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

Joost H. van den Berg [a,b,c,*], Koen Oosterhuis [c], Wim E. Hennink [b], Gert Storm [b], Leonardus J. van der Aa [d], Johan F.J. Engbersen [d], John B.A.G. Haanen [c], Jos H. Beijnen [a,e], Ton N. Schumacher [c], Bastiaan Nuijen [a]

[a] Department of Pharmacy & Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC, Amsterdam, The Netherlands
[b] Department of Pharmaceutics, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands
[c] Division of Immunology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands
[d] Department of Biomedical Chemistry, Faculty Science & Technology, University of Twente, 7500 AE, Enschede, The Netherlands
[e] Department of Bioanalysis, Utrecht Institute of Pharmaceutical Sciences, Utrecht University, Sorbonnelaan 16, 3584 CA, Utrecht, The Netherlands

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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.

1. 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 5×10^6 to 5×10^9 plasmid copies applied being taken

* Corresponding author. Department of Pharmacy & Pharmacology, Slotervaart Hospital, Louwesweg 6, 1066 EC, Amsterdam, The Netherlands. Tel.: +31 20 5124733; fax: +31 20 5124753.
E-mail address: joost.vandenberg@slz.nl (J.H. van den Berg).

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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.

2. Materials and methods

2.1. 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′*-cystamine-bisacrylamide 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.

2.2. 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 °C 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.

2.3. 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.

2.4. 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 h after surgical removal.

To obtain an epidermal cell suspension, skin was incubated for 1 h 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 °C, upon which the epidermis was mechanically peeled from skin samples. The obtained epidermal sheet was digested at 37 °C 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, 1×10^5 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 °C. After 24 h, 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.

2.5. 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, 10 μL 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 8 mm, 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 s.

After tattooing, skin samples were kept at 5% CO_2, 37 °C 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.

2.6. 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 min 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.

2.7. 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.

2.8. 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 sensitive, 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 a 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 days 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.

2.9. 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.

2.10. 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.

2.11. 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

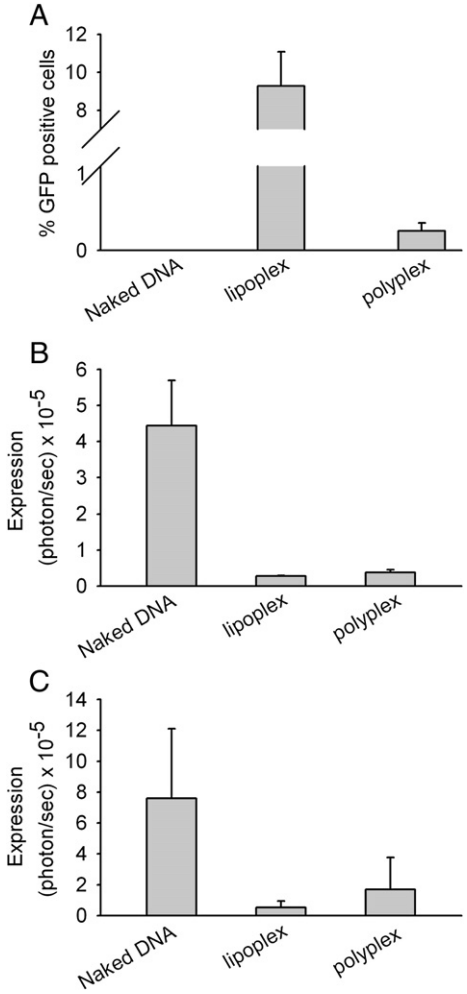

Fig. 1. Discordant *in vitro* and *in vivo* performance of cationic nanoparticles. (A) Transfection of epidermal cell suspensions with naked DNA, DOTAP–DOPE/DNA complexes ('lipoplex'), and PAA/DNA complexes ('polyplex'). Bars represent the mean + SD of three independent measurements. (B) Luciferase activity 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 h after DNA application. The same poor performance of lipoplex and polyplex DNA formulations was observed after 21 h. 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 h 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.

adjustment test was applied to correct the significant level when multiple groups were compared.

3. Results

3.1. 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 h 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, Fig. 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 Fig. 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 (Fig. 1C) and corresponding undetectable antigen-specific immune responses in vaccinated mice (see below). 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.

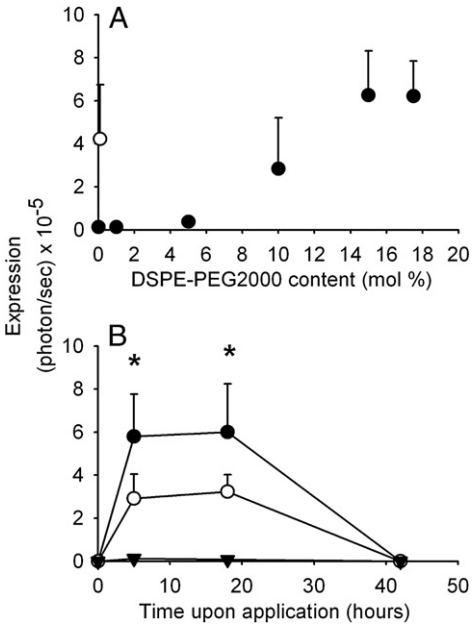

Fig. 2. Antigen expression (luciferase) upon tattoo vaccination of PEGylated lipoplexes in *ex vivo* human skin. (A) Expression induced by administration of naked DNA (○), or by administration of DOTAP–DOPE/DNA complexes as a function of DSPE-PEG content at an N/P ratio of 5 (●). Expression was measured at 5 h 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.

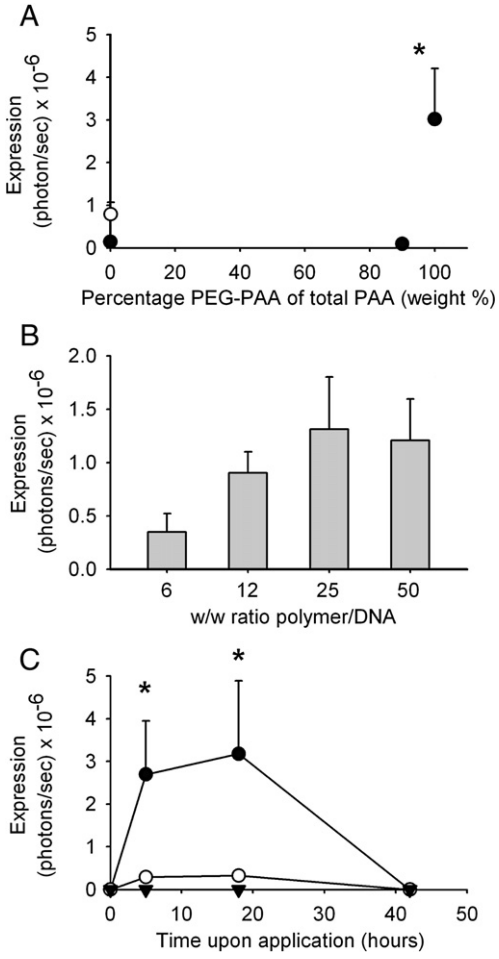

Fig. 3. Antigen expression upon tattoo vaccination of PEGylated polyplexes in *ex vivo* human skin. (A) Expression upon application of naked DNA (○), or application of PAA/DNA polyplexes as a function of the percentage of PEGylated PAA at a w/w ratio polymer/DNA of 50 (●). (B) Expression upon application of naked DNA (○), or application of PEGylated PAA /DNA polyplexes at different ratios polymer/DNA. Expression was measured at 5 h 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.

3.2. 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 Figs. 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 Figs. 2 and 3).

For the PEGylated lipoplexes, antigen expression levels reached a plateau value at a DSPE-PEG content between 15 and 17.5% (Fig. 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).

The PEGylated polyplexes showed a plateau in antigen expression levels at a polymer/DNA w/w ratio between 25:1 and 50:1 (Fig. 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.

3.3. 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)

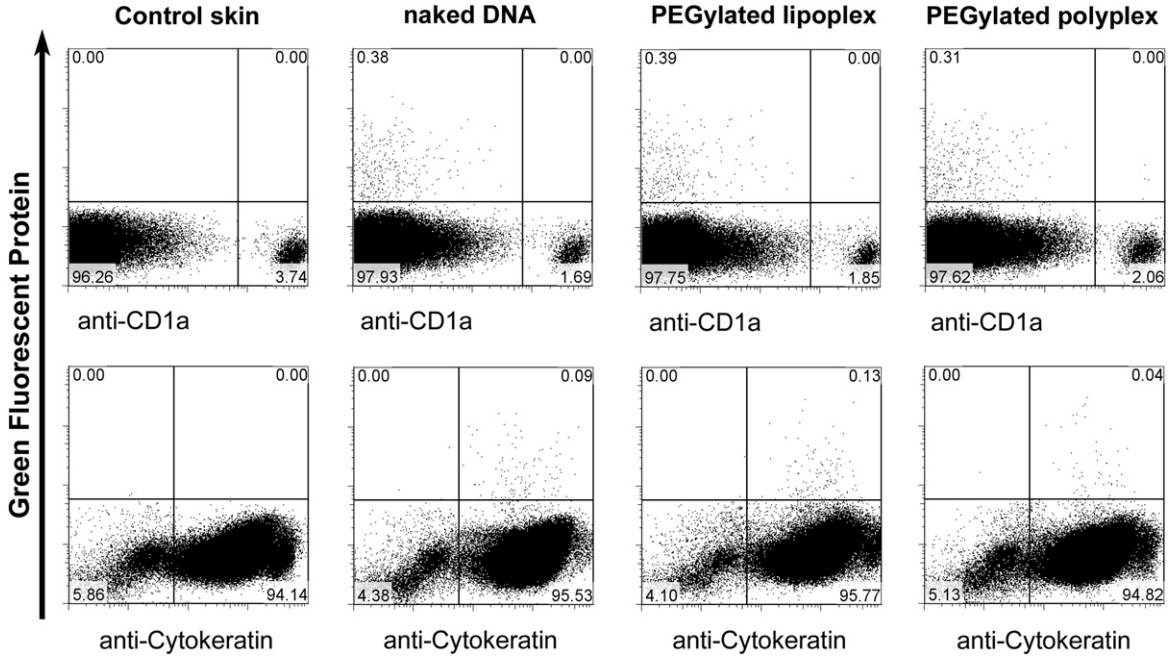

Fig. 4. Flow cytometric analysis of epidermal cell suspensions of tattooed skin. Skin was tattooed with naked GFP-encoding DNA, DOTAP–DOPE/DNA lipoplexes with 17.5 mol% DSPE-PEG (N/P ratio 5), or PEG–PAA/DNA polyplexes (w/w ratio 50). Cell suspensions of tattooed or control skin were stained with anti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

(Fig. 4). These data reveal that *ex vivo* nanoparticle administration by DNA tattooing does not result in preferential expression in epidermal LCs.

3.4. 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 days 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.

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 (Fig. 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 (Fig. 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 days 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 (Fig. 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.

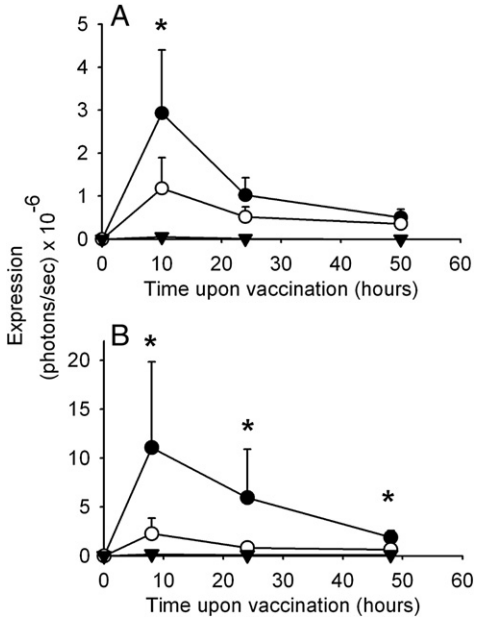

Fig. 5. *In vivo* antigen expression in mice upon tattoo vaccination of: (A) Naked DNA (○) or DOTAP–DOPE/ DNA lipoplex with (●) or without (▼) 17.5 mol% DSPE-PEG (both lipoplexes at an N/P ratio of 5). (B) Naked DNA (○), PEGylated PAA/DNA polyplexes (●) or nonPEGylated PAA/DNA polyplex (▼) (both polyplexes 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.

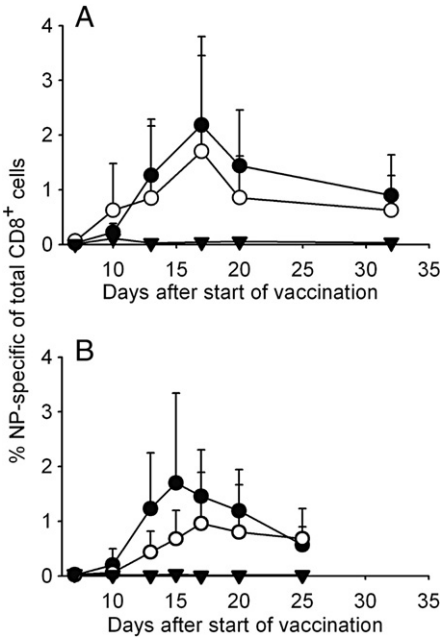

Fig. 6. T cell responses upon tattoo vaccination of DNA nanoparticles. NP_{366}-specific T cell responses upon tattoo vaccination with the Luc-NP construct are shown for: (A) Mice vaccinated with naked DNA (○) or DOTAP–DOPE/ DNA lipoplex with (●) or without (▼) 17.5 mol% DSPE-PEG (both lipoplexes at 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.

4. 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] (Fig. 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 hypothesis 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.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.09.005.

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