MHC class II molecules. These two processes are often essential for the proper subsequent activation of naïve $CD8^+$ T lymphocytes or B lymphocytes. Therefore, antigen presentation by APCs plays a key role in the induction of an immune response upon DNA vaccination. Realizing this, targeting DNA vaccines towards APCs has become a growing aim in vaccine delivery research. However, direct transfection of APCs is not always required for the generation of a solid immune response.

It is generally accepted that immunogenic proteins, originally produced in non APCs like myocytes or keratinocytes can be presented by APCs on MHC Class I via a mechanism called cross-presentation (86) (visualized in Figure 1). Upon cross-presentation, APCs can present peptides of the exogenously derived protein on MHC class I to induce a $CD8^+$ effector response. Normally, extracellular proteins are presented via MHC class II molecules. Cross-presentation is an important process in eliminating viral infections and tumors (87). It has previously been shown that exclusive transfection of epidermal keratinocytes by a keratinocyte-specific promoter is sufficient for the induction of a CTL response upon DNA tattooing (10). A similar pathway was demonstrated for intramuscular DNA vaccination (88,89).

While it is clear that expression of antigens by non APCs is sufficient, targeting to APCs can potentially increase the efficiency of DNA vaccines. A recent study by Nchinda et al. investigated the fusion of an antibody fragment towards the DC-associated DEC205 receptor into the encoding region of a DNA vaccine. In this study, a 100 fold lower vaccine dose was required to generate the same antigen specific cytotoxic T cell response as the control constructs (90). Another study successfully used a similar strategy by introducing a CTLA-4 domain in the encoding region to specifically bind B7 molecules on APCs (91). In these studies, the targeting ligand was expressed as a fusion protein with the antigen itself, most probably in myocytes. Coupling of targeting ligands to a particle is a more complex strategy. Successful targeting and internalization should be followed by transcription and translation of the encoded antigen in order to generate an antigen-specific immune response. Nevertheless, some successes has been reported by using mannose as a DC-directed ligand for the targeting of DNA vaccine particles. It was already shown that an intraperitoneal injection of a mannolysated lipoplexes resulted in more antigen mRNA than control lipoplexes (92,93) in $CD11c^+$ DCs. Recently, DermaVir, a topically applied HIV antigen encoding DNA vaccine, formulated with mannosylated polyethylenimine into nanoparticles was extensively studied as an APC-specific formulation. Topical application with DermaVir resulted in antigen expression in migrating Langerhans Cells from skin samples (94) and mRNA levels in the lymph nodes of mice (95). Nevertheless, the performance of

unmannosylated particles was not tested in these studies, making it difficult to evaluate the additional effect of the mannose ligand. For other targeting ligands, uptake by DCs has only been evaluated *in vitro*. In future studies the identity of the transfected cell type upon vaccination should be unraveled, in order to judge the additional value of targeting strategies.

The ideal particle

Setting the ideal characteristics for a DNA vaccine delivery system is important to create an optimal vaccination strategy. It is generally known from studies using fractionated particles that smaller particles have higher uptake in both non-phagocytotic cells and APCs (46,56). Higher uptake will probably also lead to higher levels of antigen expression, which is required for a robust immune response. Therefore, smaller particles (<200 nm) are probably more efficient as delivery vehicle than larger microparticles that only give low transfection efficiencies.

Since most carrier/DNA complexes are formed by spontaneous electrical interaction, the size distribution of these systems is relatively broad. This broad distribution makes is difficult to pin down the optimal size for particle systems. Therefore, it is valuable to develop formulation protocols that are able to produce particles with a narrower size distribution and in which the size can be altered by adapting the protocol. In line with that, it might be worthwhile to set up strategies able to sort carrier/DNA complexes from different sizes prior to *in vitro/ in vivo* evaluation in order to finding the optimal particle size for each application.

The *in vivo* studies summarized in Table 1 show particles of varying surface charges that have been evaluated. A positive charge generally leads to the highest transfection efficiency *in vitro*. Nevertheless, a major disadvantage of cationic particles is their interaction with serum proteins, extracellular matrix components or blood cells *in vivo*. This can lead to aggregation or immobilization of these systems (51,96), which is detrimental to their transfection properties. These unwanted interactions can be prevented by the introduction of poly(ethyleneglycol) (PEG) moieties on the surface of the particles which allows shielding of the surface charge (97). Although PEGylated particles are used for intravenous administration of poly/lipoplexes, their use in DNA vaccination is rare, until now. The probable main reason is that PEGylation is also associated with a lower transfection efficiency *in vitro*, which is due to a decreased interaction between the negative cell membranes and the DNA particle. Nevertheless, in recent experiments performed by our group, PEGylation appeared to be essential for antigen expression and immunogenicity upon dermal DNA tattoo vaccination (see Figure 2) (98). This observation stresses that a positive charge might not always be the optimal charge in vaccination protocols. Therefore, it would be worthwhile to assess the optimal charge for each formulation in relation to its route of administration. This can range from positive to neutral to even negative (99,100). In addition, PEGylated particles are a promising starting platform for active targeting strategies

as shielded particles show less cell binding and targeting ligands can easily be coupled to the terminal end of the PEG chains. Possible disadvantages of PEG coating might be lower cell interaction and pDNA release. Therefore, sheddable coatings (i.e. loss of the coating after arrival at the target site) are currently under development (101).

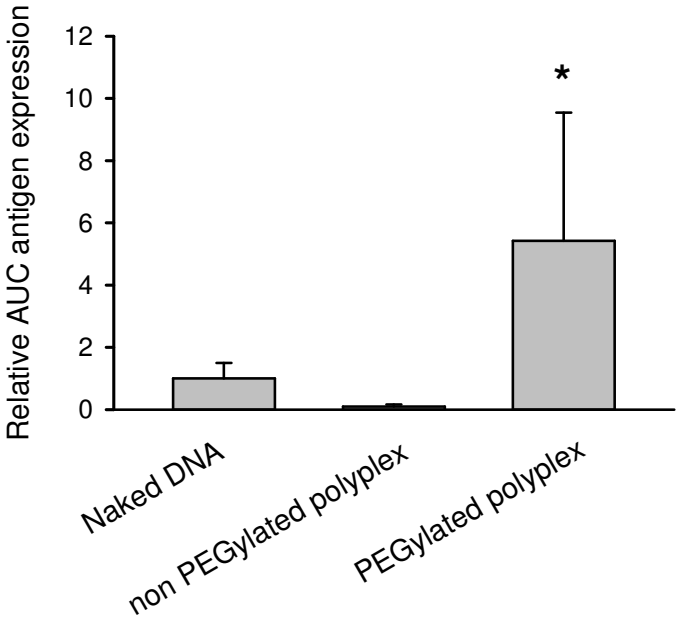

Figure 2: Importance of charge on the performance of DNA vaccines.
In vivo antigen expression upon DNA tattooing of naked DNA and polyplexes with and without PEG coating. PEGylation of polyplexes (and thus shielding of the surface charge) results in a 55 fold increase in antigen expression when compared with nonPEGylated polyplexes that exhibit a positive surface charge. ***** Value significantly higher than naked DNA control (Mann-Whitney U-test, $p < 0.05$). Data adapted from (98).

Evaluation models

Table I shows that a wide variety of pDNA constructs have been used for pre-clinical evaluation of DNA vaccine carriers. Since the expression cassette of the pDNA can heavily influence immunogenicity, it is almost impossible to compare those studies. Although difficult, it would be highly valuable if research groups utilized a common or similar construct for the evaluation of synthetic carriers to allow cross-comparison.

Several model systems can be used to evaluate the performance of synthetic particles. The most commonly used models are *in vitro* cell cultures, *ex vivo* organ cultures and *in vivo* animal models. Although in vitro cultures are widely used to evaluate transfection properties and DC activation induced by particle systems, the predictive value of such cultures for in vivo performance is very limited. This is due to the complete absence of an extracellular matrix (as discussed above) and the unnatural distribution of cells. Ex vivo organ cultures have a much better predictive value for transfection properties. Especially human or porcine skin are widely used as models for dermal vaccination because of the wide availability from surgeries or abattoirs (31,102). A major advantage of cultured skin is that it can be used as a broad screening tool for transfection properties and toxicity of a variety of particle formulations. Such a screening method can be highly valuable before evaluating formulations in *in vivo* animal models. Although the measurement of an adaptive immune response is not possible in cultured skin, the measurement of cytokine release and APC

activation markers, the first indicators for immune activation, is technically feasible (103). For the measurement of an adaptive immune response, *in vivo* animal models are essential. Because of the lower costs and the larger availability of reagents, murine models are most commonly used. Nevertheless, one should be aware that translation of murine results to large animals or humans is often difficult. For example, if a 25 gram mouse receives a pDNA dose of 50 ug, the proportional dose of a 70 kg human would require the enormous and impractical dose of 140 mg pDNA. In future experiments, it might be extra important to carefully assess the dose-response relationship for particle systems during *in vivo* evaluation. For naked pDNA, that has a very low toxicity, the more is most often the better. For a synthetic particle this might be more complicated, since the toxicity of the carrier can result in a delicate balance between increased immune activation or inhibition of antigen expression and immune activation, due to cell death.

Hurdles towards clinical application

In the past decade, numerous DNA vaccination trials have been performed, in which naked DNA in aqueous solution was almost exclusively used as the sole formulation (www.clinicaltrial.gov). Despite the extensive amount of research on non-viral gene delivery systems since the late 80's, particle systems are only sporadically used as pDNA carrier in immunotherapy trials (see table 2 for an overview). The primary reason for this is the relatively high toxicity of carriers compared with naked DNA which is, as discussed earlier in this paper, mainly due their cationic nature. Although biodegradable and less toxic carriers are currently under development to overcome this hurdle (65), they have not yet found their way to the clinic.

Another limitation may be that the majority of carrier systems that are used for pre-clinical evaluation, are synthesized at 'bench scale' under non (c)GMP conditions. In addition, synthesis is not always reproducible. Furthermore, these experimental carriers are often poorly defined in terms of contaminations, impurities or physical-chemical properties (like molecular weight distribution and charge). Also, the exact composition and appearance of the formed particles (amount of carrier/DNA molecules in a particle, shape of the particle etc.) is often poor and difficult to define.

Table 2: Overview of DNA immunotherapy studies in patients, using synthetic DNA vehicles

Formulation	Size	Charge	Route of administration	Encoding gene	Disease and patients	Performance (method used)		Reference
						Immune response	Clinical outcome	
PLG microparticle	micro	anionic	Intramuscular injection	HPV-E7epitope	Anal dysplasia, 12 patients	10/12 (ELISPOT	3/12 (histology)	(121)
PLG microparticle	micro	anionic	Intramuscular or subcutaneous injection	HPV-E7epitope	cervical intraepithelial neoplasia, 15 patients	11/15 (ELISPOT)	5/15 (histology)	(122)
DMRIE/DOPE lipoplex	nano	cationic	intralesional	HLA-B7	Stage IV melama, 10 patients	6/7 (T cell migration in lesion) 2/2 (TIL reactivity)	2/10 (local inhibition tumor growth) 1/10 (CR after TIL treatment)	(123)
DMRIE/DOPE lipoplex	nano	cationic	intratumoral	HLA-B7	Advanced squamous dell carcinoma, 69 patients	n.d.	after 1st cycle: 23/69 SD or PR after 2nd cycle: 6/23 SD, 4/23 PR, 1/23 CR	(124)
DMRIE/DOPE lipoplex	nano	cationic	intratumoral	IL-2	Metastatic renal cell carcinoma, 31 patients	4/8 increase IL-2 expression 5/8 increase T cell infiltration	2/31 PR, 1/31 CR	(71)
DC-Chol/ DOPE lipoplex	nano	cationic	cutaneous	HLA-A2 HLA-B13 H-2K^k	Different tumors with cutaneous metastases, 19 patients	Expression of HLA-A2 and HLA-B13 mRNA in biopsies	2/8 CLR, 4/8 PLR	(125)

PLG=poly (lactide-co-glycolide), DMRIE= dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium, DOPE= L-alpha-dioleoyl phosphatidylethanolamine, DC-Chol= 3β-[N-(N',N'- dimethyl-aminoethane)-carbamoyl] cholesterol, TIL= tumor infiltration lymphocyte, CR= complete respons, PR= partial response, SD= stable disease, CLR= complete local relaps, PLR= partial local relaps, IL-2= interleukin-2, n.d= not determined.

A major hurdle to clinical application of synthetic DNA vaccine particles is the low physical stability of particle systems in pharmaceutical formulations. Because nanoparticles in an aqueous environment are in a fragile equilibrium between Van der Waals attraction and electrostatic repulsion, aggregation can easily occur. This risk is highly relevant for DNA vaccination, since this application generally requires high DNA concentrations, a factor that increases the chance of aggregation. Since aggregation may severely reduce transfection efficiency, and thus vaccine efficiency, efforts should be made to promote the development of formulations with a high physical stability and shelf life, thus reducing this risk. Successes in lyophilization of polyplex and lipoplex formulations have already been reported (104,105).

Conclusion and Perspectives

Synthetic particles may prove to be a drastic improvement for the current generation of naked DNA vaccines, which have a low transfection efficiency and immunogenicity. Particles can be combined with other strategies as mentioned in the Introduction section of this review. Though a variety of systems have been tested in pre-clinical *in vivo* models, future experiments should further uncover their precise mode of action and cellular routing. In addition, particle characteristics and the pre-clinical evaluation model should be chosen carefully. After optimization of particle characteristics, the possible additional effect of targeting ligands, coupled to the particles, can also be studied.

Finally, it is important to translate pre-clinical success with DNA vaccine particles towards clinical trials. This step is essential to reveal if these systems have a distinct clinical advantage over naked DNA vaccines.

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Chapter 3

GMP production of pDERMATT for vaccination against melanoma in a phase I clinical trial

Susanne G. L. Quaak
Joost H. van den Berg
Mireille Toebes
Ton N. Schumacher
John B. A. G. Haanen
Jos H. Beijnen
Bastiaan Nuijen

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Abstract

For the treatment of melanoma DNA vaccines are a promising therapeutic approach. In our institute a plasmid encoding a melanoma associated epitope (MART-1) and an immunostimulatory sequence (tetanus toxin fragment-c) termed pDERMATT was developed. In a phase I study the plasmid will be administered intradermally using a newly developed tattoo strategy to assess the toxicity and efficacy of inducing tumor-specific T cell immunity. To facilitate this study a Good Manufacturing Practice (GMP)-compliant plasmid manufacturing process was set up and a pharmaceutical dosage form was developed. Each batch resulted in approximately 200 mg plasmid DNA of a high purity >90% supercoiled DNA, an A260/280 ratio 1.80-1.95, undetectable or extremely low residual endotoxins, E.coli host cell protein, RNA, and DNA. In the manufacturing process no animal derived enzymes like RNase or potentially harmful organic solvents are used. After sterile filtration the concentration of the plasmid solution is approximately 1.1 mg/mL.

For the scheduled phase I study a concentration of 5 mg/mL is desired, and further concentration of the solution is achieved by lyophilisation. The formulation solution is composed of 1 mg/mL pDERMATT and 20 mg/mL sucrose in Water for Injections. Upon reconstitution with a five times smaller volume an isotonic sucrose solution containing 5 mg/mL pDERMATT is obtained. Lyophilised pDERMATT is sterile with >90% supercoiled DNA, an A260-280 ratio 1.80-1.95, content 90-110% of labeled, and residual water content <2% (w/w). The product yields the predicted profile upon restriction-enzyme digestion, is highly immunogenic as confirmed in an *in vivo* mouse model, and stable for at least six months at 5°C. We have not only developed a reproducible process to manufacture pharmaceutical grade plasmid DNA but also a stable dosage form for the use in clinical trials.

Introduction

Malignant melanoma is a highly aggressive type of cancer. In the past 10 years its incidence and mortality rate are rapidly increasing compared to other cancers (1,2). Surgical resection and systemic chemotherapy are the main therapeutic strategies for the treatment of malignant melanoma. However, these approaches are insufficiently effective and may be associated with significant adverse effects.

In the past decades, evidence has accumulated for a role of T lymphocytes in the host immune response against cancer in general and against melanoma in particular (3). MART-1 is a melanoma associated antigen (melanocyte lineage-specific) that is expressed in a large fraction of melanomas and that is frequently recognized by tumor-reactive cytotoxic T lymphocytes (CTLs) in melanoma patients (4). Previous studies with MART-1 peptide vaccines have shown that it is feasible to boost immune responses against the MART-1 epitope (5,6). However, while peptide vaccination can enhance melanoma-reactive CTL responses, it has become clear that the vaccine-induced responses are insufficiently potent to induce clinical responses in advanced-stage patients (7,8).

In parallel work it has been shown that naked DNA injected into muscle tissue is expressed *in vivo* (9), and that the introduction of immunogenic sequences can result in the induction of vaccine-specific T cell and B cell responses (10,11). DNA vaccines are currently in development for the prevention and treatment of a series of human diseases, including cancer, acquired immune deficiency syndrome (AIDS), malaria and hepatitis B (10,12). Because of the potential of DNA vaccines in the induction of melanoma reactive T cell responses (13,14) and because of the marked potency of a recently developed DNA tattoo technology (15), we sought to develop a GMP production process for DNA vaccines encoding melanoma-associated antigens

To this purpose we developed a novel DNA vaccine termed pDERMATT (plasmid DNA Encoding Recombinant MART-1 and Tetanus toxin fragment-c) (Fig 1). In pDERMATT the

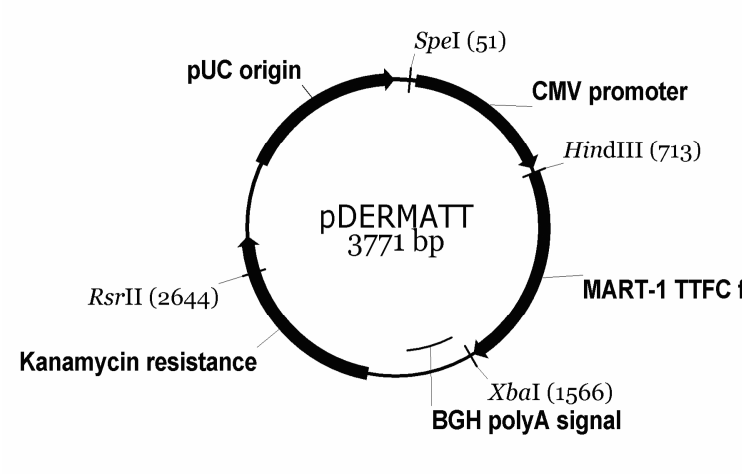

Figure 1: Plasmid DNA map of pDERMATT (plasmid DNA encoding recombinant MART-1 and tetanus toxin fragment-c) including selected restriction sites.

MART-1 $_{(26-35)}$ E27L (ELAGIGILTV) major histocompatibility complex (MHC) class I epitope is fused to the carboxyl terminus of tetanus toxin fragment-c. Prior work has demonstrated that such a genetic fusion of single epitopes to the COOH-terminus of carrier proteins yields superior CTL responses (16). Furthermore, the tetanus toxin fragment-c contains the well described "universal" helper epitope p30 (17,18). This epitope binds to a range of mouse and human MHC class II alleles (19), and the resulting $CD4^+$ T cell stimulation (20) is necessary for the induction of robust $CD8^+$ T cell responses by DNA vaccines. The plasmid that is described here will be administered intradermally using a newly developed tattoo strategy, which has been shown to lead to a rapid and sustained development of both T-and B-cell responses (15). In a phase I study toxicity and efficacy of inducing tumor-specific T cell immunity of pDERMATT will be assessed.
To facilitate this study an in-house plasmid manufacturing process to obtain pharmaceutical grade plasmid DNA was set up and a pharmaceutical dosage form was developed. The challenge was to design a scalable, robust and reproducible manufacturing process, resulting in a pharmaceutical product meeting current quality standards. This means that the pharmaceutical production of pDERMATT needs to meet the requirements for Good Manufacturing Practice (GMP) (21). The plasmid product must be of high purity, essentially in its supercoiled form and free of hosT cell proteins, chromosomal DNA, RNA (preferably without the use of ribonuclease A (22-24)) and endotoxins (23,25,26). As pharmaceutical formulation a sterile, injectable pharmaceutical dosage form containing 5 mg/mL pDERMATT was required.

In this article we describe the GMP production of pDERMATT for vaccination in which we use an RNase free, one-step purification and organic solvent free protocol. Tangential Flow Filtration (TFF) in combination with lyophilisation was used in order to achieve a highly concentrated and stable pDERMATT dosage form.

Materials and methods

General

The plasmid DNA production facility for manufacturing of the Master Cell Bank (MCB) and bulk drug consists of two class 100 (B) cleanrooms (Interflow, Wieringerwerf, The Netherlands). One room is dedicated to handling bacteria and the second room is dedicated to purification. Both cleanrooms contain a class 100 (A) biosafety cabinet and the fermentor is placed in a class 100 (A) down-flow booth. Manufacturing of pDERMATT final product is performed in a third class 100 (B) cleanroom , containing a class 100 (A) down-flow cabinet, as described earlier (27). All cleanrooms are subjected to a monitoring program for viable and non-viable particles at operating and at resting state (28).

During manufacture only product dedicated glassware and sterile disposables are used. Buffer components, pharmaceutical excipients and primary packaging materials used in the manufacture of pDERMATT were of European Pharmacopeia (Ph. Eur.) grade (if possible) and provided with a Certificate of Analysis (CoA) by the supplier. All materials and excipients were approved on the basis of in-house quality controls carried out according to monographs in the mentioned pharmacopoeia (if applicable).

Plasmid design and Master Cell Bank (MCB) generation

The insert of the pVAX-based plasmid pDERMATT (Fig 1), a plasmid of 3.8 kb with a kanamycin resistant marker, was designed at the Netherlands Cancer Institute (NKI-AvL, Amsterdam, The Netherlands) and subsequently manufactured by GeneArt (Regensburg, Germany) to obtain a small quantity of plasmid produced under GMP/Good Laboratory Practice (GLP) conditions. The insert was synthesised and cloned into a pVAX1 backbone (Invitrogen, California, USA) using *Hin*dIII and *Not*I (Roche, Mannheim, Germany).

E. coli DH5 cells (ATCC, Teddington, Middlesex, UK) were made competent with $CaCl_2$ and subsequently transformed with the plasmid pDERMATT using a standard heat shock method (29). These cells were plated on Luria Bertani (LB) plates containing 100 µg/mL kanamycin (Biotrading Benelux, Mijdrecht, The Netherlands) and grown in a Refrigerated Incubator Shaker Innova 4230 (New Brunswick Scientific BV, Nijmegen, The Netherlands). One single colony was isolated and grown in 175mL LB-Miller broth (Sigma Aldrich Chemie B.V., Zwijndrecht, The Netherlands) containing 100 µg/mL kanamycin (Roche Diagnostics Nederland B.V., Almere, The Netherlands) at 37°C in a 1L sterile baffled shake flask (Nalgene) at 200rpm. When an OD_{600} of more than 0.7 was reached, glycerol (BUFA, Uitgeest, The Netherlands) was added to the culture (30% v/v) and aliquots of 1mL were frozen to -80°C, forming the MCB.

The MCB was verified to contain kanamycin resistant *E.coli*, no adventitious viruses and no mycoplasma by MicroSafe B.V. (Leiden, The Netherlands) according to Ph. Eur. (30) and FDA guidelines (CBER, 1993) (31). Plasmid identity was confirmed by sequencing (in house).

Fermentation

The inoculum of the fermentor consisted of a shake flask culture. One vial of the MCB was transferred into a baffled shake flask containing 500mL LB-Miller with 100 µg/mL kanamycin, which was subsequently grown for approximately 8 hours at 37°C and 200rpm. This culture was inoculated into a BioFlo 3000 benchtop fermentor with a working volume of 10L (New Brunswick Scientific BV, Nijmegen, The Netherlands) containing 500mL 500g/L glucose (B. Braun, Melsungen, Germany), 100 µg/mL kanamycin (as selection agent), 5mL gamma irradiated silbione antifoaming agent (BUFA, Uitgeest, The Netherlands), and 9L LB-Miller. During fermentation, pH was controlled at 7.0 with 10% NaOH (BUFA, Uitgeest, The

Netherlands). Air inflow was set at 5 L/min and agitation speed (300-800 rpm) was automatically feed-back controlled based on dissolved oxygen (DO) at a set point of 35%.

Downstream processing

The lysis, pre clarification and purification procedures were performed according to Qiagen protocols (32). All buffers for bacterial lysis and column chromatography were prepared by the production facility of our pharmacy department. The composition of the buffers was kindly supplied by Qiagen. Equilibration buffer contained 750 mM KCl and 50 mM KAc. pH was adjusted to 5.0 – 5.2 with acetic acid and conductivity was adjusted to 90 mS/cm with 3 M KCl. Wash buffer contained 1.7 M KCl and 50 mM Tris-Cl, pH was adjusted to 7.2 with HCl. Elution buffer contained 2.0 M NaCl and 50 mM Tris-Cl, pH was adjusted to 7.5 with HCl.

Recovery

The cells were harvested from the fermentor by mechanically pumping the culture into a Flexstand Tangential Flow Filtration (TFF) system equipped with a Hollow Fibre Cartridge model UFP-300-E-6A with a membrane area of 0.28m^2 (GE Healthcare, Diegem, Belgium). The cells were concentrated from 10L to approximately 1.5L. Subsequently LB-Miller was exchanged for sterile buffer P1 (50mM Tris-HCl pH 8.0, 10mM EDTA) without addition of RNase A. After exchanging eight times, the suspension was harvested and divided equally over three 5 litre bottles (Schott). To each bottle additional P1 buffer was added to a total volume of 1.5L.

Lysis and pre clarification

Lysis was performed at room temperature by adding 1.5L of sterile buffer P2 (200 mM NaOH, 1% SDS) and inverted mixing by hand. Eight minutes after lysis, cellular debris, gDNA and proteins were precipitated by gently adding and mixing 1.5L of pre-cooled (4-10°C) buffer P3 (3 M KAc pH 5.5). The contents of the three bottles were transferred into a sterile 20L vacuum bottle (Schott) and a small vacuum of 500 mbar was applied to this bottle. After 15 min, the precipitated material floated on top and formed a distinct upper face (33). The pre-cleared lysate was harvested through a tube that reaches to the bottom of the 20 L bottle and subsequently filtered through an Opticap 4 capsule filter, 1.2/0.5/0.22 µm 9/16 in. (Millipore, Amsterdam, The Netherlands) to obtain a clear solution. To this solution 1/10th volume of endotoxin removal buffer was added and subsequently the conductivity of the solution was adjusted to approximately 90 mS/cm with a 3 M KCl solution.

Purification

Plasmid purification was performed using anion exchange chromatography. A BPG-100/500 column with 10 cm I.D. x 50 cm height and a maximum bed volume of 2 L (GE Healthcare,

Diegem, Belgium) was packed with 520 mL Ultrapure resin, containing positively charged DEAE groups on the surface (QIAGEN, Venlo, The Netherlands) according to the instructions of the manufacturer. The column was connected to an Äkta Pilot (GE Healthcare, Diegem, Belgium) and equilibrated for 20 min at a flowrate of 120 mL/min. The cleared lysate was loaded onto the column with a flowrate of 14 mL/min. The column was washed in two steps, first 20 min (120 mL/min) equilibration buffer followed by 55 min (120 mL/min) wash buffer. pDERMATT was eluted at 50 mL/min, a total of 1 L elution fraction was collected. During the run the following parameters were monitored: UV at 260 and 280nm, pH, conductivity and the pressure (△P) over the column.

Concentration and sterile filtration

After elution the plasmid solution was transferred into a Quixstand TFF system equipped with a Hollow Fibre Cartridge model UFP-300-E-3MA with a membrane area of 0.011m^2 (GE Healthcare, Diegem, Belgium) to concentrate the solution. The concentration of the elution fraction was determined with an Eppendorf BioPhotometer 6131 (Hamburg, Germany). The solution was concentrated to a concentration of approximately 1.1 mg pDERMATT/mL. Subsequently, the elution buffer was exchanged for Water for Injections (Wfl, B. Braun, Melsungen, Germany) in eight steps. After exchanging eight times the solution was sterile filtered through a 0.2µm Mini Kleenpack filter (Pall, Hitma B.V., Uithoorn, The Netherlands). The final plasmid solution was stored at -80°C in sterile United States Pharmacopeia (USP) grade poly propylene (PP)-bottles (Nalgene, VWR, Amsterdam, The Netherlands) until further processing.

Formulation

Formulation development

Small-scale test batches of lyophilised formulations of four different compositions containing sucrose (Merck, Darmstadt, Germany), trehalose (Ferro Pfanstiehl, Waukegan, IL, USA), mannitol (Bufa, Uitgeest, The Netherlands) or Polyvinyl Povidone (PVP) (Plasdone C15 (34), ISP, Waalwijk, The Netherlands) were manufactured and subjected to quality control and stability studies. Cake appearance, residual water content, pDERMATT content and purity were determined after manufacture and after 2 weeks, 1, 2, 3, and 6 months of storage at 25±2°C/60±5% relative humidity (RH), and 40±2°C/75±5% RH in climate chambers (HEKK 0057, Weiss Technik Ltd., Buckinghamshire, UK)

The formulation solutions for the test batches contained 1 mg pDERMATT/mL. Aliquots of 2.0 mL were filled into 8 mL colourless glass injection vials (hydrolytic class 1 Type Fiolax-clear, Aluglas, Uithoorn, The Netherlands) and grey butyl rubber lyophilisation stoppers (Type FM157/1, Helvoet Pharma N.V., Alken, Belgium) were inserted into each injection vial,

subsequently the formulations were lyophilised. Vials were frozen to -35°C, primary drying was started at a shelf temperature of -35°C and a chamber pressure of 0.4 mbar. During the primary drying phase temperature was elevated to -12°C. Secondary drying was carried out at a shelf temperature of 25°C and a chamber pressure of 0.2 mbar. Vials were sealed with aluminium caps.

Manufacturing process

The formulation solution contained 1 mg/mL pDERMATT and 20 mg/mL sucrose. After complete dissolution of the sucrose, the solution was adjusted to final volume with sterile water for injection and subsequently filtered through a sterilising 0.2 µm Mini Kleenpack filter (Polyethersulfone (PES) membrane). Aliquots of 2 mL were filled into the same vial-stopper system used for the manufacture of the test batches. Platinum cured silicone tubing (Watson Marlow, Cheltenham, UK) was used during filtration and filling. Three vials were equipped with thermocouples and all vials were loaded into a Model Lyovac GT 4 freeze-dryer (GEA lyophil GmBH, Hürth, Germany) at ambient temperature and lyophilised. The product temperature, shelf temperature, chamber pressure and condenser temperature were monitored. The product was sealed with flip-off caps (West Pharmaceutical, Germany) and labelled.

Quality control

All chemicals used for quality control were of analytical grade and used without further purification.

Agarose gel electrophoresis including restriction endonuclease digestion

Agarose gels were run in a Horizon 20·25 horizontal gel electrophoresis unit coupled to a Whatmann Biometra power supply (Westburg B.V., Leusden, The Netherlands).

IPCs and final product were analysed by electrophoresis using 25 cm, 1% agarose (ABGene, Epsom, Surrey, UK) self cast gels. Running buffer was a 40mM Tris-Acetate, 1mM EDTA, pH 8.3 solution and electrophoresis was carried out at 30V for 21 hours. After electrophoresis, gels were stained for 1.5 hours with a 1x Sybr green I solution (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) and then visualized and photographed under UV light (GeneGenius, Westburg B.V., Leusden, The Netherlands).

Open circular and linear standards of the plasmid DNA were prepared using *N.BstNB*I (New England Biolabs, Hertfordshire, England, UK) and *Hin*dIII (Roche Diagnostics Nederland B.V., Almere, The Netherlands), respectively, according to the instruction of the manufacturer.

For AGE the samples were diluted, if necessary, to approximately 20 ng pDERMATT/µL; for determination of residual *E.coli* host RNA/DNA in the bulk and lyophilised product the

sample was diluted to 50 ng pDERMATT/µL. Of the resulting solutions 10 µL was mixed with 2 µL of 6x loading dye and this was subsequently loaded onto the gel.

Anion exchange chromatography

Analytical chromatography was performed with an HPLC system that consisted of an 1100 series binary HPLC pump, Model G1312A (Agilent technologies, Amstelveen, The Netherlands), a model spectraSERIES AS3000 automatic sample injection device, equipped with a 20 µL sample loop (Thermo Separation Products, Breda, The Netherlands), a photodiode array detector (PDA) Model Waters™ 996 (Waters Chromatography B.V., Etten-Leur, The Netherlands). Chromatograms were processed using Chromeleon software (Dionex Corporation, Sunnyvale, CA, USA).
Plasmid purity was determined by a validated anion-exchange chromatography (AEX-HPLC) on a TSK DNA-NPR column (75mm x 4.6mm I.D. particle size 2.5µm) protected by a TSK DNA-NPR guard column (5 x 10mm, Anachem House, Luton, England). Injection of 10 µL of sample was followed by a linear gradient of 0.45-0.53 M NaCl in 20mM Tris, 10% isopropyl alcohol (IPA), pH 9.0 for 32 min. The flow rate was 0.5 mL/min. Absorbance was monitored at 260 and 280nm. Furthermore, a UV spectrum was recorded from 340-190nm using a diode-array detector. Samples were diluted, if necessary, to approximately 100 µg/mL.
Standards of plasmid pDERMATT were derived from the first plasmid batch. Under the conditions used the TSK-DNANPR column was able to resolve open circular, supercoiled and linear plasmid (manuscript in progress).

UV analysis

UV analysis was used to determine the concentration and purity of the plasmid DNA sample. The HPLC samples were diluted to approximately 30 µg/mL and measured with a Biofotometer (Eppendorf, Hamburg, Germany). Absorbance was measured at 230, 260, 280 and 320 nm.

Protein analysis

A bicinchoninic acid (BCA) assay from Pierce (Rockford, IL, USA) was used to measure residual protein content.

Endotoxin analysis

A Pyrochrome® limulus amoebocyte lysate assay (Cape Cod Associates, Cape Cod, MA, USA) was used to measure endotoxin content in the bulk material.

Sequencing

Primers were designed using Primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (35). A total of 26 primers were designed to cover the whole

sequence. First, PCR products of the forward and reverse couples were generated. Results of the PCR reaction were analysed on a 2% agarose gel. For sequencing the PCR products were purified with ExoSAP-IT (GE-Healthcare, Diegem, Belgium). After purification, DNA cycle sequencing was carried out as described by the manufacturer (Applied Biosystems, Foster City, CA, USA) in 20 µL reactions on a PTC-200 thermocycler (MJ Research, Inc., Waltham, MA, USA). The forward and reverse primers were identical to those used in the PCR amplifications. Both DNA strands were sequenced. Sequences were analysed on an Applied Biosystems 3100-Avant DNA sequencer. For sequence alignment Seqscape v2.1 (Applied Biosystems, Foster City, CA, USA) was used.

Bioburden

The bioburden of the solution was determined using the total viable aerobic count Ph. Eur. <2.6.12> using 10 mL of the bulk product.

Sterility and bacterial endotoxins

Sterility of the final lyophilised product was checked by the filtration method and the presence of bacterial endotoxins with the limulus amoebocyte lysate (LAL) test (Cape Cod Associates, Cape Cod, MA, USA), both carried out according to the Ph. Eur. (2.6.1 and 2.6.14) (30).

Mice

Human Human D^b (HHD) mice (2-4 months) transgenic for the Human Leukocyte Antigen (HLA)-$A2K^b$ fusion gene (36) were obtained from the experimental animal department of The Netherlands Cancer Institute. Animals were kept under normal conditions. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Laboratory Animal Research Committee of the Institute.

DNA immunization

Mice were vaccinated with pDERMATT final product (2 mg/mL in Wfl), or with empty pVAX vector as a control. For intradermal DNA vaccination, the hair of the left hind leg of the mice was removed with depilatory cream (Veet sensitive). Next, 10 µl of the pDERMATT solution was applied to the skin and a sterile disposable 9-needle cartridge (MT Derm, Berlin, Germany) mounted on a Aella® tattoo machine for medical use (MT Derm, Berlin, Germany) was used to apply the vaccine. Needle depth was adjusted to 1.0 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were punched into the skin by a 30 s tattoo. Mice were vaccinated with a standard vaccination scheme on day 0, 3 and 6 (15). All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment.

Cytotoxic T cell assay

To measure specific CTL responses against the MART-1 epitope, peripheral blood lymphocytes were stained at the indicated time points with Phyco Erythrin (PE)-conjugated A2K^b-ELAGIGLTV-tetramers and Allo Phyco Cyanin (APC)-conjugated CD8 (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1× Phosphate Buffered Saline (PBS), 0.5 % Bovine Serum Albumin (BSA) and 0.02 % sodium azide) as described before (37). CD8$^+$ cells expressing the MART-1 epitope-specific T cell receptor will bind to the tetramer and can be detected by flow cytometry. After incubation cells were washed three times in FACS buffer and analyzed. Living cells were selected based on propidium iodide (PI) exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) using CellQuest software (Becton Dickinson, Franklin Lakes, USA).

Results

Production process

An overview of our plasmid production process is shown in figures 2A, 2B. One fermentation run consisted of a total of 10 L media incubated for 13-15 hours. Five fermentation runs allowed for the production of 780 mg pDERMATT, a sufficient amount of plasmid DNA for the clinical trial. A total of six fermentation runs were performed (Table 1). For all fermentations the final OD_{600} was approximately 7, irrespective of OD_{600} of inoculum or fermentation time.

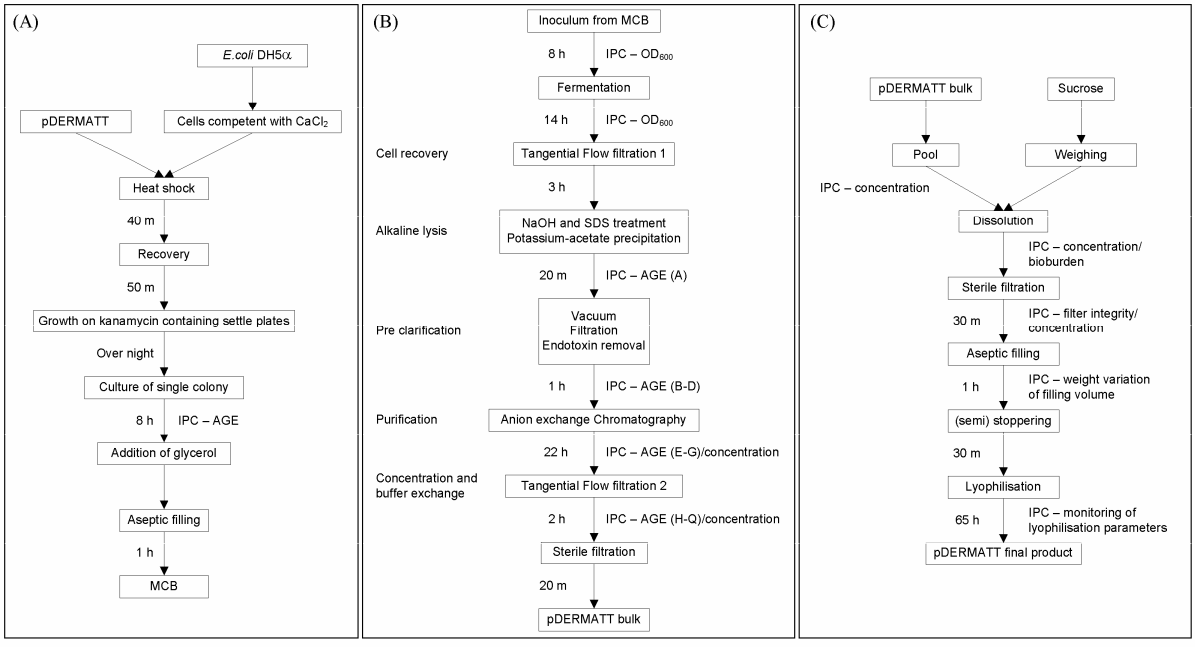

Figure 2: Proces flow sheets for the production of pDERMATT at our facility, production of Master Cell Bank (MCB) (A), pDERMATT bulk (B) and pDERMATT final product (C). Approximate time in hours (h) or minutes (m) for each step are displayed on the left of the arrow, while in process controls (IPCs) are indicated on the right side. The letters behind the IPC controls in figure B correspond to the letter in Fig. 3.

Table 1: Quality control performed on produced bulk plasmid including set specifications

Test item	Specification	Bulk product (batch number)					
		1	2	3	4	5	6
IPC							
OD_{600} inoculum	≥ 0.7	0.721	2.94	1.67	2.46	3.275	2.51
Fermentation time (hh:mm)	For information	15:07	13:27	15:40	15:45	15:20	14:51
OD_{600} fermentation	For information	6.84	7.52	7.45	6.54	7.46	7.20
$A_{260/280}$ eluate	1.80 – 1.95	1.90	1.94	1.90	1.86	1.89	1.95
Amount DNA in eluate (mg)	For information	154	201	195	202	212.8	171.1
$A_{260/280}$ after 2nd TFF	For information	1.78	1.68	1.74	1.70	1.72	1.70
Recovery after 2nd TFF (%)	≥ 85%	95.5	88.5	106.4	112	102.5	96.1
Amount DNA final product (mg)	For information	134.1	164.9	191.2	214.1	207.4	149.3
QC							
Apperance	Clear, colourless solution	conforms	conforms	conforms	conforms	conforms	conforms
Sequencing	Conform reference	conforms	conforms	conforms	conforms	conforms	conforms
HPLC analysis							
1. Identification	Rt identical to standard	conforms	conforms	conforms	double peak	conforms	conforms
2. Purity	≥ 90% supercoiled	98	90	91	88	94	96
UV analysis							
1. Concentration	For information (mg/ml)	1.048	1.024	1.180	1.252	1.152	1.044
2. Purity	$A_{260/280}$ = 1.80 – 1.95	1.86	1.88	1.83	1.84	1.84	1.87
AGE analysis							
1. Identification	3395 – 4149 bp	3700	3728	3756	3785	3756	3609
2. Restriction map	Compares to theoretical	conforms	conforms	conforms	conforms	conforms	conforms
3. Residual *E.coli* host DNA	< 5% (< 0.05 mg/mg plasmid)	< 5%	< 5%	< 5%	< 5%	< 5%	< 5%
4. Residual *E.coli* host RNA	≤ 4% (≤ 0.04 mg/mg plasmid)	< 4%	< 4%	< 4%	< 4%	< 4%	< 4%
BCA assay	< 5 µg/ml protein	n.d.	n.d.	n.d.	n.d.	n.d.	nd
Residual ER buffer	None detected	n.d.	n.d.	n.d.	n.d.	n.d.	nd
Total viable count	0 cfu	0	0	0	0	0	0
Endotoxin	< 10 EU/mg	< 2	< 2	< 2	< 2	< 2	< 2
Approved?	**Complies to all test items**	**yes**	**yes**	**yes**	**no**	**yes**	**yes**

OD_{600},= optical density at 600nm. AGE= agarose gel electrophoresis, AEX-HPLC= anion exchange high performance liquid chromatography, UV= ultra violet, BCA= bicinchoninic acid, n.d.= not detected, RP-HPLC = reversed phase high performance liquid chromatography, ER = endotoxin removal, cfu,= colony forming unit, Ph. Eur. = European Pharmacopoeia, EU= Endotoxin Unit.

With the aim to obtain higher yields in future studies, new fermentation strategies are currently being developed. Preliminary data using fed-batch culture with glycerol as carbon source and a luciferase encoding plasmid showed that an OD_{600} of 105 can be reached with a plasmid content of 135mg/ml Pre-manufacturing experiments showed that a column bed volume of 520 mL was sufficient for purification. Table 1 and figure 3 show that *E. coli* host RNA can efficiently be removed during purification, even when cellular RNA is not removed by RNase A. As is shown in Figure 3E and F *E.coli* host RNA is released from the column during two wash steps by increasing pH and salt concentration. Subsequently, the plasmid DNA is released by a further increase in salt concentration and pH (Fig 3G). HPLC and AGE data show no alterations in topology during the downstream processing (Table 1, Fig 3G vs 3H and 4A). During downstream processing, in particular with the tangential flow filtration step, the $A_{260/280}$ (DNA/protein) ratio decreases. However when the bulk product is diluted in Tris-EDTA (TE) buffer instead of Wfl the $A_{260/280}$ ratio returns to a value between 1.80 and 1.95.

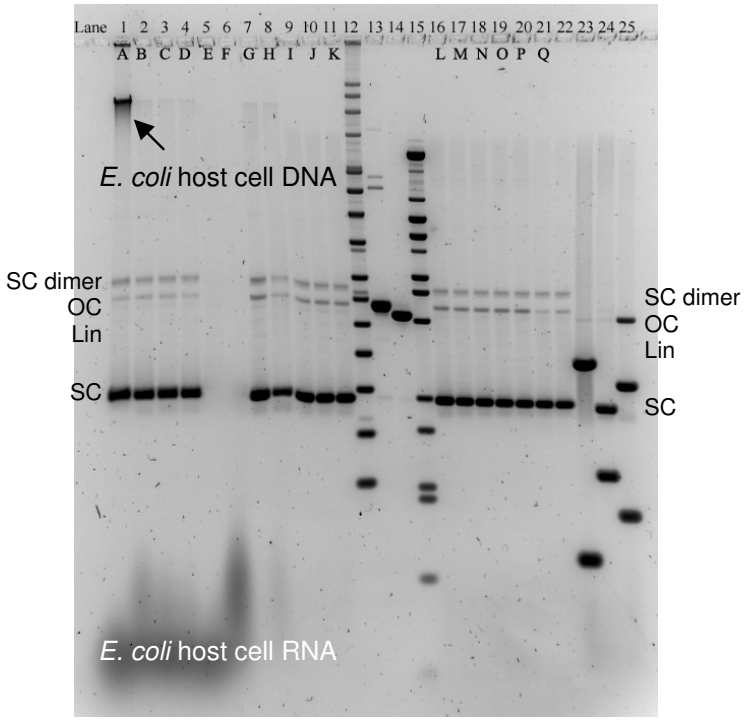

Figure 3: Analysis of IPCs, bulk product and restriction fragments by 1% agarose gel electrophoresis: lane 1-11 and 16-21 contain IPCs, letters corresponding to letters in figure 2B; lane 12 contains a supercoiled DNA ladder; lane 13 open circular standard; lane 14 linear standard; lane 15 lambda DNA *BstE*II digest; lane 22 pDERMATT bulk product; lane 23 *Hin*dIII and *Xba*I digest; lane 24 *Spe*I and *Xba*I digest; lane 25 *Rsr*II and *Spe*I digest

Table 1 represents the Quality control (QC) results of the pDERMATT batches including in-proces controls (IPCs). The specifications for pDERMATT bulk are based on specifications found in literature (38-44). As is shown in Figure 4A the topology as determined with HPLC analysis does not change during production, however one of the batches showed a double peak in the HPLC analysis (Fig 4B). AGE analysis showed a slight difference in migration between both peaks (data not shown). Additionally, the purity of this batch is below the set specification. Therefore the batch was rejected, and further research is being performed as to see what caused the formation of the second peak and to characterise this peak. Possibly

the peak consists of supercoiled plasmid DNA with a lower amount of supercoils, as has been described earlier (45). Linear plasmid was not seen in any of the samples measured. The last peak in the chromatogram still has to be fully characterised, but this is probably a

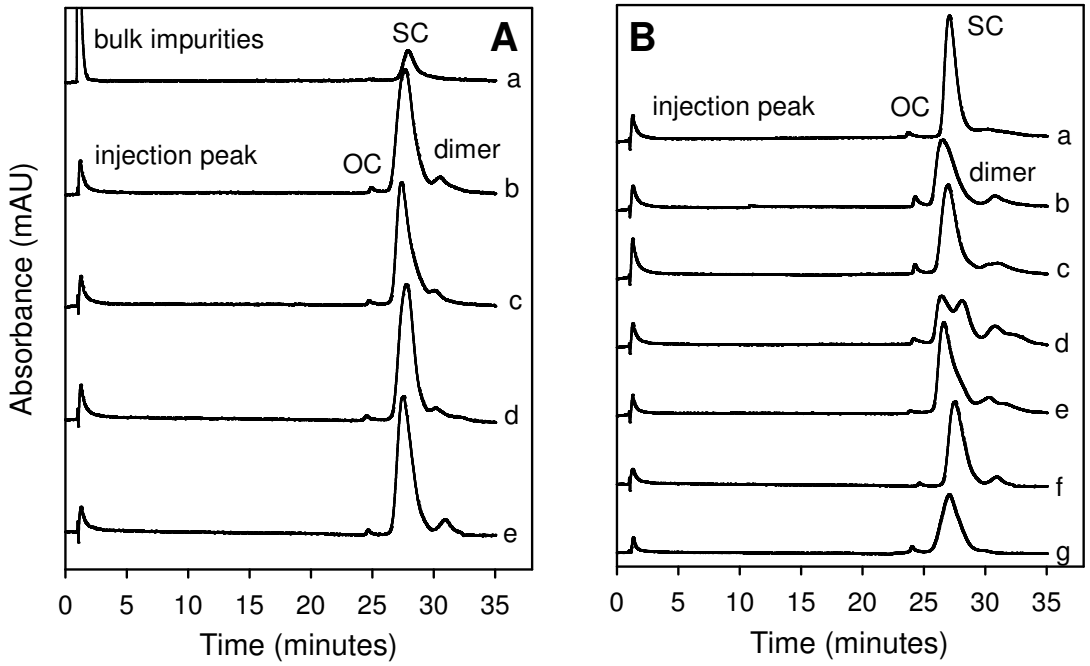

Figure 4: Typical HPLC chromatograms; IPCs and bulk product a = after lysis, b = after concentration with 2nd TFF system, c = after exchanging elution buffer for Wfl in 8 steps, d = before filtration, e = after sterile filtration (**A**), pDERMATT bulk and final product a = 1st bulk batch, b = 2nd bulk batch, c = 3rd bulk batch, d = 4th bulk batch, e = 5th bulk batch, f = 6th bulk batch, g = final product (**B**).

dimer. All samples show a band just above the open circular band in AGE. When compared to the supercoiled ladder the size of this band is approximately twice the size of the supercoiled plasmid, confirming that this is a dimer (33) (Fig 3). The endotoxin removal detergent is completely removed during chromatography, resulting in < 0.0025% (limit of detection) in the final eluate. Endotoxin level of the six batches is ≤ 2EU/mg pDERMATT (Table 1), well below the specification of <10 EU/mg.

Table 2: Composition of pDERMATT testbatches per ml of formulation solution. All formulations contain 2 mg/ml pDERMATT. Residual water content is given at t=0, just after manufacturing. The pDERMATT content (% of initial) and purity after 1 month of storage is given, with exception of formulation 1 and 4 as is indicated by asterixes. Mean values are given with the standard deviation within parenthesis.

No	Excipient	Amount (mg)	Water content (%) (t=0)	% of initial content		Purity (%)	
				25±2°C/ 60±5% RH	40±2°C/ 75±5% RH	25±2°C/ 60±5% RH	40±2°C/ 75±5% RH
1	Sucrose*	20	1.03 (0.3)	100.3 (2.3)	96.0 (1.4)	98.4 (0.6)	91.6 (0.5)
2	Trehalose	20	0.77 (0.07)	86.7 (1.0)	68.1 (1.6)	92.8 (0.5)	76.1 (1.2)
3	Mannitol	10	4.87 (0.3)	83.4 (1.9)	39.8 (1.8)	90.9 (0.3)	50.8 (1.6)
4	PVP**	20	0.89 (0.3)	63.5 (3.4)	17.6 (2.7)	67.5 (3.3)	26.7 (1.2)

* results after 6 months in the stability program

** results after 2 weeks in the stability program

Formulation development

The vials were filled with 2 mg pDERMATT and upon reconstitution with 400 µL an isotonic solution containing 5 mg/mL pDERMATT was achieved. Four different bulking agents were selected for the test batches; sucrose, trehalose and PVP (2%) as non-crystallising and mannitol (1%) as crystallising excipients. The initial pDERMATT SC content of the formulation solutions was within 95.0-105.0% with exception of the PVP formulation (90.7%). pDERMATT content and purity after one month of storage at 25±2°C/60±5% RH or 40±2°C/75±5% RH are given in Table 2. The supercoiled purities are normalised to the purity of the formulation solution. The residual water content of the mannitol formulation is relatively high. It is not clear what caused the rapid decrease in content and purity of pDERMATT in this formulation, either mannitol is not a suitable bulking agent, or the high water content or both. For the mannitol containing formulation the lyophilisation process needs to be optimised.

Even though pDERMATT content and purity decrease over time in all formulations, the formulation containing only sucrose is the most stable. Therefore development of this formulation was further pursued.

Table 3: Overview of in-process controls (IPC) and quality control (QC) results of pDERMATT 2 mg/vial final product with the derived specifications. Mean values are given with standard deviations within parentheses.

Test item	Specification	Result
IPC		
Concentration of pool (mg/ml)	1.0 – 1.1	1.039 (0.75)
Concentration formulation solution (mg/ml)	0.9 – 1.1	0.92 (1.44)
concentration after filtration (mg/ml)	0.95 – 1.05	1.001 (0.12)
Bioburden before filtration (cfu)	< 2.7 x 10^6 cfu/ml (retention capacity filter)	0
Filter integrity test, bubble point (psi)	≥ 1200	1576
Weight variation of filling volume (%)	≤ 3.0	0.31
QC		
Visual inspection	White, freeze-dried cake	Conforms
Reconstitution	Complete, leaving no visible residue as undissolved matter and resulting in a clear colourless solution	Conforms
HPLC analysis		
1. Identification	Rt reference standard = Rt final product	Conforms
2. Purity	≥ 90% supercoiled	95.7 (0.5)
UV analysis		
1. Content	90.0 – 110.0% of labelled content	99.6 (0.3)
2. Purity	$A_{260/280}$ = 1.80 – 1.95	1.8
AGE analysis		
1. Identification	3395 – 4149 bp	3773 bp
2. Residual *E.coli* host DNA	< 5% (< 0.05 mg/mg plasmid)	< 5%
3. Residual *E.coli* host RNA	≤ 4% (≤ 0.04 mg/mg plasmid)	< 4%
Uniformity of dosage units	Conforms Ph. Eur. <2.9.40> Acceptance value < 15%	Conforms 8.3%
Residual water content (%w/w)	≤ 2%	0.7 (0.07)
Endotoxin level (EU/vial)	< 10	Conforms
Sterility	Sterile	Conforms

Manufacturing process

An illustrative flowchart of the lyophilised plasmid DNA production process is shown in Fig 2C. First, the different pDERMATT bulk products were pooled and gently mixed, to ensure that the material for the phase I clinical trial originated all from the same batch. The pDERMATT concentration of the formulation solution before and after filtration and filling was shown to be equal, indicating no retention of pDERMATT in the filter or tubing. The QC results for pDERMATT 2 mg/vial final product with their specifications are presented in Table 3.

Immunogenicity

To test the immunogenicity of the produced pDERMATT DNA vaccine, HHD-transgenic mice were vaccinated with pDERMATT final product by intradermal DNA tattooing. Figure 5 shows the CTL response upon dermal vaccination. All vaccinated mice developed a strong MART-1 specific CTL response, with a peak level at day 12, similar to previous data (15). The control group did not develop a specific immune response.

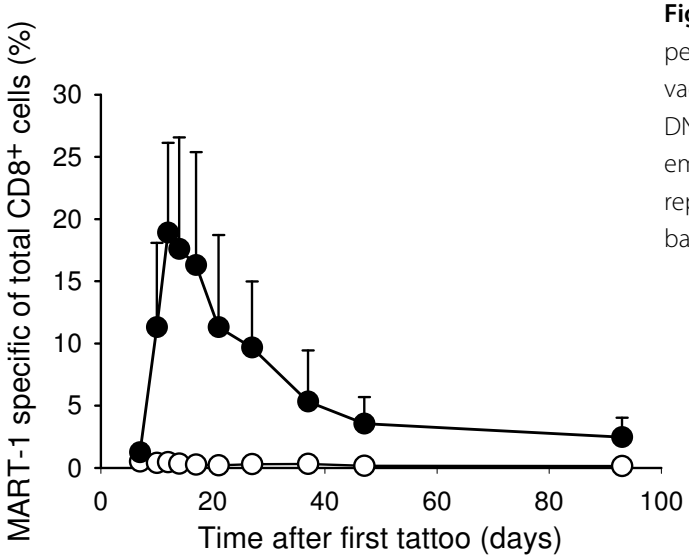

Figure 5: MART-1 specific $CD8^+$ T cell responses in peripheral blood lymphocytes in HHD mice upon vaccination with pDERMATT (n=7, filled circles) by DNA-tattooing. Control mice were vaccinated with empty pVAX vector (n=5, open circles). Values represent the Mean + Standard Deviation (error bars).

Stability upon storage

Stability of pDERMATT 2 mg/vial lyophilised product was evaluated. Samples were taken at different points in time. Stability studies were initiated at -20°C, +5±3°C and the accelerated storage condition of +25±2°C/60±5% RH (climate chamber HEKK 0057, Weiss Technik Ltd.) in the dark. Table 4 shows the shelf life results obtained thus far for the stability studies. Although the residual water content increases during storage, content and purity of all samples still comply to the specifications (content 90-110% of labelled content and purity ≥90% supercoiled). To date, pDERMATT 2 mg/vial final product proved stable for 6 months at all storage conditions.

Table 4: Stability of pDERMATT 2 mg/vial final product Lot 112106SQ1. Mean values are given with standard deviations within parentheses. All test items were analysed in triplicate.

Test item	Initial	3 months		6 months		
		5±3°C, dark	25±2°C/ 60±5% RH, dark	-20°C, dark	5±3°C, dark	25±2°C/ 60±5% RH, dark
Appearance	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
Reconstitution	Conforms	Conforms	Conforms	Conforms	Conforms	Conforms
HPLC analysis, purity (% supercoiled)	95.7 (0.5)	98.5 (0.08)	98.3 (0.09)	97.2 (0.3)	96.2 (0.4)	95.1 (1.1)
UV analysis, Content (%)	99.6 (0.3)	98.0 (0.2)	98.3 (0.2)	97.5 (0.4)	100.8 (4.7)	97.5 (0.0)
Residual water content (%w/w)	0.7 (0.07)	n.d.	n.d.	1.27 (0.05)	1.96 (0.2)	2.41 (0.2)

RH= relative humidity, n.d.= not determined.

Discussion

We chose not to make a Working Cell Bank (WCB) because a MCB batch consisting of 250 vials was deemed sufficient for the planned experiments. If more pDERMATT would be required for subsequent clinical studies, a WCB can readily be made from the existing MCB.

For fermentation a minimal medium (Luria Miller Broth), supplying only minimum nutritional requirements, was used. Glucose was added to the medium to provide an extra carbon source and extra energy for achieving a higher cell density (46). Since fermentation was performed in the batch mode, growth of bacteria was limited by the nutrition supply in the growth medium. Following fermentation bacterial cells can be harvested from the fermentor by using either centrifugation or filtration (47). In our process we used TFF for harvesting, since it gave the possibility of concentrating the cell paste and subsequently exchanging the culture medium for buffer P1, thus avoiding an additional resuspension step before lysis. Usually RNase A is added to this first buffer to ensure complete RNA removal, however the use of animal derived enzymes is not preferred for the production of clinical grade plasmid DNA (22,23,48). The suspension was harvested from the membrane and equally divided into three lysis bottles. To minimise loss of bacterial cells in the membrane, the membrane was subsequently rinsed with one litre of fresh buffer P1. Each bottle contained approximately 100g bacterial wet cell paste to ensure efficient lysis of the bacterial cells. The high salt neutralisation solution promotes the formation of large, flock-like particles of gDNA, host cell protein, and cell debris (49), some of which float while others precipitate (47). By applying a small vacuum (appr 500 mbar) to the bottle even the precipitated flocks start to float (33,50). With this separation technique air escapes from the solution under vacuum to encourage the formation of a compact bed of flocculent material. The vacuum clarification only takes 15 minutes, and pre-clears the lysate sufficiently to allow filtration using a standard, commercially available capsule filter (51).

Plasmid DNA can be purified with many different techniques (40,52), and a number of purification procedures are on the market (e.g. QIAGEN, PlasmidSelect Xtra from GE-Healthcare and Mustang Q from Pall). We selected the QIAGEN purification resin as it is a single step method (43) and approved for production of clinical grade DNA since 1996 (32). For every purification run a fresh column was prepared, since the resin cannot be sanitised with standard agents (e.g. sodium hydroxide) (51). The BPG column is easy to clean and has sanitary end fittings, all according to GMP. Instead of using the standard Qiagen endotoxin removal (ER) buffer we used a different triton-X based buffer. The advantage of this ER buffer is that endotoxin removal does not require incubation of the solution on ice, and therefore it is time saving. Furthermore cooling on ice of the cleared lysate during loading onto the column is not necessary.

The conductivity and pH of the equilibration buffer and the lysate are equal and of such a value that plasmid DNA will bind to the column material (53). Because we are using an RNase free process not only plasmid DNA but also *E.coli* host RNA will bind to the column, since both are polyanionic molecules (22). Fortunately RNA is efficiently removed as can be seen in Table 1 and figure 3. The QIAGEN resin has an exceptionally high charge density and plasmid DNA remains tightly bound to the DEAE groups over a wide range of salt concentrations. The broad separation range of the resin makes efficient separation of plasmid DNA from RNA possible (54). pH of the buffer is also important for efficient separation of RNA from DNA on DEAE resins (22). For the wash steps pH and salt concentration are chosen such that only RNA elutes from the column, while DNA stays bound to the resin. Following purification plasmid DNA is usually precipitated using ethanol or isopropanol and then resuspended in the desired volume (54). However the use of potential toxic organic solvents during manufacture of plasmid DNA intended for human applications is not preferred (22,23,55). TFF in combination with lyophilisation is used as alternative methods to concentrate the purified DNA. With the second TFF step the solution is concentrated to approximately 1 mg/mL pDERMATT, and elution buffer is exchanged for Wfl. During this buffer exchange step the $A_{260/280}$ ratio of the solution decreases. The pH of Wfl is slightly acidic compared to the elution buffer, which has a pH of 7.2. It is known that pH and ionic strength of the spectrophotometric solution significantly alters the $A_{260/280}$ ratio of nucleic acids, because the degree of ionisation of the bases is strongly pH-dependent (56). Specifications for $A_{260/280}$ are set using a slightly basic solution, therefore the $A_{260/280}$ ratio returns to a value within specifications when using TE-buffer (pH 8.3).

The final amount of DNA (134.1 – 214.1 mg) cannot be correlated to the OD_{600} (6.54 – 7.52) see Table 1. It is known that during cell division plasmid DNA, of high copy number plasmids like pVAX, is distributed randomly between the new born cells. Therefore plasmid copy number may vary among individual dividing cells in the population (57). Furthermore, fermentations can be characterised into two distinct phases. First, a phase of rapid growth occurring from inoculation, followed by a phase characterised by a reduced growth rate to

the end of the cultivation cycle. It is during the second phase that most of the plasmid production is achieved (58). Possibly some of the batches were in the second phase of fermentation for a prolonged period, thus generating more plasmid DNA.
For the scheduled phase I clinical trial a plasmid DNA solution of 5 mg/mL was required. Since the pDERMATT bulk material could only be concentrated to approximately 1 mg/mL, lyophilisation was chosen for further concentration (59). A second advantage of lyophilisation is that the product has a longer shelf life than when kept in solution (60). During storage of plasmid DNA in solution at 2-8°C a conversion of SC DNA towards OC DNA was observed within a week. Furthermore, after prolonged storage the OC DNA was converted into linear species, as was described previously (60). This was prevented by storage at -20°C, however repeated freeze thaw cycles did also affect the topology.
The four selected bulking agents in the formulation development phase are well known for protein and non-viral DNA vector stabilisation during lyophilisation (61-63). Since naked DNA is more stable than proteins or non-viral DNA vectors we assumed that the selected bulking agents would also be able to stabilise pDERMATT during lyophilisation. The lyophilisation program for the mannitol formulation contained an annealing step in order to completely crystallise the mannitol. Amorphous mannitol may crystallise during transport or storage, releasing the sorbed water. This water may then be available for interaction with the other formulation ingredients including the active pharmaceutical ingredient (API) (64).
For the final lyophilised product, containing sucrose as bulking agent, a less tight specification limit (90-110% in stead of 95-105%) concerning content is applied to prevent unnecessary batch rejection, since the product is an investigational agent (27). As additional batches will be manufactured, tightening of the specification will be considered on the basis of the resulting risk of batch failure that is estimated from the quality control results obtained (process mean, process standard deviation) (27,65).
Immunogenecity was tested using HHD-transgenic mice, which express a chimeric HLA-A2.1 heavy chain (α1 and α2 human, α3 mouse) linked to a human β_2-microglobulin with a double knock-out of mice H-2K^{b}D^b. These mice are a suitable model to test the immunogenicity of pDERMATT as the encoding MART-1 epitope is HLA-A2.1 restricted (66). Figure 5 shows that pDERMATT is a very potent DNA vaccine in this animal model. Vaccination with the lyophilised product resulted in a rapid and strong MART-1 specific CTL response against the MART-1 epitope in all vaccinated animals.

In conclusion the production process used at our facility is capable of producing pharmaceutical grade pDERMATT, under GMP conditions, at a relatively large scale. The process is generic and will be optimised in the future to obtain even higher yields. Quality control of the bulk product revealed a high purity of the plasmid even though an RNase free process is used. Furthermore the product is chemically more pure since the use of organic solvents is avoided.

A sterile and stable, injectable final product for pDERMATT has been developed. Lyophilisation of 1 mg/mL pDERMATT with sucrose as bulking agent in a concentration of 2% (w/v) resulted in a stable product. Furthermore upon reconstitution with a five times smaller volume an isotonic product with a DNA concentration of 5 mg/mL was obtained. This product showed immunogenicity and a strong MART-1 specific CTL response in a murine model. The lyophilised pDERMATT 2 mg/vial final product will soon be used in Phase I clinical trials.

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Chapter 4

Lipopolysaccharide contamination in intradermal DNA vaccination: Toxic impurity or adjuvant?

Joost H. van den Berg*
Susanne G. L. Quaak*
Jos H. Beijnen
Wim E. Hennink
Gert Storm
Ton N. Schumacher
John B. A. G. Haanen
Bastiaan Nuijen

* contributed equally

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Abstract

Purpose: Lipopolysaccharides (LPS) are known both as potential adjuvants for vaccines and as toxic impurity in pharmaceutical preparations. The aim of this study was to assess the role of LPS in intradermal DNA vaccination administered by DNA tattooing.

Method: Mice were vaccinated with a model DNA vaccine (Luc-NP) with an increasing content of residual LPS. The effect of LPS on systemic toxicity, antigen expression and cellular immunity was studied.

Results: The presence of LPS in the DNA vaccine neither induced systemic toxicity (as reflected by IL-6 concentration in serum), nor influenced antigen expression (measured by intravital imaging). Higher LPS contents however, appeared to be associated with an elevated cytotoxic T lymphocyte (CTL) response but without reaching statistical significance. Interestingly, the DNA tattoo procedure by itself was shown to induce a serum cytokine response that was at least as potent as that induced by parenteral LPS administration.

Conclusion: LPS does not show toxicity in mice vaccinated by DNA tattooing at dose levels well above those encountered in GMP-grade DNA preparations. Thus, residual LPS levels in the pharmaceutical range are not expected to adversely affect clinical outcome of vaccination trials and may in fact have some beneficial adjuvant effect. The observed pro-inflammatory effects of DNA tattoo may help explain the high immunogenicity of this procedure.

Introduction

Endotoxins, which are lipopolysaccharides (LPS) originating from gram-negative micro-organisms, are a common impurity in plasmid DNA (pDNA) preparations. This is inherently due to the fact that *E.Coli*, a gram-negative bacterium, is used for amplification in the manufacturing process of the plasmid (1,2). In the down-stream processing of pDNA, *E.Coli* cells are lysed, leading to high LPS levels originating from the outer cell membrane. LPS can be efficiently removed by different endotoxin removal steps and current protocols are capable of bringing LPS levels in pDNA preparations down to 0.01 - 100 International Units (IU)/mg, depending on the purification strategy applied (3-8).

Residual LPS in pDNA drug products can be potentially toxic, as parenteral injection of LPS can result in a septic shock like syndrome (9,10). Therefore, a limit on maximal endotoxin dose for injectable dosage forms has been established (<5 IU per kilogram of body mass per hour (11,12).

Because of its immunostimulatory properties, LPS is also seen as a potential adjuvant for vaccines (13). LPS can activate and mature dendritic cells (DCs) *in vitro* (14) and *in vivo* (15) by triggering Toll-like receptor (TLR) 4 (16,17). DCs play an important role in vaccination, as they serve as professional antigen presenting cells (APCs), necessary for antigen presentation and the initiation of adaptive immunity (18). To benefit from the adjuvant properties of LPS without encountering systemic toxicity, a detoxified form of Lipid A derived from LPS (MonoPhosphoryl Lipid A; MPLA) has been developed, and is already successfully used as an adjuvant in vaccine formulations (19,20).

Our institute recently described a novel method for intradermal DNA vaccination, called DNA tattooing. This vaccination strategy is able to induce fast cellular immune responses upon immunization with pDNA in mice and outperforms classical intradermal DNA vaccination by 10-100 fold in non-human primates (21,22). Since DNA tattoo vaccination relies on the use of a needle array for administration, we consider this technique as a parenteral administration route LPS levels in vaccine batches produced for clinical administration should be limited (7). In order to unravel the role of LPS in DNA vaccines applied by tattooing, the first aim of this study was to assess the toxicity of LPS when using this delivery technique. The second aim was to study the influence of LPS on the magnitude of the antigen-specific T cell responses induced by DNA tattoo vaccination.

Together, these results should provide information on the role of LPS during pre-clinical and clinical intradermal DNA tattoo vaccination, both from a pharmaceutical as well as from an adjuvant perspective.

Materials and methods

Mice

C57BL/6J mice (2-4 months) were obtained from the experimental animal department of The Netherlands Cancer Institute. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Institute's Animal Research Committee.

DNA vaccines

To measure *in vivo* antigen expression and cellular immune responses, a model plasmid DNA, pVAX:Luc-NP (21), was used which encodes the influenza A $NP_{366-374}$ epitope fused to the carboxy terminus of firefly luciferase inserted into the EcoRI/NotI sites of the pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). Plasmid DNA was expressed and amplified in *E. Coli* DH5. DNA was purified using either the QIAGEN Mega-kit or the Endofree™ QIAGEN Mega-kit (QIAGEN, Hilden, Germany) which resulted in plasmid preparations containing 5000IU/mg and 2IU/mg pDNA respectively as determined in duplicate by a Pyrochrome® limulus amoebocyte lysate assay (Cape Cod Associates, Cape Cod, MA, USA). Both preparations were mixed in different ratios to obtain the required range of LPS concentrations.

DNA immunization & Control groups

For intradermal DNA vaccination, 10 µl of a 2 mg/ml DNA solution in Water for Injection (Wfl) was applied to the shaved left hind leg of the mice and administered using a disposable 9-needle cartridge (MT Derm, Berlin, Germany) mounted on an Aella® tattoo machine for medical use (MT Derm). DNA vaccines were tattooed during 30 s at a needle depth of 1.0 mm, with an oscillation frequency of 100 Hz. Mice were vaccinated using a standard schedule on day 0, 3 and 6 (21) with DNA solutions containing increasing amounts of LPS (2, 100, 500 and 5000 IU/mg DNA), corresponding to an absolute amount of 0.04, 2, 10 and 100 IU LPS per dose of 20 µg plasmid.

As a positive control for IL-6 release, mice were injected intraperitoneally with 10 ng (100 IU) LPS from *E.Coli* 0113:H10 (Cape Cod Associates, Cape Cod, MA, USA) diluted in 200 µl of Phosphate Buffered Saline (PBS).

As negative controls, mice were tattooed with Wfl or were sham-treated. Sham-treated mice were shaved and anesthetized, but the mice did not receive any tattoo or injection. Table 1 shows the different groups used in this study. All groups were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA) during treatment. At the indicated time points post-

immunization, approximately 50 µl of peripheral blood was collected by tail bleeding for the measurement of serum IL-6 levels, and antigen-specific T cell responses.

Table 1: Group categorization.

Group	Compound	Administration route	Amount LPS	Number of animals
1	Luc-NP DNA vaccine 20µg	Tattoo	2 IU/mg DNA (0.04 IU per dose)	6
2	Luc-NP DNA vaccine 20µg	Tattoo	100 IU/mg DNA (2 IU per dose)	6
3	Luc-NP DNA vaccine 20µg	Tattoo	500 IU/mg DNA (10 IU per dose)	6
4	Luc-NP DNA vaccine 20µg	Tattoo	5000 IU/mg DNA (100 IU per dose)	6
Wfl	Wfl 10µl	Tattoo	<0.01 IU/ml* (<2 x10^{-4} IU per dose)	4
i.p. (pos control)	Purified LPS	i.p. injection	100 IU per dose	4
Sham (neg control)	-	-	-	4

** according Certificate of Analysis*
LPS= lipopolysaccharide, IU= International Units, Wfl= Water for Injections, i.p= intraperitoneal

IL-6 ELISA

The concentration of the pro-inflammatory cytokine IL-6 in serum was measured using an IL-6 ELISA kit (R&D systems, Minneapolis, MN USA). Serum samples were diluted 1:10 with PBS in order to keep the results within the linear range of the assay, as determined by the calibration curve. The sensitivity of the assay was 1.6 pg/ml according to the calibration curve.

Antigen expression using intravital imaging

To monitor antigen expression upon DNA vaccination, a light-sensitive camera was used for longitudinal measurement of *in vivo* firefly luciferase activity. Mice were anesthetized with isoflurane. A solution of the firefly luciferase substrate luciferin in PBS (150 mg/kg, Xenogen, Hopkinton, USA) was injected intraperitoneally and 18 min later the luminescence produced by active luciferase was acquired during 30 s in an IVIS® system 100 CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Cytotoxic T cell assay

To measure Cytotoxic T Lymphocyte (CTL) responses, peripheral blood lymphocytes were stained with PE-conjugated H-2D^b/NP$_{366–374}$-tetramers and APC-conjugated CD8α antibody (BD Pharmingen, San Jose, USA) at 20°C for 15 min in FACS buffer (1× PBS, 0.5 % BSA and 0.02 % sodium azide) as described previously (23). Subsequently, cells were washed three

times in FACS buffer and analyzed by flow cytometry. Living cells were selected based on propidium iodide exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) using Summit analysis software (Dako, Glostrup, Denmark).

Statistical analysis

Data were analyzed using a ANOVA and Tukey's test and P values <0.05 (tested two-sided) were considered statistically significant.

Results and Discussion

The aim of the present study was to investigate the role of LPS in genetic vaccination by DNA tattoo administration. Since LPS is a potent immunostimulator (9,10) the effect of LPS on toxicity, antigen expression and cellular immunity was investigated. For this purpose, mice were immunized with a construct encoding the fusion protein of luciferase and a MHC class I restricted influenza A NP epitope (Luc-NP), to allow the simultaneous measurement of antigen expression and vaccination-induced T cell responses. DNA vaccines were prepared with increasing amounts of residual LPS, ranging between 2 IU/mg (highly purified) and 5000 IU/mg (unpurified product). 100 IU/mg was chosen as the pharmaceutical upper level and 500 IU/mg as intermediate LPS content. The pharmaceutical upper level was set based on an average patient weight of 70 kg. A patient of that weight is allowed to receive a maximum of 70 kg * 5 IU/kg/h = 350 IU LPS per hour. As all DNA vaccine doses are normally administered within an hour, an LPS content of 100 IU/mg pDNA allows a maximum of 3.5 mg pDNA per dose. This maximum injectable dose of 3.5 mg pDNA is sufficient to cover current intradermal clinical DNA vaccination protocols (24-28).

Effect of LPS on serum IL-6

To determine the systemic toxicity of LPS, serum IL-6 levels of the treated animals were measured using an IL-6 ELISA, the first 24 hours after administration. It is known that systemic administration of LPS results in the production of pro-inflammatory cytokines, such as IL-6, by activated APCs. Furthermore, these cytokines are important mediators in the fever-like response upon LPS administration (10,29) and therefore a good predictor for toxicity.

Mice receiving an intraperitoneal injection of 100 IU LPS (positive control) showed a peak in IL-6 concentration 2 hours after injection, similar in kinetics and magnitude as reported previously (Fig 1) (10,30). The sham-treated group (negative control) showed a small rise in serum IL-6 levels on the day of treatment, reflecting the effect of handling stress, including anaesthesia, shaving and tail bleedings (31).

The control group which received a tattoo with Wfl showed a strong increase in IL-6 serum levels, with a peak 7 hours after administration, presumably caused by damage of the skin by tattooing. It is possible that the measured IL-6 is released by damaged keratinocytes, since it is known that these cells can release IL-6 upon stress exposure (32). All mice receiving DNA tattoo vaccines containing increasing amounts of LPS showed the same, tattoo-associated, serum IL-6 peak, 7 hours after administration. Importantly, tattoo administration showed no differences in both kinetics and magnitude of IL-6 serum level between the different amounts of LPS. Moreover, none of the mice showed any physical signs of illness. This suggests that even the highest concentration of LPS is not associated with an increase in systemic toxicity for this intradermal route of genetic vaccination.

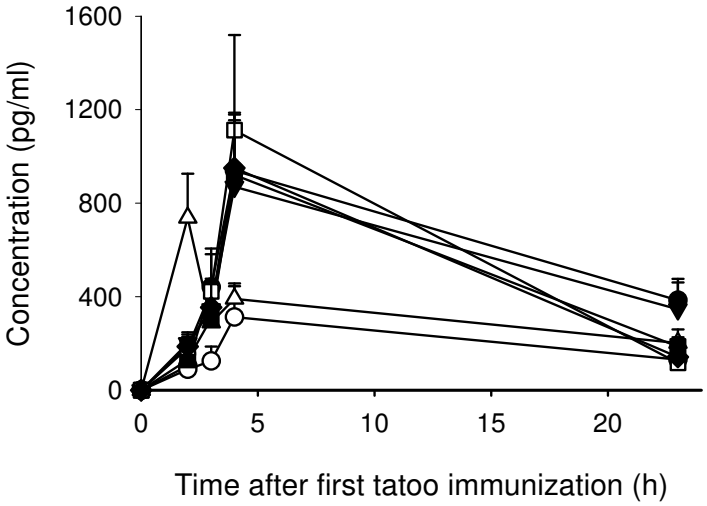

Figure 1: IL-6 response in serum after DNA tattoo immunization. Cohorts of mice (n=6) were immunized by DNA tattooing with the Luc-NP vaccine batches containing an increasing amounts of LPS (2 IU ● , 100 IU ■, 500IU ▼ or 5000 IU ♦ per mg pDNA). As controls, cohorts of mice (n=4) received a tattoo with Wfl (□), 100 IU purified LPS intraperitoneally (Δ) or were sham-treated (○).Values represent the mean + standard error of the mean (error bar) for each treatment group.

These observations showed that high levels of LPS in dermal vaccines applied by DNA tattooing does not result in additional release of IL-6 above the release already provoked by the tattooing technique itself. It is important to emphasize that humans are 250 times more sensitive to a systemic LPS challenge than mice, based on acute inflammatory response (10). However, since patients will be vaccinated with purified pDNA with LPS levels ≤2 IU/mg, inflammatory reactions provoked by this concentration of residual LPS are unexpected, since mice vaccinated with 250 to 2500 fold higher levels (500 and 5000 IU/mg) did not show any additional IL-6 release in this experiment either.

Effect of LPS on antigen expression

To study the effect of LPS on *in vivo* antigen expression, luciferase expression was measured with a light sensitive CCD camera on day 1 and 3 after DNA tattooing with the Luc-NP construct. All groups showed a similar luciferase expression profile upon vaccination (Figure 2) with no significant effect of administered LPS concentration on luciferase expression.

In literature, the presence of LPS is associated with a decrease in transfection efficiency of pDNA in eukaryotic cells in vitro (33,34) and *in vivo* (35). In addition, it is known that very high LPS concentrations (26.000 IU/mg pDNA) can inhibit DNA vaccination-induced gene expression after intratumoral injection in mice (35). However, at the LPS levels used in this study, no substantial effect of LPS on intradermal antigen expression was observed.

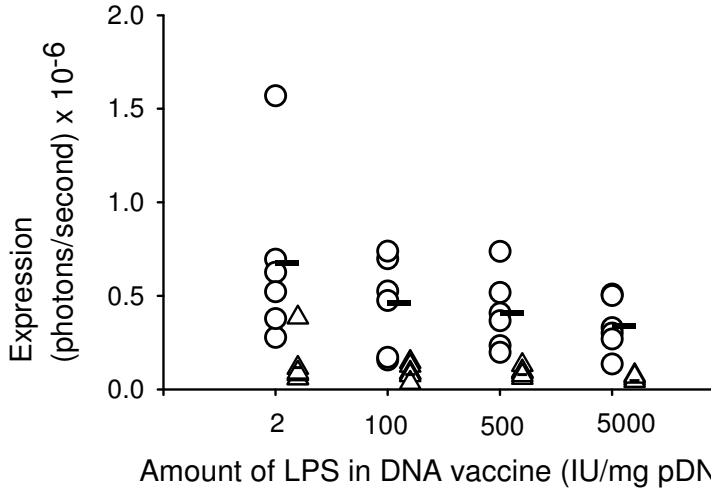

Figure 2: Effect of LPS content on *in vivo* antigen expression level after DNA tattoo immunization, as determined by a light-sensitive camera on day 1 (○) and 3 (Δ). Antigen expression levels of individual mice are plotted together with group averages (—).

Effect of LPS on CTL response

The effect of LPS on cellular immunity was assessed by determining the percentage of $NP_{366–374}$ specific $CD8^+$ T cells in peripheral blood at the peak of the DNA vaccine-induced T cell responses, 13 days after start of vaccination (21). All mice showed a strong epitope specific T cell response upon DNA tattooing (Figure 3). Mice receiving vaccinations with a high content of LPS (500 or 5000 IU per mg pDNA) showed a higher, but also a more variable CTL response. ANOVA analysis showed only a significant relationship ($p<0.05$) between de groups receiving 2 IU and 500 IU per mg.

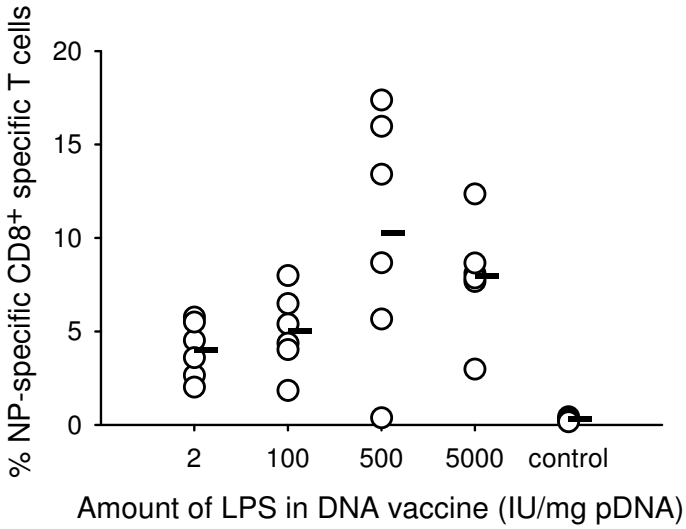

Figure 3: $NP_{366-374}$ specific T cell responses in cohorts of mice (n = 6) upon tattoo vaccination on day 0, 3 and 6 with Luc-NP vaccine batches containing an increasing amount of LPS per dose of pDNA. Control mice were tattooed with Wfl (n=4). NP_{366}-specific T cell responses were determined on day 13 by MHC tetramer staining of peripheral blood lymphocytes. The percentage of NP_{366}-reactive $CD8^+$ T cells of total $CD8^+$ T cells for individual mice is plotted (○) together with the group averages (—).

LPS is widely studied as an adjuvant for DNA vaccination. It has previously been shown that LPS is able to increase IgG responses upon intradermal injection with a DNA vaccine, without affecting CTL responses (36). Previous experiments with intradermal gene-gun DNA vaccination did not show a relationship between CTL responses and LPS level either (13). In that study, LPS levels up to 56.000 IU/mg pDNA were tested. Our results show that high concentrations of LPS in pDNA vaccines for tattoo-administration might act as an adjuvant, associated with a higher CTL response, but lacks statistical power to validate this finding. Importantly, LPS variations within the pharmaceutical spectrum (between 2 and 100 IU/mg pDNA) do not have any beneficial or toxic effects when using dermal tattoo administration. This means that batch to batch variations in LPS level within pharmaceutical accepted ranges in pDNA products will probably not influence the outcome of clinical trials.

We have previously established procedures for the large scale production of clinical grade pDNA under GMP conditions (7). By means of an endotoxin removal step, the endotoxin level of the pDNA produced is reduced to ≤2 IU/mg. In a recently started clinical trial pDNA dose will be escalated from 0.5 mg to 8 mg, corresponding to administration of a maximum of 1 IU to 16 IU LPS per dose. Based on the data presented here, this amount of LPS will, most probably, not affect toxicity, expression or immunogenicity of the administered pDNA.
A remarkable observation in this study was that the tattooing procedure itself resulted in a systemic IL-6 release that reached higher concentrations than an intraperitoneal injection with LPS. Possibly, this IL-6 release is an explanation for the high immunogenicity of DNA vaccines administered by tattooing.

In conclusion, in this specific route of pDNA vaccine administration, LPS serves as a non toxic contaminant. However, LPS concentrations above pharmaceutical limits have the potential to act as adjuvant. In addition, the results of the present study might also be extrapolated to other dermal DNA vaccination techniques, such as jet injector, gene gun, microneedles or electroporation.

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Chapter 5

Optimization of intradermal vaccination by DNA tattooing in human skin

Joost H. van den Berg
Bastiaan Nuijen
Jos H. Beijnen
Andrew Vincent
Harm van Tinteren
Jörn Kluge
Leonie A. E. Woerdeman
Wim E. Hennink
Gert Storm
Ton N. Schumacher
John B. A. G. Haanen

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Abstract

The intradermal administration of DNA vaccines by tattooing is a promising delivery technique for genetic immunization, with proven high immunogenicity in mice and in non-human primates. However, the parameters that result in optimal expression of DNA vaccines that are applied by this strategy to human skin are currently unknown. To address this issue we set up an *ex vivo* human skin model in which DNA vaccine-induced expression of reporter proteins could be monitored longitudinally.

Using this model we demonstrate the following: First, the vast majority of cells that express DNA vaccine-encoded antigen in human skin is formed by epidermal keratinocytes, with only a small fraction (~1%) of antigen-positive epidermal Langerhans cells. Second, using full randomization of DNA tattoo variables we show that an increase in DNA concentration, needle depth and tattoo time all significantly increase antigen expression ($p<0.001$), with DNA concentration forming the most critical variable influencing the level of antigen expression.

Finally, in spite of the marked immunogenicity of this vaccination method in animal models, transfection efficiency of the technique is shown to be extremely low, estimated at approximately 2-2000 out of $1x10^{10}$ copies of plasmid applied. This finding, coupled to the observed dependency of antigen expression on DNA concentration, suggests that the development of strategies that can enhance *in vivo* transfection efficacy will be highly valuable.

Collectively, this study shows that an *ex vivo* human skin model can be used to determine the factors that control vaccine-induced antigen expression and define the optimal parameters for the evaluation of DNA tattoo or other dermal delivery techniques in phase I clinical trials.

Introduction

In the past years, skin has become increasingly used as a successful delivery route for DNA vaccines (1). The excellent immunogenicity of dermal DNA vaccination is probably related to the high prevalence of Antigen Presenting Cells (APCs) in the skin, in the form of Langerhans Cells (LC) in the epidermis and dendritic cells in the dermis (2,3). Several techniques have been developed for the intradermal administration and cellular uptake of naked DNA, such as gene gun and particle injection systems (1,4,5), jet injectors (1,6), electroporators (7-10) and a technique that was recently developed by our institute that has been phrased DNA tattooing (11,12). DNA tattooing delivers naked plasmid DNA into the skin through thousands of punctures using a multiple needle tattoo device. We have demonstrated that DNA tattooing results in local transfection and expression of the encoded antigen by cells in murine skin. More importantly, the efficacy of DNA tattooing in inducing strong vaccine-specific immune responses has been established in murine models (11) and the superiority of DNA tattooing over intramuscular DNA vaccination has recently been demonstrated in non-human primates (13).

A major difficulty when translating novel dermal delivery techniques, such as DNA tattoo towards clinical application is that they have been developed and optimized in non-human skin. Since mice and macaque skin have a higher density of hair follicles than humans and a different thickness when compared to humans (14), direct translation of vaccination protocols to clinical application is difficult. To prepare for the utilization of DNA tattoo vaccination in a phase I clinical trial, we have therefore developed an *ex vivo* human skin model that allows one to measure vaccine-induced gene expression real-time. Having established this *ex vivo* human skin model, we have utilized the model to define optimized conditions for DNA tattoo vaccination of human skin.

Materials and methods

Plasmids

DNA vaccines were generated by the insertion of reporter genes in the minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). pVAX:Luc was generated by insertion of the gene encoding firefly luciferase in the EcoRI/NotI site of pVAX1. pVAX:GFP was generated by inserting Green Fluorescence Protein (GFP) encoding DNA into the BamHI/NotI site of pVAX1. pVAX:LacZ, encoding ß-galactosidase was purchased from Invitrogen. Histone- 2B-GFP (H2B-GFP) fusion protein encoding DNA cloned in the pN1 vector (Clontech) was described previously (15). pVAX: Luc was produced in one large batch for all experiments with a uniform process as described before(16). All other plasmids were purified using Endofree Plasmid kit (Qiagen, Hilden, Germany). All plasmids were dissolved in Water for

injections (Braun, Melsungen, Germany). The purity and concentration of plasmid DNA was assessed by agarose gel electrophoresis and UV-spectroscopy, respectively.

Tattooing and injection of human skin

Healthy human abdominal skin from female patients (41-63 years) was obtained from the plastic surgery department, according to institutional guidelines. Subcutaneous fat was directly removed by blunt dissection. Skin was transported on ice and used within 2 hours after surgical removal.

Before DNA tattooing, skin was cleaned with sterile PBS and pinched onto a polypropylene board with drawing pins. Next, a black marker was used to apply a chess board pattern on the skin to define the different areas for tattooing. DNA tattooing was performed by application of 10µl of DNA solution onto the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8mm, surface 50mm^2). The droplet of DNA was subsequently administered into skin using an Aella® or Cheyenne® tattoo machine (both machines and needles, MT Derm GmbH, Berlin, Germany). For all tattoos, 9-needle cartridges and an oscillating frequency of 100Hz were used. During the experiments, the tattoo depth, tattoo duration, DNA concentration and the two tattoo machines were varied using a randomization protocol (see Table 1). Needle amplitude was adjustable with an accuracy of 0.1mm using a custom built device that contained a stroboscope and microscope (MT Derm GmbH). For intradermal injections, 50µl of DNA solution at a concentration of 1 mg/ml was injected intra epidermally with a 29g 12mm needle in a side by side comparison with a tattoo of 10µl 1 mg/ml DNA solution at 1.5mm needle depth for 20 second with the Aella® machine (n=3, performed two times in separate experiments).

After tattooing or injection, skin samples were kept at 5% CO2, 37ºC in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25µg/ml amphotericin B (all Invitrogen). During this incubation, skin was cultured at the air-medium interface with the epidermis exposed to the air to mimic the natural situation.

For histology and flow cytometry experiments tattooed areas of interest were removed from the intact skin with a 6mm biopsy punch and transferred into 48-well plates.

Table 1: Fixed effects (tattoo variations) tested in linear effect model skin tattooing.

Effect	**Parameters**		
DNA concentration (mg/ml)	0.2	1	5
Tattoo duration (seconds)	5	10	20
Tattoo depth (mm)	0.5	1.0	1.5
Tattoo machine	Aella®	Cheyenne®	

Histology

Four-micrometer cryostat cross-sections of skin tattooed with ß-galactosidase encoding plasmid (pVAX:LacZ) were prepared. Cryostat sections were fixed in acetone for 10 min and washed for 10 min in PBS. Sections were stained for 10 min with X-gal staining solution (Roche Applied Science, Indianapolis, USA) to visualize ß-galactosidase expression. Subsequently, sections were stained with haematoxylin and eosin according to standard procedures.

Flow cytometric analysis of DNA vaccine induced antigen expression

Directly after tattooing with pVAX:GFP, skin samples were incubated for 1 hour in 10 mg/ml dispase II (Sigma Aldrich, St. Louis, MO, USA) in keratinocyte medium at 37ºC, upon which the epidermis was mechanically peeled from skin samples. The obtained epidermal sheet and dermis were cultured overnight in complete keratinocyte medium to allow accumulation of vaccination induced GFP expression. 18 hours later, epidermal sheets were digested at 37ºC in complete keratinocyte medium containing 0.05% trypsin and 300U/ml DNaseI (Roche). After 15 min, the epidermis was disrupted with a glass pipette and 10% FCS was added to the medium. Dermal samples were digested in 50 mg/ml collagenase type IV (Sigma) at 37ºC for 3 hours, after which the cells were filtered through 70µm nylon gauze to remove debris. Filtered epidermal cell suspensions and dermal cell suspensions were washed with PBA (1x PBS, 0.5% BSA and 0.02% sodium azide) prior to antibody staining. The antibodies used were mouse anti-human CD1a phycoerythrin (PE) (Immunotech, Prague, Czech Republic), mouse anti-human CD1a allophycocyanin (APC) (Immunotech) and mouse anti human cytokeratin (equal mixture of clone LP34 and MNF116 (both Dako, Glostrup Denmark)), labelled with Alexa Fluor 647 (Invitrogen) according to manufacturer's protocol. Prior to cytokeratin staining, epidermal cell suspensions were permeabilized using the BD Cytofix/Cytoperm kit (BD Sciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. Cell suspensions were analyzed and sorted using a FACSCalibur or a FACSAria (BD Sciences) and Summit analysis software (Dako). In case of anti CD1a staining, live cells were selected based on propidium iodide exclusion.

Confocal laser scanning microscopy

Skin samples were tattooed with a H2B-GFP encoding construct as described above. The next day, epidermal cell suspensions were prepared and GFP positive epidermal keratinocytes and Langerhans cells were isolated by fluorescence-activated cell sorting on the basis of CD1a expression and subsequently analyzed by confocal laser scanning microscopy (Leica SP2) in PBA.

Calculation of transfection efficiency

The transfection efficiency upon DNA tattooing was calculated by determining the number of GFP expressing cells per tattoo area using flow cytometry. The amount of administered molecules of plasmid DNA was calculated using the molecular weight of the construct ($2.26x10^3$ kD, 3724 base pairs) and the used dose per tattoo. Transfection efficiency was calculated by:

*(cells transfected *estimated number of plasmid molecules per transfected cell /plasmid molecules administered by tattooing)*100%.* [1]

Imaging of luciferase expression

The expression of luciferase was measured in intact skin samples at 3, 18, 24, 48 and 72 hours after tattooing. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/ml. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 minutes after the addition of luciferin, luminescence produced by active luciferase was acquired during 30 s with an IVIS system 100 CCD camera (Xenogen, Hopkinton, USA).
Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. During each measurement, background luminescence was measured to allow correction during data analysis. After each measurement, medium was refreshed to remove residual luciferin.

Linear mixed effects model

To study the effect of different tattoo parameters on the level of antigen (i.e. luciferase) expression, the natural log transform of the area under the curve (AUC) over the 72 hour period was analyzed. To account for possible within skin correlation of the repeated measurements of antigen expression (at different parameter levels), a linear mixed effects model was constructed. Fixed effects included the four variables of primary interest: the DNA concentration, the duration of tattooing, the depth of tattoo, and the type of tattoo machine (see Table 1). Patient age, background luminescence (measured in non-tattooed regions), the location of the tattoo in the piece of skin (edge vs. centre) and the time from surgery to tattooing were also included as fixed effects to adjust for any possible confounding influences. Patient identifier was implemented as the random grouping variable. Pair-wise interactions were examined between all fixed effects. Backward stepwise selection was performed, removing terms at the 0.05 significance level. Conventional residual analysis was performed to assess model fit. Data analysis was performed with Splus v6.2 pro (Insightful, Seattle, USA).

Results

Histology of DNA tattoo-treated *ex vivo* human skin

In view of the documented value of DNA tattoo in murine and non-human primate models (11-13) we set out to further develop this technique towards clinical application. As the morphology of animal skin differs substantially from human skin, an *ex vivo* human skin model was deemed essential to allow translation of this new DNA vaccination technique towards clinical testing. In a first set of experiments we aimed to determine whether DNA tattoo results in transfection of human skin cells and to assess the effect of DNA tattoo application on general skin structure and transfection. To this purpose, a ß-galactosidase encoding plasmid was introduced into *ex vivo* human skin by DNA tattoo application. Application of DNA tattoo to human skin with a needle depth of 1.0mm resulted in a substantial disruption of the epidermal layer of human skin, but only a slight disturbance of the underlying dermal layer (Figure 1). Furthermore, consistent with the data obtained in mouse models (11), DNA tattooing resulted in the sporadic transfection of cells in the epidermis (Figure 1). Transfection of cells in the dermal layer was not observed.

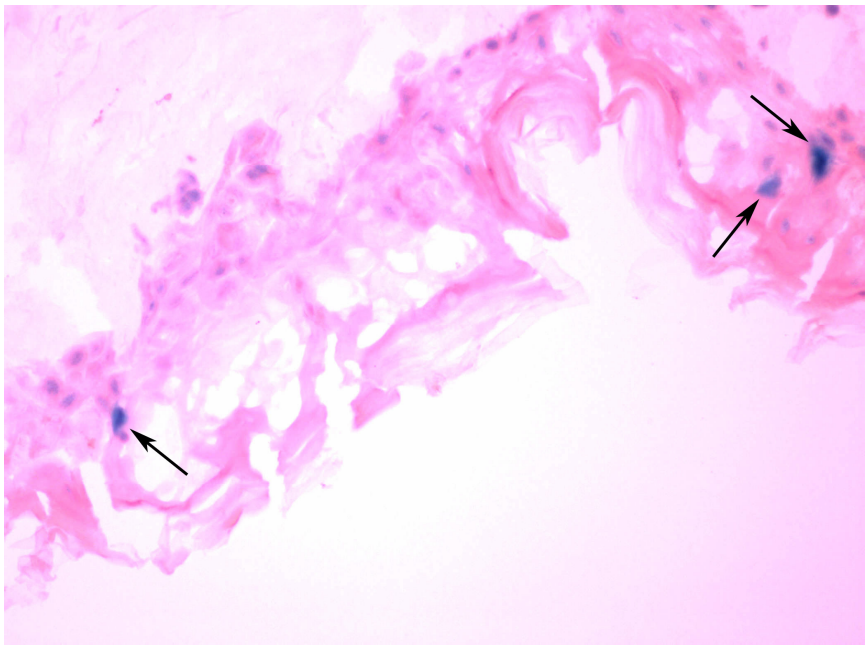

Figure 1: Expression of LacZ in a cryosection (20x magnification) of human skin after tattooing of LacZ DNA. 18 hours after tattooing, cross sections of skin were prepared and stained with X-gal solution to generate a blue precipitate in transfected cells (indicated by arrows). The skin was tattooed for 20 seconds with the Aella® tattoo machine at a depth of 1.0mm with 5 mg/ml pVAX:LacZ.

Visualization of vaccine-induced antigen expression in human skin

To determine which specific cell type(s) expressed the vaccine-encoded antigen following DNA tattoo vaccination, skin samples were tattooed with a GFP-encoding plasmid and cell suspensions of these samples were analyzed by flow cytometry, using specific markers for epidermal keratinocytes and LCs.

Of the viable cells (75.3% ± 6.3 of total cells, based op PI exclusion, mean ± SD of 3 separate experiments) recovered from epidermal preparations, approximately 2% expressed the

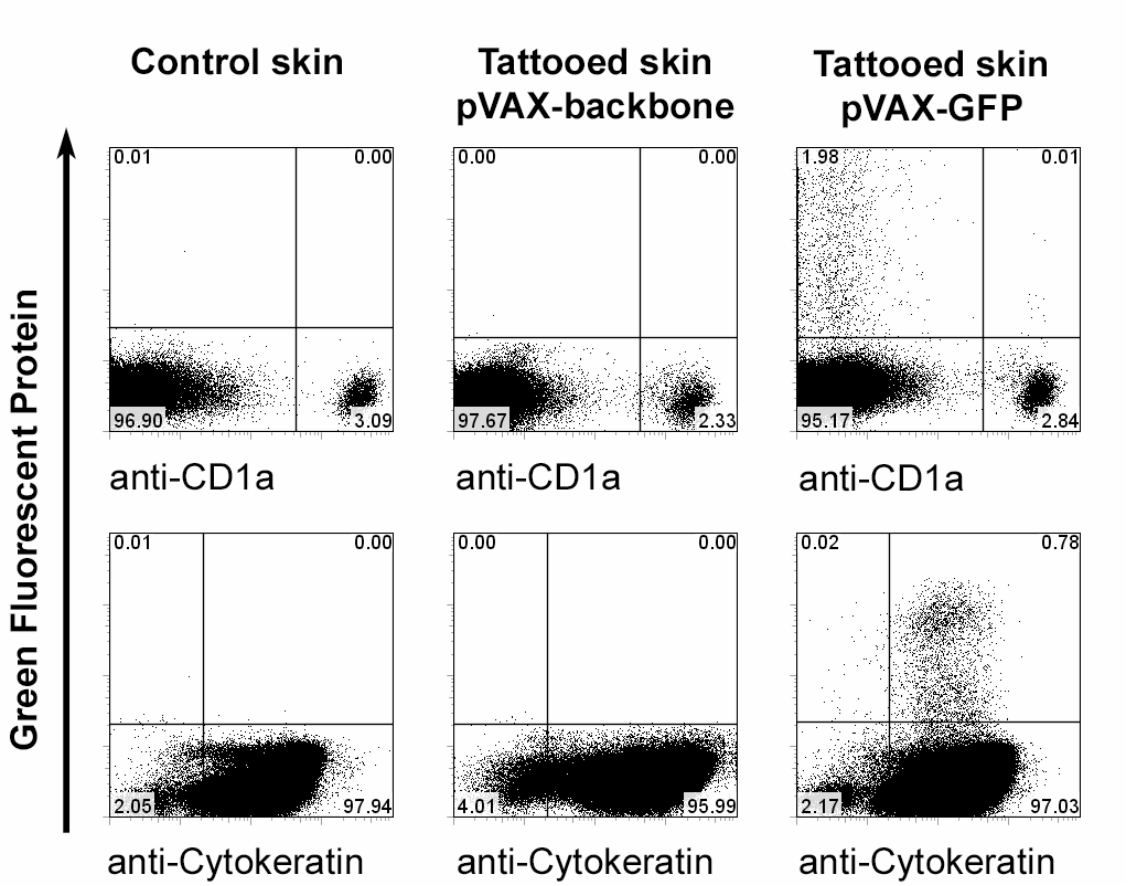

Figure 2: Flow cytometric analysis of epidermal cell suspension of tattooed skin. Control skin or skin tattooed at 1.5mm for 20 seconds per 50mm^2 with 5 mg/ml pVAX:GFP or 5 mg/ml empty pVAX backbone was stained with anti CD1a antibody or with anti-cytokeratin antibody.

vaccine-encoded GFP (1.80 ± 1.35%, mean ± SD of 3 different patients, all measured in triplicate). The vast majority (>98%) of these epidermal GFP$^+$ cells appeared to be keratinocytes, as based on expression of cytokeratin (Figure 2). The fact that antigen-expression is almost exclusively restricted to keratinocytes was confirmed by the observation that of all GFP$^+$ cells, only 1% (1.29 ± 0.53% (mean ± SD, 3 different patients, all in triplicate)) expressed the Langerhans cell marker CD1a. In the dermal layer of the skin only a small number of GFP$^+$ cells could be detected (approximately 2.5% of all GFP$^+$ cells in the skin sample were detected in the dermis, measured at a tattoo depth of 1.5mm, data not shown). Collectively, these data indicate that transfection upon DNA tattooing is almost exclusively restricted to keratinocytes within the epidermal layer. Transfection of CD1a$^+$ LCs is also observed but is relatively rare, and essentially proportional to the relative frequency of LC and keratinocytes in human skin (a ratio of 2:98).

The quantification of the number of cells expressing the vaccine-encoded antigen allowed us to determine the "biological availability" of DNA vaccines, administered with the tattoo technique. For this calculation, we assumed that the bulk of the GFP expressing cells was directly transfected with the plasmid, as there is no evidence in literature that keratinocytes

(which constitute > 98% of the GFP positive cells) are able to cross-present antigens. The total number of GFP-expressing cells per tattoo area of 50mm^2 was 2848 ± 762 (mean ± SD, 3 different patients, all in triplicate) using a DNA concentration of 5 mg/ml (20 sec application at needle amplitude of 1.5mm, using the Aella® tattoo machine). Prior work has shown that upon tattooing of a mixture of two different fluorescent reporter plasmids in mouse skin, co-expression of two reporter genes in one single cell occurs in some but not all cells (17). Based on this observation and on the fact that the log difference in GFP expression is around 3 fold (see Figure 2), we consider it reasonable to assume that a single transfected cell can take up approximately 1-1000 DNA molecules, indicating that the 2.8x10^3 antigen-expressing cells were transfected by a total of 2.8x10^3 - 2.8x10^6 DNA molecules. As the number of administered molecules of plasmid DNA per tattoo was calculated to be 1.33x10^{13}, this observation indicates that the *in vivo* transfection efficiency is between 2x10^{-8} and 2x10^{-5} % (i.e. 2 to 2000 out of 10,000,000,000 applied plasmid copies are taken up and translated into protein). These data indicate that transfection of epidermal cells upon DNA tattooing forms a highly inefficient process, when compared the currently available in vitro cell transfection methods. As an example, upon transfection of cells in *in vitro* systems, using cationic liposomes, approximately 8% of administered DNA molecules has been shown to become expressed, a 4x10^5-4x10^8 fold higher efficiency than that observed here upon intradermal DNA vaccination (18).

Longitudinal measurements of gene expression

To explore the possibility of following antigen expression in a longitudinal fashion, human skin was tattooed with firefly luciferase reporter plasmid (pVAX:Luc) and expression was measured at several time points in intact skin by optical imaging.

Luciferase expression could readily be detected by a light-sensitive camera and expression was restricted to the areas of tattooing (Figure 3B). Expression was observed at significant levels as early as 2 hours after tattooing, indicating that DNA transfection, translation and expression of the protein take place very rapidly after DNA tattooing. Luciferase expression peaked between 2 to 18 hours after tattooing and remained detectable for approximately 2-3 days (Figure 4). Based on the above data it seems plausible that the longitudinal measurement of gene expression may be used as a preclinical model to assess different methods of intradermal genetic vaccination (1,19). As a first step towards such a comparison, we evaluated the capacity of intradermal tattoo and intradermal DNA injection to induce luciferase expression in intact skin. Remarkably, upon intradermal injection, luciferase expression levels were not above background levels (where a tattoo with the same solution gives a expression level that is at least 10 to 20 fold higher as background), indicating that within *ex vivo* human skin, the expression upon DNA tattooing is at least 10-fold higher than that obtained upon classical intradermal injection.

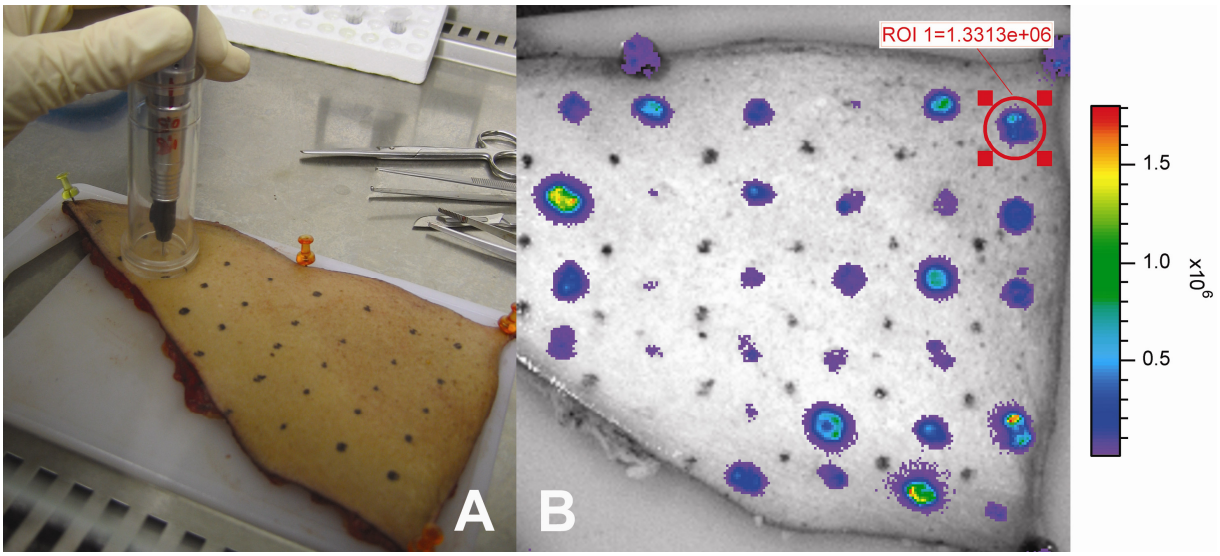

Figure 3: Tattooing procedure of the human skin (A) and typical expression of luciferase (B), visualized with a light sensitive camera, 18 hours after tattooing. Each area of 50mm^2 was tattooed with a different tattoo setting. Note the marked variation in luciferase signal obtained with different vaccination conditions.

Optimizing of DNA tattooing

Having established the feasibility of performing longitudinal measurements of DNA vaccine-induced antigen expression in human skin, we aimed to optimize variables that we considered likely to influence the efficiency of DNA vaccination. To this purpose, a total of 428 skin areas with a constant surface of 50mm^2 were tattooed using a luciferase encoding plasmid, using 10 samples of healthy abdominal skin. The tattoo variables tested for were: DNA concentration, duration of tattooing, needle depth and tattoo machine (see Table 1).

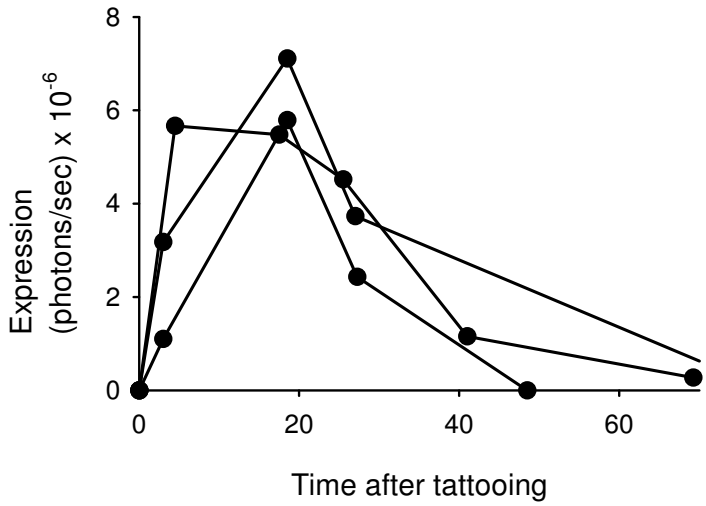

Figure 4: Typical longitudinal luciferase expression kinetics in intact *ex vivo* human skin upon DNA tattooing. Lines represent expression in 3 different tattooed areas of 50mm^2, placed on 3 different skin explants. The skin was tattooed for 20 seconds with the Aella® tattoo machine at a depth of 1.5mm with 5mg/ml pVAX:Luc. Note that the kinetics of luciferase expression are comparable in skin samples derived from different donors.

The relationship between log AUC and the different fixed effects tested for the Aella® machine is shown in Figure 5 and data obtained using the Cheyenne® machine showed a

similar profile (data not shown). All the tested fixed effects (Table 1), except the two different tattoo machines, had a significant effect ($p<0.001$) on antigen expression. Specifically, within

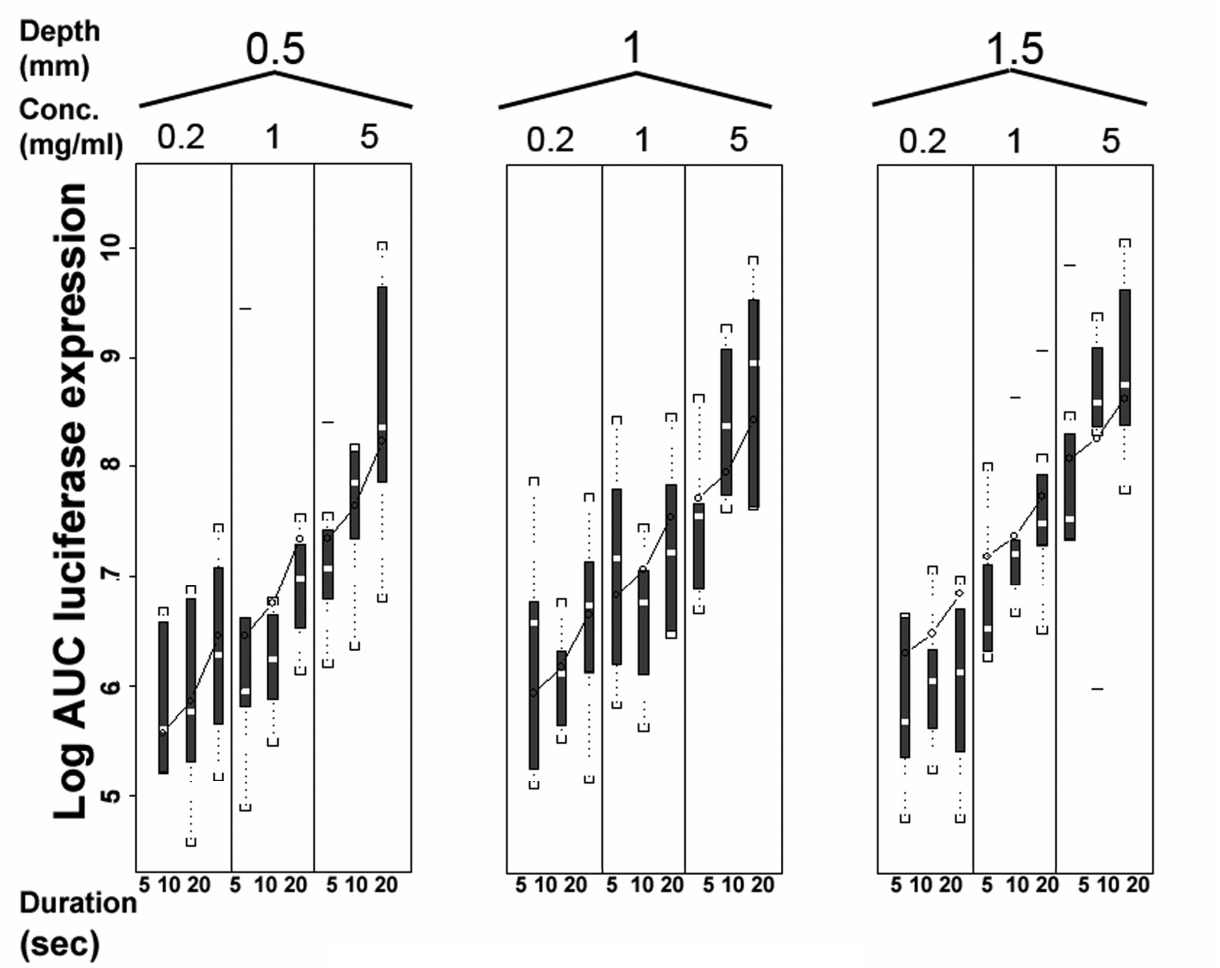

Figure 5: Results of the randomization study, demonstrating the relationship between the log Area Under the Curve (AUC) and the pVAX:Luc DNA concentration, needle depth and tattoo duration for the Aella® tattoo machine. Log AUC is visualized as a box-plot (showing the lowest observation, the lowest 25% of the data, median, the upper 25% of the data and largest observation). Lines visualize the predicted AUCs obtained from the model equation.

the tested range of values an increase in both DNA concentration, needle depth and tattoo time resulted in an increase in antigen expression.

Patient age and tattoo location (edge or centre) were found not to be significantly related to gene expression AUC. The interactions that were significant were between tattoo machine and surgery to tattoo time ($p=0.01$), between tattoo machine and tattoo depth ($p=0.0007$), between the log of DNA concentration and surgery to tattoo time ($p=0.002$) and between tattoo time and tattoo depth ($p=0.03$). However, none of these interactions had an influence on the tattoo settings that resulted in maximum antigen expression. For both tattoo machines the relationship between the AUC of antigen expression and the variations is expressed by the equation:

$$log(AUC) = log(10^4) + (0.35+0.13*A)* log(Conc) + (0.07-0.023*C)*B + X \quad [2]$$

where X is different for the different tattoo machines:

Aella®: $X= 6.9 – 0.76*A + 0.84*C$ [3]

Cheyenne®: $X= 7.1 – 0.47*A + 0.41*C$ [4]

in which A is pre-tattoo time (hrs), B is tattoo duration (sec) and C is tattoo depth (mm).

When varying each term separately (and fixing the others at their median values), the AUC changes by a factor of 5.8 over the range of DNA concentrations tested (0.2 to 5 mg/ml), 2.0 over the range of tattoo durations (5 to 20sec), 1.8 and 1.2 over the range of tattoo depths (0.5 to 1.5mm) for tattoo machine Aella® and Cheyenne®, respectively, and 1.2 for Cheyenne® versus Aella®. These data demonstrate that, of the variables tested, the concentration of the DNA solution that is applied is by far the major determinant of antigen expression in human skin (see Figure 6). The lines in Figure 5 visualize the predicted AUC of antigen expression obtained from the model. This figure shows that the obtained equation has a good fit with the experimental data.

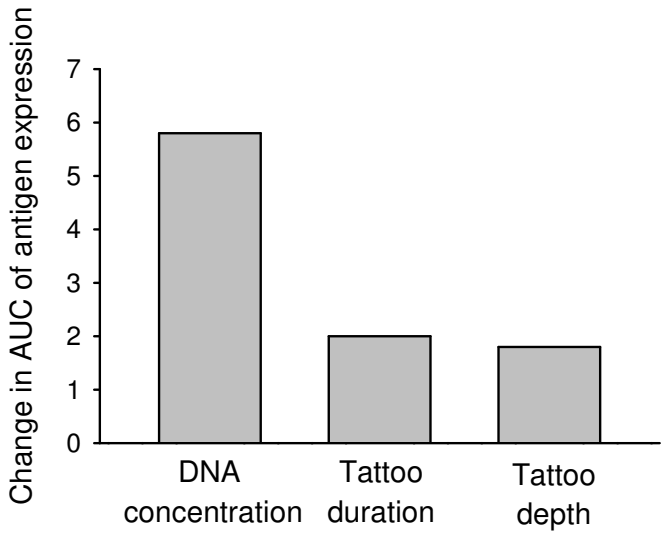

Figure 6: Results from the linear mixed effect model, demonstrating the influence of changing each tattoo variation over its full range (with fixing the others at median value) on change in AUC of antigen expression for the Aella® tattoo machine.

Discussion

Several dermal delivery techniques are currently moving from pre-clinical to clinical evaluation, and consequently there is a strong need to optimize these methods in a clinically relevant model. To allow such evaluation, we developed an *ex vivo* human skin model and used this model to characterize and optimize a recently developed DNA tattooing strategy for clinical application.

As a first step towards developing this model we analyzed vaccination-induced antigen expression by flow cytometry and histology. Flow cytometric analysis of tattooed human skin showed that only a small fraction of the epidermal cells, approximately 2%, is transfected. This low frequency of transfection was confirmed with an analysis of treated skin sections by histology that also showed an infrequent transfection of cells in this cell layer. This rather inefficient transfection of cells in the epidermis is comparable to that observed with other intradermal delivery techniques, such as gene gun (1) and to a prior analysis of DNA tattoo-induced transfection in murine skin (11). We assume that the large amount of DNA that is not taken up by skin cells is rapidly degraded in the skin by endonucleases, similar to observations in murine skin (20).

We subsequently demonstrated that vaccination-induced antigen expression can be quantified by longitudinal monitoring of luciferase activity in human skin samples. Tattooing of firefly luciferase encoding DNA in intact skin showed a fast and reproducible expression of the antigen, which we could measure longitudinally and was retained for 2-3 days. This kinetic profile is comparable to the *in vivo* antigen kinetics observed upon DNA tattooing of mice (11). This rather transient nature of vaccine-induced antigen expression in skin may possibly be explained by the high turnover of the epidermis (21) but could also be due to gene silencing. Importantly, it has previously been shown that fresh skin in culture does not lose its viability in the first 30h of culturing, with a viability decrease of 50% after 60h in medium at 37^{o}C (22,23). Therefore measurement of *ex vivo* antigen expression over a maximum time span of 3 days in this study was deemed appropriate.

As the level and duration of vaccination-induced antigen expression is correlated with the magnitude of vaccine-specific CTL response in mice upon tattoo immunization (11), we have used the *ex vivo* human skin model to determine the relationship between different vaccination parameters and antigen expression. From these experiments, analyzed in a linear mixed effect model, we conclude that the effect of DNA concentration forms the most important factor influencing the AUC of antigen expression in human skin (Figure 6). We did not reach saturation for DNA concentration in these experiments. This observation is in line with data obtained in mouse models, in which intramuscular or intradermal injections of naked DNA were utilized (24). In contrast, it was recently reported that transfection upon intradermal injection of messenger RNA is saturated at a concentration of 0.05 mg/ml. This

suggests a different uptake mechanism between intradermal mRNA injection and the tattooing of dsDNA (25).

Using the *ex vivo* human skin model, we also showed that the effects of tattoo time and tattoo depth on antigen expression are significant, but less dominant. The fact that transfection of skin cells is almost exclusively observed in the epidermal layer of the skin may explain why an increase in needle depth has only a small effect on antigen expression. The epidermis of the abdominal skin samples used in this study was between 200 and 300µm in depth. Thus, the minimal needle depth of 0.5mm that was utilized here should be enough to reach the complete epidermal layer when considered a fixed object. However, it is important to stress that injection depth is probably lower than the needle depth, due to the flexibility and resistance characteristics of the skin.

To what extent can data on vaccination-induced antigen expression obtained in the *ex vivo* human skin model that we have developed here be expected to translate to vaccination-induced antigen expression and vaccine-induced immune responses in human subjects? A first issue of relevance here is whether the observed luciferase signal that is primarily derived from keratinocytes forms a good measure of vaccine efficacy. Specifically, can expression in keratinocytes be expected to correlate with immunogenicity?

Murine studies using a plasmid carrying a K14 promoter, which is only active in keratinocytes, suggest that cross-presentation is an important route in the induction of cytotoxic T cell responses by DNA tattooing (26). These findings were in line with a study that showed that exclusive transfection of DCs (using a CD11c promoter) upon gene gun immunization cannot trigger strong CD4 and CD8 responses in mice (27), suggesting that antigen expression in non-APC cell types may be required for the generation of strong cellular immunity. Thus, antigen expression within keratinocytes is likely to be in large part responsible for the immunogenicity of intradermal DNA vaccines and the measurement of antigen expression in human skin samples is therefore likely to have predictive value. Interestingly, it has previously been shown that phagocytosed GFP is quenched in the acidic environment of the endosomes (28). As the small population of GFP positive LCs that is observed in human skin samples does show a lower GFP intensity than the GFP positive keratinocyte population (Fig. 2), it may be speculated that the GFP in these LCs may possibly be derived from phagocytosed material. To further unravel the source of the GFP signal observed in LCs, we tattooed skin with a construct encoding a histone-GFP fusion protein. Direct transfection of cells with this construct results in expression of GFP exclusively in the nucleus of the cell, where GFP taken up by cross presentation should be present in the endosomal pathway and possibly also in cytosol/ nucleus. By sorting GFP^+ $CD1a^-$ cells and GFP^+CD1a^+ cells and analysing these separated populations by confocal laser microscopy, we aimed to reveal the subcellular localization of the GFP expressed in these cells. As shown in Figure 7, GFP expression in CD1a-negative keratinocytes is clearly restricted to the nucleus**,** demonstrating their direct transfection. Unfortunately, due to their very low

numbers and fragility during the sorting procedure, we were not able to successfully visualize GFP positive Langerhans cells by confocal laser scanning microscopy. Therefore, the source of their GFP signal remains presently unknown.

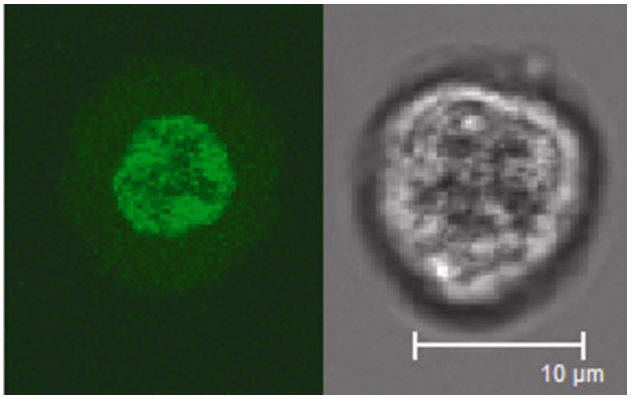

Figure 7: Localisation of GFP in keratinocytes upon tattooing at 1.5mm for 20 seconds per 50mm^2 with 5 mg/ml of H2B-GFP encoding DNA, visualized by confocal laser scanning microscopy. GFP expression (left panel) is clearly localized to the nucleus, confirming that this cell type was directly transfected upon DNA tattoo.

Accepting that the antigen expression observed in *ex vivo* skin samples has predictive value for *in vivo* immunogenicity it is also of importance to consider the effect of the variables that have been tested here. With regard to the influence of DNA concentration on antigen expression, it may be expected that the influence of this parameter on antigen expression will be similar *in vitro* and *in vivo*, as an increase in DNA concentration is not expected to alter the location of antigen expression or cell type involved. In addition, a high antigen expression due to an increased DNA concentration is likely to directly translate into increased antigen presentation and immunogenicity. Also with respect to the influence of DNA tattoo time on antigen expression, the *in vitro* skin model is likely to translate well as the site of antigen expression and cell types involved are unlikely to be influenced by this parameter. It does however seem possibly that the effect of DNA tattoo time on immunogenicity is larger than predicted by the monitoring of *ex vivo* antigen expression, as the increased tissue damage that occurs upon prolonged vaccination may have an adjuvant effect. Finally, it may be argued that the effect of tattoo depth on antigen expression measured here may perhaps have the smallest predictive value for *in vivo* antigen expression and immunogenicity, as the flexibility of skin in patients may well be larger than that of the fixed *ex vivo* skin samples used here.

Based on the analyses performed in this study we will initiate a phase I clinical trial, in which we will administer a DNA vaccine at a concentration of 5 mg/ml for 20s per 50mm^2 of skin surface, at a needle depth of 1.5mm, the settings that showed the maximum level of antigen expression in this study. In this trial, a DNA vaccine encoding the HLA-A2 restricted MART-1 epitope will be administered to patients with metastatic melanoma. This will be a dose escalation study, in which an increased dose of the DNA vaccine will be achieved by a simple increase in the skin area used for DNA tattoo application, starting from 4cm^2 up to 32cm^2.

In conclusion we here demonstrate that *ex vivo* human skin is an adequate model for the characterization and optimization of intradermal DNA vaccines. Furthermore, we have shown that at fixed volumes, DNA concentration is the most important parameter influencing vaccination-induced antigen expression. In ongoing experiments, the skin model developed in this study is used to determine the value of non-viral DNA carriers and other dermal delivery techniques for their ability to improve dermal antigen delivery. It seems reasonable to assume that the preclinical testing of such DNA vaccine formulations in this *ex vivo* human skin model will form an efficient strategy to select promising vaccination strategies for subsequent testing in clinical trials.

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Chapter 6

Shielding the cationic charge of nanoparticle-formulated dermal DNA vaccines is essential for antigen expression and immunogenicity

Joost H. van den Berg
Koen Oosterhuis
Wim E. Hennink
Gert Storm
Leonardus J. van der Aa
Johan F. J. Engbersen
John. B. A. G. Haanen
Jos H. Beijnen
Ton N. Schumacher
Bastiaan Nuijen

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Abstract

Nanoparticle-formulated DNA vaccines hold promise for the design of *in vivo* vaccination platforms that target defined cell types in human skin. A variety of DNA formulations, mainly based on cationic liposomes or polymers, has been investigated to improve transfection efficiency in *in vitro* assays.

Here we demonstrate that formulation of DNA into both liposomal and polymeric cationic nanoparticles completely blocks vaccination-induced antigen expression in mice and *ex vivo* human skin. Furthermore, this detrimental effect of cationic nanoparticle formulation is associated with an essentially complete block in vaccine immunogenicity. The blocking of DNA vaccine activity may be explained by immobilization of the nanoparticles in the extracellular matrix, caused by electrostatic interactions of the cationic nanoparticles with negatively charged extracellular matrix components. Shielding the surface charge of the nanoparticles by PEGylation improves *in vivo* antigen expression more than 55 fold. Furthermore, this shielding of cationic surface charge results in antigen-specific T cell responses that are similar as those induced by naked DNA for the two lipo- and polyplex DNA carrier systems. These observations suggest that charge shielding forms a generally applicable strategy for the development of dermally applied vaccine formulations. Furthermore, the nanoparticle formulations developed here form an attractive platform for the design of targeted nanoparticle formulations that can be utilized for *in vivo* transfection of defined cell types.

Introduction

Non-viral carrier systems are widely used as transfection reagents to deliver nucleic acids for both *in vitro* and *in vivo* applications. In these systems, negatively charged DNA is bound by electrostatic interaction to an excess of a positively charged carrier. In this complexation process, DNA is condensated into positively charged, nanosized particles and protected from nuclease degradation, resulting in substantially higher transfection efficiencies compared to naked nucleic acids in *in vitro* assays. The two most frequently used carriers to enhance transfection efficiency are cationic lipids and cationic polymers, and the resulting DNA nanoparticles are referred to as lipoplexes and polyplexes, respectively (1-4). In addition to the beneficial effect on *in vitro* transfection efficiency, formulation of DNA into cationic particles has also been shown to result in a higher transfection efficiency than naked DNA upon intramuscular injection (5,6).

While DNA vaccines were first described using intramuscular injection as an administration route (7), a growing interest has developed into intradermal DNA vaccine delivery. Specifically, because of its natural barrier function, the skin is perceived as a site that is well-equipped for the induction of adaptive immune responses and the high density of antigen-presenting cells in skin provides indirect support for this notion. Dermal DNA vaccines can be applied by various methods, including classical intradermal injection, gene gun and DNA tattoo (8). The latter strategy delivers naked plasmid DNA into the skin through thousands of punctures using an oscillating multiple needle tattoo device. DNA tattooing leads to the induction of strong and rapid antigen-specific cellular immune responses in mice (8). Furthermore, the immunogenicity of DNA tattoo is approximately 10-100 fold higher than that of classical intramuscular DNA vaccination in non-human primates (9). Importantly, in spite of the high immunogenicity of DNA tattoo, the *in vivo* transfection efficiency of naked DNA with this technique is extremely low, with approximately 1 out of $5x10^6$ to $5x10^9$ plasmid copies applied being taken up, transcribed and translated (10). Based on the strongly positive effect on transfection that is generally observed for DNA encapsulation in cationic lipo- and polyplexes in *in vitro* assays, we aimed to determine whether such formulations could also result in improved transfection and subsequent immune response for intradermal vaccines applied by DNA tattooing. Development of these nanoparticle-formulated DNA vaccines forms an essential first step towards the further development of targeted intradermal DNA vaccines.

Materials and methods

Materials

The pVAX:Luc-NP plasmid (8) encodes the influenza A $NP_{366-374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). pVAX:GFP was generated by inserting Green Fluorescence Protein (GFP) encoding DNA into the BamHI/NotI site of pVAX1. Plasmids were expressed and amplified in *E. Coli* DH5 and were purified by Endofree™ QIAGEN® Mega-kit (QIAGEN®, Hilden, Germany). 1,2-dioleoyl-oxypropyl-3-trimethyl-ammonium chloride (DOTAP) was obtained from Avanti Polar lipids (Alabster, AL, USA). Dioleoylphosphatidyl-ethanolamide (DOPE) and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG) were a kind gift from Lipoïd GmbH (Ludwigshafen, Germany). NonPEGylated and PEGylated poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible disulfide linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of *N,N'*-cystaminebisacrylamide with the appropriate amine according to the procedure described previously (11). The PEGylated analog was prepared using 11 mol% of MeO-PEG-NH_2 in the total amino monomer feed during the PAA synthesis. All other chemicals were of analytical grade.

Liposome preparation

NonPEGylated liposomes, composed of DOTAP-DOPE, were prepared in a 1:1 molar ratio. For PEGylated liposomes, DOPE was replaced by DSPE-PEG at different concentrations to keep the total molarity of lipids constant. Lipid mixtures were dissolved in chloroform/methanol (1:1 v/v) and mixed in a round-bottomed flask. Organic solvents were evaporated at 40 ºC using a vacuum evaporator and the obtained lipid films were purged with nitrogen for 30 min. Lipid films were rehydrated in 20 mM HEPES, pH 7.4, 10% sucrose, to give a final lipid concentration of 35 mM. The resuspended lipids were extruded 8 times through two stacked polycarbonate membranes (Poretics, Livermore, USA, 200 and 100 nm) to obtain small unilamellar vesicles of 100 nm.

Lipoplex and polyplex preparation and characterization

Lipo- and polyplexes were prepared by mixing an equal volume of plasmid and cationic liposomes or dissolved polymer. All formulations were prepared in 20 mM HEPES pH 7.4, 10% sucrose buffer with a high viscosity and a low ionic strength, conditions previously shown to be favourable for obtaining small and stable DNA complexes (12). Formulation characterizations were performed with the Luc-NP construct.

N/P ratios were defined as the charge ratio between cationic nitrogen residues in DOTAP or PAA and anionic phosphate groups in the DNA. Ratios were calculated assuming that 302

and 532 g/mol correspond with each (protonable) nitrogen containing-repeating unit of PAA and PEG-PAA, respectively. For DOTAP 699 g/mol is the mass bearing one cationic nitrogen. For plasmid DNA 330 g/mol corresponds with the average mass of a repeating unit bearing one negative phosphate group. For polyplexes, weight ratios polymer/DNA are also provided. All complexes were formulated with a final DNA concentration of 0.5 mg/mL. Control naked DNA was diluted to the same concentration in the same buffer. The size of obtained particles was measured in 20 mM HEPES pH 7.4, 10% sucrose, with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4, using a Zetasizer Nano Z (Malvern Instruments). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound DNA was visualized by electrophoresis at 85 V using a 1% agarose gel containing 0.5 µg/mL etidium bromide. Only particle formulations shown not to be aggregated and containing no free DNA were used in further experiments.

Transfection of epidermal cell suspensions

Healthy human abdominal skin from female patients (41-63 years) was obtained from the plastic surgery department of the institute according with the guidelines of the Antoni van Leeuwenhoek Hospital/ The Netherlands Cancer Institute. Subcutaneous fat was directly removed by blunt dissection. Skin was transported on ice and used within 2 hours after surgical removal.

To obtain an epidermal cell suspension, skin was incubated for 1 hour in 10 mg/mL dispase II (Sigma Aldrich, St. Louis, MO, USA) in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25 µg/mL amphotericin B (all Invitrogen) at 37 ºC, upon which the epidermis was mechanically peeled from skin samples. The obtained epidermal sheet was digested at 37 ºC in complete keratinocyte medium containing 0.05% trypsin. After 15 min, the epidermis was disrupted with a glass pipette and 10% FCS was added to the medium, after which the cells were filtered through 70 µm nylon gauze to remove debris.

Per well, 1 x 10^5 cells of a freshly prepared epidermal cell suspension were seed in 24-well tissue culture plates in complete keratinocyte medium. Cells were incubated with 50 µl naked pVAX:GFP or the indicated nanoparticle formulation (all at 0.04 mg/mL DNA to obtain a final concentration of 1 µg DNA/well) at 2% CO_2, 37 ºC. After 24 hours, cells were harvested and analyzed for GFP expression using a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) and data were analyzed using Flowjo software (Three Star, Ashland, USA). Live cells were selected based on propidium iodide exclusion.

DNA tattooing of *ex vivo* human skin

Formulations and naked DNA controls encoding firefly luciferase were administered to intact skin by DNA tattooing (8) to allow longitudinal luciferase expression measurements. Alternatively, formulations and naked DNA controls encoding GFP were used for flow cytometric analysis of transfected cell types. The skin model used in these experiments has been described previously for the optimization of tattooing of naked DNA in skin (10). In brief, 10µl of the indicated formulation at a final DNA concentration of 0.5 mg/ml was applied to the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8mm, surface 50 mm^2). The formulation was subsequently administered into the skin using a Permanent Make Up (PMU)® tattoo machine (kindly provided by MT Derm GmbH, Berlin, Germany). For all tattoos, 9-needle cartridges at an oscillating frequency of 100 Hz were used. The needle depth was adjusted to 1.5 mm and tattoo duration was 20 seconds.

After tattooing, skin samples were kept at 5% CO_2, 37 ºC in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 0.25 µg/mL amphotericin B (all Invitrogen) to allow longitudinal expression measurements of luciferase. During this incubation, skin was cultured at the air-medium interface with the epidermis exposed to the air to mimic the natural situation.

Measurement of antigen expression using intravital imaging of *ex vivo* human skin

The expression of luciferase was measured in intact skin samples at the indicated time points after tattooing. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/mL. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 minutes after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS system 100 CCD camera (Xenogen, Hopkinton, USA).

Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis. After each measurement, medium was refreshed to remove residual luciferin.

Flow cytometric analysis of DNA vaccine induced antigen expression

For flow cytometry experiments, tattooed areas of interest were removed from the intact skin with a 6 mm biopsy punch and transferred into 48-well plates. Directly upon tattooing, epidermal sheets were removed as described above and incubated overnight at 37 °C. After incubation, epidermal sheets were digested and stained with antibodies. The antibodies

used were mouse anti-human CD1a allophycocyanin (APC) (Immunotech) and mouse anti human cytokeratin (equal mixture of clone LP34 and MNF116 (both Dako, Glostrup Denmark)), labelled with Alexa Fluor 647 (Invitrogen) according to manufacturer's protocol. Prior to cytokeratin staining, epidermal cell suspensions were permeabilized using the BD Cytofix/Cytoperm kit (BD Sciences, Franklin Lakes, NJ, USA) according to the manufacturer's protocol. In case of anti CD1a staining, live cells were selected based on propidium iodide exclusion.

DNA immunization

C57BL/6J mice (6-8 weeks) were obtained from the experimental animal department of The Netherlands Cancer Institute. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the NKI-AVL Animal Research Committee.

To allow simultaneous measurement of antigen expression and T cell responses, mice were immunized by DNA tattooing with formulations containing the pVAX:Luc-NP construct. Before intradermal DNA vaccination, the hair at the administration sites was removed with depilatory cream (Veet sensitve, Reckitt Benckiser, Hull, UK). During immunization, 15 µl of lipo- or polyplex formulation or naked DNA solution at a final concentration of 0.5 mg/ml was applied to the skin of the hind leg and administered using a disposable 9-needle cartridge mounted on an PMU® tattoo machine. DNA vaccines were tattooed during 30 s at a needle depth of 1.0 mm, and the needle bar oscillated at 100 Hz. Using this needle depth setting, cells in both the epidermis and upper layer of the dermis are transfected (8). Mice were vaccinated on day 0, 3 and 6. All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment. At the indicated time points after immunization, approximately 50 µl of peripheral blood was collected by tail bleeding for the measurement of T cell responses.

Measurement of antigen expression using intravital imaging

Antigen expression upon DNA vaccination was measured by a light-sensitive camera to allow longitudinal *in vivo* expression of firefly luciferase. Mice were anesthetized with isoflurane. A solution of the substrate luciferin in PBS (150 mg/kg, Xenogen) was intraperitoneally injected and after 18 min, the luminescence produced by active luciferase was acquired during 30 s in an IVIS® system 100 CCD camera (Xenogen). Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Antigen-specific T cell assay

To measure antigen-specific T cell responses, peripheral blood lymphocytes were stained on different time points with H-2D^b/NP$_{366–374}$-tetramers and APC-conjugated CD8α antibody (BD Pharmingen, San Jose, USA) at 20 °C for 15 min in FACS buffer (1× PBS, 0.5% BSA and 0.02% sodium azide) as described before (13). Cells were washed three times in FACS buffer and analyzed by flow cytometry. Live cells were selected based on propidium iodide exclusion.

Statistical Analysis

A two-tailed Mann-Whitney U-test was used for statistical analysis and a value of $p < 0.05$ was considered significant. A Bonferroni adjustment test was applied to correct the significant level when multiple groups were compared.

Results

Cationic nanoparticles increase transfection efficiency in epidermal cell suspensions but decrease antigen expression in *ex vivo* human skin and in mice

In order to develop nanoparticle formulations for intradermal application of DNA vaccines, DNA was complexed with cationic DOTAP-DOPE liposomes or with cationic poly(amidoamine) (PAA) polymers, to form lipoplexes and polyplexes, respectively. DOTAP-DOPE was chosen since this is the most commonly used composition in liposomal based transfection experiments. PAA was chosen as a novel and biodegradable polymeric carrier system. As DNA vaccination is known to require high DNA concentrations (14-16), complexes were formulated with a final DNA concentration of 0.5 mg/mL, which is relatively high for these systems. The obtained lipoplexes and polyplexes were characterized for particles size and surface charge (as reflected by the zeta potential). Both types of DNA-nanoparticles had particle sizes below 240 nm, did not contain free DNA (as determined by gel electrophoresis) and exhibited a zeta potential above +40 mV.

To determine the effect of DNA formulation into nanoparticles on *in vitro* transfection efficiency, fresh suspensions of non-transformed human epidermal cells were used. These target cells were transfected *in vitro* with either lipoplex or polyplex nanoparticles that had been formulated with a GFP encoding construct, and transfection-induced GFP expression was analyzed 24 hrs after addition to the cells, by flow cytometry. For both types of nanoparticles, nanoparticle formulation resulted in marked increase in transfection efficiency when compared with naked DNA (by a factor of >26 and >900 for polyplexes and lipoplexes respectively, Figure 1A). These data demonstrate that the superior *in vitro* transfection properties of formulated cationic nanoparticles previously shown for human cell lines also apply to non-transformed human skin cells.

To study the performance of cationic DNA-nanoparticles in a clinically more relevant model, antigen expression was measured in intact *ex vivo* human skin upon tattooing of nanoparticles formulated with a luciferase encoding construct. Luciferase expression was measured with a light sensitive CCD camera. Surprisingly, antigen expression induced by application of both lipoplex and polyplex DNA nanoparticles was extremely low (see Figure 1B). In contrast, intradermal application of naked DNA resulted in robust levels of antigen expression, as observed previously (10). Consistent with the data obtained in human skin, application of lipo- or polyplexes to murine skin also yield very low levels of antigen expression (Figure 1C) and corresponding undetectable antigen-specific immune responses in vaccinated mice (see below).

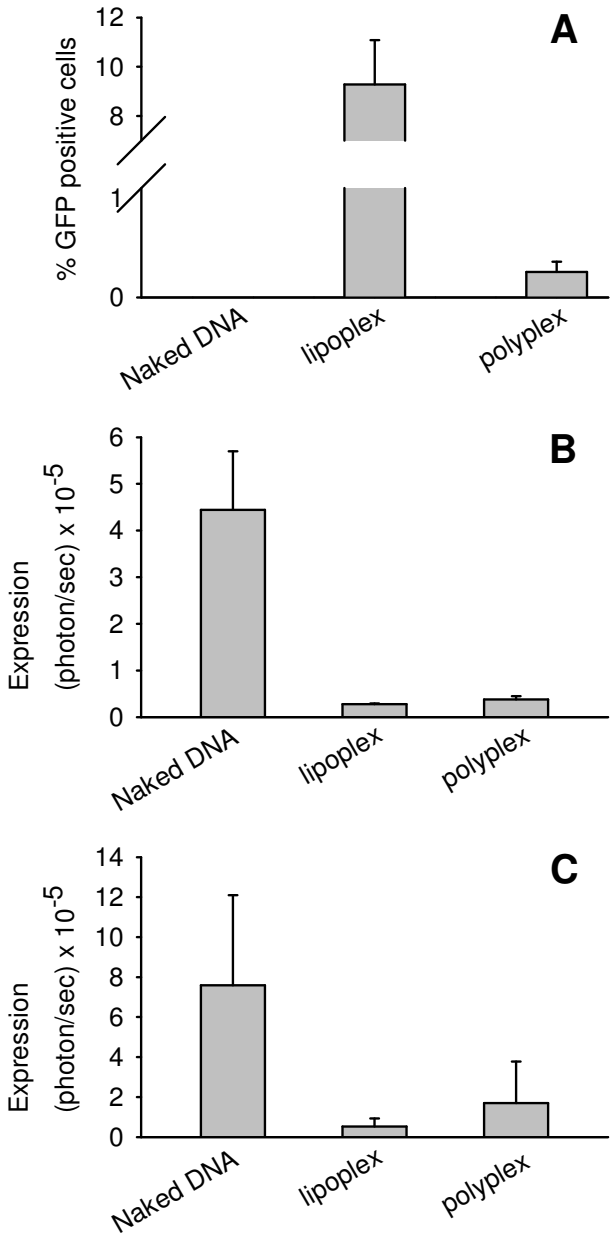


Figure 1. Discordant *in vitro* and *in vivo* performance of cationic nanoparticles.

(**A**) Transfection of epidermal cell suspensions with naked DNA, DOTAP-DOPE/DNA complexes ('lipoplex'), and PAA/DNA complexes ('polyplex'). Bars represent the mean + SD of three independent measurements.

(**B**) Luciferase activity upon application of naked DNA, lipoplex or polyplex formulation to intact *ex vivo* human skin by DNA tattoo. Data shown depict luciferase activity measured 5 hours after DNA application. The same poor performance of lipoplex and polyplex DNA formulations was observed after 21 hrs. Bars represent the mean + SD of 3-5 measurements.

(**C**) Luciferase activity upon application of naked DNA, lipoplex or polyplex formulation to murine skin. Data shown depict the poor performance of lipoplex and polyplex DNA formulation when compared with naked DNA. Expression was measured 8 hours after tattooing. Bars represent the mean + SD of 5-8 mice.

All formulations were prepared at a charge (N/P) ratio of 5 (lipoplexes) or 55 (polyplexes), to obtain particles with sizes below 240 nm and a zeta potential above +40 mV.

These results demonstrate that *in vitro* transfection data of these positively charged DNA vaccine formulations bear little, if any, predictive value for *in vivo* expression in either murine or human skin. Furthermore, the data provide the more general indication that cationic nanoparticles are ill-suited for the intradermal application of DNA vaccines.

Shielding of the cationic surface charge restores transfection efficiency of nanoparticles in *ex vivo* human skin

To determine the underlying reason for the discordance between the effectiveness of DNA nanoparticles in *in vitro* and *in/ ex vivo* assays we focussed on potential differences between intact skin and skin cell cultures. First, the presence of the extracellular matrix (ECM) in skin tissue conceivably reduces free diffusion of particles in intact skin. As condensation of DNA into nanoparticles results in a reduced size compared to free DNA it is unlikely that a sieve function of the ECM is responsible for the reduction in effectiveness *in vivo*. However, several ECM components carry a net negative charge and are likely to interact with the positively charged nanoparticles, with the result that the nanoparticles become immobilized in the ECM. Thus, while in *in vitro* cultures the cationic charge of the nanoparticles is positively contributing to the transfection process by promoting binding to cell surfaces, this positive effect is most likely surpassed *in vivo* by electrostatic interactions with anionic ECM components and consequent immobilization of these nanoparticles in the ECM, preventing the particles to reach their target.

In order to evaluate whether the positive particle charge is responsible for the absence of vaccination-induced antigen expression in intact skin, we introduced poly (ethylene glycol) (PEG) moieties to both types of nanoparticles, a strategy that is known to reduce the surface charge of lipo-and polyplexes. The presence of increasing concentrations of PEG in both lipo- and polyplexes resulted in a reduction of the surface charge in a dose-dependent manner from +40-60 to close to neutrality for both formulations, together with a modest (2-fold or less) reduction in particle size (see supplementary Figures 1-3).

Subsequently, PEGylated nanoparticles were applied to intact human skin by DNA tattooing and vaccination-induced antigen expression was analyzed. In agreement with our hypothesis that blocking of the intradermal expression as observed for the cationic nanoparticles is due to their positive surface charge, the PEGylated nanoparticles showed a very marked increase in antigen expression (~50-fold and ~20-fold for lipo- and polyplexes, respectively, see Figures 2 and 3).

For the PEGylated lipoplexes, antigen expression levels reached a plateau value at a DSPE-PEG content between 15 and 17.5% (Figure 2A) with an expression level that was 1.6 ± 0.3 fold (mean ± SD, measured in three independent pieces of skin) higher at the peak of expression than naked DNA. At a DSPE-PEG content of 10%, no difference in antigen expression was observed between N/P ratio 2 and 5 (data not shown).

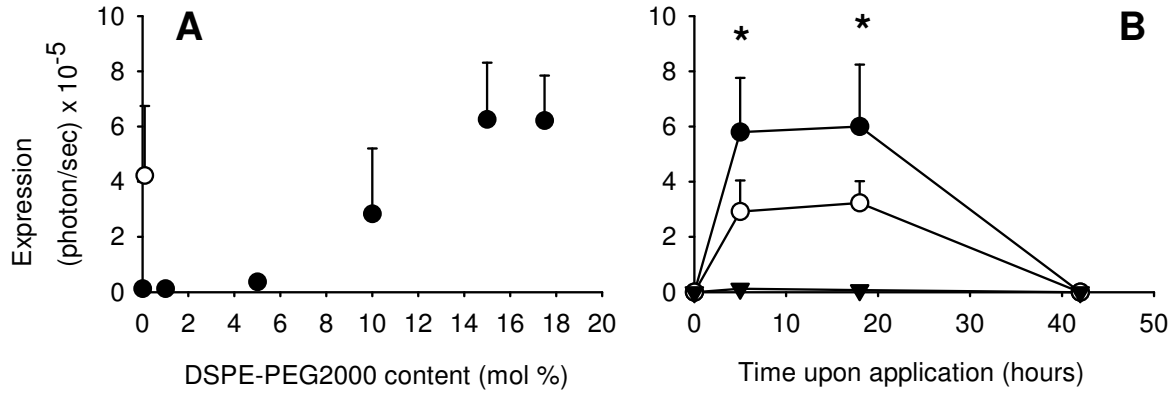


Figure 2: Antigen expression (luciferase) upon tattoo vaccination of PEGylated lipoplexes in *ex vivo* human skin. (**A**) Expression induced by administration of naked DNA (○), or by administration of DOTAP-DOPE/DNA complexes as a function of DSPE-PEG content at an N/P ratio of 5 (●). Expression was measured at 5 hrs post DNA application. (**B**) Longitudinal expression upon application of naked DNA (○) or application of DOTAP-DOPE/DNA lipoplexes (N/P of 5) with (●) or without (▼)17.5 mol % DSPE-PEG. Each point represents the mean + SD of 3-8 data points, randomized tattooed over one biopsy of skin. All experiments were performed in triplicate.
* Values significantly different from naked DNA control.

The PEGylated polyplexes showed a plateau in antigen expression levels at a polymer/DNA w/w ratio between 25:1 and 50:1 (Figure 3B) that was 8.5 ± 4.4 fold (mean ± SD, measured in three independent pieces of skin) higher than naked DNA.

Finally, to determine whether the physical incorporation of PEG into nanoparticles is essential to restore antigen expression, a control experiment was performed in which unbound PEG 2000 was added to the nonPEGylated lipo- and polyplexes in the same concentrations as used in the PEGylated particles. Application of these formulations to human skin resulted in non-detectable levels of antigen expression levels (data not shown). This demonstrates that the observed effects are due to the PEG modification of the particles rather than to the presence of PEG itself in the formulation solutions.

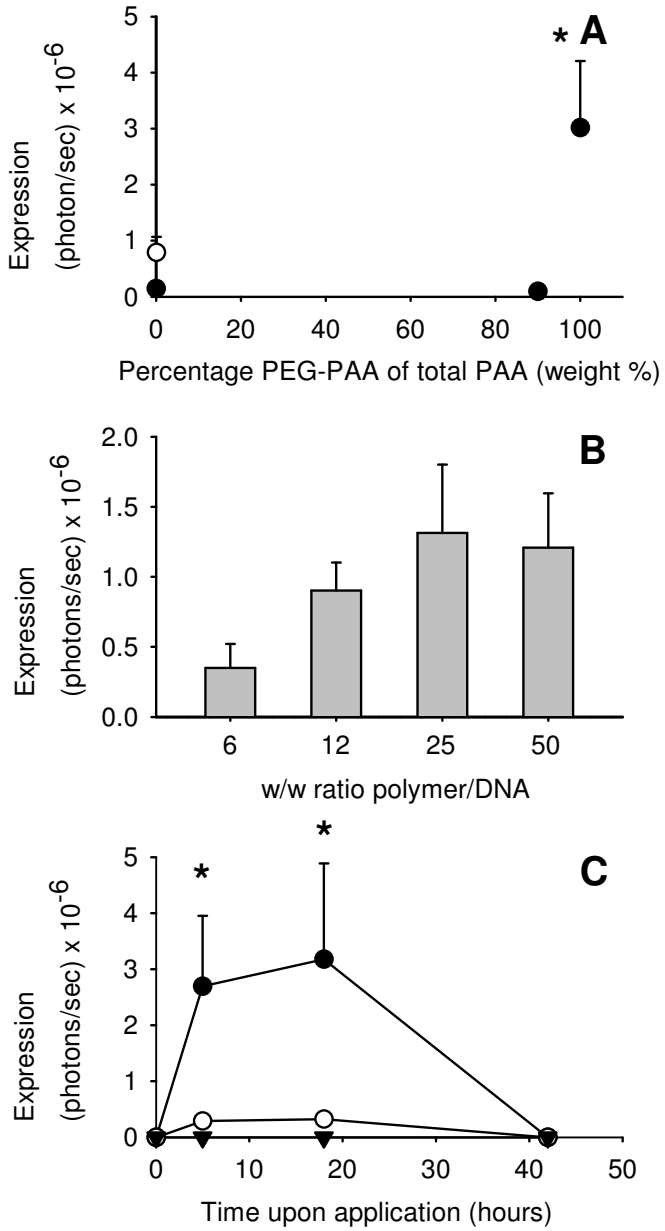


Figure 3: Antigen expression upon tattoo vaccination of PEGylated polyplexes in *ex vivo* human skin.

(**A**) Expression upon application of naked DNA (○), or application of PAA/DNA polyplexes as a function of the percentage of PEGylated PAA at a w/w ratio polymer/DNA of 50 (●).

(**B**) Expression upon application of PEGylated PAA /DNA polyplexes at different ratios polymer/DNA. Expression was measured at 5hrs post DNA application.

(**C**) Longitudinal expression of luciferase in human skin upon application of naked DNA (○), PEGylated PAA/DNA polyplexes at a w/w ratio polymer/DNA of 50 (●) or nonPEGylated PAA/DNA complexes at a w/w ratio polymer/DNA of 50 (▼). Each point represents the mean + SD of 3-8 data points, randomized tattooed over one biopsy of skin. All experiments were performed in triplicate.

* Values significantly different from naked DNA control.

PEGylated nanoparticles and naked DNA primarily transfect epidermal keratinocytes

It has been reported that vaccination by nanoparticles can result in preferential targeting of Antigen Presenting Cells (APCs) (17-19). Therefore, it is of interest to evaluate which type of cells are transfected upon DNA tattoo vaccination with PEGylated lipo-and polyplexes. To this purpose, a GFP encoding plasmid was applied by DNA tattooing to human skin biopsies, either as uncomplexed DNA, or encapsulated in PEGylated lipo- or polyplexes. After DNA application, the epidermis of the skin was removed and digested to a single cell suspension. Cells were subsequently stained with anti-cytokeratin and anti-CD1a antibodies to reveal transfection of cytokeratin positive epidermal keratinocytes and CD1a positive Langerhans Cells, respectively. Flow cytometric analysis of obtained cell populations

demonstrated that, as is the case for uncomplexed DNA (10), intradermal application of DNA encapsulated into PEGylated lipoplexes or polyplexes resulted in the near-exclusive transfection of keratinocytes, with at most a sporadic GFP positive Langerhans Cell (LCs) (Figure 4). These data reveal that *ex vivo* nanoparticle administration by DNA tattooing, does not result in preferential expression in epidermal LCs.

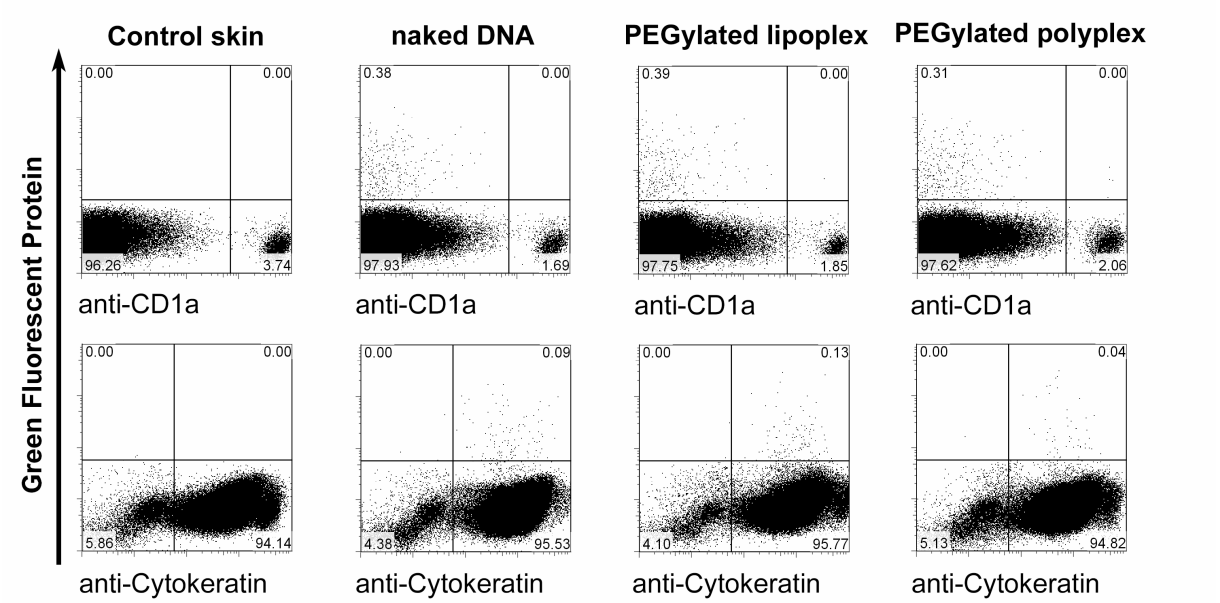


Figure 4: Flow cytometric analysis of epidermal cell suspensions of tattooed skin. Skin was tattooed with naked GFP-encoding DNA, DOTAP-DOPE/DNA lipoplexes with 17.5 mol % DSPE-PEG (N/P ratio 5), or PEG-PAA/DNA polyplexes (w/w ratio 50). Cell suspensions of tattooed or control skin were stained with anti CD1a (top) antibody or with anti-cytokeratin antibody (bottom).

Shielding of the cationic surface charge of nanoparticles results in an increased transfection efficiency and preserved immunogenicity of lipoplexes upon *in vivo* tattooing

The *in vivo* antigen expression and immunogenicity of the PEGylated nanoparticles was studied in C57/B6 mice. To this purpose, mice were vaccinated with naked DNA or lipo- or polyplex (both nonPEGylated and PEGylated) formulations using a standard tattoo vaccination protocol, with DNA administrations on day 0, 3 and 6 (8). To allow the simultaneous detection of vaccination-induced antigen expression and vaccination-induced antigen-specific T cell responses, a pVAX:Luc-NP model DNA vaccine was utilized. Use of this model DNA vaccine permits the monitoring of *in vivo* antigen expression by assessment of luciferase activity, while vaccine immunogenicity can be determined by monitoring of T cell responses against the vaccine-encoded influenza $NP_{366-374}$ epitope.

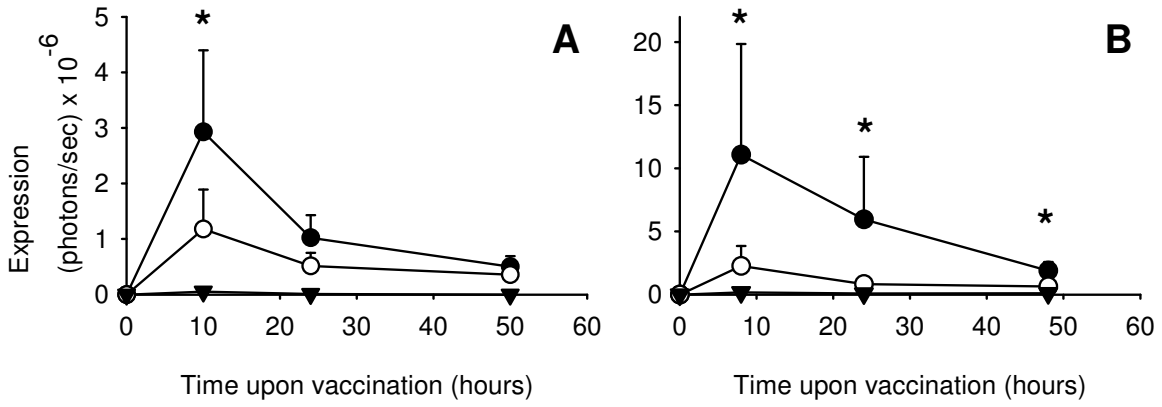


Figure 5: *In vivo* antigen expression in mice upon tattoo vaccination of:
(**A**) Naked DNA (○) or DOTAP-DOPE/ DNA lipoplex with (●) or without (▼) 17.5 mol % DSPE-PEG (both lipoplexes at an N/P ratio of 5). (**B**) Naked DNA (○), PEGylated PAA/DNA polyplexes (●) or nonPEGylated PAA/DNA polyplex (▼) (both polyplexes at a w/w ratio polymer/DNA 50). Expression of the vaccine-encoded antigen (luciferase) was measured at the indicated time points upon tattooing with a light sensitive camera. * Values significantly different from naked DNA control. Each point represents the mean + SD of 8 mice.

Consistent with the results from the *ex vivo* human skin model, PEGylation of nanoparticles was essential to obtain substantial antigen expression by either lipoplexes or polyplexes (with an increase in AUC of 73-fold and 55-fold by PEGylation for lipoplexes and polyplexes, respectively). Furthermore, PEGylated lipo- and polyplexes showed a significant increase in antigen expression as compared to the naked DNA control (Figure 5), where again the PEGylated polyplexes induced higher expression levels as compared to PEGylated lipoplexes. When compared to naked DNA, the AUC of antigen expression were 2.1 and 5.4 fold higher for lipo- and polyplexes, respectively.

To investigate whether shielding of the surface charge is sufficient to restore the immunogenicity of nanoparticle-formulated DNA vaccines, vaccine-induced, antigen-specific T cell responses were measured directly *ex vivo* in peripheral blood by staining with MHC tetramers (Figure 6). We focused exclusively on effector T cell immunity in this study since we are developing DNA tattooing as a method for therapeutic tumor immunization (20), which aims for high T cell titres (21). As expected, due to the absence of substantial levels of antigen expression, no significant T cell responses were detected in animals vaccinated with nonPEGylated nanoparticles. In contrast, vaccination with the matched PEGylated nanoparticles resulted in a strong T cell response that peaked between day 15 and 17, similar to that observed for the naked DNA control group. The magnitude of the antigen-specific T cell response induced by vaccination with naked DNA or with PEGylated lipoplexes or polyplexes was similar, with no significant difference (Figure 6). These data establish that the presence of the PEG moieties on the DNA nanoparticles is not only

sufficient to restore vaccination-induced antigen expression in human and murine skin but also leads to a full restoration of vaccine immunogenicity.

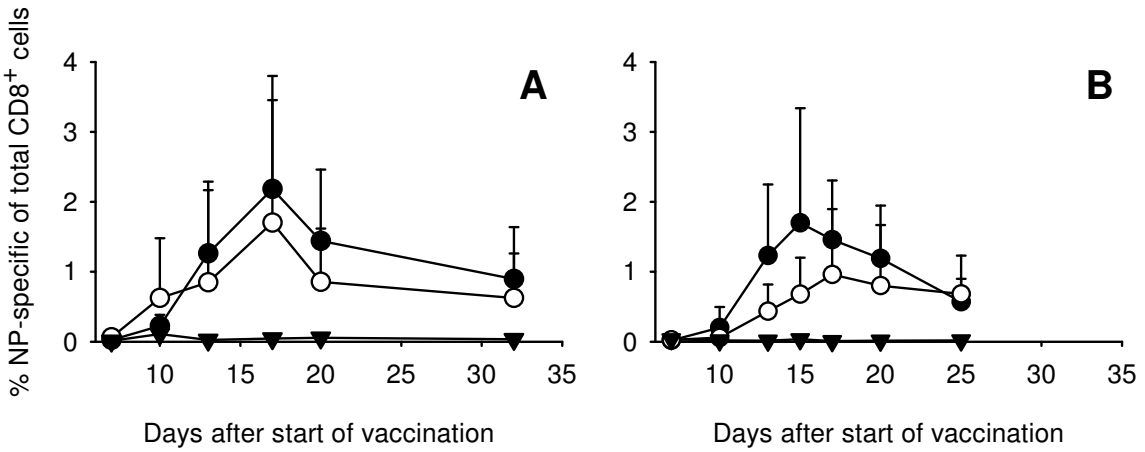


Figure 6: T cell responses upon tattoo vaccination of DNA nanoparticles. $NP_{366-374}$ specific T cell responses upon tattoo vaccination with the Luc-NP construct are shown for: (**A**) Mice vaccinated with naked DNA (○) or DOTAP-DOPE/ DNA lipoplex with (●) or without (▼)17.5 mol % DSPE-PEG (both lipoplexes at an N/P ratio of 5). (**B**) Mice vaccinated with naked DNA (○), PEGylated PAA/DNA polyplexes (●) or nonPEGylated PAA/DNA polyplexes (▼)(both polyplexes at a w/w ratio polymer/DNA of 50). NP_{366}-specific T cell responses were measured by direct *ex vivo* MHC tetramer staining of peripheral blood lymphocytes. Each point represents the mean + SD of 8 mice.

Discussion

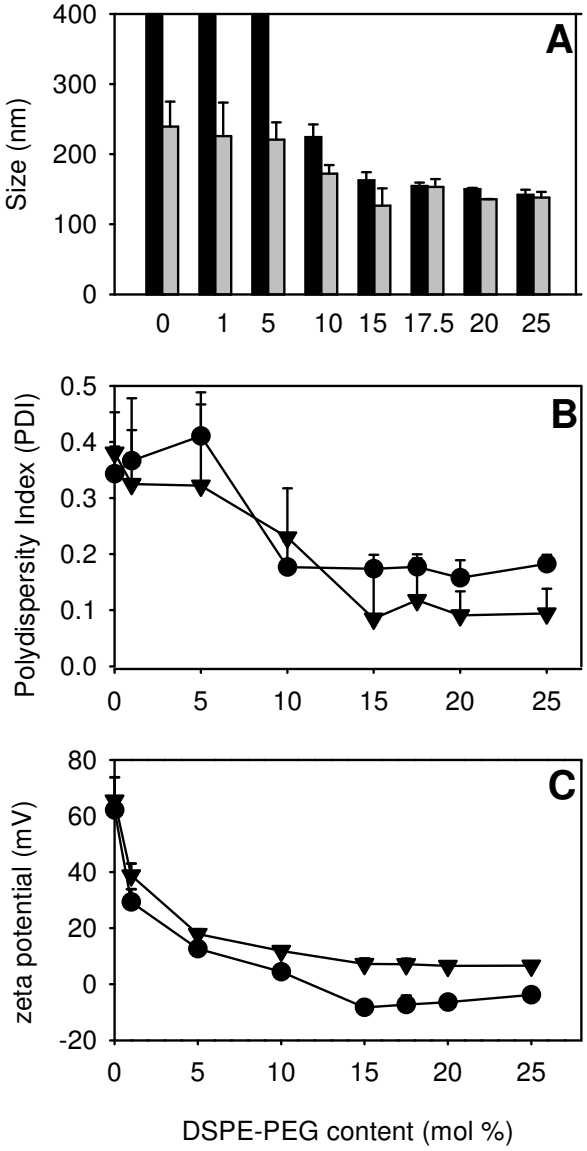
Incorporation of DNA in nanoparticles may offer the possibility to enhance cellular uptake and may offer the opportunity to develop intradermal DNA vaccines that are amenable to target specific cell types. A first requirement in this research is the development of particles that can be active *in vivo*. This study shows that cationic lipo- and polyplexes that are highly active in *in vitro* assays (22) (Figure 1A) yield only marginal vaccination-induced antigen expression in either murine or human intact skin. We demonstrate that the poor performance of cationic nanoparticles in the latter cases can be significantly improved by shielding the positive surface charge of the nanoparticles by PEGylation to generate near-neutrally charged nanoparticles that yield robust vaccination-induced antigen expression in both murine and human skin.

A possible explanation for the marked discordance between the effectiveness of cationic nanoparticles in cell culture and intact skin is the presence of ECM in intact skin. It is known that major ECM components in the skin (like proteoglycans and hyaluronic acid) have a negative charge at physiological pH (23). These negatively charged components may be responsible for electrostatic binding and immobilisation of the positively charged nanoparticles in the matrix after intradermal administration. Indirect support for this hypothese is also provided by the reported observation that inclusion of ECM components in the transfection medium can inhibit cellular uptake of lipo-and polyplexes in *in vitro* assays (24-26). Although PEGylated particles induce low levels of *in vitro* transfection their

performance upon *in vivo* tattooing was markedly improved compared to the unPEGylated particles and naked DNA. It is known that PEGylation of particles affects *in vitro* transfection both at the level of cellular uptake and intracellular trafficking. The transfection efficiency of PEGylated particles is generally 2-fold lower compared to nonPEGylated particles due to a decrease in cell binding and uptake (27,28). In contrast, the mobility of PEGylated particles through the cytosol upon *in vitro* microinjection is 2-fold faster than nonPEGylated particles (29). During intracellular trafficking, both PEGylated and nonPEGylated complexes are thought to be unpacked similar in the cytosol before the DNA can enter the nucleus (28). In our view, these *in vitro* data do not explain the marked difference in transfection properties observed between PEGylated and nonPEGylated particles upon *in vivo* tattooing but do indeed suggest that nonPEGylated particles do not reach the skin cells upon tattooing.

The current data demonstrate that PEGylation of the nanoparticles to a level that allows a near complete shielding of the surface charge suffices to restore and even enhance antigen expression in intact skin. These PEGylated nanoparticles give higher antigen expression than naked DNA controls in the *ex vivo* and *in vivo* experiments. It seems plausible that further optimization of nanoparticle properties and dosing may yield shielded formulations that give a further increase in *in vivo* antigen expression and immunogenicity.

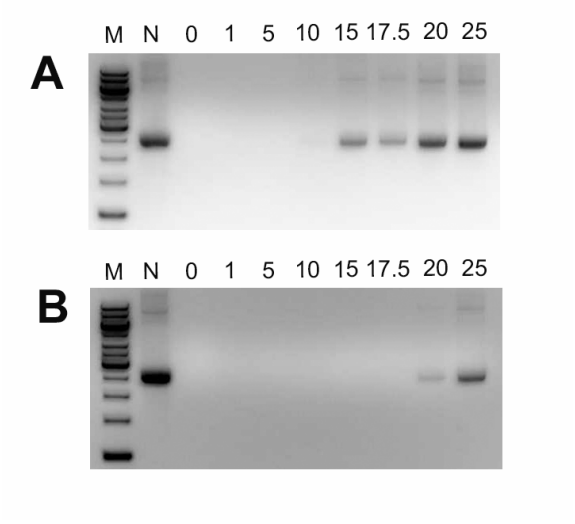
In addition to the optimization of the properties of shielded nanoparticle formulations to enhance vaccination-induced antigen expression, a second key step is the introduction of defined ligands within these formulations (30-32). The current shielded nanoparticles form a highly suited platform for such introduction, as ligands can readily be attached to the terminal ends of the PEG chains, using standard protocols. Two specific goals may be achieved by introduction of such ligands. First, introduction of ligands for defined cell surface receptor may enhance cellular uptake or may be used to target defined epidermal cell types (for example epidermal LCs). Second, the introduction of Toll-like receptor ligands or ligands for other pathogen-associated molecular pattern receptors on the surface of the nanoparticles is an attractive option to further enhance the immunogenicity of shielded nanoparticle vaccines, by providing an intrinsic danger signal.



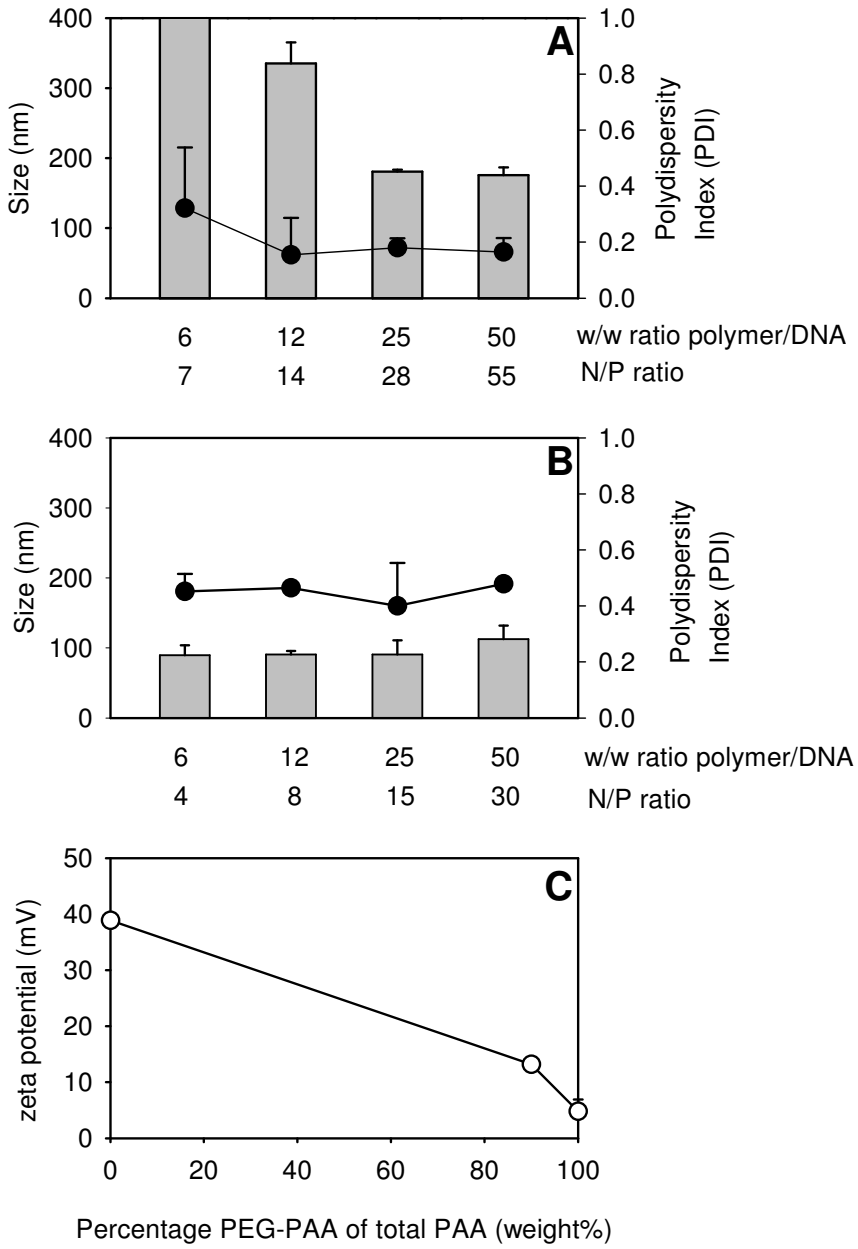
Supplementary Figure 1:. Lipoplex characteristics**:** effect of increasing DSPE-PEG content on size **(A)**, Polydispersity Index (PDI) (**B**) and ζ potential (**C**) of DOTAP-DOPE/DNA lipoplexes. Complexes were prepared at a charge (N/P) ratio of 2 (black bars or ●) and 5 (grey bars or ▼) at 0.5 mg/mL DNA in 20 mM HEPES pH 7.4, 10% sucrose.

Bars or symbols represent the mean + SD of three independent measurements.

Aggregated lipoplexes are indicated by bars that reach the upper axis.



Supplementary Figure 2: Condensation of plasmid DNA with DOTAP-DOPE liposomes containing increasing concentration of DSPE-PEG at a charge (N/P) ratio of 2 (**A**) or 5 (**B**). Each lane was loaded with 2 µl formulation, corresponding to 1 µg DNA, and samples were analyzed by gel electrophoresis. Lane M; l/Hind III DNA molecular-weight markers; lane N, naked DNA control; lane 0 to 25, DOTAP-DOPE/ DNA lipoplexes prepared with an increasing mol percentage of DSPE-PEG. Data demonstrate that up to a DSPE-PEG percentage of 10% (for N/P 2) or 17.5% (for N/P 5) all DNA is complexed as no free DNA is detected.



Supplementary Figure 3: Polyplex characteristics: effect of PEGylation on particle size (bars) and Polydispersity Index (PDI) (lines) of polymer/DNA polyplexes. Polyplexes were prepared at the indicated polymer/DNA ratios, either with nonPEGylated PAA polymer (**A**) or with PEGylated PAA polymer (**B**). (**C**) PEGylated and nonPEGylated PAA polymer was mixed at the indicated ratios (always in a 50/1 w/w ratio polymer/DNA) to obtain particles with a range of ζ potentials. Bars or symbols represent the mean + SD of three independent measurements. All complexes were prepared at 0.5 mg/mL DNA in 20 mM HEPES pH 7.4, 10% sucrose. Aggregated polyplexes are indicated by bars that reach the upper axis. Gel electrophoresis showed no free DNA in all polyplexes formulated at the tested ratios (gel not shown).

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Chapter 7

DNA tattoo vaccination: effect on plasmid purity and transfection efficiency of different topoisoforms

Susanne G. L. Quaak
Joost H. van den Berg
Koen Oosterhuis
Jos H. Beijnen
John. B. A. G. Haanen
Bastiaan Nuijen

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Abstract

Recently, DNA tattooing was introduced as a novel intradermal administration technique for plasmid DNA (pDNA) vaccines. The aim of this study was to determine if tattooing affects the integrity of the pDNA (reduction in supercoiled (SC) content) and whether a change in pDNA topology would affect antigen expression and immune response. We show that 1.) *in vitro* tattooing of pDNA solutions results in minor damage to pDNA (≤3% SC pDNA reduction) and only open circular (OC) pDNA formation, 2.) antigen expression and T cell responses upon DNA tattoo administration of SC and OC pDNA are equal in a murine model, 3.) SC pDNA gives a significantly higher antigen expression than OC and linear pDNA in *ex vivo* human skin, 4.) pDNA topology does not influence antigen expression when formulated as PEGylated polyplexes. We concluded that a 3% reduction in SC purity most likely will have little or no effect on clinical antigen expression and T cell responses. For intradermal tattoo administration the *ex vivo* skin model might be more suitable than the standard murine model for distinguishing subtle alterations in antigen expression of clinical pDNA formulations. The results from this study enable justification of release and shelf-life specifications of pDNA products applied by this specific route of administration, as requested by the regulatory authorities (≥ 80% SC).

Introduction

For the administration of nucleic-acid-based vaccines several routes have been investigated, including intramuscular (im), subcutaneous (sc), intravenous (iv), intradermal (id), nasal, and oral (1-3). Iv injection of naked DNA into the systemic circulation leads to its rapid clearance from the blood and degradation due to the presence of serum nucleases (4,5). In contrast to iv injection, im injection of naked DNA has proven successful in inducing high levels of antigen expression, however sustained immune responses are difficult to achieve (6,7). Dermal administration, such as gene-gun or jet-injection, is growing in popularity since the skin is rich in Antigen Presenting Cells (APCs), which are crucial in inducing a vaccine specific immune response (8). It has been shown that a single administration can already provide a full humoral and cellular response, with antibody titers being higher than those achieved after im injection (2,8). Recently DNA tattooing has been described as a simple and promising id DNA vaccination technique (9,10). Using this tattoo technique, pDNA encoding for a specific antigen is punched into the skin with an oscillating needle array, where expression of the encoded antigen leads to a rapid and robust induction of both T- and B-cell responses (9). At our institute a phase I clinical trial with pDNA encoding recombinant MART-1 and tetanus toxin fragment-c (pDERMATT) (11) for the treatment of melanoma, using tattoo administration, is currently ongoing.

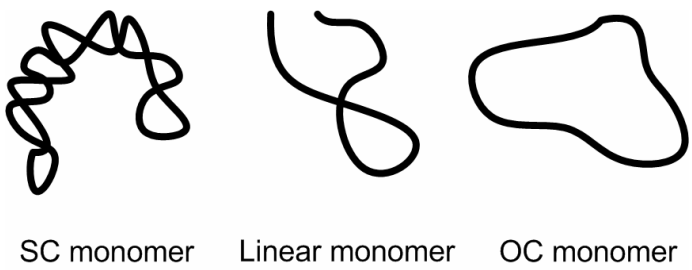

Figure 1: Topoisoforms of pDNA. All forms can also exist as dimmers.

pDNA is considered a very stable molecule, however during production and administration it can undergo various types of stress leading to degradation (12). Degradation is initiated by disruption of the tertiary structure of the molecule, which can result in supercoiled (SC) conversion into open circular (OC) or linear pDNA (Figure 1). This can be caused enzymatically (e.g. by nucleases), chemically (e.g. by high pH) or physically (e.g. by high shear stress) (12,13). It is known that gene transfer methods like jet injection cause pDNA damage as an effect of shear forces (14,15). Since SC pDNA is the only intact and undamaged plasmid form (16), this form is considered to have the highest transfection efficiency and is therefore most appropriate for therapeutic applications (17,18). However, this assumption is still controversial since contradictory results have been published. Depending on the used carrier or cell line in *in vitro* experiments SC pDNA performs better (17,19) or similar (20,21) to OC in transfection efficiency. In addition, *in vivo* experiments showed that the SC topoisoform of naked pDNA is more effective (up to three times) (20,22) upon im injection, whereas no difference between the topoisoforms was observed when

complexed with a cationic lipid carrier (21) upon intra tumoral injection. The European Medicines Agency (EMEA) requires specifications for acceptable proportions of the molecular forms of the plasmid which contribute to efficacy of the pDNA product (www.emea.europe.eu/pdfs/human/bwp/308899en.pdf). In line with this, the US Food and Drug Agency (FDA) recommends that the fraction of the SC topoisoform in pDNA vaccines is at least 80% (www.fda.gov/cber/gdlns/plasdnavac.htm). In this study we assessed if tattooing induced any damage to pDNA in terms of degradation from SC to OC and linear forms, using pDERMATT as template. Secondly, we assessed the effects of an increased OC content on biological activity upon DNA tattooing of both mice and in *ex vivo* human skin, in order to assess the potency of each topoisoform for this specific route of administration.

Materials and methods

Chemicals

The pVAX:Luc-NP plasmid (9) contains the influenza A $NP_{366-374}$ epitope as a genetic fusion with firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). pDERMATT (3771 bp) and pVax:Luc-NP (4706 bp) were produced in house as described before (11). Tris, NaCl and water for chromatography were obtained from Merck (Darmstadt, Germany). Sterile water for injections (Wfl) was purchased from B. Braun (Melsungen, Germany). Isopropanol and 25% (w/v) HCl from Biosolve B.V. (Amsterdam, The Netherlands). Poly amidoamine (PAA) copolymers possessing protonable amino groups and bioreducible disulfide linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of *N,N'*-cystaminebisacrylamide with the appropriate amine according to the procedure described previously (23). The poly ethylene glycol (PEG)ylated analogue was prepared using 11 mol% of MeO-PEG-NH_2 in the total amino monomer feed during the PAA synthesis. All chemicals obtained were of analytical grade.

Chromatography

pDERMATT purity was determined by a validated anion-exchange high pressure liquid chromatography (AEX-HPLC) assay as described previously (24). Additionally capillary gel electrophoresis (CGE) of pDERMATT and pVAX:Luc-NP was performed at PlasmidFactory (Bielefeld, Germany) as published earlier (25).

Agarose gel electrophoresis

Agarose gels were run in a Horizon 20·25 horizontal gel electrophoresis unit coupled to a Whatmann Biometra power supply (Westburg B.V., Leusden, The Netherlands).
Analysis was performed by electrophoresis using 25cm, 1% agarose (ABGene, Epsom, Surrey, UK) self cast gels. Running buffer was a 40mM Tris-Acetate, 1mM EDTA, pH 8.3 solution and

electrophoresis was carried out at 30V for 21 hours. After electrophoresis, gels were stained for 1.5 hours with a 1x Sybr green I solution (Sigma Aldrich Chemie, Zwijndrecht, The Netherlands) and then visualized and photographed under UV light (GeneGenius, Westburg B.V., Leusden, The Netherlands). The samples were diluted, if necessary, to approximately 20 ng pDNA/µl. Of the resulting solutions 10µl was mixed with 2µl of 6x loading dye and this was subsequently loaded onto the gel.

In vitro tattooing experiment

50µl of a solution containing 5 mg/ml pDNA in Wfl or in a 10% sucrose solution was transferred into a well plate. Subsequently these solutions were exposed to an oscillating 9 needle array set at 100 Hz (Permanent Make Up (PMU)® tattoo device, kindly provided by MT Derm GmbH, Berlin, Germany) for 0, 20 or 120 seconds (n=2 for each solution).

Preparation of linear and open circular pDNA

Linear pDNA species were generated by incubating SC pVAX:Luc-NP with *Bam*HI (cuts between CMV promoter and insert), *Nru*I (cuts between pUC origin of replication and CMV promoter), or *Xba* (cuts after insert), all single-cutting restriction enzymes, (New England Biolabs, Hertfordshire, England, UK) according to the instruction of the manufacturer. Open circular (OC) pDNA was generated using *N.BstNB*I (New England Biolabs, Hertfordshire, England, UK) according to the instruction of the manufacturer. The efficiency of the sample digestion was confirmed using agarose gel electrophoresis. Subsequently the OC pDNA was precipitated and reconstituted with Wfl in such a volume that the final concentration was 1 mg/ml. For administration in the *ex-vivo* human skin model this solution was mixed with a 1 mg/ml SC solution to obtain ratios of approximately 75, 80, 85, 90, and 95% SC pVAX:Luc-NP. These solutions were tattooed into the *ex vivo* human skin model using a randomisation protocol. For the murine model, 100% OC and 100% SC solutions in a concentration of 1 mg/ml were used.

Preparation of polyplexes

Polyplexes were prepared by mixing an equal volume of SC or OC plasmid (pVAX:Luc-NP) and PEG-PAA, resulting in polyplexes with an optimal charge ratio carrier/pDNA of 30 (36). All formulations were prepared in 20 mM HEPES pH 7.4, 10% sucrose buffer with a high viscosity and a low ionic strength, conditions previously shown to be favourable for obtaining small and stable DNA complexes (26). All complexes were formulated with a final DNA concentration of 0.5 mg/mL. Control naked DNA was diluted to the same concentration in the same buffer.

Characterization of polyplexes

The size of obtained particles was measured in 20 mM HEPES pH 7.4, 10% sucrose, with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4, using a Zetasizer Nano Z (Malvern Instruments). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound DNA was visualized by electrophoresis at 85 V using a 1% agarose gel containing 0.5 µg/mL etidium bromide.

In vivo experiments

Mice

C57BL/6J mice (6-8 weeks) were obtained from the experimental animal department of The Netherlands Cancer Institute. All animal procedures were performed according to approved protocols and in accordance with recommendations for the proper use and care of laboratory animals. All animal experiments were approved by the Institute's Animal Research Committee.

DNA immunization

Mice were vaccinated with pVAX:Luc-NP (1 mg/mL in Wfl) 100% OC or 100% SC. For intradermal DNA vaccination, the hair of the left hind leg of the mice was removed with depilatory cream (Veet sensitive). Next, 10 µl of the pVAX:Luc-NP solution was applied to the skin and tattoo administered. Needle depth was adjusted to 1.0 mm, and the needle bar oscillated at 100 Hz. DNA vaccines were punched into the skin by a 30 s tattoo. Mice were vaccinated with a standard vaccination scheme on day 0, 3 and 6 (9). All mice were anesthetized with isoflurane (Abbott Laboratories, Illinois, USA), during treatment.

Longitudinal measurement of antigen expression in mice

Mice were anesthetized with isoflurane. A solution of the firefly luciferase substrate luciferin in PBS (150 mg/kg, Xenogen, Hopkinton, USA) was injected intraperitoneally and 18 min later the luminescence produced by active luciferase was acquired during 30 s with the IVIS® system 100 CCD camera. Signal intensity was quantified as the sum of all detected light within the region of interest, after subtraction of background luminescence.

Antigen specific T cell assay

To measure antigen specific $CD8^+$ T cell responses, peripheral blood lymphocytes were stained with PE-conjugated H-$2D^b$/$NP_{366–374}$-tetramers and APC-conjugated CD8α antibody (BD Pharmingen, San Jose, USA) at 20°C for 15 min in FACS buffer (1× PBS, 0.5 % BSA and

0.02 % sodium azide) as described previously (27). Subsequently, cells were washed three times in FACS buffer and analyzed by flow cytometry. Living cells were selected based on propidium iodide exclusion. Data acquisition and analysis was done with a FACSCalibur (Becton Dickinson, Franklin Lakes, USA) and data were analyzed using Flowjo software (Three Star, Ashland, USA).

Ex vivo human skin model

Tattooing of *ex vivo* human skin

Healthy human abdominal skin from female patients was obtained from the plastic surgery department, according to institutional guidelines. Subcutaneous fat was directly removed by blunt dissection. DNA solutions were administered into the intact skin, within 2 hours after surgical removal, by DNA tattooing to allow longitudinal luciferase expression measurements. This skin model was described before for the optimization of tattooing of naked DNA in skin (28). In brief, 10µl of the formulation was applied onto the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8 mm, surface 50 mm^2). The droplet of formulation was subsequently administered into skin using a PMU® tattoo machine. The needle depth was adjusted to 1.5 mm and tattoo duration was 20 seconds. After tattooing, skin samples were kept at 5% CO_2, 37ºC in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 2.5 µg/ml amphotericin B (all Invitrogen). During this incubation, skin was cultured at the air-medium interface with the epidermis exposed to the air to mimic the natural situation.

Longitudinal measurement of antigen expression in human skin

The expression of luciferase was measured in intact skin samples at 3, 18, 24 and 48 hours after tattooing. As literature shows that human skin in culture does not lose its viability in the First 30 hr of culturing, with a viability decrease of 50% after 60 hr in medium at 37°C, measurements of antigen expression levels over the time period used in these experiments is considered to be appropriate (28-30). The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/ml. 30 minutes after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS system 100 CCD camera (Xenogen, Hopkinton, USA). Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. During each measurement, background luminescence was measured to allow correction during data analysis. After each measurement, medium was refreshed to remove residual luciferin.

Linear mixed effect model

To study the effect of different amounts of SC pDNA on the level of antigen (i.e. luciferase) expression, the luciferase expression over the 48 hour period was analysed in human skin. The natural log transform of the area under the curve (AUC) was plotted versus the amount

of SC pDNA in the solution. To account for possible within skin correlation of the repeated measurements of antigen expression, a linear mixed effects model was constructed. The fixed effect was the SC density. Patient identifier was implemented as the random grouping variable. Pair-wise interactions were examined between all fixed effects. The significance level was set at 0.05. Conventional residual analysis was performed to assess model fit. Data analysis was performed with Splus v6.2 pro (Insightful, Seattle, USA).

Statistical analysis

A two-tailed Mann-Whitney U-test was used for statistical analysis other than the linear mixed effect model and a value of $p < 0.05$ was considered significant.

Results

pDNA damage by tattooing

In a phase I clinical trial at our institute, the plasmid pDERMATT is administered using a dermal tattoo device equipped with a 9 needle (0.3 mm diameter, solid) array at a concentration of 5 mg/ml and an effective tattoo time of 20 sec/10 µl. To mimic the clinical application *in vitro*, 5 mg/ml pDERMATT solutions were exposed to the tattoo device for 0, 20 or 120 seconds in a well plate. Potential damage in terms of reduction in SC pDNA levels was determined using agarose gel electrophoresis (AGE), CGE and AEX-HPLC analysis. In Table 1 it is shown that degradation of pDERMATT in this tattoo-timeframe is very limited with a maximal decrease in SC content of approximately 3%. Also, no significant effect of sucrose, present as excipient in the pDERMATT formulation, is seen. Figure 2 gives CGE (Fig 2A), HPLC (Fig 2B) and AGE (Fig 2C) data of tattoo stressed samples. It is shown that exposure of SC pDNA to the tattoo device causes an increase in OC forms. No linear forms were detected and the amount of SC dimer remains constant. Also, no significant difference in degradation of pDERMATT compared to pVAX:Luc-NP was seen in this experiment. Since pVAX:Luc-NP is a larger plasmid compared to pDERMATT (4.7 vs 3.7 kD), this molecule is possibly less resistant to shear stress and thus more susceptible to degradation (12).

Table 1: Percentage of topoisoforms of pDERMATT and pVAX:Luc-NP normalised to purity at t=0 after different *in vitro* tattooing times (5 mg/ml in Wfl or 10% sucrose), measured with capillary gel electrophoresis (CGE).

Plasmid (solute)	Tattoo time (sec)	SC purity (%)
pDERMATT (Wfl)	20	98.7
	120	98.6
pDERMATT (sucrose)	20	97.0
pVAX:Luc-NP (Wfl)	20	96.8

Wfl= water for injections, SC= supercoiled

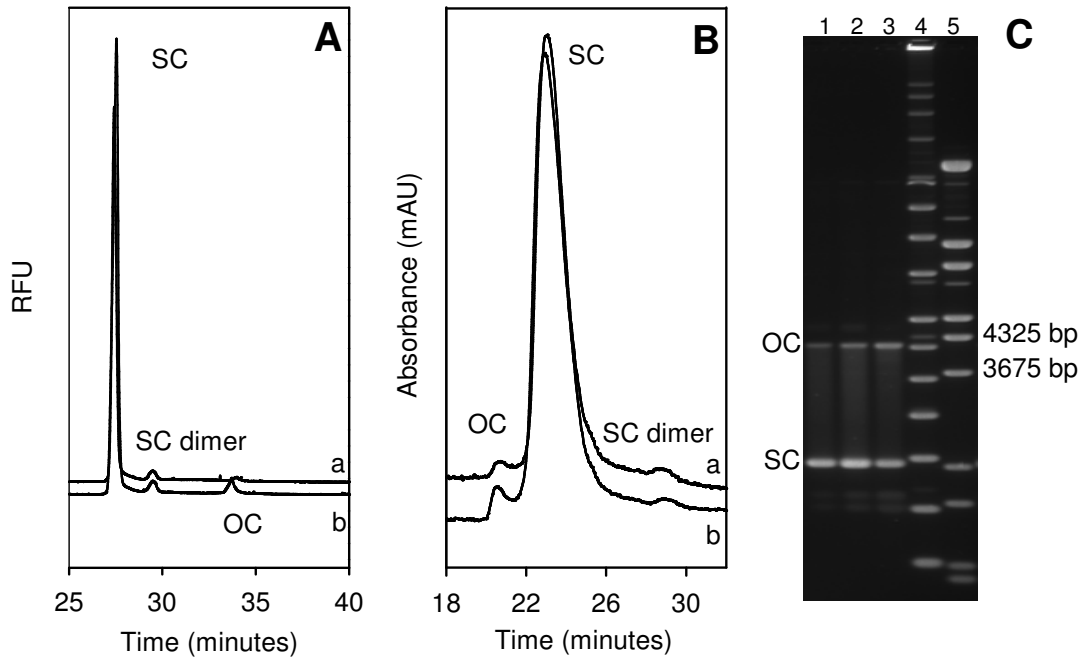

Figure 2: Representative chromatograms of pDERMATT (5 mg/ml in Wfl) after 120 sec *in vitro* tattoo stress (b) compared to the reference (a). CGE, 100 ng (**A**); HPLC, 1 µg and 260 nm detection (**B**); and AGE picture (**C**) of pDERMATT samples, 200 ng after 0 (lane 1), 20 (lane 2) and 120 sec (lane 3) *in vitro* tattoo stress; lane 4, supercoiled DNA ladder; lane 5 linear ladder (Lambda DNA *BstE*II digest). If linear pDERMATT (3771 bp) was present it would be visible just above the 3675 bp band of the linear ladder.

Antigen expression and immunogenicity of pDNA topoisoforms in mice

pDNA topology is thought to affect biological activity of pDNA products. To assess the influence of pDNA topology on *in vivo* antigen expression and immunogenicity of DNA vaccines applied by DNA tattooing, mice were vaccinated with 100% OC or 100% SC pDNA (as determined by HPLC and AGE analysis) using a standard tattoo vaccination protocol (DNA administration on day 0, 3 and 6) (9).

To allow the simultaneous detection of vaccination-induced antigen expression and vaccination-induced $CD8^+$ T cell responses, a pVAX:Luc-NP model DNA vaccine was utilized. Use of this model DNA vaccine permits the monitoring of *in vivo* antigen expression by assessment of luciferase activity, while vaccine immunogenicity can be determined by monitoring of $CD8^+$ responses against the vaccine-encoded influenza $NP_{366-374}$ epitope. Surprisingly, the murine model showed no significant difference in antigen expression upon the administration of OC or SC pDNA (Figure 3A), as measured by intravital imaging. This resulted in a comparable antigen-specific T cell response after vaccination with OC or SC pDNA (Figure 3B), as determined directly *ex vivo* in peripheral blood by staining with MHC tetramers. T cell response reached a maximum 16 days after vaccination.

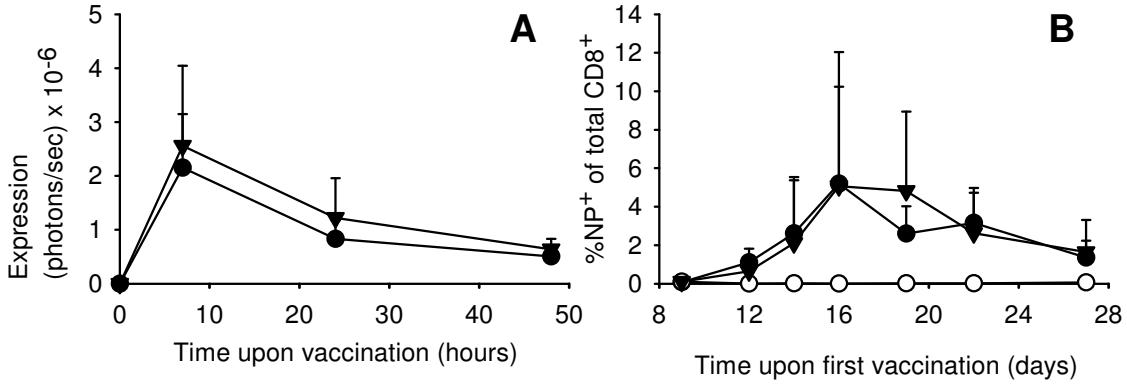

Figure 3: *In vivo* antigen expression (**A**) and antigen specific $CD8^+$ T cell responses (**B**) in mice upon tattoo vaccination of 100% supercoiled DNA (●), 100% open circular DNA (▼) or unvaccinated controls (○). DNA was used at a concentration of 1 mg/ml in water for injection. Expression of the vaccine-encoded antigen (luciferase) was measured at the indicated time points upon tattooing with a light sensitive camera. $NP_{366-374}$ specific T cell responses were measured by direct *ex vivo* MHC tetramer staining of peripheral blood lymphocytes. Each point represents the mean + SD of 15 mice.

Antigen expression of pDNA topoisoforms in *ex vivo* human skin

To assess the effect of pDNA degradation on antigen expression upon DNA tattooing in *ex vivo* human skin, pVAX:Luc-NP solutions in Wfl containing 100% OC and 100% linear DNA were prepared. Three different linear preparations were made to examine if the location of the strand break has an influence on antigen expression.

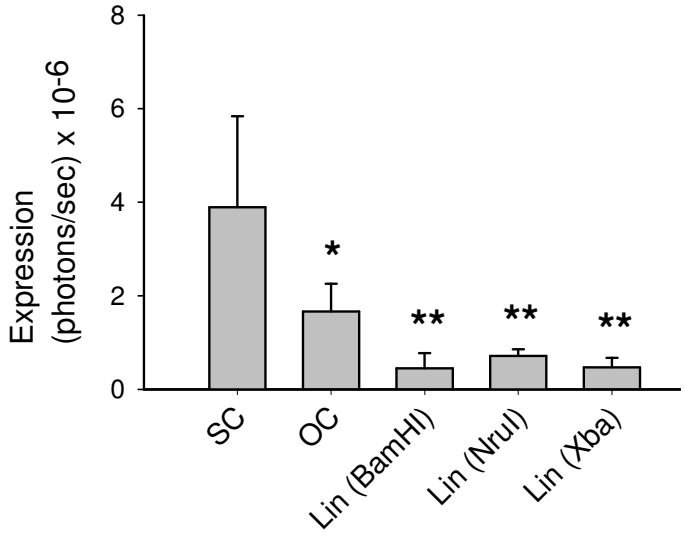

Figure 4: Luciferase expression in human skin after tattooing different topoisoforms of pVAX:Luc-NP (t=21 h after administration). Linear DNA is cut between promoter and copy DNA (*Bam*HI), pUC origin of replication and promoter (NruI) or after copy DNA (Xba). pDNA concentration is 1 mg/ml. Abbreviations: SC = supercoiled, OC = open circular, Lin = linear. Each bar represents the mean + SD of 5 measurements (* p = 0.045; ** p = 0.006 compared to SC control).

As can be seen in Figure 4, and in contrast to the *in vivo* experiment, SC pDNA significantly gives the best antigen expression after tattoo administration compared to the other formulations. OC pDNA still has about half the efficiency of SC pDNA, and interestingly, all three forms of linear pDNA give antigen expression, even when cut between promoter and copy DNA (possibly due to self-ligation), although only at low levels. Additionally, the antigen expression of mixtures of OC and SC naked pDNA containing 75, 80, 85, 90, 95 and 100% SC pDNA were assessed as this concentration range is relevant from a pharmaceutical

perspective (release and shelf-life specifications) as well as from a clinical perspective (degradation upon administration). For this experiment, a total of 69 skin areas, divided over 4 samples of healthy abdominal skin, were tattooed with pVAX:Luc-NP. Linear mixed effect data analysis showed that with a decreasing SC content, the antigen expression decreases significantly ($p < 0.01$) (Figure 5). A reduction in SC content of 25% results in a reduced antigen expression of approximately 10%. This non-proportional decrease is due to the fact that OC pDNA still gives relatively high levels of antigen expression (Figure 4). From the relationship between SC content and antigen expression it is extrapolated that a decrease of 3% in SC form, the maximal conversion found in the *in vitro* experiment, will lead to a decrease of only 1% in antigen expression in *ex vivo* human skin.

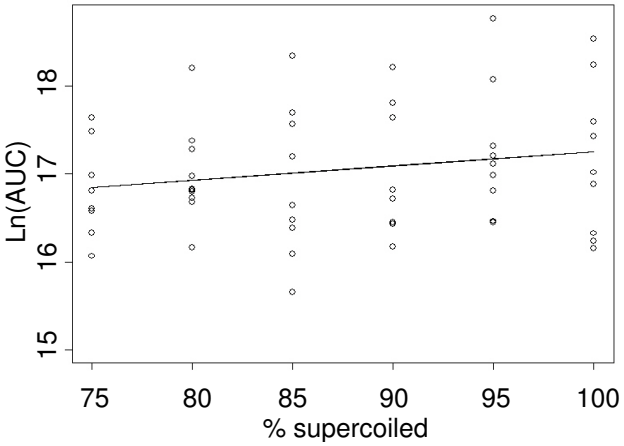

Figure 5: AUC values of luciferase expression (photons/second) in human skin after tattoo administration of DNA at different supercoil levels (1 mg/ml in Wfl, needle depth 1.5 mm, 100 Hz, 20 sec). The line visualises the predicted AUCs obtained from the model equation

Recently we obtained very promising results with tattoo administration of pDNA complexed with poly ethylene glycol (PEG)ylated poly amidoamine (PAA) copolymers (36). Since these polyplexes show a substantially higher expression compared to naked pDNA in the *ex vivo* human skin model, we examined if topology of the complexed pDNA has an influence on antigen expression. The polyplexes were formulated with a final pDNA concentration of 0.5 mg/ml and characterised for particle size and surface charge (as reflected by the zeta potential). Both OC and SC DNA-nanoparticles had particle sizes below 130 nm, did not contain free DNA (as determined by gel electrophoresis) and exhibited an almost neutral zeta potential (table 2).

Table 2: Characteristics of pDNA polyplexes with carrier/pDNA ratio of 30. The results are expressed as mean values of 3 measurements ± (standard deviation).

Plasmid DNA	Size of polyplexes (nm)	Polydispersity Index (PDI)	ζ-potential (mV)
SC pDNA	121.4 (13.5)	0.48 (0.01)	6.4 (2.1)
OC pDNA	125.5 (7.3)	0.46 (0.02)	8.6 (0.7)

Interestingly, tattoo administration of polyplexes containing 100% OC or 100% SC pDNA did not result in a significant difference in antigen expression (Figure 6). Moreover, in line with previous findings (36) antigen expression of both OC and SC polyplexes was significantly (3-

fold) higher than naked SC pDNA. And control naked OC pDNA resulted in approximately 50% of the naked SC pDNA response.

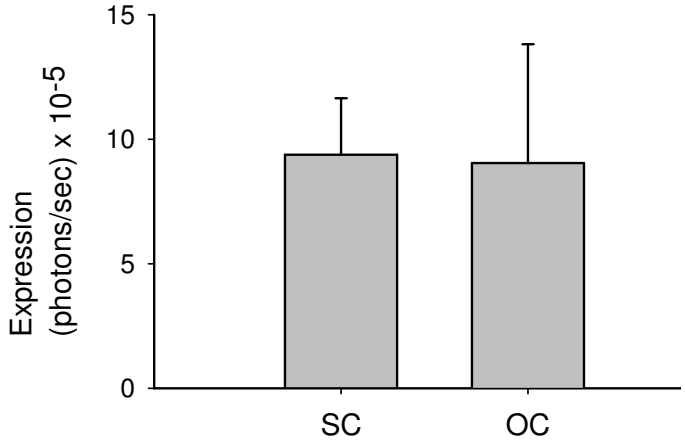

Figure 6: Luciferase expression in human skin after tattooing complexed open circular (OC) and supercoiled (SC) pVAX:Luc-NP (t=22 h after administration). pDNA concentration is 0.5 mg/ml. Each bar represents the mean + SD of 8 measurements.

Discussion and Conclusion

Novel dermal gene transfer methods are likely to cause more stress to pDNA than the classical single needle injection as these administration techniques either use a high pressure (jet injection - 3 bar) (31) or high voltage (electroporation - up to 1000V/cm skin) (32-34). For example, for a jet-injection application a decrease of 20% in SC content has been reported (14). During our DNA tattoo administration technique in a "mimic-the-clinic" experiment only a limited reduction in SC pDNA content (3%) was observed with formation of only OC forms but no linear DNA forms. Therefore, compared to jet-injection tattooing can be considered mild. Upon intramuscular injection of naked pDNA it has been shown that the SC topoisoform is up to three times more efficacious than OC DNA (20,22). However, when we adminstered 100% OC pDNA to mice using DNA tattooing, no difference in both antigen expression and vaccine-induced immune responses could be demonstrated compared to the use of 100% SC pDNA, indicating that for DNA tattooing the DNA topoisoform is much less critical for induction of vaccine-specific immunity. This, however, may be dependent on the model since tattooing of OC and SC pDNA in an *ex vivo* human skin model (28) showed that OC pDNA led to about half the amount of antigen expression of SC pDNA. This difference in transfection efficiency observed between murine and *ex vivo* human skin might be due to inter species skin variation. Murine skin has a much thinner stratum corneum and epidermis than human skin (35), therefore permeation rates of the epidermis are generally higher in murine skin. Indeed, tattoo administration transfects both epidermal and dermal cells in murine skin (9), whereas it almost exclusively transfects epidermal cells in human skin (28). Although also in murine skin keratinocytes are transfected upon DNA tattooing, we cannot exclude that the transfection of other cell types, especially those residing in the dermal skin layer, are responsible for the observed difference in antigen expression of OC DNA in murine versus human skin. Based on these results we consider that although murine experiments are crucial for studying immunogenicity of

newly designed DNA vaccines, the *ex vivo* human skin model is thus far the strongest and clinically most relevant tool to optimize antigen expression properties of pDNA vaccines.
We show in the *ex vivo* human skin model that when the percentage of SC pDNA is lowered from 100% to 75%, antigen expression drops significantly, but only with 10%. This limited loss in antigen expression may be explained by the fact that OC pDNA still gives relatively high levels of antigen expression (Fig 4), since the encoding region is not damaged (17). From the relationship between extend of SC pDNA and antigen expression, the reduction in transfection efficiency can be deduced. With a maximum decrease of 3% measured in SC content that is attributed to the tattoo administration, the effect on antigen expression will only be marginal (approximately 1%). Most likely, this very small decrease in antigen expression will have no significant effect on T cell response in clinical vaccination, as the size of the induced vaccine-specific T cell response is proportional to the level of antigen expression (9). Therefore, the requested SC purity of ≥ 80% SC pDNA for clinical use of a DNA vaccine may result in ≤8% reduction in antigen expression when using tattooing, which seems a very acceptable figure considering that in most clinical settings the SC DNA content of the vaccine will be >90%. Interestingly, when pDNA is formulated into PEGylated PAA polyplexes, pDNA topology does not influence transfection efficiency (Fig 6). This is in contrast to previously reported *in vitro* data, were polyplexes formulated with OC pDNA showed lower transfection efficiencies than polyplexes formulated with SC pDNA (19). Possibly, the electrostatic interactions between the cationic polymer and the anionic pDNA recompact the open circular topoisoform, making it again as efficacious as SC pDNA. This result may be translated to other nonviral vector systems, e.g. cationic lipids. Therefore, when formulating pDNA with these nonviral vectors, specifications for the used plasmid can be less stringent with respect to SC purity, but this needs to be determined on a case by case basis.
We conclude that the reduction in SC purity observed by tattoo administration most likely will have little or no effect on clinical antigen expression and T cell responses. In addition, for the intradermal tattooing administration technique the *ex vivo* skin model might be more suitable for distinguishing subtle alterations in antigen expression properties of clinical pDNA formulations as compared to the standard murine model. The results of this study enable justification of release and shelf-life specifications of pDNA products applied by this specific route of administration, as requested by the regulatory authorities (≥ 80% SC).

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Chapter 8

Combining DNA tattooing, electroporation and non-viral nanoparticles for the intradermal delivery of DNA vaccines

Joost H. van den Berg
Wim E. Hennink
Gert Storm
Jos H. Beijnen
John. B. A. G. Haanen
Ton N. Schumacher
Bastiaan Nuijen

Manuscript in preparation

Abstract

Both tattooing and electroporation are promising techniques for the intradermal delivery of DNA vaccines. To increase transfection efficiency of DNA vaccines applied by tattooing, the present study introduces electroporation into the current tattooing procedure ("electrotattooing").

First, we demonstrated that DNA tattooing immediately followed by electroporation with a common plate-fork electrode improved antigen expression in an *ex vivo* human skin model by 3-fold. After that, the electrotattooing strategy was combined with a previously described non-viral PEGylated polymeric poly(amido amine) (PAA) gene carrier (PEG-PAA/DNA polyplex). The combination of electrotattooing and the carrier had a synergistic effect on vaccine-induced antigen expression: where electrotattooing increased expression by a factor of 3 and the PEGylated PAA/DNA polyplex increased it by a factor of 7, the combination of the two resulted in a 20-fold increase in expression.

These data demonstrate that electrotattooing is a promising strategy to further increase the transfection efficiecy of DNA vaccines applied via tattooing.

Introduction

DNA vaccination is an attractive approach for the induction of an antigen specific immune response against viral infections and tumors. Although intramuscular injection forms the classical route of administration (1), the interest in the delivery of DNA vaccines by intradermal administration is now growing. Because of its natural barrier function and high density of antigen-presenting cells, the skin is well-equipped for the induction of an adaptive immune response.

Among the different strategies described for the dermal delivery of DNA vaccines, DNA tattooing appeared to be one of the most potent (2). By using a common tattoo device, DNA can efficiently be delivered by thousand of punctures into the epidermis of the skin (3). DNA tattooing leads to the induction of strong and rapid antigen specific cellular immune responses in both mice (2) and non-human primates (4). Despite this high immunogenicity, we previously calculated that the *in vivo* transfection efficiency of naked DNA by tattooing is extremely low. At most, 1 out of $5x10^6$ plasmid copies applied is taken up, transcribed and translated (3). Therefore, we are currently exploring possibilities to enhance the transfection efficiency of DNA tattooing, thereby aiming to increase the immunogenicity of the technique.

One potent strategy to increase the transfection of naked DNA is electroporation (5). Electroporation uses short electrical pulses to destabilize cell membranes. Under optimal conditions, this will lead to the formation of reversible pores which allows the entrance of macromolecules such as DNA into the cell. This electropermeabilization is followed by electrophoretic displacement of the negatively charged DNA molecule into the cytoplasm of the cell (6). Electroporation is a widely accepted technique to increase transfection efficiency *in vitro* and *in vivo* and already used in clinic trials (7,8). Most DNA electroporation protocols consist of an intramuscular injection followed by the application of an electric field (9-11). In line with the current shift towards dermal DNA vaccination, recent animal studies described the combination of an intradermal injection with intradermal electroporation (12-16), to increase dermal gene expression.

Another possible approach to increase transfection of naked pDNA is the use of a non-viral carrier. Electrostatic interaction of e.g. a cationic lipid or polymer with negatively charged DNA leads to the formation of a nanoparticle (lipoplex and polyplex, respectively) [16]. By using nanoparticles cellular uptake of plasmid DNA can be increased and degradation by endonucleases can be prevented (17). Indeed, we have recently shown that such nanoparticles, when grafted with poly (ethylene glycol) (PEG), can significantly increase antigen expression levels compared to naked DNA upon tattooing (18).

The aim of the current study was to explore if electroporation can be combined with DNA tattooing ("electrotattooing") in order to increase the epidermal transfection efficiency of

both uncomplexed (naked) DNA vaccines as well as DNA vaccines complexed with a carrier (polyplex).

Materials and methods

Materials

The pVAX:Luc plasmid (2) encodes the firefly luciferase gene, inserted in the EcoRI/NotI site of minimal pVAX1 plasmid backbone (Invitrogen, Carlsbad, USA). Poly(amido amine) (PAA) copolymers possessing protonable amino groups and bioreducible disulfide linkages in the main chain and hydroxybutyl groups in the side chains (CBA-ABOL), were synthesized by Michael addition polymerization of *N,N'*-cystaminebisacrylamide with the appropriate amine according to the procedure described previously (19). The poly ethylene glycol (PEG)ylated analogue was prepared using 11 mol% of MeO-PEG-NH_2 in the total amino monomer feed during the PAA synthesis. All chemicals obtained were of analytical grade.

Preparation and characterization of polyplexes

Polyplexes were prepared by mixing an equal volume of plasmid and PAA or PEG-PAA, resulting in polyplexes with an optimal w/w ratio carrier/pDNA of 50 (18). All formulations were prepared in 20 mM HEPES pH 7.4, 10% sucrose buffer with a high viscosity and a low ionic strength, conditions previously shown to be favorable for obtaining small and stable DNA complexes (20). All complexes were formulated with a final DNA concentration of 0.5 mg/mL. Control naked DNA was diluted to the same concentration in the same buffer.

The size of obtained particles was measured in 20 mM HEPES pH 7.4, 10% sucrose, with dynamic laser scattering using an ALV/GCS-3 (Malvern Instruments, UK). Particle size distribution is described using the polydisperity index (PDI), ranging from 0 for a monodisperse to 1 for a heterodisperse preparation. The zeta potential of obtained particles was determined in 20 mM HEPES pH 7.4, using a Zetasizer Nano Z (Malvern Instruments). Both instruments were calibrated using polystyrene latex beads of defined size and electrophoretic mobility. The presence of unbound DNA was visualized by electrophoresis at 85 V using a 1% agarose gel containing 0.5 µg/mL etidium bromide.

In vitro electroporation experiment

40 µl of formulation (naked DNA or polyplexes) was transferred into a electroporation cuvette with a gap size of 2 mm, (Harvard Apparatus, Inc, Holliston, MA, USA). Subsequently, these formulations were exposed to electrical pulses, applied with a BTX ECM 830 electroporator (Harvard Apparatus).

DNA administration and electroporation of *ex vivo* human skin

Healthy human abdominal skin from female patients was obtained from the plastic surgery department of the institute according with the guidelines of the Antoni van Leeuwenhoek Hospital/ The Netherlands Cancer Institute. Subcutaneous fat was directly removed by blunt dissection. Skin was transported on ice and used within 2 hours after surgical removal. This skin model was described before for the optimization of tattooing of naked DNA in skin (3). For intradermal tattoo administration, 10µl of the formulation was applied onto the skin into a custom fabricated mould to keep the area of tattooing constant (diameter 8mm, surface 50mm2). The droplet of formulation was subsequently administered into skin using a Cheyenne® tattoo machine (kindly provided by MT Derm GmbH, Berlin, Germany). The needle depth was adjusted to 1.0 mm and tattoo duration was 20 seconds. A CUY663 stainless steel plate-fork electrode (Sonidel, Dublin, Ireland), consisting of a rectangular plate (10-mm long and 5-mm wide), and a fork consisting of three straight needles (10 mm long, 0.5 mm in diameter at 2.5-mm distance) was used for the delivery of electrical pulses. Immediately after tattooing, the fork electrode was inserted into the tattooed area, parallel to the skin surface. The plate electrode, sprayed with electrode solution (SignaSpray, Parker Laboratories inc, Fairfield, NJ, USA) was then placed on the tattooed skin surface, sandwiching the skin between both electrodes. The field distance between the fork and the plate was approximately 2 mm. Two series of four consecutive pulses of opposite polarity were administered to each site at a rate of 1 pulse/s.

After treatment, skin samples were kept at 5% CO2, 37ºC in complete keratinocyte serum free medium (SFM) containing 1% penicillin/streptomycin and 2.5 µg/ml amphotericin B (all Invitrogen). During this incubation, skin was cultured at the air-medium interface with the epidermis exposed to the air to mimic the natural situation (3).

Measurement of antigen expression using intravital imaging of *ex vivo* human skin

The expression of luciferase was measured in intact skin samples at the indicated time points after administration. The substrate luciferin (Xenogen, Hopkinton, USA) was added to the medium in a final concentration of 45 µg/mL. During this procedure extra medium was added to the box in which skin was incubated, to cover the complete epidermis of skin samples with fluid to guarantee full accessibility of luciferin to the tattooed areas. 30 minutes after the addition of the substrate, luminescence produced by active luciferase was acquired during 30 s with an IVIS system 100 CCD camera (Xenogen, Hopkinton, USA).

Signal intensity was quantified as the sum of all detected light within the tattoo area of interest. In all measurements, background luminescence was determined for non-treated skin to allow correction during data analysis. After each measurement, medium was refreshed to remove residual luciferin.

Statistical Analysis

A two-tailed Mann-Whitney U-test was used for statistical analysis and a value of $p < 0.05$ was considered significant.

Results

DNA tattooing followed by electroporation ("electrotattooing") results in higher gene expression than tattooing alone

To examine the effect of the combination of DNA tattooing and electroporation, human skin was tattooed with a luciferase encoding construct. Directly after tattooing, the tattooed area was sandwiched between a plate-and-fork electrode, followed by the administration of electrical pulses. Luciferase expression was measured with a light sensitive CCD camera. Combinations of a low voltage and a long pulse duration (75V/cm, 50ms) and a high voltage and short pulse duration (5000V/cm, 10µs) were tested, since both combinations have previously been described as optimal for intradermal electroporation of mouse skin (12,14,15). Figure 1 shows that antigen expression in *ex vivo* human skin increased more than 3-fold using this electrotattooing strategy as compared to DNA tattooing alone. This increase, however, was only observed when using an electroporation protocol with a high voltage and short pulse duration.

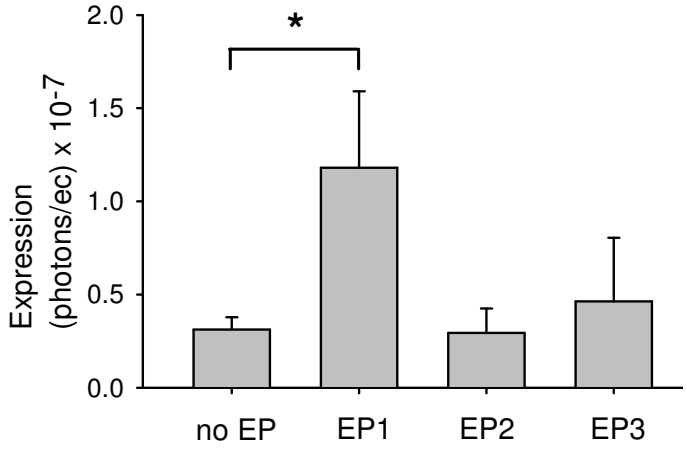

Figure 1: The combination of DNA tattooing with electroporation (EP).
Antigen expression (luciferase) upon tattoo administration of naked plasmid DNA in *ex vivo* human skin with or without EP. 50 µg of pVAX:Luc in 10 µl Water for injections (Wfl) was administered by intradermal tattoo administration, followed by immediately EP using a plate-and-fork electrode (condition EP1: 5000V/cm,10 µs, 8 pulses. condition EP2: 75 V/cm, 100 µs, 8 pulses. condition EP3: 75 V/cm, 50 ms, 8 pulses). Each bar represents the mean + SD of 3-4 data points (randomized over one skin biopsy). Data represent expression levels measured after 24 hours.
* Values significantly different from control without EP ($p<0.05$)

The combination of electrotattooing and a polymeric nanoparticle-formulation has a synergistic effect on antigen expression

We recently demonstrated that complexation of naked DNA with a PEGylated poly(amido amine) (PAA) (19) carrier resulted in the formation of nanoparticles which significantly enhanced transfection efficiency of naked DNA upon tattooing in *ex vivo* human skin and mice (18). To explore the effect of the combination of electrotattooing and the nanoparticle delivery strategy, PEG-PAA/ DNA and PAA/DNA nanoparticles were tattooed into *ex vivo* human skin with or without the application of electric pulses by a plate-and-fork electrode.

Figure 2 shows that normal tattooing of PEGylated nanoparticles resulted in a 7-fold increase in antigen expression, as observed previously (18). Electrotattooing of these PEGylated nanoparticles resulted in an additional 3-fold increase in antigen expression, similar to the relative effect of electrotattooing on antigen expression of naked DNA compared to normal tattooing. This means that combining electrotattooing with nanoparticle delivery resulted in an overall 20-fold increase in antigen expression compared to the same amount of naked DNA administered by just DNA tattooing.

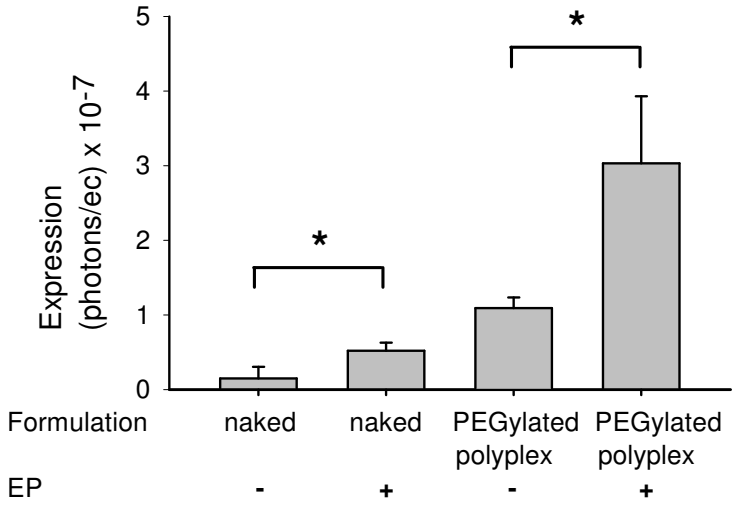

Figure 2: Antigen expression (luciferase) upon tattoo administration of naked plasmid DNA or PEGylated PAA/DNA polyplexes in *ex vivo* human skin with or without electroporation (EP). 10 µl of the indicated formulation with a final DNA content of 5 µg was administered by intradermal tattoo administration, followed by immediately EP using a plate-and-fork electrode (condition 5000V/cm,10 µs, 8 pulses). Each bar represents the mean + SD of 4-5 data points (randomized over one skin biopsy). Data represent expression levels measured after 18 hours. The experiment was repeated in duplicate.
* Values significantly different from control without EP ($p<0.05$).

Electrotattooing of non-PEGylated PAA/DNA polyplexes with an unshielded, positive surface charge (see Table 1) did not result in significant antigen expression (data not shown). This is in line with previous results which showed very low expression levels for this formulation after tattooing only, emphasizing the necessity of shielding the surface charge of these nanoparticles (18).

To study the integrity of the nanoparticles and naked DNA upon electroporation, formulations were electroporated *in vitro*, to mimic the conditions during administration. Electroporation did not affect particle sizes, zeta potential or DNA integrity (gel not shown) of the formulations tested, as shown in Table 1.

Table 1: Effect of *in vitro* electroporation on polyplexes and uncomplexed DNA. The results are expressed as mean values of 3 measurements ± (standard deviation).

Formulation		Particle size	PDI	Zeta potential
uncomplexed DNA	before EP	n.d.	n.d.	- 16.9 (0.5)
	after EP	n.d.	n.d.	- 17.4 (4.8)
PEGylated polyplex	before EP	105 (14)	0.46 (0.02)	+ 6.9 (0.2)
	after EP	105 (9)	0.49 (0.02)	+ 6.4 (0.9)
nonPEGylated polyplex	before EP	176 (4)	0.21 (0.05)	+ 41.8 (1.7)
	after EP	179 (8)	0.24 (0.03)	+ 42.1 (2.2)

PDI= polydisperity index, EP= electroporation, n.d. = not determined

Discussion

Electroporation is a technique that can potentially increase the transfection efficiency of dermally applied DNA vaccine formulations (12-16). In order to increase the low transfection efficiency of DNA tattooing, we tested if the introduction of electroporation into the tattooing protocol would lead to higher levels of transgene expression in *ex vivo* human skin (3). In these proof-of-concept experiments we showed that co-administration of electrical pulses during DNA tattooing resulted in a significant increase in antigen expression, compared to tattooing without electroporation.

Furthermore, we showed that electrotattooing of a PEGylated PAA/DNA nanoparticle formulation had a synergistic effect on gene expression in human skin (Figure 2), resulting in a 20-fold increase in antigen expression compared to tattooing of naked DNA. Since electroporation resulted in a similar increase in tattoo-induced antigen expression for both naked DNA and the PEGylated nanoparticles, the partly shielded (due to the PEG) and positive (due to the excess of cationic carrier) surface charge of the particles compared to the negatively charge naked DNA (see Table 1) probably did not have an inhibitory effect on the electromobilization of the particles into the electroporated skin cells, which is a prerequisite for successful electroporation (6). It has previously been shown that electroporation of cationic lipoplexes in tumor tissue resulted in similar (21) or lower (22) levels of gene expression compared to electroporation of uncomplexed DNA. The electroporation of murine muscle resulted in a similar level of expression for lipoplexes or uncomplexed DNA (22). Thus, the effect of electroporation on DNA nanoparticle mediated transfection probably strongly depends on the used carrier and target cell.

In conclusion, we showed that the combination of electroporation and tattooing ("electrotattooing") significantly increases antigen expression in *ex vivo* human skin. Furthermore, electrotattooing of a PEGylated PAA/DNA polyplex formulation showed the highest increase in antigen expression thus far. Optimization of the electrotattooing protocol and nanoparticle formulation together with the development of electrotattoo arrays that can cover larger areas of skin, is warranted in order to further translate these promising electrotattoo strategy towards clinical application.

Acknowledgements

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Chapter 9

Summary and Perspectives

Summary

DNA vaccination is an appealing strategy of active vaccination against viral infections and cancer. The delivery of bacterial plasmid DNA leads to the intracellular production of the encoding antigen which results in an efficient activation of an antigen specific immune response. Intradermal DNA tattooing was recently developed as a simple and robust method to induce antigen expression in the skin, followed by a systemic antigen specific T cell response (1). This thesis focused on optimizing 1) the pharmaceutical production and formulation and 2) the delivery of DNA vaccines administered by DNA tattooing.

After a general introduction (**Chapter 1**), **Chapter 2** of this thesis provides an overview of the literature on the use of synthetic vehicles as gene carriers to improve the performance of DNA vaccines. Besides an overview of current knowledge, this chapter gives future recommendations for the formulation, targeting, testing and clinical application of these carrier systems.

In **Chapter 3**, a generic production process is described for the small scale production of naked DNA vaccines for phase I and II clinical trials. The developed production process was reproducible, with a constant high quality, stability and yield of plasmid DNA. The manufactured melanoma DNA vaccine, pDERMATT (encoding Tetanus Toxin Fragment C and a MART-1 epitope), showed a robust epitope specific T cell response in a humanized murine model vaccinated by DNA tattooing and the product is currently under clinical evaluation in a phase I trial.

The effect of lipopolysaccharide (LPS), a common contamination of plasmid DNA, was assessed in **Chapter 4**. The presence of LPS (up to 5000 IU/mg DNA) in DNA vaccines, applied via DNA tattooing, neither induced systemic toxicity (as reflected by IL-6 concentration in serum), nor influenced antigen expression (measured by intravital imaging) in mice. LPS content was not associated with a statistically significant increase in cytotoxic T lymphocyte (CTL) response. Interestingly, the DNA tattoo procedure by itself was shown to induce serum IL-6 responses that were at least as potent as that induced by parenteral LPS administration. Probably, this IL-6 release was induced by local skin damage.

Chapter 5 describes the development of an *ex vivo* human skin model that allows the longitudinal measurement of vaccine induced antigen expression in intact skin. This skin model demonstrated that the majority of cells transfected by DNA tattooing are epidermal keratinocytes, with only a small fraction (~1%) of antigen-positive epidermal Langerhans cells. Furthermore, using full randomization of DNA tattoo variables we showed that an increase in DNA concentration, needle depth and tattoo time all significantly increase

antigen expression ($p<0.001$), with DNA concentration forming the most critical variable influencing the level of antigen expression. Also, we calculated that the transfection efficiency of DNA tattooing is extremely low, only 1 out of $5x10^6$ to $5x10^9$ copies of plasmid applied is taken up, transcribed and translated.

In an attempt to increase this low transfection efficiency, we developed a nanoparticle formulation for the delivery of dermal DNA vaccines (**Chapter 6**). This chapter shows that the shielding of the surface charge of a cationic nanoparticle (both lipoplexes and polyplexes) via PEGylation is essential to induce both antigen expression and immunogenicity. Shielding the surface charge of nanoparticles by PEGylation improves *in vivo* antigen expression more than 55-fold compared to nonPEGylated nanoparticles. Non-PEGylated particles carry a positive zeta potential and therefore likely bind to negatively charged tissue matrix macromolecules preventing cellular binding and internalization. Our observations suggest that charge shielding is a general prerequisite for dermally applied vaccine formulations.

In **Chapter 7** the effect of the DNA tattooing on plasmid DNA integrity (supercoiled (SC) content) was assessed. DNA tattooing only resulted in minor damage to pDNA (≤3% SC pDNA reduction) and only open circular (OC) pDNA formation. Surprisingly, antigen expression levels and immunogenicity upon DNA tattooing of SC or OC pDNA were equal in a murine model, whereas tattooing in *ex vivo* human skin resulted in a significant higher level of antigen expression for the SC form. DNA topology did not influence expression in human skin when formulated as PEGylated polyplexes.

In an attempt to further increase the transfection efficiency of DNA tattooing, **Chapter 8** describes the combination of DNA tattooing with electroporation. First, it was shown that tattooing immediately followed by electroporation ("electrotattooing") can increase antigen expression levels in *ex vivo* human skin by a factor of 3. Even more interestingly, electrotattooing of a polyplex formulation boosts antigen expression up to levels 20-fold higher compared to tattooing of naked DNA.

Perspectives

Human or murine skin?

The *ex vivo* skin model developed in Chapter 5 was used throughout this thesis for the evaluation of expression properties of DNA vaccine formulations and administration techniques. It was demonstrated that the results obtained from *ex vivo* human skin can be different than results obtained in an *in vivo* murine model. In some cases these differences were only moderate, for example in Chapter 6, where the increase in antigen expression properties of PEGylated nanoparticles, compared to naked DNA, are slightly different between mice and man. In other experiments, the differences between human and murine skin were more striking. In Chapter 7, a difference in expression properties of OC DNA and SC DNA was observed in *ex vivo* human skin, whereas in mice these two topoisoforms induced similar expression levels. Since the differences in morphology between mice and human skin are obvious, I believe that the *ex vivo* human skin model is the best predictive model for the clinical setting. An additional advantage of this skin model is a reduction in the required number of test animals. We roughly estimate that the use of *ex vivo* human skin, a waste product from the surgery department, for the experiments described in this thesis, saved several hundreds of laboratory animals. This obviously has both ethical and economical benefits.

Nevertheless, the *ex vivo* skin model also has some clear drawbacks. First, since the viability of skin in culture is limited, this model is not suitable for the evaluation of systems that aim for long term gene expression. To overcome this issue, a xenograft model, in which human skin is transferred to nude mice can be used to allow long-term expression measurements (2). Nevertheless, the set-up of such a model is laborious and expensive. A second disadvantage of the *ex vivo* human skin model is the absence of a fully operational immune system. Therefore, it is only possible to measure expression levels of antigen and local immune activation (e.g. cytokine release and Antigen Presenting Cell (APC) activation and migration) but not the influx of cells from the adaptive immune system. However, as antigen expression is the first requirement for an immune response and since we have previously demonstrated the positive correlation between antigen expression and CTL response in mice (1), the skin model has a clear value as a screening tool for strategies that aim to increase immune responses by increasing antigen expression. In addition, the model is helpful for the evaluation of targeting strategies since antigen expression in different cells types can be distinguished. For the evaluation of strategies that aim to increase the immunogenicity of DNA vaccines by other means than antigen expression levels (for example addition of adjuvants or the introduction of more $CD4^+$ help), the *ex vivo* human skin model is less suitable.

Improving formulation and delivery

Chapter 6 describes the first steps towards the development of a nanoparticulate formulation for the improved delivery of DNA vaccines via DNA tattooing. We have demonstrated that shielding of the cationic surface charge (by PEGylation) is essential in order to obtain antigen expression and antigen specific T cell responses. Until presently, we could only demonstrate that the nanoparticles significantly increase antigen expression upon DNA tattooing compared to naked DNA, in both *ex vivo* human skin and mice. We were not able to demonstrate a significant increase in T cell response in the animal model. Nevertheless, thus far only one PEGylated polyplex and lipoplex formulation was tested *in vivo* and additional testing of modified formulations is necessary to fully evaluate and optimize the immunogenicity of these particles. Formulation parameters that could be modified are: composition of the cationic backbone or lipids, length and density of the PEG side chain, ratio DNA/carrier, used buffer and the concentration of the particle. Nanoparticle optimization should aim for formulations that yields the highest expression in the *ex vivo* human skin model, followed by a confirmation of their immunogenicity *in vivo*.

Next, a PEGylated nanoparticle is a perfect starting platform for the development of a targeted DNA vaccine formulation. It is fairly simple to integrate PEG-chains with a functional group to the terminal end (such as maleimide or an amine) enabling the chemical coupling of a targeting ligand (for example an antibody or peptide). For dermal administration, targeting to epidermal Langerhans cells (LCs) or dermal dendritic cells (DCs) is the most logical strategy since these cells are crucial in antigen presentation and T cell activation and are abundantly present in the skin. Nevertheless, as discussed in Chapter 2 and previously shown *in vivo* (3), cross-presentation of antigens from transfected keratinocytes to LCs or dermal DCs is also very effective in generating an antigen specific immune response upon DNA tattooing. Therefore, the evaluation of non-specific cell-targeting ligands (4) is also highly interesting.

In a first attempt to develop a targeted particle, we chemically coupled a LC-specific CD1a antibody to the terminal end of a PEGylated lipoplex. As visualized in Figure 1, incubation with an epidermal cell suspension showed preferential binding of these particles to LCs, demonstrating that the chemically coupled antibody had considerable affinity for this cell type. Although it is described that the CD1a receptor is internalized into LCs (5), the observed specific binding of the targeted nanoparticles did not result in preferential expression of the encoded protein in LCs (not shown). The best continuation for these type of experiments might be the screening of a large panel of ligands for APC specific receptors (for example C-type lectin receptors (6)). Possible hurdles for targeted particles might be that the different skin cells are not easily accessible in intact skin due to the extracellular matrix and that the particles need to be internalized after binding to the surface receptor, followed

by intracellular dissociation of the DNA/carrier complex in order to obtain antigen expression.

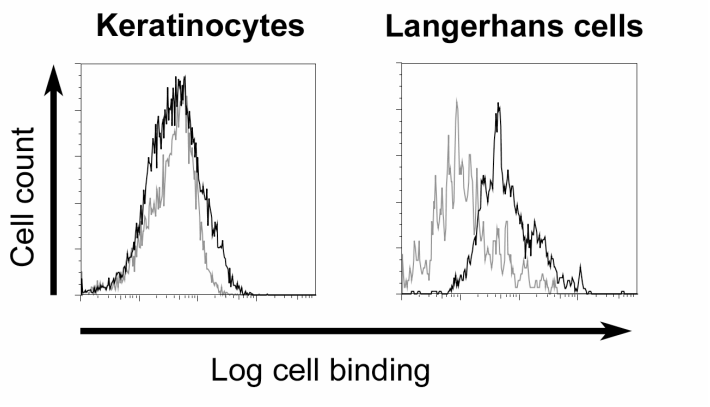

Figure 1: Towards targeted DNA vaccine nanoparticles.
Specific cell binding of PEGylated lipoplexes formulated with fluorescently (Cy5) labelled DNA. Langerhans cells-specific CD1a-antibody was chemically coupled to the terminal end of the PEG-liposome (described in Chapter 6) before DNA complexation and incubated with fresh epidermal cell suspensions. After 15 minutes of incubation on ice, specific cell binding was measured with flow cytometry (▬ targeted PEGylated lipoplexes, ▬ control PEGylated lipoplexes). Langerhans cells were distinguished from keratinocytes by CD1a staining.

In Chapter 8 it is shown that a combination of DNA tattooing and electroporation can increase antigen expression levels in the skin. Although the electrotattooing of naked DNA results in only a limited increase in antigen expression, the electrotattooing of nanoparticles showed high antigen expression levels is therefore a very promising approach and should be further explored in future experiments. For clinical application, however, this also involves the development of an electrode that is able to cover a larger skin areas. An additional advantage of introducing electroporation in the DNA tattoo vaccination protocol may be its positive effect on cytokine release and the expression of co-stimulatory molecules on APCs, which could enhance immunogenicity.

For the further optimization of DNA vaccine constructs, formulations and delivery techniques it is important to unravel the cellular routing of DNA vaccines applied via tattooing. It is known that the cellular uptake of carrier/DNA complexes *in vitro* is mediated via different endocytotic pathways (7). Electroporation of naked DNA in cell cultures leads to the direct transfer of DNA into the cytosol (8). The exact cellular routing of DNA upon tattooing is presently unknown. However, since antigen expression is already measurable a few hours after tattooing and since negatively charged naked DNA performs surprisingly well, we hypothesize that upon tattooing naked DNA can directly passes the negatively charged cell membrane and enters the cytosol. This transfer might be provoked by friction, intracellular injection or cell activation caused by the energy provided by the oscillating tattoo needles. Once inside the cytosol, the DNA has to pass the nuclear envelope in order to obtain antigen expression. Most probably, nuclear entry takes place upon cell division of skin cells, when the nuclear envelope is temporarily broken down, allowing the entry of

large molecules like DNA. In addition, entry of DNA through the nuclear pore complex can not be excluded (9). Since we do not observe a clear difference in expression kinetics of DNA when formulated as a nanoparticle or upon electrotattooing, we expect that these strategies do not alter the cellular routing followed by DNA vaccines. The increase in antigen expression observed for nanoparticles may be due to the decrease in size compared to naked DNA, thus simplifying cellular entry or cytosolic trafficking. In addition, the shielded surface charge might reduce the electrostatic repulsion between the vaccine and the cells. The increase in expression induced by the addition of electroporation could be due to more cellular influx of DNA surrounding the cells, via pores induced by electroporation. Future experiments, for example by using *in vivo* or *in situ* confocal laser scanning microscopy, should further unravel the precise cellular routing of DNA tattoo vaccines.

In conclusion, this thesis shows that optimizing both the formulation and the delivery technique can increase the performance of dermal DNA vaccines administered via DNA tattooing. In addition, we have observed that a combined approach of enhanced delivery (via the introduction of electrotattooing) and enhanced formulation (via nanoparticle formulation) was superior compared to naked DNA in terms of the highest increase in antigen expression in *ex vivo* human skin. Therefore, this combination represents be the most promising future direction to increase the performance of dermal DNA vaccines. In order to translate the results of this research towards a clinical application, future experiments should focus on the optimization of the composition, characterization, upscaling and stability of the particles as well as the improvement of electrotattooing protocols and arrays.

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Appendices

Samenvatting

DNA vaccinatie is een aantrekkelijke methode voor de actieve immunisatie tegen virale infecties en kanker. De toediening van bacterieel plasmide DNA (pDNA) leidt tot de intracellulaire productie van het coderende antigeen, wat resulteert in een antigeen specifieke, cellulaire immuunrespons. Intradermale DNA tatoeage is recentelijk ontwikkeld als een simpele en robuuste methode om antigeen expressie te induceren in de huid. Deze lokale expressie resulteert in diermodellen in een antigeen specifieke T cel respons. Dit proefschrift richt zich op de farmaceutische formulering en de toediening van DNA tatoeage vaccins.

Na een algemene introductie (**Hoofdstuk 1**), geeft **Hoofdstuk 2** van dit proefschrift een literatuuroverzicht van studies waarin synthetische DNA dragers gebruikt worden om de werking van DNA vaccins te verbeteren. Naast een overzicht van de huidige kennis, geef dit hoofdstuk aanbevelingen voor de formulering, "targeting", evaluatie en klinische applicatie van deze dragersystemen.

Een generiek proces voor de productie van naakt pDNA voor gebruik in fase I en II klinische trials is beschreven in **Hoofdstuk 3**. Het ontwikkelde proces is reproduceerbaar en resulteert in een constante hoge kwaliteit, stabiliteit en opbrengst van het pDNA. Het geproduceerde DNA vaccin pDERMATT (coderend voor Tetanus Toxine fragment C en een MART-1 epitoop) gaf een robuuste, epitoop specifieke, T cel respons in een gehumaniseerd muismodel en wordt op dit moment klinisch toegepast in een fase I studie.

Lipopolysaccharide (LPS), een component uit het celmembraan van *Escherichia coli*, is een veel voorkomende vervuiling in pDNA producten. Dit is inherent aan het gebruik van *E. Coli* als productiecel. In **Hoofdstuk 4** wordt een studie beschreven waarin het effect van een toenemende hoeveelheid LPS in plasmide DNA vaccins op systemische toxiciteit, antigeen expressie en cellulaire immuunrespons wordt onderzocht. De aanwezigheid van LPS resulteert niet in systemische toxiciteit (gemeten d.m.v. interleukine 6 (IL-6) concentratie in serum) of verandering in antigeen expressie (gemeten *in vivo* met een lichtgevoelige camera). LPS vervuiling blijkt niet geassocieerd met een statistisch significante toename in T cel respons. Opmerkelijk is dat de tatoeage procedure zelf ook een IL-6 respons veroorzaakt die minstens even sterk is als een respons geïnduceerd door een parenterale LPS injectie. Deze tatoeage geassocieerde IL-6 vrijgifte wordt waarschijnlijk veroorzaakt door schade aan de huid door de tatoeage.

Hoofdstuk 5 beschrijft de ontwikkeling van een *ex vivo* humaan huidmodel waarin vaccin geïnduceerde antigeen expressie over een langere tijd gemeten kan worden. Met dit

huidmodel hebben we laten zien dat de meerderheid van de getransfecteerde huidcellen na DNA tatoeage epidermale keratinocyten zijn, met slechts een kleine fractie (~1%) antigeen positieve epidermale Langerhans cellen. Gebruik makend van een randomisatie van de voor tatoeage relevante variabelen, is vervolgens bepaald dat een toename van DNA concentratie, naalddiepte en tatoeagetijd allen significant bijdroegen aan de toename in antigeen expressie ($p<0.001$). DNA concentratie blijkt daarbij de grootste invloed op antigeen expressie te hebben. Daarnaast is berekend dat de transfectie efficiëntie van DNA tatoeage bijzonder laag is. Slecht 1 van de $5x10^6$ tot $5x10^9$ toegediende plasmide kopieën wordt daadwerkelijk opgenomen, omgezet in mRNA en vertaald in eiwit.

Hoofdstuk 6 beschrijft de ontwikkeling van een DNA nanodeeltje, met als doel de lage transfectie efficiëntie van DNA tatoeage te verbeteren. In dit hoofdstuk is te lezen dat het afschermen van de oppervlakte lading (zeta potentiaal) van kationе deeltjes (zowel lipoplexen als polyplexen), door het koppelen van poly (ethyleenglycol) ketens (PEGylering), noodzakelijk is om antigeen expressie en immuunresponsen te induceren. Afschermen van de oppervlakte lading door PEGylering verbetert de *in vivo* antigeen expressie meer dan 55 maal vergeleken met niet gePEGyleerde nanodeeltjes. De niet gePEGyleerde nanodeeltjes hebben een positieve zeta potentiaal waardoor ze waarschijnlijk binden aan negatief geladen weefselcomponenten. Deze immobilisatie remt hoogstwaarschijnlijk cellulaire binding en opname. Deze bevindingen suggereren dat het afschermen van een positieve lading een eerste vereiste is voor dermaal toegediende vaccinformuleringen.

In **Hoofdstuk 7** is het effect van DNA tatoeage op de integriteit van het plasmide onderzocht. pDNA bestaat in meerdere vormen zoals de lineaire, open circulaire (OC) en supercoiled (SC) vorm, waarbij gedacht wordt dat de SC vorm over de hoogste biologische activiteit beschikt. DNA tatoeage resulteert slechts in een geringe afname (≤3%) in SC concentratie en enkel in de vorming van OC pDNA. Opmerkelijk is dat de tatoeage van SC en OC pDNA in een muizenmodel resulteert in een gelijke antigeen expressie en T cel respons. Daar tegenover staat dat in het *ex vivo* humane huid model SC pDNA een significant hogere expressie geeft dan OC en lineair DNA. Dit verschil in expressie tussen de verschillende DNA vormen kan worden opgeheven door pDNA te verpakken in een gePEGyleerd nanodeeltje (polyplex).

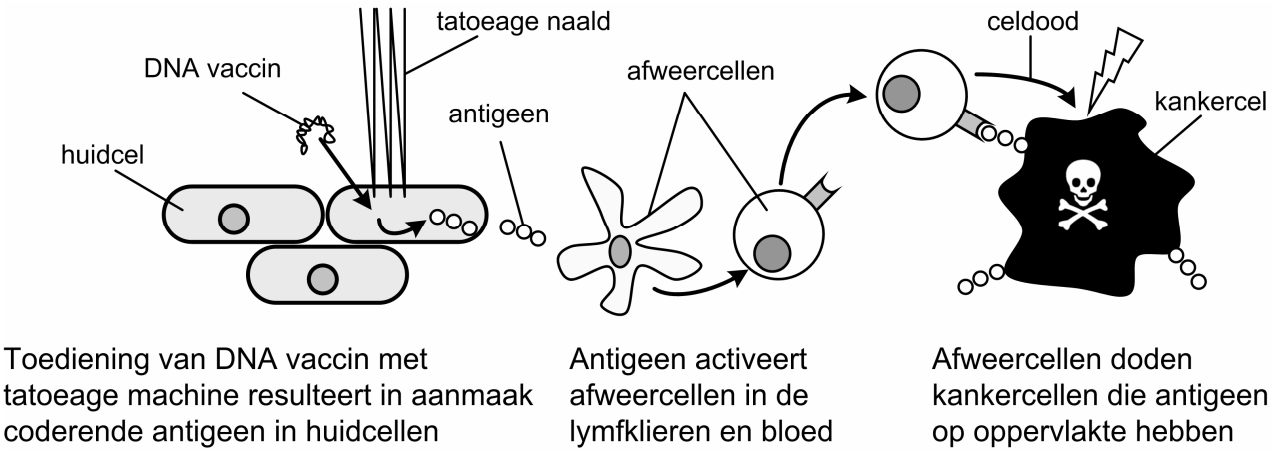
In een poging om de transfectie efficiëntie van DNA tatoeage verder te vergroten, beschrijft **Hoofdstuk 8** de combinatie van DNA tatoeage met electroporatie. Allereerst werd aangetoond dat tatoeage direct gevolgd door electroporatie ("electrotatoeage") antigeen expressie significant kan verhogen met een factor 3 in *ex vivo* humane huid. Nog interessanter was dat de electrotatoeage van een polyplex formulering resulteert in een 20 keer verhoogde antigen expressie vergeleken met tatoeage van naakt DNA.

Samenvattend wordt in dit proefschrift aangetoond dat zowel het optimaliseren van de vaccin formulering als toediening de werking van dermale DNA vaccins kunnen verbeteren. Het gebruik van een nanodeeltje in combinatie met de electrotatoeage strategie resulteerde daarbij in de grootste toename in antigeen expressie. Om in de toekomst te komen tot een klinische toepassing van deze systemen zal gewerkt moeten worden aan zowel de optimalisatie van de compositie, karakterisering, opschaling en stabiliteit van de nanodeeltjes alsmede de verbetering van electrotatoeage protocollen en apparaten.

Samenvatting voor niet-ingewijden

Bij **vaccinatie** of inenting wordt het afweersysteem van het lichaam geactiveerd met een vaccin om besmetting met bepaalde ziektemakers (**pathogenen**) in de toekomst te voorkomen, of de symptomen van besmetting minder ernstig te maken. Een vaccin bestaat uit een verzwakte of gedode vorm van een pathogeen of uit een onderdeel van het pathogeen waartegen het afweersysteem een reactie kan vormen. Zo'n onderdeel van een pathogeen wordt ook wel een **antigeen** genoemd. Antigenen zijn meestal eiwitten die uniek zijn voor een bepaald pathogeen. Vaccinatie is een zeer succesvolle methode gebleken om ziekten te bestrijden, zo zijn ziektes als polio en pokken zelfs helemaal verdwenen dankzij vaccinatie.

In dit proefschrift is onderzoek gedaan naar **DNA vaccins**. DNA vaccins bestaan uit stukjes DNA, die de genetische code voor een eiwit bevatten. Als een DNA vaccin in het lichaam wordt gespoten worden de stukjes DNA opgenomen door cellen. Deze cellen kunnen vervolgens de genetische code aflezen en het coderende eiwit (= antigeen) gaan maken. Hierdoor maken onze eigen cellen dus antigeen aan waartegen een afweerreactie opgeroepen kan worden. Het voordeel van de DNA vaccinatie strategie ten opzichte van "gewone" eiwit vaccinatie is dat het DNA eenvoudig veranderd kan worden in het laboratorium en dat het vaccin relatief eenvoudig te produceren is.
DNA vaccinatie kan onder andere gebruikt worden voor de behandeling van **kanker**. Dit komt omdat kankercellen vaak antigenen aan hun celoppervlak hebben, waartegen het lichaam potentieel een afweerreactie kan vormen. In diermodellen is aangetoond dat DNA vaccinatie inderdaad een goede afweerreactie kan uitlokken tegen kanker. Momenteel ontwikkelen wij DNA vaccinatie als methode om een afweerreactie op te wekken tegen tumoren waarvan de antigenen bekend zijn. Het werkingsmechanisme van DNA vaccinatie tegen kanker is schematisch weergeven in Figuur 1.



Figuur 1: Werkingsmechanisme DNA vaccins, toegediend via DNA tatoeage.

DNA vaccins kunnen op verschillende manieren toegediend worden aan een patiënt. Veelal worden zij in een spier gespoten. In dit proefschrift maken wij gebruik van een **tatoeage** machine om DNA vaccins toe te dienen. Tatoeage van DNA vaccins in de huid resulteert in de opname van het vaccin in huidcellen die vervolgens het antigeen gaan produceren. In vergelijking met een injectie in de spier, is de afweerreactie na DNA tatoeage veel sterker gebleken. Dit komt waarschijnlijk doordat de huid van nature veel afweercellen bevat en de beschadiging van de huid door de tatoeage het immuunsysteem activeert.

Na een overzicht van de vakliteratuur in **Hoofdstuk 2**, hebben wij in dit proefschrift eerst gekeken hoe wij DNA vaccins het beste kunnen produceren in een eigen productie faciliteit **(Hoofdstuk 3)**. Het is daarbij van belang om te voldoen aan alle regelgeving van overheden zodat deze vaccins in patiënten gebruikt kunnen worden. Het eerste DNA vaccin wat wij geproduceerd hebben wordt op dit moment getest bij patiënten met huidkanker en wordt door middel van tatoeage aan de patiënten toegediend. Vervolgens hebben wij het effect van een veelvoorkomende onzuiverheid op de werking van DNA vaccins onderzocht in een muizenmodel **(Hoofdstuk 4)**.

Alle vaccins worden eerst getest in diermodellen voordat ze bij patiënten toegediend kunnen worden. Veelal wordt hier gebruik gemaakt van muizen. Aangezien de muizenhuid sterk verschilt van mensenhuid wordt in **hoofdstuk 5** van dit proefschrift een model gepresenteerd waarbij de werkzaamheid van DNA vaccins, toegediend via DNA tatoeage, getest wordt op mensenhuid. Deze mensenhuid blijft over na ingrepen van de plastische chirurgie in het ziekenhuis. Uit onze experimenten bleek dat deze mensenhuid een uitstekend model is om de werkzaamheid van onze vaccins te testen. Dit model geeft hoogstwaarschijnlijk een goede voorspelling van de werkzaamheid in patiënten en beperkt het gebruik van proefdieren in dit type onderzoek. Het huidmodel is vervolgens gebruikt om de DNA tatoeage methode te optimaliseren en om te kijken welke huidcellen het vaccin opnemen.

Uit het huidmodel bleek dat er slechts een zeer klein gedeelte van het toegediende DNA vaccin daadwerkelijk wordt opgenomen door de huidcellen (ongeveer 1 op 5 miljoen tot 5 miljard aangebrachte moleculen). Om dit percentage te verbeteren hebben wij in **Hoofdstuk 6** het DNA ingepakt in hele kleine bolletjes van slechts een paar honderd nanometer groot (een nanometer is een miljoenste deel van een millimeter). Het bleek dat deze nanobolletjes, gemaakt van vet of een polymeer, de opname van DNA vaccins in huid sterk kunnen verbeteren (ongeveer 8 keer beter dan het standaard vaccin). Het is dan wel noodzakelijk om de elektrische lading die deze bolletjes hebben af te schermen. Zonder

deze afscherming “plakken” de nanobolletjes waarschijnlijk aan huidbestanddelen waardoor zij niet meer opgenomen kunnen worden door de huidcellen.

DNA is een groot molecuul (DNA lijkt op een hele lange kralenketting), dat op verschillende manieren opgevouwen kan zijn. In **hoofdstuk 7** hebben wij gekeken hoe deze verschillende manieren van opvouwen de werking van DNA vaccins beïnvloeden. Het bleek dat de tatoeage procedure de vouwing van DNA nauwelijks beïnvloedde. In het muismodel bleek bovendien geen enkel verschil te bestaan in werkzaamheid van verschillend opgevouwen vormen DNA. Verrassend genoeg was er wel een verschil waarneembaar in het mensenhuid model.

Een andere manier om de opname van DNA te vergroten is het toedienen van stroomschokjes (electroporatie). Daarom worden er in **Hoofdstuk 8** stroomschokjes op de plek van DNA tatoeage toediening gezet. Deze stroomschokjes kunnen de opname van DNA in huidcellen verder vergroten door de celwand tijdelijk doorlaatbaar te maken. Uit deze experimenten bleek dat de introductie van stroomschokjes de opname van DNA vaccins 3 maal kan verhogen in mensenhuid. Door deze stroomschokjes te combineren met de nanobolletjes uit Hoofdstuk 6 wordt de opname zelfs met een factor 20 verhoogd.

Samenvattend laat dit proefschrift zien dat zowel het optimaliseren van de farmaceutische verpakking (nanobolletjes) als het optimaliseren van de toediening (introductie van stroomschokjes) de werking van onze DNA vaccins kunnen verbeteren. In de toekomst willen wij deze technieken verder ontwikkelen zodat zij bij patiënten kunnen worden toegepast.

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Joost

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Curriculum Vitae

Joost van den Berg was born on the 2nd of June in Leidschendam, The Netherlands. After finishing secondary school at the Oranje Nassau College in Zoetermeer he started studying pharmacy in 1999 at Utrecht University. During his study, he performed a research project on meningitis B vaccine formulations at the Department of Pharmaceutics, Utrecht University and the Laboratory of Process and Product Development, Netherlands Vaccine Institute, Bilthoven. After graduating as a pharmacist he started with his PhD project described in this thesis. This research was a collaboration between the Department of Pharmacy and Pharmacology, Slotervaart Hospital/ Netherlands Cancer Institute, Amsterdam, the Department of Pharmaceutics, Utrecht University and the Division of Immunology, Netherlands Cancer Institute, Amsterdam and was supervised by prof. dr. J.H. Beijnen, prof. dr. ir. W.E. Hennink, prof. dr. G. Storm, dr. B. Nuijen and prof. dr. J.B.A.G. Haanen. The author is currently working as a postdoctoral researcher at the Division of Immunology, Netherlands Cancer Institute and The Netherlands Commission on Genetic Modification (COGEM) under the supervision of prof. dr. J.B.A.G. Haanen.

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