

DNA Vaccines and Intradermal Vaccination by DNA Tattooing

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Abstract Over the past two decades, DNA vaccination has been developed as a method for the induction of immune responses. However, in spite of high expectations based on their efficacy in preclinical models, immunogenicity of first generation DNA vaccines in clinical trials was shown to be poor, and no DNA vaccines have yet been licensed for human use. In recent years significant progress has been made in the development of second generation DNA vaccines and DNA vaccine delivery methods. Here we review the key characteristics of DNA vaccines as compared to other vaccine platforms, and recent insights into the pre-requisites for induction of immune responses by DNA vaccines will be discussed. We illustrate the development of second generation DNA vaccines with the description of DNA tattooing as a novel DNA delivery method. This technique has shown great promise both in a small animal model and in non-human primates and is currently under clinical evaluation.

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1 An Introduction on Two Decades of DNA Vaccination

It is now two decades ago since it was first demonstrated that injection of naked plasmid DNA into mouse muscle results in expression of the encoded protein (Wolff et al. 1990). Soon thereafter it was demonstrated that both cellular and humoral immune responses can be elicited against DNA vaccine-encoded proteins, when applied intradermally using a ‘gene gun’ (Tang et al. 1992) or upon intramuscular (IM) injection (Wang et al. 1993; Ulmer et al. 1993). Furthermore, these DNA vaccination-induced immune responses were shown to confer protection in various preclinical disease models, including models of viral, bacterial, and parasitic diseases and various tumor models [reviewed by Donnelly et al. (1997), Gurunathan et al. (2000)]. Based on these encouraging preclinical data and a number of perceived advantages of DNA-based vaccines (see below), a series of clinical trials was initiated during the late 1990s that evaluated the efficacy of DNA vaccines in the induction of immune responses against pathogen- (HIV, malaria, hepatitis B) and cancer-associated antigens (Donnelly et al. 2003; Liu and Ulmer 2005; Lu et al. 2008). While these trials provided overwhelming evidence for the overall safety of DNA vaccines (Donnelly et al. 2003; Liu and Ulmer 2005), immunogenicity of this first generation DNA vaccines was at best modest.

Following the observation of low immunogenicity of DNA vaccines in the early human trials, the field has taken two directions. (1) It has been argued that while DNA vaccines may not induce high-level immune responses as a single modality,

these vaccines would nevertheless be valuable to provide low-level priming. Such low-level immune responses can then subsequently be amplified by administration of a virus-based vaccine (McConkey et al. 2003; Lu et al. 2008). Such DNA-prime viral vector-boost regimens can reduce the issue of vector-specific immune responses that are a common problem in viral vector-based vaccines. (2) As a second and more ambitious goal, a large effort has been made to develop (what we here will loosely call) “second generation DNA vaccines” that should be able to induce robust immune responses without a requirement for booster vaccination by virus-based vaccines. In these vaccines, optimization has either focused on (i) improvement of the expression vectors, (ii) improvement of the vaccine formulation, (iii) enhancement of the immunogenicity of the vaccine-encoded antigen, or (iv) the provision of molecular adjuvants in order to boost immunogenicity. A selected set of examples of such optimizations will be provided. Furthermore a large effort has been made to develop novel physical delivery methods that aim to increase DNA vaccine efficiency, of which intradermal (ID) DNA tattooing forms an example.

Is it plausible that DNA vaccines will become available for human use in the foreseeable future? The licensing of three different DNA vaccines in the field of veterinary medicine (against West Nile virus in horses, against infectious haematopoietic necrosis virus in salmon and for treatment of melanoma in dogs) (Redding and Weiner 2009), and a recent report showing DNA vaccination-mediated protection of human subjects against influenza challenge (Jones et al. 2009), both illustrate the therapeutic potential of DNA vaccines as single modalities. Owing to this, there is presently renewed optimism that DNA vaccines may within the next years be approved for applications in humans (Lu et al. 2008; Kutzler and Weiner 2008).

2 Advantages of DNA Vaccination Compared to Conventional Vaccine Platforms

DNA vaccines have a number of attractive properties that contribute to the strong interest in their development. Among these properties are the ease and speed of vaccine production, the ability to induce both cellular and humoral immunity and the favorable safety profile as compared to other gene-based vaccine platforms that are able to induce strong cellular immunity. These aspects are discussed in more detail below.

2.1 Ease and Speed of Production

Plasmid DNA is relatively easy to produce in small to large quantities in a generic way, with little if any need for adaptation of the production process for different

individual plasmids. This is in sharp contrast to in particular protein-based vaccines, for which the production process needs to be specifically designed for each new vaccine. Moreover, since DNA vaccine-encoded proteins are synthesized by the host cells upon delivery, difficulties associated with recombinant protein-based vaccine production, such as protein folding and post translational modifications (e.g., glycosylation) are circumvented (Abdulhaqq and Weiner 2008; Jechlinger 2006). Another important advantage of DNA vaccines is the excellent stability of DNA as compared to other vaccine modalities, thereby likely circumventing the need for a ‘cold chain’ for vaccine distribution.

2.2 Ability to Induce Cellular Immunity

While direct experimental evidence is limited, there is some reason to assume that DNA vaccines are more suitable for the induction of CD8⁺ (‘cytotoxic’) T cell immunity than recombinant peptide or protein vaccines (Gurunathan et al. 2000; Rice et al. 2008; Nagata et al. 2004; Liu 1997). Due to the fact that by definition, vaccination-induced antigen expression takes place by host cells, there is ample opportunity for the transfected cells to present peptide fragments of the antigen in MHC-class I molecules at the cell surface. In contrast, in many other vaccine formats such as protein, peptide, or inactivated pathogen-based vaccines, antigen is offered within the extracellular space. As extracellular antigens are mainly presented via MHC-class II molecules, induction of CD4⁺ (‘helper’) T cell and antibody responses can be expected to predominate (Liu 1997). This discussion is somewhat complicated by the observation that induction of T cell responses upon DNA vaccination occurs at least in part by cross-priming rather than direct interaction between naive CD8⁺ T cells and transfected skin or muscle cells (see below). However, as cross-priming is also more efficient for cell-associated than for soluble antigens (Li et al. 2001), the advantage of vaccine formats that induce intracellular antigen expression remains.

2.3 Lack of Vector-Specific Immune Responses

While the presumed advantage of DNA vaccines in the induction of CD8⁺ T cell responses is shared with live attenuated viral vaccines or viral vector-based vaccines, the latter modalities bear greater risks in terms of production and safety (Draper and Heeney 2010; Robert-Guroff 2007). Furthermore, viral vector-based vaccines such as recombinant adenovirus or vaccinia virus can suffer from pre-existing immunity toward the vector or can induce vector-directed immunogenicity, thereby preventing repeated administration of these vectors (Limbach and Richie 2009). In the case of DNA vaccines the only immunogenic structure produced is the antigen itself, thereby allowing repeated administration.

2.4 *Favorable Safety Profile*

For the large-scale use of new vaccine formats in the general population their safety profile obviously needs to be well-established (Wilson and Marcuse 2001; O'Hagan and Rappuoli 2004). Because of their non-infectious and non-replicating nature, DNA vaccines are considered safer than live attenuated viruses or recombinant viral vectors. Furthermore, DNA vaccines have proven to be well tolerated and non-toxic in both preclinical- and clinical studies (Lu et al. 2008; Kutzler and Weiner 2008; Schalk et al. 2006; Parker et al. 2001; Parker et al. 1999). However, a few safety issues unique to plasmid DNA vaccines may potentially hamper their widespread use.

The main safety concern associated with DNA vaccines is the risk of genomic integration into the host genome. Genomic integration could potentially lead to activation of oncogenes, inactivation of tumor suppressor genes, or, when integrated into the chromosomal DNA of germ line cells, to vertical transmission. Several studies have examined the frequency of integration upon DNA vaccination. Collectively, these studies indicate that integration can occur but with a frequency that is manifold (around three orders of magnitude, depending on the system) lower than the spontaneous gene-inactivating mutation frequency of the genome. (Schalk et al. 2006; Ledwith et al. 2000; Wang et al. 2004). Vertical transmission due to genomic integration in germ line cells has been observed after direct injection of DNA into the gonads (Gao et al. 2009). However, genomic integration into germ line cells has not been observed after DNA vaccination at sites distant from the gonads (Parker et al. 1999; Manam et al. 2000). In conclusion, because of the low frequency of genomic integrations at the vaccination site and the absence of integrations in germ line cells, the risks associated with genomic integration upon DNA vaccination are at present considered negligible. An important exception to this is formed by DNA vaccines that encode proteins with known or suspected transforming activity (e.g., the HPV E6 and E7 oncoproteins). Proteins with transforming activity are attractive targets for vaccination as they can serve as unique tumor associated antigens. However, for such DNA vaccines, the survival advantage of cells that express the encoded proteins could conceivably lead to outgrowth of those (extremely) few cells in which genomic integration has occurred (Jeon et al. 1995). Because of this concern, the use of engineering strategies that abolish the transforming properties of the vaccine-encoded antigen should be considered essential.

A second potential safety concern in the use of DNA-based vaccines is the induction of anti-DNA antibodies and the subsequent development of autoimmune disease. This concern is increased by the fact that the bacterial derived DNA contains unmethylated phosphodiester-linked cytosine and guanine (CpG) motifs in the plasmid backbone that have an immunostimulatory activity via triggering of Toll-like receptor 9 (TLR9) (Krieg 2002), see also below. Anti-DNA antibodies are considered a hallmark of certain autoimmune diseases such as systemic lupus erythematosus (SLE), as most (but not all) patients manifest this

characteristic of disease (Donnelly et al. 1997; Isenberg et al. 2007). Although induction of anti-DNA antibodies has been observed in some animal models after injection of plasmid DNA, thus far no evidence has been found that these antibodies are associated with the development of systemic autoimmune diseases, either in healthy animals or in animals that are at risk for the development of autoimmune disease [reviewed by Schalk et al. (2006), Smith and Klinman (2001)]. Furthermore, in human DNA vaccination trials no statistically significant increase in the presence of antinuclear antibodies and anti-DNA antibodies among vaccinees has been detected (Schalk et al. 2006).

In conclusion, all preclinical and clinical studies that have aimed to evaluate potential safety concerns of DNA vaccines have not provided any compelling evidence for substantial risks associated with the use of DNA vaccines. Because of this, we currently see no major obstacles for the application of DNA vaccines for therapeutic purposes, or for prophylaxis against high-risk disease. It is noted, however, that the potential toxicities of DNA vaccines would primarily concern long-term effects that may be difficult to address in the studies discussed above. Because of this, it would seem prudent to await the long-term outcome of clinical trials for high-risk indications before widespread application of DNA vaccination for low-risk disease is considered.

3 Mechanism of T cell Priming upon DNA Vaccination

At first glance, the general mechanism by which plasmid DNA vaccines induce immunity seems straightforward. Upon administration the plasmid DNA is taken up by host cells, leading to production of the antigen by these cells and to the release of ‘danger’ signals as dictated by the danger model. However, there is still substantial uncertainty about the antigen-presentation pathway that leads to the display of antigen-derived epitopes to naïve T cells and also by which molecular mechanisms ‘danger’ is perceived upon DNA vaccination. Importantly, a better understanding of both of these factors is likely to result in more efficient DNA vaccine formats.

3.1 Direct- Versus Cross-priming

Through the use of bone marrow chimeras it has been demonstrated that the induction of cellular and humoral immune responses upon DNA vaccination is absolutely dependent on antigen presentation by bone marrow-derived professional antigen-presenting cells (APCs) (Iwasaki et al. 1997). On the other hand, for various routes of administration it has been demonstrated that antigen expression upon DNA vaccination primarily results in antigen expression in non-immune cells in peripheral tissues, such as myocytes in the muscle and keratinocytes in the

skin (Dupuis et al. 2000; Porgador et al. 1998). An important question, therefore, is whether immune activation primarily occurs by the action of a small number of APCs that have become directly transfected, or whether antigen produced by the much larger number of non-immune cells serves as a source of antigen that is handed over to APCs that subsequently present the antigen (a process termed cross-presentation in the case of CD8⁺ T cell activation). This issue is of more than academic interest as it has previously been demonstrated that the efficiency with which antigens are cross-presented can vary markedly depending on the context in which an epitope is provided (see also below) (Norbury et al. 2004; Wolkers et al. 2004).

Most DNA vaccination studies performed to address this question have used gene gun or IM needle injection as a delivery platform. From these studies there is clear evidence that both direct presentation of antigen by transfected APCs (Porgador et al. 1998; Condon et al. 1996; Chattergoon et al. 1998; Torres et al. 1997) and cross-presentation of antigen acquired from non-immune cells (Wolff et al. 1990; Cho et al. 2001; Ulmer et al. 1996) can occur *in vivo* after DNA vaccination. The design of most of these studies, however, does not allow a conclusion on the relative contribution of these two processes to CD8⁺ T cell activation *in vivo*. An exception to this is formed by a study in which a DNA vaccine encoding the influenza A nucleoprotein (NP) under control of either the keratinocyte-specific K14 promoter or the APC-specific CD11b promoter was applied via gene gun (Cho et al. 2001). This study revealed that keratinocyte-directed transgene expression induced both higher cellular and humoral immune responses than APC-directed transgene expression, thus providing strong evidence for a dominant role for cross-presentation in CD8⁺ T cell priming upon gene gun immunization. These data are in apparent contrast to a second study that—again using gene gun application—provided evidence for a dominant role for directly transfected APCs in CD8⁺ T cell activation (Porgador et al. 1998). In this study, co-transfection, but not co-immunization of plasmids encoding co-stimulatory molecules was shown to restore the immunogenicity of an otherwise non-immunogenic nuclear protein (NP) variant. This observation seems most consistent with antigen presentation by directly transfected APCs, as cross-presentation would not be expected to result in cell surface expression of the vaccine-encoded costimulatory molecules on the APC. It is noted however, that the NP variant used in the latter study may form a poor substrate for cross-presentation, as the mutations within this antigen may prevent proper folding and thereby reduce antigen accumulation within the donor cell or by other means disrupt the transfer of antigen from the antigen-producing cells to specialized APCs (see below) (Iwasaki et al. 1997).

Taken together, to date no definitive answer exists regarding the exact mechanism of T cell priming upon DNA vaccination (Shedlock and Weiner 2000; Laddy and Weiner 2006), and it is plausible that the mechanism of immune induction will differ between different methods of immunization (Torres et al. 1997; Heath and Carbone 2001), between target tissues (e.g., skin vs. muscle) (Torres et al. 1997), and between different DNA vaccine designs.

3.2 Influencing Antigen Properties

Several strategies have been developed in which an antigen of interest is genetically fused to a ‘carrier’ protein. Carrier proteins that have been shown to (sometimes strongly) increase the immunogenicity of the fused antigen include tetanus toxin fragment C (TTFC), heat shock protein 70 (HSP 70), MHC-class II invariant chain (Ii), calreticulin (CRT), herpes simplex virus viral protein 22 (HSV VP 22) and *E. coli* β -glucuronidase (Table 1). The exact mechanism(s) by which these carrier proteins enhance the immunogenicity of the fused antigen remain largely unclear and may vary between different carrier molecules. However, based on our current understanding of DNA vaccines, two broad categories are likely to play dominant roles.

Provision of CD4⁺ T cell help: There is abundant evidence that CD8⁺ T cell responses induced by DNA vaccination are dependent on CD4⁺ T cell help (Maecker et al. 1998). However, CD4⁺ T cell responses are likely to be weak or lacking when using DNA vaccines that either encode self proteins or single CD8⁺ T cell epitopes. In such cases, the provision of CD4⁺ T cell help via carrier encoded helper epitopes is likely to be an important factor in the immune-enhancing effect of foreign carrier molecules, like TTFC and *E. coli* β -glucuronidase (Stevenson et al. 2004; Smahel et al. 2004).

Enhancement of antigen presentation: There is strong evidence that improvement of antigen stability enhances DNA vaccine immunogenicity. First, many of the above-mentioned fusions result in increased steady state antigen levels (Brulet et al. 2007; Smahel et al. 2004; Michel et al. 2002). Second, formal evidence for the notion that the stability of DNA vaccine-encoded antigens in the transfected cell contributes to vaccine immunogenicity has been provided using a set of engineered luciferase variants with a variable in vivo half-life (Bins et al. 2007). For this set of variants, immunogenicity was directly correlated to antigen stability. Also the observation that covalent linkage of an epitope toward a carrier protein, but not the simultaneous expression of the epitope and the carrier using a bicistronic vector, improves vaccine immunogenicity is consistent with the notion that carrier proteins can influence vaccine immunogenicity by increasing antigen half-life (Wolkers et al. 2002). At present, the most straightforward explanation for the observed effect of antigen stability on vaccine immunogenicity is that it would enhance cross-presentation, although a direct analysis of epitope density on APCs would be required to provide formal evidence for this model. Genetic fusion to carrier proteins may also influence antigen presentation through other mechanisms. For VP-22 it has been proposed that it enhances antigen spreading to neighboring cells (Michel et al. 2002). For HSP-70 it has been proposed that it increases uptake of the antigen by APCs via a HSP specific receptor (Wu 2007). Finally, some carrier molecules such as Ii (Brulet et al. 2007) and calreticulin (Cheng et al. 2001) alter the subcellular localization of an antigen and might thereby improve the immunogenicity of the DNA encoded antigen. This is in line with the finding that the sole addition of signals influencing subcellular localization

Table 1 Selection of methods to enhance DNA vaccine potency

Type of optimization	Method	Proposed mode of action ^a	Reference
Improvement of the vector	Gene optimization	Stabilization of RNA; more efficient translation of RNA	Liu et al. (2002), Lin et al. (2006)
	Addition of viral post-transcriptional regulatory elements	Increased cytoplasmic mRNA levels	Sun et al. (2009), Garg et al. (2004)
Improvement of vaccine formulation	Formulation of naked DNA into nano/micro particles	Increased cellular uptake of DNA	van den Berg et al. (2010a, b)
Improvement of antigen immunogenicity	TTFC fusion	Provision of CD4 ⁺ help	Hedley et al. (1998), Klencke et al. (2002)
	HSP-70 fusion	Improved cross-presentation of antigen	Stevenson et al. (2004)
	Calreticulin fusion	Targeting of antigen for antigen processing and presentation	Chen et al. (2000)
	Invariant chain fusion	Enhanced stability/changed subcellular localization	Cheng et al. (2001)
	<i>E. coli</i> β -glucuronidase fusion	Changed subcellular localization of antigen, provision of CD4 ⁺ help	Brulet et al. (2007)
	HSV VP 22 fusion	Improved antigen spreading	Smahel et al. (2004)
Enhancement of immune activation by addition of adjuvants	Co-delivery of pro-inflammatory cytokines (GM-CSF, IL-2, IL-12)	Recruitment, expansion and activation of APCs	Kim et al. (2004), Michel et al. (2002)
	Co-delivery of chemokines (CCL-21, CCL27, CCL-28, CCL-5)	Attraction of immune cells to the site of vaccination	Scheerlinck (2001), Barouch et al. (2004), Abdulhaqq and Weiner (2008)
	Co-delivery of co-stimulatory genes (CD80, CD86)	Improvement of co-stimulation	Kutzler et al. (2010), Yamano et al. (2006), Calarota and Weiner (2004)
	HMGB-1 co-delivery	Recruitment, expansion and activation of APCs	Calarota and Weiner (2004), Iwasaki et al. (1997)
	TLR agonists (imiquimod,CpG)	Activation of APCs	Muthumani et al. (2009)
			Thomsen et al. (2004), Schneeberger et al. (2004)

^a For most of these methods, evidence that the increase in vaccine immunogenicity is indeed due to the proposed mechanism is at best circumstantial. Furthermore, only for selected strategies their added value has been confirmed in independent studies

(such as ER targeting signals) to DNA vaccine-encoded antigens can improve their immunogenicity (Michel et al. 2002; Boyle et al. 1997; Kim et al. 2006; Rice et al. 1999). Also in this case, enhanced immunogenicity may be due to increased cross-presentation, but again, formal evidence is lacking. Clearly, improved insight into the mechanisms by which different carrier influence vaccine immunogenicity will enable more rational DNA vaccine optimization and should be an important area of future research.

4 Origin of the “Danger Signal” in DNA Vaccines

Although the addition of various adjuvants (Table 1) can enhance their immunogenicity, DNA vaccines are also able to induce strong immune responses in animal models without the addition of adjuvants that provide inflammatory signals. As the induction of adaptive immune responses requires not only the presence of antigen, but also the presence of signals that induce APC activation (something often referred to as the danger model) (Heath and Carbone 2001; Matzinger 2002; Pulendran and Ahmed 2006), this implies that either DNA vaccines themselves or the DNA vaccination procedure provides elements that result in a sense of danger.

4.1 *Danger in ‘Naked’ DNA*

For many years it has been assumed that unmethylated CpG motifs were the primary source of danger in DNA vaccine preparations. Unmethylated CpG motifs form one of the so called ‘pathogen-associated molecular patterns’ (PAMP) that are recognized by pattern recognition receptors (PRR), in the case of CpG the TLR9. TLR9 is expressed in the endocytic pathway, providing endocytosing cells with the ability to detect CpG motifs within ingested material. Triggering of TLR9 initiates a cascade of signaling events that leads to NF- κ B and activator protein 1 (AP-1) activation, and the subsequent induction of a pro-inflammatory response characterized by the release of cytokines and chemokines, e.g., type I interferons (IFNs), interleukin (IL)-6, IL-12 and tumor necrosis factor (TNF)- α (Tang and Pietersz 2009). In early work, the inclusion of additional CpG motifs within the plasmid backbone was shown to improve DNA vaccine efficiency after ID vaccination in a murine melanoma model (Schneeberger et al. 2004). As TLR9 is differentially expressed between mice (all dendritic cell subsets) and men (only plasmacytoid dendritic cells) (Iwasaki and Medzhitov 2004), it has been suggested that a reduced ability to initiate a CpG-dependent danger response could explain the poor track record of DNA vaccines in humans. However, several studies have shown that both the induction of cellular as well as humoral immune responses is unaffected in TLR9-deficient mice (Babiuk et al. 2004; Spies et al. 2003).

Assuming that TLR9 forms the sole receptor for CpG, these data suggest that danger in DNA vaccination must (also) be sensed by other means.

Recently, evidence has been provided indicating that double stranded DNA (dsDNA) in the B form (right-handed helical structure) functions as an intrinsic adjuvant in DNA vaccines [reviewed by Tang and Pietersz (2009), Coban et al. (2008)]. Two dsDNA sensors have been identified thus far, namely DAI (DNA-dependent activator of IFN-regulatory factors) and AIM 2 (absent in melanoma-2). Contrary to TLR9, these dsDNA sensors are expressed within the cytosol, providing transfected cells with the ability to detect incoming DNA. DAI-induced immune activation is mediated through the activation of IFN-regulatory factor 3 (IRF3) and NF- κ B and results in the production of type I IFNs (Takaoka et al. 2007). AIM 2 has recently been described as the cytosolic DNA sensor that is responsible for activation of the inflammasome, thereby resulting in the production of active IL-1 β , IL-18 and IL-33 (Schroder et al. 2009). However, as optimal DNA vaccine immunogenicity requires type I IFNs (Ishii et al. 2008) and AIM2 is not required for type I IFN production, it is considered to have a secondary role in the DNA-induced adjuvant response (Tang and Pietersz 2009). An important study by Ishii et al. has demonstrated a pivotal role for TANK-binding kinase 1 (TBK-1), a non-canonical I κ B kinase, in mediating the adjuvant effect of DNA vaccines. In the presence of dsDNA, TBK-1 activates IRF3 and IRF7, leading to the production of type I IFNs. Notably, TBK-1 deficient mice were unable to generate antigen-specific humoral and cellular immune responses upon vaccination with a DNA vaccine delivered by IM injection followed by electroporation (Ishii et al. 2008). In contrast, DNA vaccine-induced immune responses were not affected by DAI deficiency and from this observation it was concluded that TBK-1 but not DAI is essential to the DNA vaccine mediated adjuvant response. Recently evidence was provided for the involvement of another signaling component named stimulator of IFN genes (STING) in TBK-1 mediated dsDNA sensing (Ishikawa et al. 2009). STING assembles with TBK-1 after dsDNA stimulation (Saitoh et al. 2009) and TBK-1 trafficking is blocked in the absence of STING (Ishikawa et al. 2009). Moreover STING is essential for intracellular DNA-mediated type I IFN production and STING deficient mice showed an almost complete inhibition of both humoral and cellular immune responses upon DNA vaccination. Notably, despite the increasing knowledge on the signaling route that controls cellular responses upon cytosolic DNA encounter, the critical element recognizing dsDNA in this pathway still needs to be identified. Our current knowledge on intracellular DNA sensors is summarized in Table 2.

4.2 Administration-Induced Danger

While recognition of the introduced DNA forms one route through which DNA vaccination results in a danger response, the physical damage induced by the administration procedure itself is likely to be a second factor. Sensing of physical

Table 2 Cellular DNA sensing elements and their importance in DNA vaccination-induced immune responses

Pattern recognized	DNA recognizing element	Signaling components involved	Mediators released	Relevance for DNA vaccination	Reference
CpG motifs	TLR9	MyD88	IL-6, IL-12, TNF- α , type 1 IFN	Little/moderate	Krieg (2002), Babiuk et al. (2004), Spies et al. (2003)
dsDNA	AIM2	Inflammasome	IL-1 β , IL-18, IL-33	Little	Schroder et al. (2009)
	DAI	TBK-1/IRF3	Type 1 IFN	Little	Takaoka et al. (2007)
	Unknown	TBK-1/ STING/ IRF3	Type 1 IFN	High	Ishii et al. (2008), Ishikawa et al. (2009)

damage seems likely to be of particular importance for ID delivered DNA vaccines, as the skin has an important barrier function in host defense and is densely populated with immune cells. Therefore, administration procedure-induced local skin injury is likely to result in an inflammatory response that can boost vaccine immunogenicity (Nestle et al. 2009). This notion is supported by a recent report demonstrating that epidermal injury during poxvirus immunization is crucial for the generation of protective T cell mediated immunity (Liu et al. 2010). Furthermore, delivery-induced damage has also been suggested to play a role following electroporation-mediated IM delivery (Chiarella et al. 2008; Peng et al. 2007) and even following simple IM injection in mice, as the injection volume used (usually about 50 μ l) exceeds the fluid capacity of the muscle resulting in local tissue damage (Rice et al. 2008; Dupuis et al. 2000).

What are the molecular mediators of the inflammatory response that is induced by physical damage? First, cell death that occurs during vaccination may lead to the release of intracellular molecules (with high-mobility group protein B1 (HMGB-1) as a prototype) that can be recognized by neighboring cells, or can result in the formation of uric acid crystals. This class of endogenous indicators of danger, sometimes referred to as alarmins [reviewed by Matzinger (2002), Pulendran (2004)] is likely to grow further in coming years, and it seems plausible that the role of individual alarmins as indicators of danger will depend on the strategy used for DNA vaccine delivery. In the case of ID DNA vaccine delivery, the vaccination-induced damage may also result in a danger response through an indirect mechanism. Specifically, the disruption of the skin barrier will create opportunities for pathogens/skin-resident microorganisms to locally invade the epidermal or dermal layer. As a consequence, immune activation can be expected to occur via the sensing of one of the many identified PAMPs, such as LPS, peptidoglycans, flagellin, etc. (Pulendran and Ahmed 2006).

While there is increasing interest in the role of adjuvant signals provided by the DNA itself, little attention has thus far been given to the contribution of the DNA vaccination procedure-induced damage to vaccine immunogenicity. Furthermore,

our understanding of the contribution of different danger signals (be they either DNA- or damage-induced) to different types of adaptive immune responses (humoral, Th1, Th2, Th17, cytotoxic) is still limited.

5 Optimizing DNA Vaccination by Intradermal Tattooing

Given the poor performance of DNA vaccines (mostly IM delivered) in non-human primates and early clinical trials we set out to develop an improved strategy for DNA vaccine delivery. First, we postulated that a strategy in which DNA vaccines are introduced into the skin by a multitude of needle injections rather than a single injection would be superior. This method, in which DNA is delivered to the epidermal skin layer by many thousands of injections using a permanent make-up or tattoo device has been named DNA tattooing (Bins et al. 2005). Secondly, by measuring DNA vaccination-induced antigen expression *in vivo* using a firefly luciferase-encoding DNA, the kinetics of antigen expression could be followed. Notably, despite the fact that antigen expression after ID tattoo was approximately 10- to 100-fold lower and of much shorter duration than after IM injection, presentation of the vaccine-encoded epitope to CD8⁺ T cells was shown to be markedly better. Based on the observation that DNA tattoo-induced antigen expression was restricted to approximately 96 h, a vaccination schedule was developed in which DNA is applied three times with 2 day intervals. Using this short-interval ID DNA delivery schedule, robust CD8⁺ T cell responses that can readily be measured directly *ex vivo* could be induced within 2 weeks. In contrast, IM vaccination with this short-interval regimen did not lead to detectable T cell responses. Furthermore, in comparison to IM DNA vaccination, DNA tattooing was shown to mediate substantially better protection in mouse models of influenza A infection and HPV 16-associated cancer. A likely explanation for the higher immunogenicity of DNA tattoo vaccination is that skin is a better equipped for the induction of immune responses. In contrast to muscle, skin is rich in APCs (Zaba et al. 2009) and is the body's first line of defense against many pathogens (Nestle et al. 2009). Also, since the tattoo procedure inflicts thousands of skin perforations it is likely to result in the release of many more danger signals than simple IM or ID injection, thereby serving as a potent adjuvant (see below).

Interestingly, ID tattoo vaccination has also been applied to other vaccine modalities. For peptide-based vaccines it was shown that ID tattooing was more efficient than a subcutaneous (SC) injection (Pokorna et al. 2009). Also adenoviral vectors have been administered via ID tattoo in a side-by-side comparison with SC injection. In contrast to the results obtained with DNA vaccines, delivery of adenovirus via ID tattoo immunization did not provide any obvious advantage over delivery via ID injection (Potthoff et al. 2009). A possible explanation for this lack of superiority of ID tattoo vaccination is that the uptake of the adenovirus into host cells is much more efficient obviating the need for a more sophisticated delivery procedure and/or that viral particles themselves serve as a strong adjuvant, thereby making the tattoo procedure redundant.

6 Mechanism of Immune Induction upon DNA Tattooing

6.1 Antigen Expression and Priming

How does ID DNA tattooing induce CD8⁺ T cell responses? Upon ID DNA tattoo vaccination, antigen expression is largely confined to cells within the epidermal layer, as revealed by beta-Gal staining (Bins et al. 2005). Furthermore, by flow cytometric analysis of single cell suspensions of tattooed ex vivo human skin (see below for more details on this model) it was shown that the vast majority of transfected cells consists of keratinocytes. Sporadic transfection of Langerhans cells (LCs) in the epidermis could also be observed (approximately 1% of transfected cells, more or less proportional to their frequency in human skin cell preparations) (van den Berg et al. 2009). Notably, the fact that only few antigen-expressing LCs could be recovered from human skin could not be explained by rapid migration of these cells after DNA administration. Does the fact that antigen expression upon DNA tattoo is largely restricted to keratinocytes indicate a dominant role for this cell type in the induction of immune responses? When vaccination-induced antigen expression is restricted to keratinocytes by the use of the K14 promoter, CD8⁺ T cell responses could still be induced by this strategy for DNA vaccination in a murine model (Bins et al. 2007). As there is no evidence for migration of keratinocytes to the skin-draining lymph nodes, nor for na T cell priming at the site of vaccination, these data strongly suggest that the induction of a vaccine-specific CD8⁺ T cell response upon DNA tattooing is at least partially due to cross-priming. A schematic representation of the different possibilities that lead to CD8⁺ T cell priming upon DNA tattooing is provided in Fig. 1.

6.2 Provision of Danger Signals

As discussed above, recognition of danger signals upon DNA vaccination may either involve the direct recognition of the introduced DNA, or the detection of physical damage caused by DNA introduction. Thus far, only the role of TLR9 in sensing unmethylated CpG motifs upon DNA tattoo has been evaluated. Consistent with data from studies that have evaluated the role of TLR9 in other DNA vaccination modalities, the magnitude of CD8⁺ T cell responses induced by DNA tattoo in wild type and in TLR9^{-/-} mice were identical, demonstrating that—at least in mice—TLR9 mediated signaling is not essential for the induction of immune responses by DNA tattoo vaccination (Bins et al. 2005). Evidence for or against a role for different cytosolic dsDNA sensing systems in DNA tattoo vaccination-induced immunity is at present lacking. However, as this DNA vaccination strategy relies on the generation of thousands of skin perforations, a contribution of vaccination-induced skin damage to the immunogenicity of DNA tattooing, therefore, seems plausible. Support for the notion that inflammatory signals inflicted by tissue damage contribute to the immunogenicity of DNA tattoo

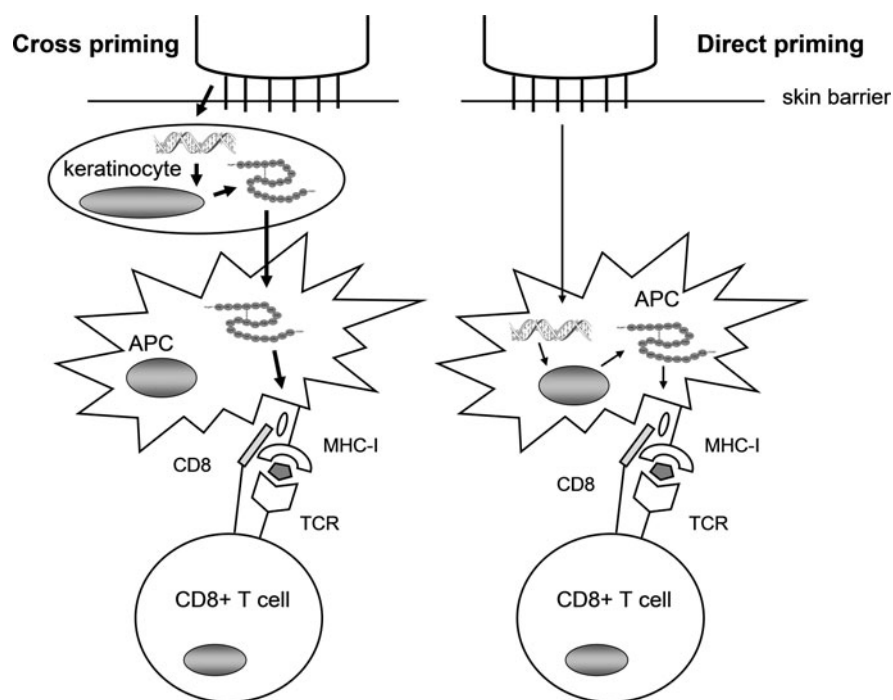


Fig. 1 Possible routes for the priming of CD8+ cytotoxic T cells upon ID DNA tattoo vaccination. Upon DNA tattooing mainly keratinocytes are transfected and produce antigen that is acquired by professional APCs, leading to cross-presentation to CD8+ cytotoxic T cells. As an alternative, APCs can become directly transfected, leading to direct priming of CD8+cytotoxic T cells. Based on available evidence, cross-presentation is considered the predominant route (see text)

is provided by a study in which we measured serum IL-6 levels upon tattoo application of either DNA or water-for-injections (WFI). Notably, systemic IL-6 levels were increased to the same extent in both groups of mice and exceeded those seen upon intraperitoneal delivery of 100 IU of LPS [a known inducer of IL-6 (Harden et al. 2006)]. These data suggest that administration-induced danger signals form a major factor in the immunogenicity of DNA tattoo (van den Berg et al. 2010c). A schematic representation of the different routes by which danger can be sensed upon DNA tattooing is provided in Fig. 2.

7 DNA Tattoo Versus Other DNA Delivery Techniques

To date, a large number of different delivery methods for DNA vaccines have been developed. In the following section a selection of these methods is discussed and their pros and cons relative to DNA tattooing are evaluated.

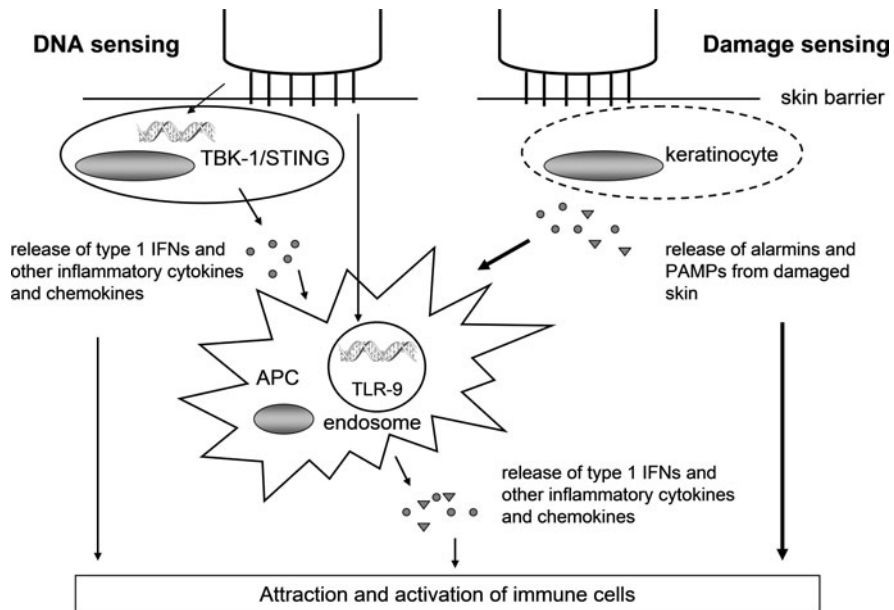


Fig. 2 Routes by which danger can be sensed upon intradermal DNA tattoo vaccination. Danger can be sensed via detection of dsDNA by cytosolic DNA sensors and signaling via TBK-1/STING, or via detection of CpG motifs by endosomal TLR9 (although the latter route is not critical). On the other hand, the tattoo procedure can induce damage to the skin leading to the release of PAMPs and alarmins. The thus released mediators can activate skin-resident APCs and attract/activate other immune cells. The fact that tattooing without any DNA already results in strong immune activation as measured by serum IL-6 levels (see text) suggests that damage-induced danger signals may play a dominant role

7.1 Intramuscular Injection

IM injection is one of the first routes of administration used for the delivery of naked plasmid DNA (Wolff et al. 1990) and the ease and simplicity of the method are particularly attractive for large-scale use. Although the method has proven effective in small animal models, the results obtained in studies in non-human primates and clinical trials have been disappointing even when doses up to 5 mg plasmid were used (Lu et al. 2008; Dean et al. 2003; Johnston et al. 2002). This translational block has been referred to as the “simian barrier” (Johnston et al. 2002) and is possibly explained by the impossibility to scale-up the injection volume used in mice (50 μ l) to non-human primates and humans. Based on the difference in body weight (20 g vs. 80 kg) an injection volume of about 200 ml would be needed for a linear scale-up. Inability to perform such scale-up may be a particularly important factor as it has been suggested that the tissue damage inflicted by injection of a large volume of DNA relative to the volume of the

injection site contributes to the immunogenicity of IM DNA vaccination in mice (Rice et al. 2008; Dupuis et al. 2000).

As described above we have performed an extensive comparison of the efficiency of ID tattoo vaccination and classical IM DNA vaccination in murine models. These data demonstrated that DNA tattoo outperformed IM DNA vaccination both with respect to the speed of CD8⁺ T cell induction and with respect to the magnitude of this immune response. The superiority of DNA tattoo in mice has since then been confirmed in a study by Pokorna et al., in which previously described strategies to enhance the potency of IM DNA vaccination (cardiotoxin pretreatment or GM-CSF DNA co-delivery) were also included. In this study, ID DNA tattooing elicited significantly higher L1 specific humoral and cellular immune responses as compared to all IM conditions evaluated, even if the number of IM injections exceeded the number of tattoo administrations (Pokorna et al. 2008).

Having demonstrated the superiority of DNA tattoo in small animal models we set out to determine if the translational block described for IM delivery would also apply to DNA tattoo. To this end we performed a study in rhesus macaques, in which we delivered an HIV clade C DNA vaccine via ID DNA tattooing and compared the results with a prior study using exactly the same vaccine and vaccine dose but delivered by IM injection. This study demonstrated a 10- to 100-fold increase in the magnitude of vaccine-specific T cell responses in peripheral blood from rhesus macaques vaccinated by DNA tattoo, as compared to T cell responses in animals immunized via the IM route. Furthermore an increase in the fraction of animals responding to the immunogens was also observed. In conclusion, DNA tattoo outperforms IM DNA vaccination in both small and large animal models, warranting its further testing in humans (Verstrepen et al. 2008).

7.2 Particle-Mediated Epidermal Delivery

Particle-mediated epidermal delivery (PMED) comprises the bombardment of the skin with gold particles coated with DNA and is often referred to as ‘gene gun’ vaccination. Gene gun-mediated gene transfer is the first method that was successfully used for DNA vaccination in murine models (Tang et al. 1992). The method, which was originally developed for the transfection of cells in vitro, has been extensively studied in human subjects. Within these studies, both cellular and humoral vaccine-specific responses have been demonstrated and—even though a side-by-side comparison has to our knowledge not been performed in clinical trials—the method is generally considered more efficient than IM injection (Dean et al. 2003; Dean et al. 2005). A comparison of the efficiency of gene gun and ID tattoo vaccination (using the same short-interval administration schedule) has demonstrated that the two methods are equipotent in CD8⁺ T cell activation in a murine model.

The doses of DNA required to induce immune responses by gene gun administration are surprisingly low, about 1 µg/dose, being approximately 100–1,000 fold lower than that used for IM injection, and this holds true for both murine and larger animal models (Dean et al. 2003; Dean et al. 2005). Moreover, also in human clinical trials, immune responses have been detected with doses below 10 µg (Dean et al. 2005). A possible explanation for this high efficiency is that gene gun is believed to directly deliver the DNA into the intracellular environment, in contrast to any other DNA delivery method (Dean et al. 2003). However, as the capacity of the current delivery devices is also low (1–2 µg of DNA per ‘shot’) the scaling of gun vaccination from mice to human application may still form an issue (Jechlinger 2006; Dean et al. 2005). Specifically, taking into account the difference in body surface (0.0075 vs. 1.85 m²), approximately 250 vaccinations would be required to achieve the same dose per body surface. A second drawback is that the costs per immunization may be substantial (in particular when such scaling is performed), because of the need for formulating the DNA onto gold particles (Jechlinger 2006).

7.3 Electroporation-Mediated Gene Transfer

Electroporation (EP) is successfully used as a strategy for the transfection of cells in vitro (Bodles-Brakhop et al. 2009). EP uses short electrical pulses to destabilize cell membranes. While the precise mechanism is unclear, EP is thought to promote cellular uptake of DNA through permeabilizing cell membranes and driving DNA entry via an electrophoretic process (Mir 2008). As it is believed that the poor performance of DNA vaccines in larger animals and humans can at least in part be explained by the low transfection efficiency upon needle mediated delivery of naked DNA (Babiuk et al. 2002; Nishikawa and Huang 2001), EP has been extensively evaluated for its potential to increase in vivo transfection. Several devices for EP-assisted DNA vaccination have been developed and EP has been shown to result in an increase in antigen expression and vaccine immunogenicity in murine models when combined with either IM (Dupuis et al. 2000; Widera et al. 2000) or ID DNA injection (Roos et al. 2006; Roos et al. 2009). In a direct comparison, the combination of IM injection and EP was shown to be more efficient than gene gun-mediated DNA administration (Best et al. 2009). EP mediated DNA vaccination has also been shown to increase antigen expression levels and vaccine immunogenicity in large animals (Babiuk et al. 2002; Hirao et al. 2008). Based on these highly promising preclinical data EP is now also being evaluated in clinical trials [reviewed by Bodles-Brakhop et al. (2009)].

A slight complication of this technique is that there are many variables such as pulse duration, pulse strength and the number of pulses that need to be optimized. It has been demonstrated that EP settings that result in high expression levels are not necessarily those that induce the highest immune responses (Roos et al. 2006).

Furthermore, the optimal settings may also differ depending on the array used and the targeted tissue (Mir 2008; Rabussay 2008).

A safety concern that has been associated with the improved transfection efficiency upon EP is a possible increase of the number of chromosomal integrations (Schalk et al. 2006; Smith and Klinman 2001). In one study by Wang et al. (2004), it was observed that EP markedly increased the amount of plasmid associated with high molecular weight (i.e., genomic) DNA. Furthermore, using a newly developed PCR method, four independent integration events were detected in electroporated muscle, providing direct proof for genomic integration upon DNA vaccination. However, other studies have shown no increase in the amount of plasmid DNA associated with high-molecular-weight DNA after EP in combination with IM delivery (Luckay et al. 2007). More importantly, it seems plausible that the risk of genomic integration will scale proportionally with any improvement in DNA vaccine delivery strategies and this risk is, therefore, unlikely to be unique to EP.

7.4 Jet Injection

Jet injection is a needle-free technique in which fluid is injected under high pressure and this technique is suitable for both IM and ID administration. Jet injectors have successfully been used for immunizing humans with live attenuated vaccines against measles and smallpox, as well as inactivated live vaccines against cholera, hepatitis B, influenza and polio (Mitragotri 2005). Advantages of the method are that it avoids the use of sharps and its compatibility with existing vaccine formulations that have been developed for needle-based administration. Disadvantages of the method include higher levels of pain and more frequent side reactions than observed with needle-based vaccine delivery (Mitragotri 2005). In a report by Trimble et al., CD8⁺ T cell responses and antitumor effects generated by a DNA vaccine administered ID via gene gun or Biojector® (a jet injector suitable for ID delivery) and IM via needle injection were directly compared in a murine model (Trimble et al. 2003). In this comparison, gene gun vaccination formed the most potent method of immunization. Furthermore, in non-human primates IM jet injection with the Biojector® or Mini-JectTM was not more efficient than simple IM injection with respect to the induction of both cellular and humoral immune responses (Rao et al. 2006). Based on these data it can be concluded that jet injection does not significantly improve the immunogenicity of IM administered DNA vaccines.

7.5 Microneedle-Assisted Gene Transfer

Microneedles are small needles with a size between 200 and 400 µm that have been designed to deliver drugs to the epidermal layer of the skin, without

stimulating the pain receptors that populate the underlying dermis (Birchall et al. 2005). Microneedles have been shown to be useful for the delivery of protein-based vaccines in clinical trials as delivery of the seasonal influenza vaccine via microneedles has been shown to be more effective than simple IM delivery (Arnou et al. 2009; Van Damme et al. 2009). A recent study by Zhou et al. demonstrated that microneedle-based delivery of a hepatitis B virus DNA vaccine in mice resulted in higher levels of humoral and cellular immune responses when compared to IM injection with the same DNA construct (Zhou et al. 2010). However, protein expression levels upon DNA application with microneedles are also reported to be unpredictable and difficult to control, and because of this, further optimization is likely to be required for the future development of this strategy into a robust DNA vaccination platform (Birchall et al. 2005).

7.6 Concluding Remarks on the Different DNA Vaccine Delivery Methods

Compared to the above-mentioned administration techniques (summarized in Table 3), DNA tattoo stands out by its simplicity. There is no need for formulation of the DNA as is the case for PMED. As compared to jet injection, EP and PMED, the required equipment is relatively simple and cheap. More importantly, linear scale-up from mice to man can simply be done by vaccination of larger skin areas. This notion is supported by the promising results of DNA tattoo in non-human primates (Verstrepen et al. 2008). Furthermore, as the tattoo procedure causes substantial damage to the skin requirement for the inclusion of adjuvants may not be needed, making clinical translation relatively straightforward. By the same token, it is noted, however, that the invasiveness of the method and also the requirement for repeated administration are likely to limit the current DNA tattoo strategy to high-risk diseases.

8 Clinical Translation of Intradermal DNA Tattooing

8.1 Ex Vivo Human Skin Model

On the basis of the promising preclinical data both in mice and in non-human primates an effort has been made to translate DNA tattoo vaccination into clinical application. However, before initiation of studies in humans it was important to determine the optimal settings for DNA tattoo in human skin, in particular because the physiology of human skin has obvious differences compared to that of furred murine and non-human primate skin (Godin and Touitou 2007). To address this issue we have analyzed the parameters that result in optimal expression of

Table 3 Advantages and limitations of different DNA delivery methods

Gene transfer method	Advantages	Limitations	Ref.
IM injection	Ease of the method, low costs of equipment	Poor track record in larger animals and human subjects	Wolff et al. (1990), Johnston et al. (2002), Verstrepen et al. (2008), McCluskie et al. (1999)
PMED ('gene gun')	High potency in relation to dose, extensive preclinical and clinical experience	Complex gold particle-based formulation/ high cost of the equipment, dose limitation to microgram range	Jechlinger (2006), Dean et al. (2005), Mitragotri (2005)
Electroporation	Extensive preclinical and clinical experience, can be combined with other delivery methods	High cost of the equipment	Wang et al. (2004), Bodles-Brakhop et al. (2009)
Jet injection	Needle-free method, ability to work with existing formulations, and success with many forms of vaccines	High cost of the device, higher levels of pain than with needles No evidence for superior performance relative to IM DNA vaccination	Mitragotri (2005), Ren et al. (2002)
Microneedle-based application	Favorable patient acceptability, possibility for self administration	Limited experience in DNA vaccination, low protein expression levels	Birchall et al. (2005)
DNA tattoo	Linear scale-up from mice to men possible, relatively cheap/ portable instrument	Invasiveness of the method may limit patient acceptability	Bins et al. (2005), Verstrepen et al. (2008)

vaccine-encoded antigens applied to human skin by DNA tattoo (van den Berg et al. 2009). For this purpose, we have developed an ex vivo human skin, in which DNA vaccines encoding reporter proteins are applied via ID tattoo. These studies revealed that gene expression upon ID DNA tattoo of human skin is almost exclusively restricted to the epidermal layer. Furthermore, consistent with the data obtained in mice, the vast majority of transfected cells consisted of keratinocytes. In order to optimize variables that we considered likely to influence the efficiency of DNA vaccination we have tattooed a total of 428 skin areas with luciferase-encoding DNA, thereby examining the effect of variations in (1) DNA concentration, (2) the duration of tattooing, (3) needle depth and (4) the type of tattoo machine. From these experiments, analyzed in a linear mixed effects model, it was concluded that DNA concentration is the most important factor influencing antigen expression in human skin. Furthermore, it was shown that also tattoo time and

tattoo depth had significant effects on antigen expression. These data have been instrumental for the design of the first clinical trial of DNA tattoo and we speculate that this in vitro model will also be of value for the preclinical optimization of other DNA vaccine delivery strategies.

8.2 Ongoing and Planned Clinical Trials

Currently a first phase I clinical trial is ongoing to evaluate the safety and tolerability of ID DNA tattoo for the treatment of HLA-A2 positive advanced stage melanoma patients. Melanoma forms an interesting target for therapeutic vaccination as there is evidence to suggest that cellular immune responses contribute to the spontaneous regressions that are sporadically observed (van Oijen et al. 2004). Furthermore, a large number of melanoma-associated antigens (such as MART-1, tyrosinase, and gp100) have been identified and a substantial number of cytotoxic T cell epitopes from these antigens have been mapped. The DNA vaccine that is being used within this first trial encodes a modified (affinity-enhanced) MART-1 epitope fused to tetanus toxin fragment C. The plasmid DNA for this trial was manufactured in our in-house GMP production facility (Quaak et al. 2008), illustrating that clinical translation of DNA vaccines is relatively straightforward as compared to most other vaccine formats. Thus far, the tattoo procedure is well tolerated and no obvious toxicity has been observed. MART-1-specific T cell immunity will be assayed in peripheral blood samples and on skin biopsies from the vaccination site, using both MHC-tetramer staining and IFN- γ ELISPOT. Furthermore, serum antitetanus toxin antibody titers will be measured in order to monitor the induction of humoral immune responses by the vaccine.

In the near future we will also initiate a phase I clinical trial to evaluate DNA tattoo for the treatment of HPV-16 positive penile- and cervical cancer. HPV-induced malignancies form an excellent target for immunotherapy as the transformed cells express viral proteins, thereby enabling recognition of malignant cells without the danger of targeting healthy cells (Frazer 2004). For this trial we have developed two DNA vaccines directed against the HPV 16 E6 and E7 oncogenes and clinical grade production of these plasmids is currently ongoing.

9 Opinion on Usefulness of Intradermal DNA Vaccination, Large-Scale Use of DNA Tattoo, and Future Perspectives

As mentioned above, disadvantages of DNA tattoo are the invasiveness of the method, the fact that the tattoo procedure is more time-consuming than simple injection and that multiple administrations are required in order to induce high-level immune responses. Owing to this we consider the method currently well suitable for the development of therapeutic vaccines for high-risk diseases, but not

for (prophylactic) mass vaccination. However, when tattoo time can be shortened and/or the number of administrations can be reduced, for example, by improving transfection efficiency and construct immunogenicity, the method may become more suitable for large-scale application. One of the strategies to further improve the efficiency of DNA tattoo vaccination, which is currently under evaluation, is briefly discussed below.

Thus far, most of our studies have focused on the use of naked DNA. However, from studies in the *ex vivo* human skin model, it has been calculated that the transfection efficiency of naked DNA upon tattooing is extremely low: estimated between 1 out of 1×10^{-6} to 1×10^{-9} copies applied (van den Berg et al. 2009). Therefore, there is ample room for improving the transfection efficiency of DNA vaccines applied by DNA tattoo. In a recent study we have evaluated the use of cationic nanoparticles as synthetic delivery vehicles for DNA vaccines (van den Berg et al. 2010a). Interestingly, these studies revealed that the positive charge of such particles that dramatically enhances transfection efficiency in cell culture systems essentially prevents transfection in human skin. Only when the cationic surface charge of these particles was shielded with polyethylene glycol (PEG), transfection in human skin was apparent. Delivery of a model vaccine using these PEGylated nanoparticles resulted in an increase in transfection efficiency as compared to naked DNA both in *ex vivo* human skin and in mice (in the latter about two- to fivefold depending on the type of nanoparticle). Unfortunately, for this first variant DNA formulation that we have analyzed, no significant increase in immunogenicity was observed in spite of these higher expression levels. These data do, however, illustrate the value of the combined use of these two models for preclinical DNA vaccine delivery optimization. First, the human skin model allows one to rapidly identify vaccine formulations that yield substantial antigen expression (going from the reasonable assumption that in the absence of substantial antigen expression immunogenicity will be poor). Second, those selected formulations that yield substantial antigen expression in human skin can subsequently be analyzed for immunogenicity within the murine model. As an example, skin electroporation-mediated DNA delivery has shown great promise in both small and large animal models (Roos et al. 2009), and the combined use of DNA tattoo and electroporation may well be evaluated by combining these two models.

10 Conclusion

DNA tattoo has progressed from the first preclinical evaluation to clinical testing in a period of approximately 5 years, and based on its preclinical track record ID DNA tattoo can be considered a promising strategy for DNA vaccination. At present the two main priorities will be to evaluate the current strategy for DNA tattoo in clinical trials, while at the same time developing optimized strategies in preclinical models that can be evaluated in follow-up trials.

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