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Mechanistic studies on transcutaneous vaccine delivery : microneedles, nanoparticles and adjuvants

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Chapter 10

Adjuvant effect of cationic liposomes and CpG depends on administration route: intranodal, intradermal, transcutaneous and nasal immunisation in mice

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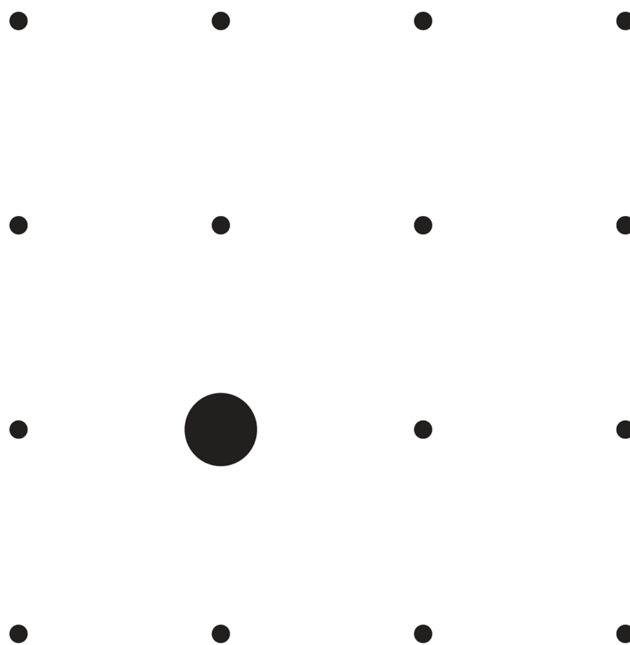
* Authors contributed equally



Submitted for publication

Abstract

In this study we explored the immunisation route-dependent adjuvanticity of cationic liposomes loaded with an antigen (ovalbumin; OVA) and an immune potentiator (CpG). Mice were immunised intranodally, intradermally, transcutaneously (with microneedle pre-treatment) and nasally with liposomal OVA/CpG or OVA/CpG solution.

In vitro, OVA/CpG liposomes showed enhanced uptake by DCs of both OVA and CpG compared to OVA+CpG solution. A similar enhanced uptake by DCs was observed *in vivo* when fluorescent OVA/CpG liposomes were administered intranodally. However, after transcutaneous and nasal application a lower uptake of OVA/CpG liposomes compared to an OVA+CpG solution was observed. Moreover, the IgG titres after nasal and transcutaneous administration of OVA/CpG liposomes were reduced compared to administration of an OVA+CpG solution. Although serum IgG titres may suggest limited added value of liposomes to the immunogenicity, for all routes, OVA/CpG liposomes resulted in elevated IgG2a levels, whereas administration of OVA+CpG solutions did not.

These data show that encapsulation of antigen and adjuvant into a cationic liposome has a beneficial effect on the quality of the antibody response in mice after intranodal or intradermal immunisation, but impairs proper delivery of antigen and adjuvant to the lymph nodes when the formulations are administered transcutaneously or nasally.

Introduction

Vaccine development has shifted focus from the classical live-attenuated and inactivated vaccines to the development of subunit vaccines. Subunit vaccines, consisting of purified proteins, are safer than live-attenuated and inactivated vaccines, but lack strong immunogenicity. A common strategy to improve their immunogenicity is encapsulation of the antigen into nanoparticles. Antigen encapsulation offers the advantages of protection from enzymatic breakdown, sustained antigen release [1, 2], enhanced uptake by professional antigen presenting cells such as DCs [3] and the possibility of co-encapsulation of immune potentiators [4, 5]. Liposomes are a type of nanoparticles that have been described to enhance the immune response because of the above mentioned advantages [6-11]. Cationic liposomes are considered the most effective vaccine delivery systems for administration via injection [12-14]. To improve the immunogenicity of liposomes, adjuvants can be co-encapsulated together with the antigen [15, 16]. Recently we showed that intradermal vaccination in mice with 250-nm sized cationic liposomes containing ovalbumin (OVA) and CpG, a Toll-like receptor 9 (TLR9) ligand, induced strongly elevated IgG2a titres and IFN-γ production by restimulated splenocytes [17].
Besides the attention given to vaccine formulation, interest is aroused for vaccine delivery via non-invasive routes, such as the nose and the skin [18, 19]. Both organs are in direct contact with the environment and therefore densely populated with immune cells to protect the body against pathogens. The nasal epithelium is equipped with the nasal associated lymphoid tissue (NALT) and the skin is densely populated with epidermal Langerhans cells (LCs) and dermal dendritic cells (DCs). The presence of high numbers of DCs at these delivery sites could facilitate the liposome's full potential to enhance antigen uptake by DCs and induce a potent, protective immune response. Moreover, liposomes can protect the antigen from enzymatic activity, which is especially an issue for nasal vaccination. Nonetheless, the beneficial effect of liposomes and nanoparticles in general for these delivery routes is under debate [20-23]. For instance, (nano)particulate matter could have more difficulties crossing the skin barrier [23], even when microneedles are employed [24] and to a lesser extent the nasal epithelium [25] (figure 1).
The aim of the present study was to investigate the impact of encapsulating an antigen, ovalbumin (OVA), and an adjuvant, CpG, in cationic liposomes on the (antibody mediated) immune response and on the transport to the lymph nodes after administration via four immunisation routes. We explored the influence of liposomal antigen and adjuvant co-encapsulation on the biodistribution and immunogenicity of the vaccine, by applying liposome via different administration routes. The transport of the vaccine through the epithelium (transcutaneous and nasal administration), to the lymph nodes (intradermal administration) and the uptake in the lymph nodes (intranodal administration) were

studied by quantifying the amount of OVA and CpG positive DCs (CpG^+ or OVA^+) in the draining lymph nodes. The added value of OVA- and CpG-containing cationic liposomes (OVA/CpG liposomes) on immunogenicity was evaluated, by determining the OVA specific serum IgG, IgG1, IgG2a levels and secretory IgA levels in nasal washes. We show that cationic liposomes enhance the concomitant uptake of OVA and CpG by DCs *in vitro* and *in vivo,* when injected intranodally. Moreover co-encapsulation of OVA and CpG seems imperative for the induction of a Th1 type immune response. However concerning transcutaneous or nasal administration, encapsulation of OVA and CpG led to reduced numbers of antigen and/or adjuvant positive DCs in the draining lymph nodes. Also lower antibody titres compared to an OVA+CpG solution were observed, indicating a negative effect of cationic liposomes for these alternative administration routes.

Material and Methods

Materials

Soybean phosphatidylcholine (PC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3-ghosphoethanolamine (DOPE) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Endotoxin free OVA was purchased at Merck (Darmstadt, Germany). Anti CD11c-PE/Cy7, CD86-FITC was acquired from Becton Dickinson (Franklin Lakes, NJ USA). Invitrogen (Breda, The Netherlands) supplied fluorescein isothiocyanate AlexaFluor647 labelled OVA (OVA_{AF647}), bovine serum albumin (BSA), chromogen 3, 3', 5, 5'-tetramethylbenzidine (TMB) and the substrate buffer. Polyclonal rabbit anti-OVA IgG and goat anti-rabbit IgG-HRP conjugate were acquired from Southern Biotech (Birmingham, AL, USA). CpG-ODN 2006 and its fluorescein isothiocyanate labelled equivalent (CpG_{FITC}) were purchased at Invivogen (Toulouse, France). Nimatek® (100 mg/ml Ketamine, Eurovet Animal Health B.V., Bladel, The Netherlands), Oculentum Simplex (Farmachemie, Haarlem, The Netherlands), Rompun® (20 mg/ml Xylazine, Bayer B.V., Mijdrecht, The Netherlands) and phosphate buffered saline (PBS 0.9% NaCl) were obtained from a local pharmacy. All other chemicals were purchased at Sigma-Aldrich (Zwijndrecht, The Netherlands), unless stated otherwise.

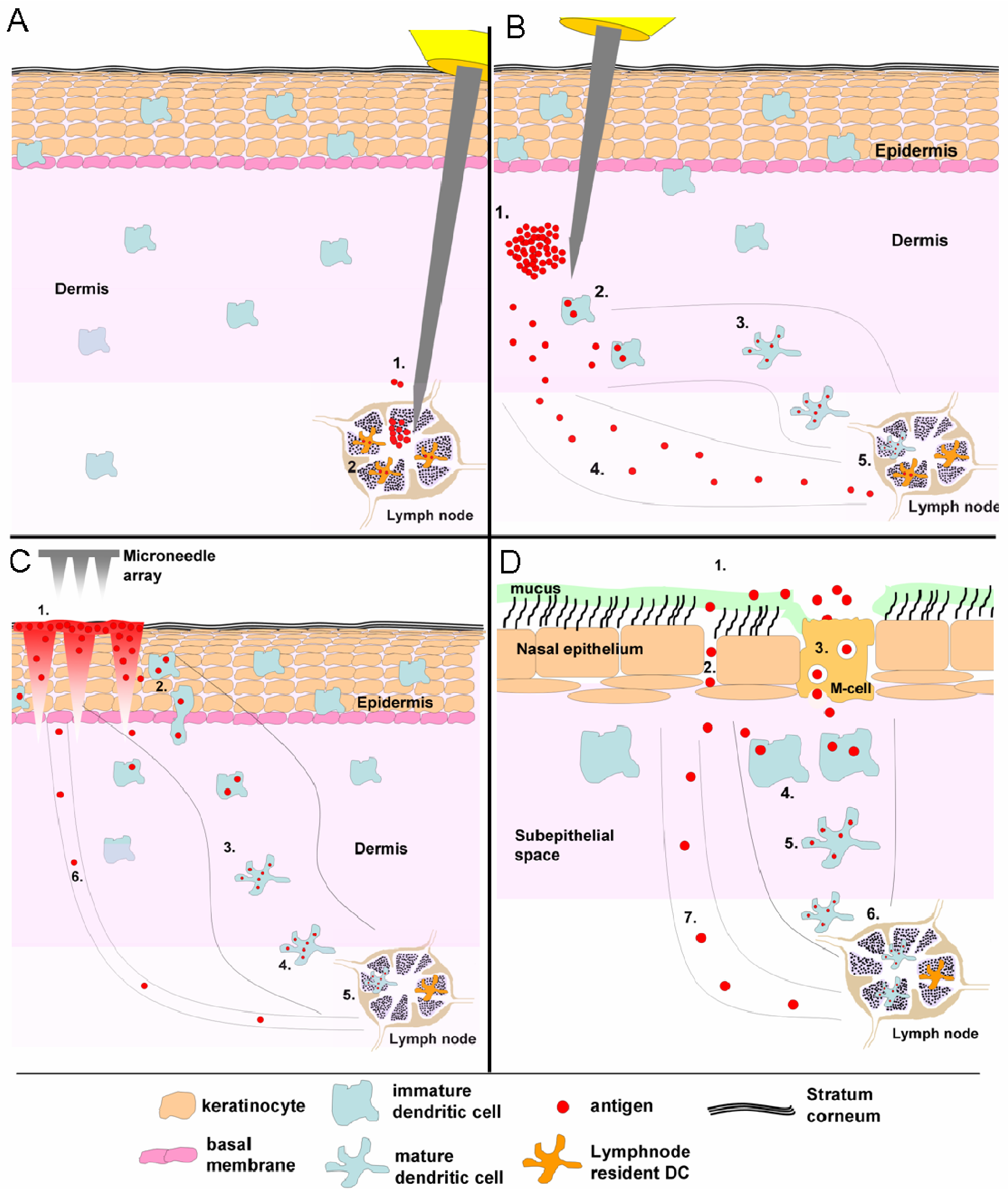

A
Dermis
1.
2.
Lymph node
B
Epidermis
1.
Dermis
2.
3.
4.
5.
Lymph node
C
Microneedle array
1.
2.
Epidermis
3.
Dermis
6.
4.
5.
Lymph node
D
1.
mucus
Nasal epithelium
2.
3.
M-cell
4.
Subepithelial space
5.
7.
6.
Lymph node
keratinocyte
immature dendritic cell
antigen
Stratum corneum
basal membrane
mature dendritic cell
Lymphnode resident DC

Figure 1. Antigen transport after immunisation via different administration routes. A) After intranodal injection (1) the antigen will directly be taken up by lymph node resident DCs (2). B) After intradermal injection (1) the antigen can be taken up by immature DCs (2) which mature and migrate to the lymph node (3) or the antigen drains directly to the lymph node (4). This results in both peripheral as well as lymph node resident DCs that are antigen positive (5). C) Transcutaneous immunisation: microneedle application creates small conduits through which the antigen can diffuse and bypass the stratum corneum (1). Immature DCs are abundantly present in the epidermis and dermis and will take up the antigen (2,) and subsequently cross the basal membrane (3), mature (4) and reach the lymph node (5). Because of the long route the antigen has to take, direct drainage of the antigen to the lymph node (6) may be limited due to degradation of the antigen. D) After nasal administration (1) the antigen can pass the epithelium either by paracellular diffusion between the epithelial cells (2) or through active transport by M-cells (3) and be taken up by immature mucosal DCs (4) which mature (5) and drain to the lymph node (6). Direct drainage to the lymph node is possible (7), but may be limited.

Preparation and characterization of liposomes

Cationic liposomes were prepared using the film hydration method [26], followed by extrusion as described previously [17]. Briefly, a thin lipid film was made of PC, DOTAP and DOPE (9:1:1 molar ratio) by evaporating the chloroform using a rotary evaporator followed by flushing with nitrogen. To prepare empty liposomes, the film was rehydrated in a 10 mM phosphate buffer pH 5 (0.1 mM Na_2HPO_4 and 9.9 mM NaH_2PO_4), whereas OVA loaded liposome were prepared by rehydration in a 10 mM phosphate buffer pH 7.4 (7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4), containing 1.5 mg/ml OVA. The final concentration of lipids was 5% (w/v).

To obtain liposomes of an equal size the solution was extruded (LIPEX™ extruder, Northern Lipids Inc., Canada) 4 times through a carbonate filter with a pore size of 400 nm and 4 times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). For adjuvanted liposomes, after rehydration CpG (final concentration of 1.5 mg/ml) was added and the dispersions were freeze-dried followed by rehydration and extrusion as described above.

After monodisperse, unilamellar liposomes were obtained, non-encapsulated antigen and adjuvant were separated from the liposomes by a Vivaspin 2 centrifugal concentrator (PES membrane, MWCO 300 kDa, Sartorius Stedim, Nieuwegein, The Netherlands) and (by using their fluorescently labelled analogues) quantified with a FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK). The same technique was applied to investigate the association of OVA with empty liposomes. The size of the liposomes was determined by dynamic light scattering (DLS) and the zeta potential was measured by laser Doppler velocimetry using a Zetasizer® Nano ZS (Malvern Instruments, UK).

Vaccination

8 week old female Balb/c mice (Charles River, Maastricht, The Netherlands) received OVA or OVA- and CpG-containing formulations via transcutaneous, nasal, intradermal or intranodal administration. Based on literature and earlier studies from our group [27-29] the antigen dose and volume was adjusted to the administration route. The dose was chosen is such a way that the IgG response after immunisation with a solution of OVA would be minimal and allow optimal discrimination between administration of OVA alone and liposomal OVA formulations.

For transcutaneous vaccination mice were shaved before pre-treatment with microneedles as described by Ding et al. [30]. Assembled metal microneedle arrays (4x4) with a length of 300 µm were used and applied with an electrical applicator as described before [24]. The formulations were applied occlusively on the skin (~2 cm^2 area restricted by a metal ring). After 2 h the abdominal skin of the mice was washed extensively with lukewarm water. Intranodal injections in the inguinal lymph nodes were performed as described by Johansen et al [31]. Intradermal and nasal immunisations were carried out as described previously [27, 28]. After 3 weeks, blood samples were drawn from the tail vein and mice received a booster dose. After 6 weeks blood samples were drawn from the femur artery and mice were sacrificed. All animal experiments were approved by the Ethical Committee of the Leiden University Medical Centre in accordance to the Dutch Animal Protection Act.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Microtitre plates (Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 ng OVA per well in a 100 mM carbonate buffer pH 9.4. Wells were blocked with 1% BSA in PBS for 1 hour at 37°C. Serial dilutions were applied for 1.5 hours after which OVA-specific antibodies were detected using HRP conjugated goat anti-mouse IgG, IgG1, IgG2a or IgA. Enzyme activity was determined by incubating with TMB/H_2O_2 in 100 mM acetate buffer pH 5.5 for 15 min at room temperature. Reaction was stopped with 2 M H_2SO_4 and absorbance was determined at 450 nm with an EL808 microplatereader (Bio-Tek Instruments, Bad Friedrichshall, Germany).

In vitro uptake by dendritic cells

Peripheral blood mononuclear cells (PBMCs) were isolated from fresh human blood using a Ficoll gradient as previously described [32]. Subsequently, monocytes were isolated from the PBMCs using a Percoll gradient as previously described [33]. After isolation, monocytes were adhered on 24-well plates by incubation for 1 hr at 37°C and 5% CO_2, and depleted of platelets by washing. Monocytes were differentiated into immature DCs by incubation for 6 days with RPMI 1640 medium supplemented with 10% foetal bovine serum, 2 mM

glutamine, 1 mM sodium pyruvate, 500 U/L penicillin/streptomycin, 250 U/ml GM-CSF and 100 U/ml IL-4.
Immature DCs were exposed for 4 h at 37°C to 0.5 µg/ml CpG_{FITC} and/or 0.5 µg/ml OVA_{AF647} in free or encapsulated form. Cells were washed three times with FACS buffer (1% w/v BSA in PBS with 2% v/v foetal bovine serum), and the number of FITC or AF647 positive DCs (CpG^+ or OVA^+) was quantified with a flow cytometer (FACSCanto II, Becton Dickinson) using quadrant analysis.

Determination of antigen specific DCs in lymph node

Mice were vaccinated with the same formulations as described in Table I, but OVA was substituted with OVA_{AF647} and CpG with CpG_{FITC}. After 4 or 24 h mice were sacrificed, the draining lymph nodes (for transcutaneous, intradermal and intranodal administration the inguinal and for nasal the cervical lymph nodes) were removed and single cell suspensions obtained. Cells were washed with FACS buffer and stained with anti-CD11c-PE-Cy7 to allow detection of DCs. Using flow cytometry (FACSCantoII, Becton Dickinson) the amount of OVA_{AF647}^+ and CpG_{FITC}^+ DCs was determined.

Statistics

All the data were analyzed with a one-way ANOVA with Bonferroni's post-test, with the exception of the antibody titres, which were processed with a Kruskal-Wallis test with Dunn's post-test. Statistics were performed using GraphPad 5.0 for Windows.

Results

Cationic liposomes improve uptake of OVA and CpG by DCs in vitro

Consistent with our previous study [17], OVA-containing liposomes with an average size of 130 nm bearing a positive zeta potential of 23 mV (loading efficiency 98%) and OVA/CpG liposomes with a size of 263 nm and a zeta potential of 18 mV were obtained (loading efficiency OVA 72%, CpG 61%). To assess whether our cationic liposomes increase the uptake of OVA and CpG, the uptake by human monocyte derived DCs was measured *in vitro*. The cationic liposomes significantly enhanced the uptake of their encapsulated cargo by DCs (figure 2). Plain OVA was readily taken up by DCs, as approximately 20% of the DCs was OVA^+ after 4 h of incubation with a mixture of an OVA and CpG solution (OVA+CpG; figure 2A), but when encapsulated into liposomes, the number of OVA^+ DC significantly increased ($p<0.001$), reaching a value of approximately 50%. In contrast to OVA, non encapsulated CpG was hardly taken up by DCs in its plain form as only 6% CpG^+ DCs were observed with OVA+CpG (figure 2B). Encapsulation of CpG in cationic liposomes however

increased the number of CpG$^+$ DCs with an order of magnitude. Co-encapsulation of OVA and CpG$^+$ in cationic liposomes caused a major improvement in the number of OVA/CpG$^{+/+}$ DCs (figure 2C), compared to addition of OVA+CpG. In conclusion, co-encapsulation of OVA and CpG in cationic liposomes enhanced their concomitant uptake by DCs *in vitro*.

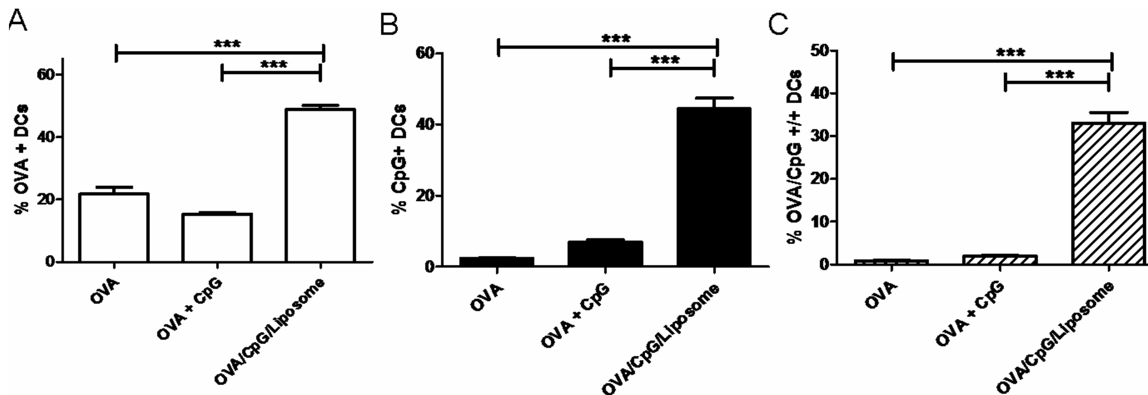


Figure 2. Uptake of a) OVA and b) CpG by human monocyte derived DCs determined by FACS analysis. c) number of OVA/CpG$^{+/+}$ double positive DCs after 4 h exposure. n=3 Average + SEM. *** $p<0.001$

Intranodal vaccination

Liposomes enhance the uptake of OVA and CpG by lymph node resident DCs after intranodal administration

Because of the large numbers of T cells present in the lymph nodes, activated DCs that have taken up an antigen have a good chance of encountering antigen specific T cells. This makes the draining lymph node the most prominent site of T cell activation after immunisation. So, direct injection of a vaccine into the lymph nodes could be a very efficient method of administration, requiring only very low amounts of antigen to result in an effective immune response, as processes like peripheral uptake of the antigen by DCs and drainage to the lymph nodes are circumvented (figure 1A). Indeed after intranodal injection a rapid uptake of fluorescently labelled OVA and CpG by DCs was observed as can be inferred from the high percentage of OVA$^+$ and CpG$^+$ DCs already after 4 h (figure 3). Liposomal co-encapsulation of OVA and CpG led to significantly elevated numbers of OVA$^+$, CpG$^+$ and OVA/CpG$^{+/+}$ DCs compared to the administration of a physical mixture of CpG and OVA. The percentage of DCs that had taken up both OVA and CpG increased by 4-fold compared to injection of OVA+CpG. After 24 h the levels had decreased drastically, suggesting that OVA and CpG had been processed. So, cationic liposomes have the potential to increase the delivery of the antigen and the adjuvant, both *in vitro* and *in vivo*.

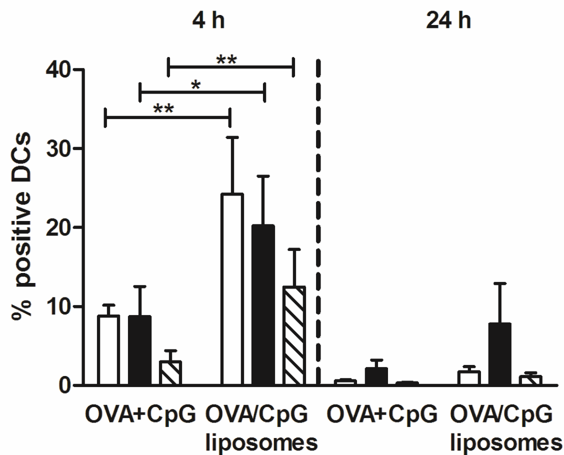


Figure 3: Quantification of OVA⁺ (white bars), CpG⁺ (black bars) and OVA/CpG$^{+/+}$ (striped bars) DCs in the draining lymph nodes 4 and 24 h after intranodal vaccination Bars represent the mean n=3 + SEM. *p<0.05, **p<0.01.

High IgG2a titres after intranodal injection of OVA/CpG liposomes

For immunisation purposes, besides OVA+CpG and the OVA/CpG liposomes, also OVA and OVA loaded liposomes (OVA liposome) as controls were included. Furthermore, empty liposomes were co-administered with OVA (OVA+liposome) to study the adjuvant effect of liposomes themselves. Despite the favourable effect of liposomal co-encapsulation of OVA and CpG on DC uptake, intranodal vaccination showed similar total serum IgG titres for all formulations (figure 4A). No effect of antigen encapsulation was observed, as OVA liposomes induced similar IgG titres as empty liposomes co-administered with OVA and an OVA solution. Similarly no effect of the addition of CpG, encapsulated (OVA/CpG liposome) or non-encapsulated (OVA+CpG) was observed, indicating that an antigen injected directly into the lymph node does not need a delivery vehicle or an adjuvant to induce a humoral immune response. However, whereas IgG titres provide information about the extent of the antibody response, subtyping of the IgG response can give insight into the type of immune response elicited. Therefore, IgG1 levels, indicative of a Th2 type response, and IgG2a titres, indicative of a Th1 type response [34, 35], were quantified to investigate the quality of the immune response after the boost immunisation (figure 4B). IgG1 titres appeared to be virtually in synchronicity with the IgG titres, again indicating that all formulations triggered the humoral immune response. However, co-encapsulation of CpG and OVA in liposomes drastically increased IgG2a levels compared to OVA solution (p<0.001) and OVA+CpG (p<0.001). As such OVA/CpG liposomes caused a decrease in IgG1/IgG2a ratio, compared to all other formulations. This shows that intrinsically, OVA/CpG liposomes are very immunogenic and can induce a mixed Th1/Th2 type immune response.

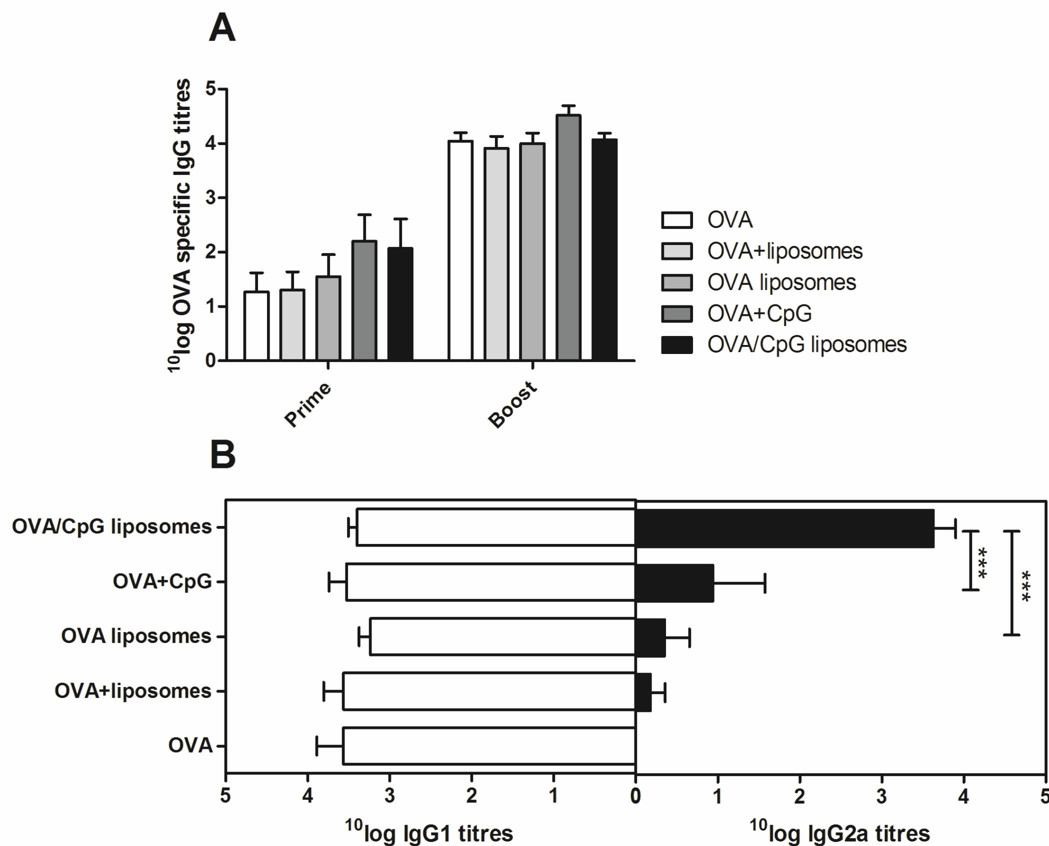


Figure 4. OVA-specific serum IgG, IgG1 and IgG2a titres after intranodal vaccination. A: IgG titres after prime and boost. B: IgG1 (white bars) and IgG2a (black bars) titres after booster immunisation. Bars represent SEM of n=5 (A and B). *** p<0.001.

Intradermal vaccination

Cationic liposomes reduce direct drainage of OVA and CpG to the lymph node

In contrast to intranodal injection, antigen that is administered intradermally can reach the lymph nodes in two ways: it can either directly drain in a quick manner to the nearest lymph node via the interstitial fluid and lymphoid vessels or it can be taken up by local DCs and transported to the draining lymph nodes in a process that takes longer (figure 1B).
Intradermal injection of fluorescent OVA+CpG seems to reveal the presence of both these routes; already 4 h after administration, OVA^+ DCs could be detected in the lymph nodes (figure 5). This indicates that a part of the OVA is directly drained to the lymph node via the interstitial flow. Uptake of OVA by dermal DCs and subsequent migration to the lymph node would require more time [36]. However, after 4 h hardly any lymph node resident DC was positive for CpG (figure 5). Liposomal delivery to the lymph nodes after intradermal administration was a slower process, as not 4 h but only 24 h after injection OVA^+ and CpG^+

DCs were found in the draining lymph nodes. This suggests that both OVA and CpG were retained at the injection side and reached the lymph node via uptake by dermal DCs. Indeed an antigen depot was visible up to 24 h after injection, as deposition of OVA_{AF647} caused a clear blue stain at the injection site. Liposomal encapsulation did not further increase the number of OVA^{+} and CpG^{+} DCs after 24 h compared to intradermal administration of non encapsulated OVA and CpG.

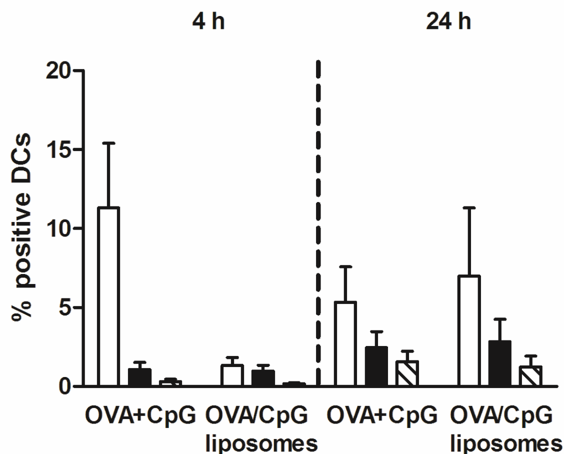


Figure 5. Quantification of OVA^{+} (white bars), CpG^{+} (black bars) and $OVA/CpG^{+/+}$ (striped bars) DCs in the draining lymph nodes 4 and 24 h after intradermal vaccination Bars represent the mean n=3 + SEM.

Co-encapsulation of OVA and CpG increases IgG2a levels significantly after intradermal administration

Vaccination via the intradermal route showed that cationic liposomes can have an adjuvant effect when mixed with OVA ($p<0.01$), whereas encapsulated liposomal OVA did not lead to a significant increase in IgG levels (figure 6A). Addition of CpG increased the antibody levels compared to OVA after the first ($p<0.001$), as well as the second immunisation ($p<0.001$) and also compared to OVA+liposomes after the first vaccination ($p<0.05$). Intradermal application of OVA/CpG liposomes increased the IgG levels to a similar extent as OVA+CpG.

After intradermal administration IgG1 titres mimicked the IgG titres and IgG2a levels were very low with the exception of those mice immunized with OVA/CpG liposomes (figure 6B). Just like after intranodal vaccination, significantly higher IgG2a titres were measured in these mice compared to those receiving plain OVA, OVA liposomes or OVA+CpG ($p<0.001$). This demonstrates that co-encapsulation results in a shift in the IgG1/IgG2a ratio, also for the intradermal route.

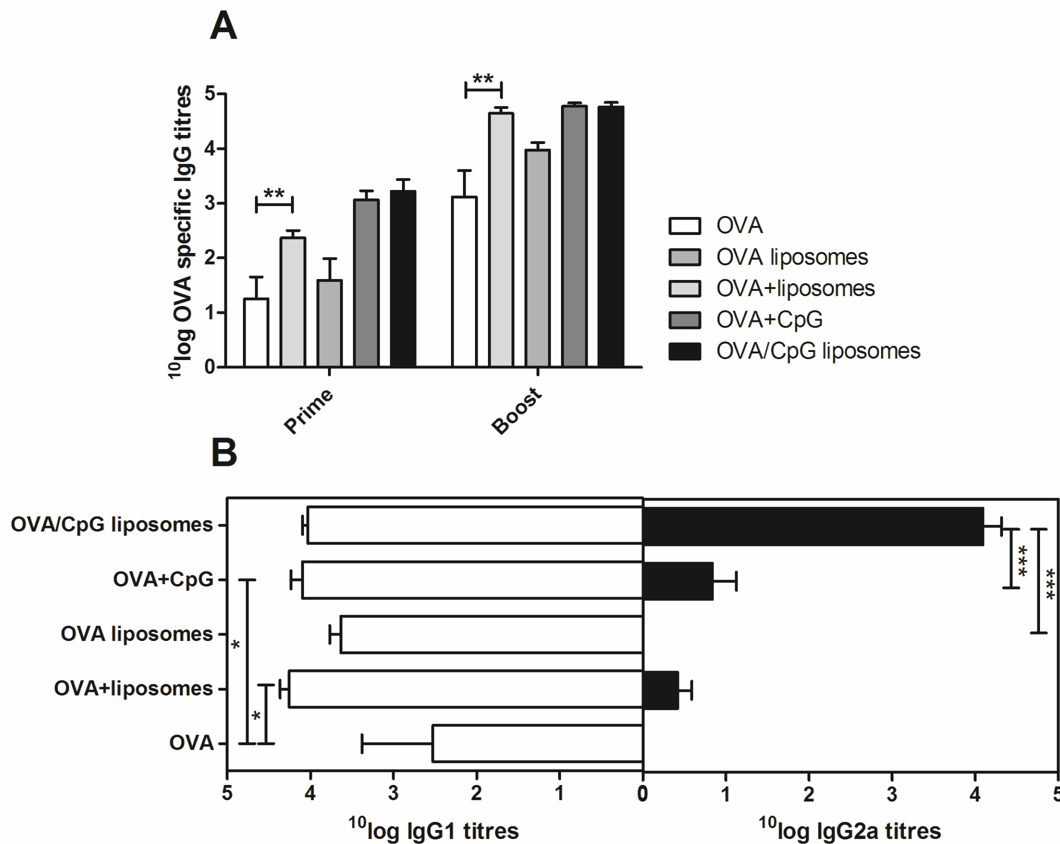


Figure 6. OVA-specific serum IgG, IgG1 and IgG2a titres after intradermal vaccination. A: IgG titres after prime and boost. B: IgG1 (white bars) and IgG2a (black bars) titres after booster immunisation. Bars represent SEM of n=5 (A and B). * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Transcutaneous immunisation

Cationic liposomes reduce DC uptake of OVA and CpG when administered transcutaneously

Transcutaneous administration involves an extra transport step across the epidermis compared to intranodal and intradermal injection (figure 1C). This is likely the reason why after 4 h no OVA^+ or CpG^+ DCs were found in the draining lymph node. Only after 24 h measurable OVA and CpG levels were observed (figure 7). A clear detrimental effect of liposomal encapsulation on the amount of OVA and CpG reaching the lymph nodes is shown, as application of OVA+CpG resulted in significantly higher numbers of OVA^+ and CpG^+ DCs than administration of OVA/CpG liposomes.

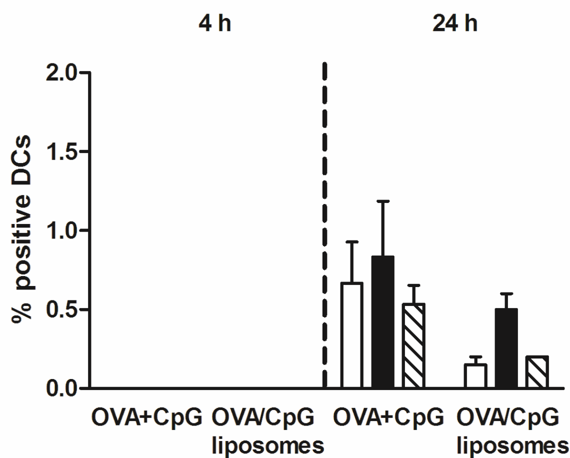


Figure 7. Quantification of OVA^+ (white bars), CpG^+ (black bars) and $OVA/CpG^{+/+}$ (striped bars) DCs in the draining lymph nodes 4 and 24 h after transcutaneous vaccination with microneedle pre-treatment, Bars represent the mean n=3 + SEM.

Liposomal co-encapsulation of OVA and CpG reduce IgG titres, but affects IgG1/IgG2a ratio

The immune enhancing effect of cationic liposomes mixed with OVA, as observed after intradermal administration was not apparent after transcutaneous administration on microneedle pre-treated skin. Encapsulating OVA in liposomes or mixing OVA with liposomes did not increase the IgG titres compared to plain OVA (figure 8A). Contrarily, administration of OVA+CpG resulted in strongly enhanced IgG titres compared to an OVA solution both after the prime and subsequent booster vaccination ($p<0.05$). However, this effect was abolished by encapsulation of antigen and adjuvant into liposomes ($p<0.01$). As far as the subtitres are concerned, unlike after intranodal and intradermal vaccination, transcutaneous immunisation with encapsulated and non-encapsulated CpG did not significantly elevate OVA-specific IgG2a titres (figure 8B), but encapsulated CpG reduced the IgG1 levels ($p<0.01$), thereby causing a decrease in the IgG1/IgG2a ratio.

Nasal vaccination

Liposomes reduce transport of OVA and CpG through the nasal epithelium

Comparable to transcutaneous vaccination, nasal administration involves an extra transport step across the epithelium (figure 1D). Ergo, 4 h after nasal application of fluorescent OVA and CpG no OVA^+ and CpG^+ DCs were detected in the cervical lymph nodes (figure 9). After 24 h DCs had taken up OVA and CpG, but similarly as after transcutaneous administration; the numbers of OVA^+ and CpG^+ DCs in the draining (cervical) lymph node were reduced when a liposomal formulation was used.

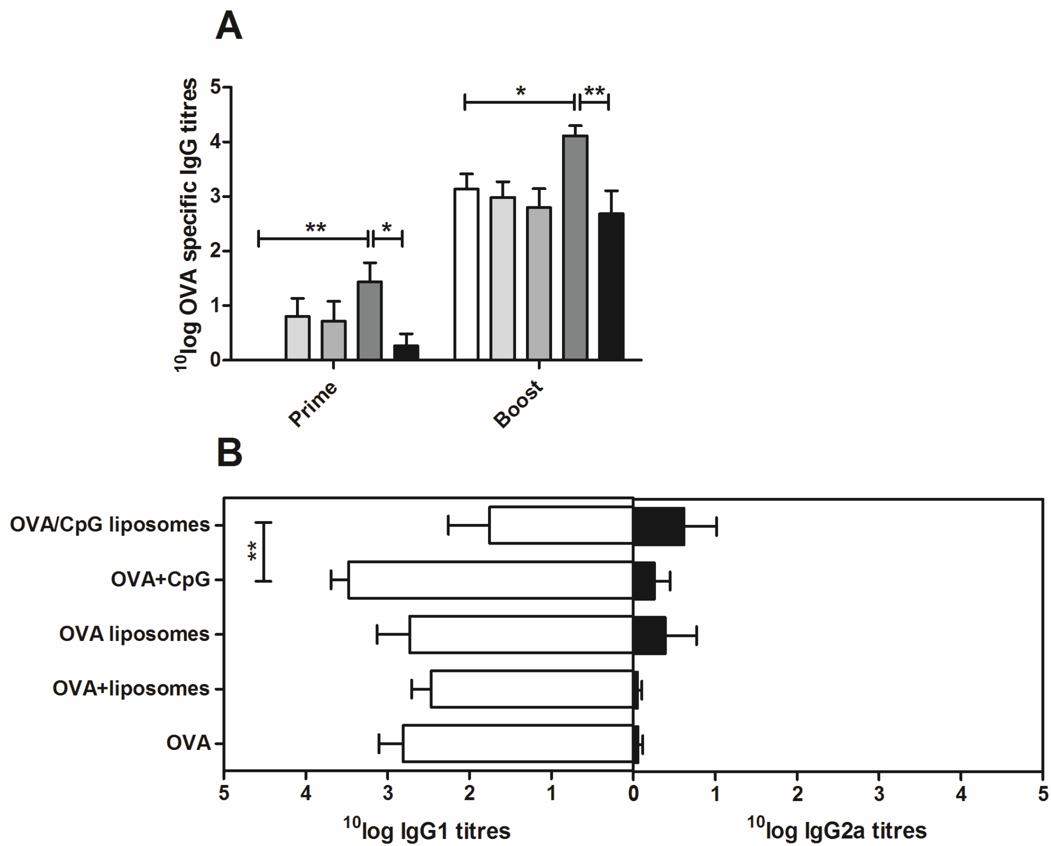


Figure 8. OVA-specific serum IgG, IgG1 and IgG2a titres after transcutaneous vaccination. A: IgG titres after prime and boost. B: IgG1 (white bars) and IgG2a (black bars) titres after booster immunisation. Bars represent SEM of n=8 (A and B). * p<0.05, ** p<0.01.

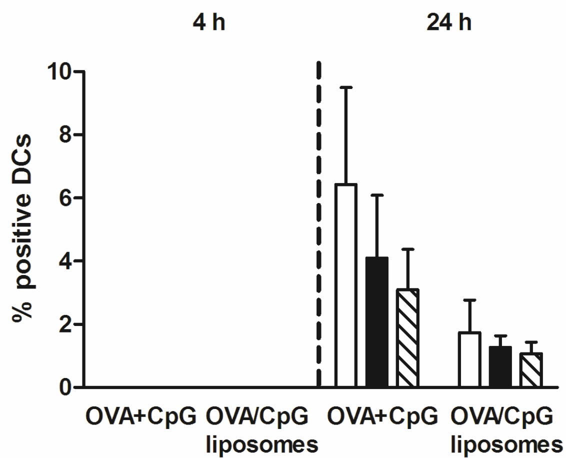


Figure 9. Quantification of OVA^{+} (white bars), CpG^{+} (black bars) and OVA/CpG$^{+/+}$ (striped bars) DCs in the draining lymph nodes 4 and 24 h after nasal vaccination Bars represent the mean n=3 + SEM.

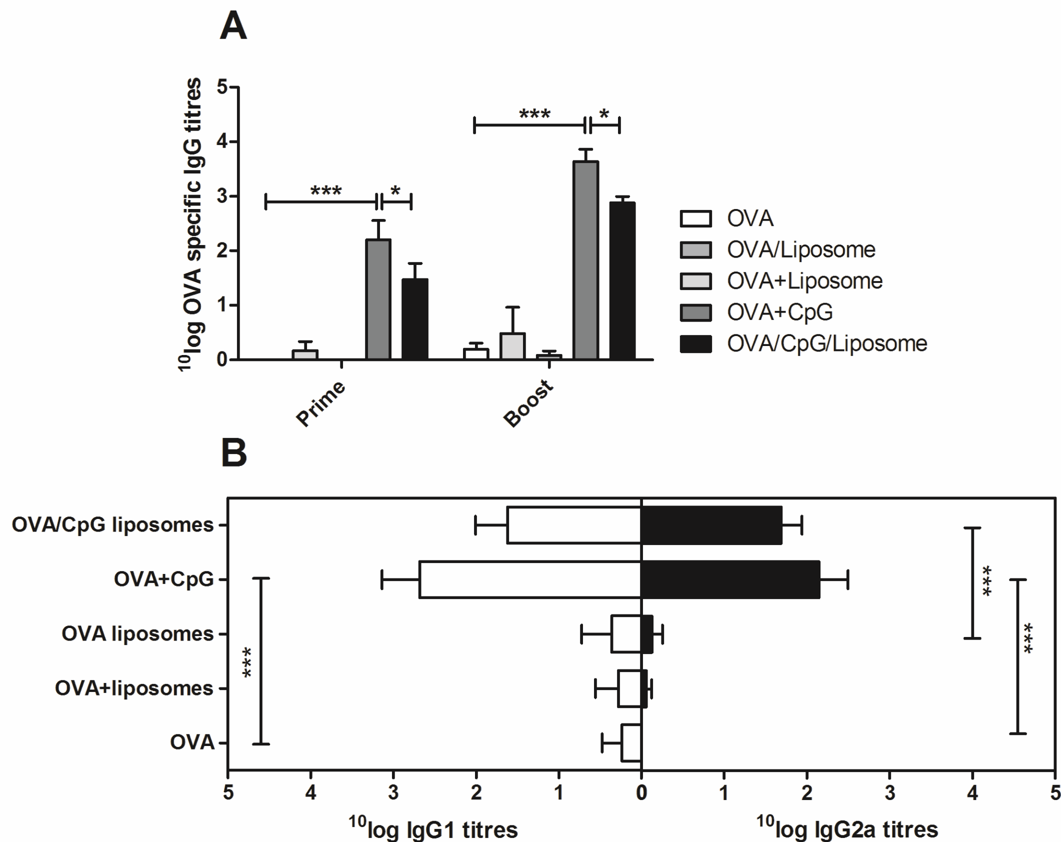


Figure 10. OVA-specific serum IgG, IgG1 and IgG2a titres after nasal vaccination. A: IgG titres after prime and boost. B: IgG1 (white bars) and IgG2a (black bars) titres after booster immunisation. Bars represent SEM of n=8 (A and B). * p<0.05, *** p<0.001.

Liposomal co-encapsulation of OVA and CpG not necessary to induce IgG2a titres

Mice receiving the formulations nasally showed a similar IgG pattern compared to after transcutaneous vaccination (figure 10A): liposomes did not stimulate the anti-OVA IgG response and OVA+CpG induced the strongest response already after a priming dose (p<0.001 compared to OVA). The effect of CpG was reduced when it was co-encapsulated with OVA in liposomes. In contrast to transcutaneous vaccination, nasal administration of OVA/CpG liposomes did elevate the serum antibody levels as compared to OVA alone. Nasal administration of OVA+CpG significantly increased the IgG2a levels compared to OVA (figure 10B, p<0.001). Co-encapsulation of CpG and OVA in liposomes also increased the IgG2a titres compared to encapsulation of OVA alone (p<0.001), but did not result in significantly higher IgG2a titres compared to OVA+ CpG, as observed for intradermally and intranodally vaccinated mice.

Finally, only nasal administration resulted in detectable levels of secretory IgA (sIgA) in nasal washes of the mice. Nasal immunisation with both encapsulated as well as free OVA

and CpG induced significantly higher levels of sIgA than vaccination with OVA alone (figure 11).

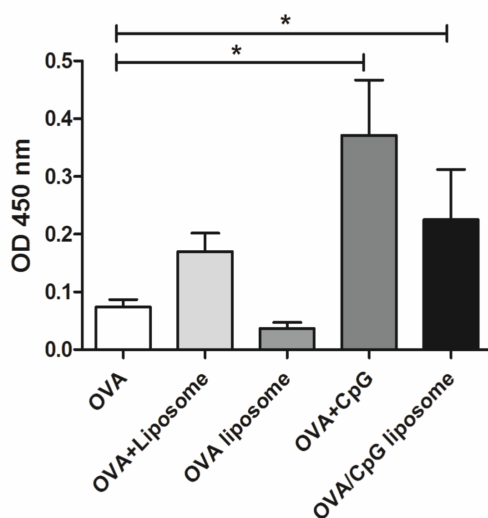


Figure 11. OVA-specific secretory IgA in nasal washes after nasal vaccination. Bars represent SEM of n=8 * p<0.05.

Discussion

Nasal and microneedle-based transcutaneous vaccination potentially provides a safe and patient friendly alternative to classical vaccine injection via the needle. However, vaccination via non-invasive routes is challenging as the antigen first has to pass a barrier (nasal epithelium or the skin, figure 1), which limits the amount of antigen that reaches the DCs. The use of particulate delivery systems has been shown to drastically decrease the effective dose after parenteral administration as they can opsonise the uptake of antigen by DCs and the addition of adjuvants can stimulate DC maturation or attract more lymphocytes to the application site [4, 37]. Consequently, an abundant amount of literature on the use of adjuvanted and non-adjuvanted particulate delivery systems for nasal and transcutaneous vaccination exists [18, 22, 38, 39]. However an effective delivery system for parenteral administration does not ensure effectiveness via non-invasive routes. For instance, PLGA particles and lipid based vesicles have been shown to improve antibody titres against OVA after intramuscular administration, but after nasal [28] or transcutaneous vaccination with microneedles [23, 24] no such effect was observed. In this study we compared the intranodal, intradermal, transcutaneous (with microneedle pre-treatment) and nasal route, not only regarding the antibody titres, but also the transport of antigen and adjuvant to the lymph nodes. Lymph node trafficking offers the possibility to study the benefit of co-encapsulating the antigen and adjuvant in cationic liposomes compared to a physical mixture of the two and allows comparison of different immunisation routes.

Using this approach we show that next to enhancing the uptake of their cargo by DCs *in vitro* (figure 2), also *in vivo* the cationic liposomes increased the number of OVA^+ and CpG^+ DCs, when directly injected into the lymph node (figure 3). This had a positive effect on the immunogenicity of OVA after intranodal or intradermal administration, although this was not directly apparent from IgG levels. IgG titres after intranodal injection were comparable with or without liposomal encapsulation and with or without the addition of an adjuvant. This may be related to the large antigen dose present in the lymph node and to the injection itself, which will already induce a danger signal to the residing DCs, thereby inducing DC activation and maturation [40], sufficient to induce a humoral response [29, 41]. Also after intradermal administration no effect of antigen or adjuvant encapsulation on the IgG titres was observed as administration of an OVA+CpG solution and OVA/CpG liposomes provoked similar IgG levels. On the contrary, empty liposomes with OVA co-administrated induced higher IgG titres than OVA loaded liposomes. Whereas application of OVA liposomes cause the formation of a depot, intradermal injection of OVA+liposomes led to both direct drainage of the antigen to lymph node and the formation of an antigen depot in the skin. According to Henriksen-Lacey et al*.* after application of an antigen liposome mixture a depot could be detected up to 14 days post intramuscular or subcutaneous injection and promoted the immunogenicity of the antigen [10]. The combination of direct drainage and prolonged antigen release can induce two distinct waves of antigen reaching the lymph nodes, and may be imperative for provoking a good (memory) immune response [36].

Cationic liposomes with encapsulated OVA and CpG did however have a substantial effect on the IgG2a titres; intranodal or intradermal injection of OVA/CpG liposomes strongly boosted IgG2a titres (indicative for a Th1 response), whereas administration of non-encapsulated OVA+CpG did not. Co-encapsulation of antigen and adjuvant results in the concomitant delivery to DCs, which has been described as crucial for a potent immune response [37, 42-44]. Here we show that OVA/CpG liposomes greatly enhanced the number of $OVA/CpG^{+/+}$ DCs *in vitro* (figure 2c) as well as *in vivo* (figure 3), which may account for the increased IgG2a titres. Furthermore, the IgG2a boosting effect may have been enhanced by the site of action of the adjuvant used. CpG is a ligand for TLR9, which is localised in the endosomal compartment of APCs. It is therefore imperative for CpG to be endocytosed by the APC, in order to be able to interact with TLR9, a process which is promoted by the use of liposomes [45]. Indeed it has been reported that OVA/CpG liposomes induce DC maturation, whereas a solution of CpG did not [17]. In contrast, a TLR ligand targeting a TLR expressed on the cell membrane, like PAM_3CSK_4 (TLR2), does not require a delivery into a liposome to induce DC maturation [17].

Despite all the beneficial effects of cationic liposome after intranodal and intradermal administration, nasally and especially transcutaneously, liposomes were found to decrease

rather than improve the immunogenicity. Although nasal administration of OVA/CpG loaded liposomes did show an increase in IgG titres compared to administration of OVA alone, this was due to the presence of CpG rather than its formulation into liposomes, as nasal administration of OVA+CpG induced superior antibody titres. After transcutaneous vaccination with microneedles, encapsulation of CpG into liposomes even completely inhibited the positive effect of the adjuvant.

The lymph node trafficking studies show that it is likely that the low immunogenicity of cationic liposomes via the nasal or transcutaneous route is a result of a reduced capacity of the antigen to pass respectively the nasal epithelium or the epidermis. Most probably the concomitant size increase, caused by the encapsulation into the liposome, and the positive charge of the delivery system obstructed the transport of the antigen and adjuvant to the lymph node [46, 47]. Lymph node trafficking of transcutaneously applied antigen has not yet received a lot of attention. The group of Glenn et al. did show that transcutaneously applied OVA and heat labile enterotoxin could be traced in the draining lymph nodes after 24 to 48 h [48, 49]. Here we report for the first time that particulate encapsulation diminishes the antigen uptake in the lymph nodes after transcutaneous administration.

The reduced DC uptake in the lymph nodes for encapsulated antigen after nasal vaccination seems rather surprising as a variety of nanoparticulate carrier systems have been reported to penetrated the nasal epithelium very effectively and to enhance the immune response [21]. This is often attributed to transport by M-cells, which can effectively transcytose particulate matter to the luminal side of the nasal epithelium [18, 38]. However, only few papers investigated the antigen accumulation in the NALT or lymph nodes after nasal vaccination. Vila et al. established that PLA-PEG nanoparticles increased antigen deposition in the lymph node (authors did not specify which lymph node) almost 6-fold and observed an enhancement of the serum IgG levels after nasal administration of PLA-PEG particles [50]. However in the same study it was also shown that PLA particles without a PEG coating did not increase antigen deposition the lymph node [50, 51]. More contrasting results, concerning little particle uptake by the NALT and lower antibody responses after antigen encapsulation have also been reported [52, 53]. Very recently our group showed that although N-trimethyl chitosan nanoparticles enhance immune response after nasal vaccination, this is most likely not due to enhancement of antigen reaching the cervical lymph nodes [25]. This seems to indicate that in contrast to general believe, particles do not easily pass the nasal epithelium to reach the cervical lymph nodes.

An interesting difference between the nasal and the transcutaneous route was observed; whereas after transcutaneous immunisation a solution of OVA or non-adjuvanted liposomes were capable of inducing seroconversion in all mice, nasal vaccination required the addition CpG to induce measurable antibody titres. This may be related to the nasal epithelium being a rather tolerogenic immunisation site [54], making the activation of DCs

with an adjuvant an important requirement for the induction of antibodies. The skin DCs however, are known to not only play a role in tissue homeostasis, but also having a strong pro-inflammatory function [55, 56]. This may have an evolutionary purpose as a micro-organism that has breached the skin barrier is more dangerous that an organism that has ended up in the nasal epithelium.
Although after nasal and transcutaneous vaccination the quantity of the humoral immune response did not benefit from co-encapsulation of OVA and CpG into liposomes, liposomal co-encapsulation still had a pronounced effect on the type of immune polarization. Nasal and transcutaneous administration of OVA/CpG liposomes induced relatively more IgG2a compared to IgG1 than the administration of a physical mixture of these components. Co-localization of antigen and adjuvant therefore still remains an important tool to enhance the immunogenicity of a non-injectable subunit vaccine, but should not be established by using a particulate delivery system. Antigen-adjuvant conjugates or Fc-receptor immune complexes have been reported to very efficiently target DCs [57-59] and due to their smaller size, may be more suitable for nasal and transcutaneous administration.

Conclusion

Despite the advantages of using cationic liposomes as a vaccine adjuvant, careful consideration should be given when such systems are designed for transcutaneous and nasal vaccination. These data show that, intrinsically, liposomes containing both the antigen as well as the adjuvant enhance the immunogenicity of the antigen and promote the induction of both IgG1 and IgG2a type antibodies. However, likely due to poor penetration of the microneedle pre-treated skin and nasal mucosa, they are unsuitable for application via the transcutaneous or nasal route.

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