Challenges and opportunities in nasal subunt vaccine delivery : mechanistic studies using ovalbumin as a model antigen

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Adjuvant effect of cationic liposomes and CpG depends on administration route.

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Abstract

In this study we explored the immunization route-dependent adjuvanticity of cationic liposomes loaded with an antigen (ovalbumin; OVA) and an immune potentiator (CpG). Mice were immunized 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 titers after nasal and transcutaneous administration of OVA/CpG liposomes were reduced compared to administration of an OVA+CpG solution. Although serum IgG titers 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 immunization, 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 i) protection from enzymatic breakdown, ii) sustained antigen release over time [1, 2], iii) enhanced uptake by professional antigen presenting cells such as DCs [3] and iv) possibility of co-encapsulation of adjuvants [4, 5]. Liposomes are a type of nanoparticles that have been widely studied as antigen carriers and their usage in vaccination studies dates back to 1974 [6]. Although liposomes themselves are not very immunogenic, they 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 titers 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 the nose and the skin 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, 24] and to a lesser extent (because of possible M-cell transport [3, 4]) the nasal epithelium [25] (figure 1).

The aim of the present study was to investigate the impact of encapsulating the antigen and the adjuvant in cationic liposomes on the (antibody mediated) immune response after administration via several immunization routes. We explored the influence of liposomal

antigen and adjuvant co-encapsulation on both the transport through the epithelium and transport of the vaccine to the lymph nodes, by quantifying the amount of OVA and CpG positive DCs (CpG^+ or OVA^+) in the draining lymph nodes after intranodal, intradermal, transcutaneous (in combination with microneedle pre-treatment) and nasal administration. The added value of OVA- and CpG-containing cationic liposomes on immunogenicity was evaluated, by determining the total serum IgG levels and secretory IgA levels in nasal washes. Finally, the quality of the immune response was addressed by measuring IgG subtypes (IgG1 and IgG2a).

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 labeled 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.

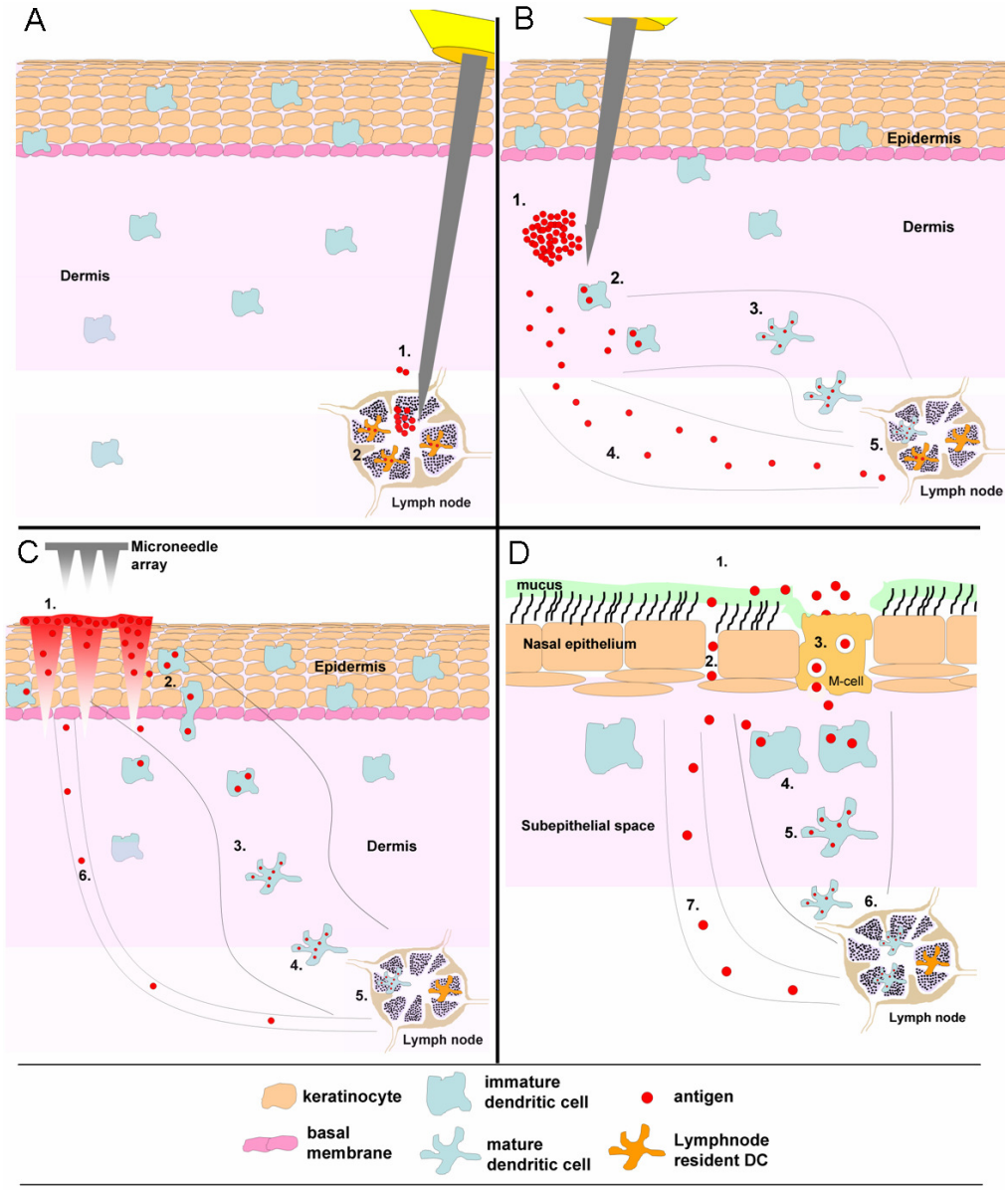

***Figure 1**: Schematic illustration (relative size of organs and cells not on scale) of antigen transport mechanisms after immunization via different administration routes. A) After intranodal injection (1) the antigen will directly be taken up by lymphnode resident DCs (2). B) After intradermal injection (1) the antigen can be taken up by an immature DC (2) which matures (3) or drains directly to the lymph node (4). This will result in both peripheral as well as lymph node resident DCs that are antigen positive (5). C) Transcutaneous immunization: microneedle application creates small conduits through which the antigen can diffuse (1). Immature DCs are abundantly present in the epidermis and dermis and will take up the antigen (2,) and will subsequently cross the basal membrane (3), mature (4) and reach the lymph node (5). Because the long route the antigen has to take, direct drainage of the antigen to the lymph node (6) may be limited. 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 lymphnode is an option (7), but might be limited.*

Preparation and characterization of liposomes

Cationic liposomes were prepared using the film rehydration 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^{TM} 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, unencapsulated 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 immunization 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 immunizations 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.

Determination of serum IgG, IgG1, IgG2a and secretory IgA

Microtiter 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% fetal 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 fetal 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 titers, 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

To assess whether our cationic liposomes increase the uptake of OVA and CpG, the uptake by human monocyte derived DCs was measured *in vitro*. Consistent with our previous study [17], OVA-containing liposomes with an average size of 130 nm bearing a positive zeta potential (23 mV) and OVA/CpG liposomes with a size of 263 nm and a zeta potential of 18 mV were obtained. The cationic liposomes significantly enhanced the uptake of their encapsulated cargo by DCs (figure 1). Plain OVA was readily taken up by DCs, as approximately 20% of the DCs was OVA^{+} after 4 h of incubation with OVA (figure 2A), but when encapsulated into liposomes, the number of OVA^{+} DC was significantly increased ($p<0.001$), reaching a value of approximately 50% OVA^{+} DCs. In contrast to OVA, non encapsulated CpG was hardly taken up by DCs in its plain form as only 6% CpG^{+} DCs were observed (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 a solution of OVA and CpG. In conclusion, co-encapsulation of OVA and CpG in cationic liposomes enhanced their concomitant uptake by DCs *in vitro*.

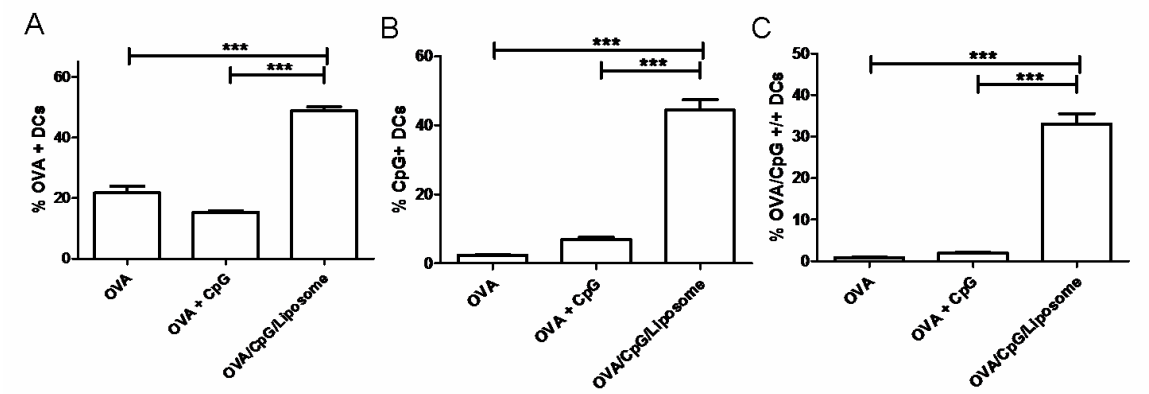

***Figure 2**: Uptake of a) OVA and b) CpG by human monocyte derived DCs determined by FACS analysis. c) number of OVA and CpG double positive DCs after 4 h exposure. n=3 Average + SEM. *** p<0.001*

Intranodal vaccination

Liposomes enhance the uptake of OVA and CpG by lymph node resident DCs.

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 finding antigen specific T cells there. Therefore the draining lymph node is the most prominent site of activation for T cells after immunization and can be considered the actual target of vaccines. As a consequence, 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 lymphnodes are circumvented (figure 1A). Indeed after intranodal injection a rapid uptake of fluorescently labeled 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 encapsulation of OVA and CpG led to significantly elevated numbers of OVA^+, CpG^+ and $OVA/CpG^{+/+}$ DCs compared to the administration of a soluble mixture of OVA and CpG. The percentage of DCs that had taken up both OVA and CpG increased by 4-fold compared to injection of a physical mixture of CpG and OVA. After 24 h the levels had decreased drastically, suggesting that OVA and CpG had been processed. Alas, next to in vitro, also in vivo cationic liposomes have the potential to increase the delivery of the antigen and the adjuvant.

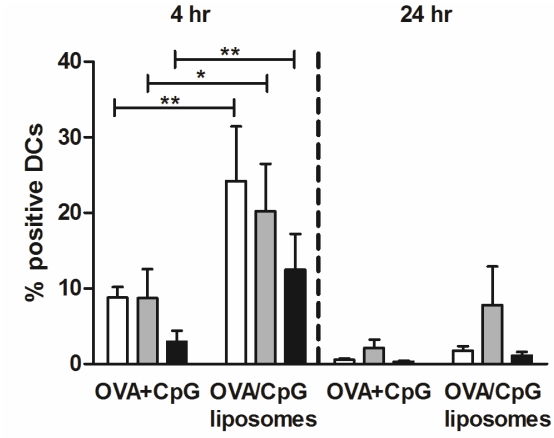

***Figure 3**: Quantification of OVA$^+$ (white bars), CpG$^+$ (grey bars) and OVA/CpG$^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after intranodal vaccination, Bars represent the mean n=3 + SEM. *p<0.05, **p<0.01.*

High IgG2a titers after intranodal injection of OVA/CpG liposomes

In spite of the favorable effect of liposomal co-encapsulation of OVA and CpG on DC uptake, intranodal vaccination showed similar total serum IgG titers for all formulations (figure 4A). Both after the prime and booster immunization no effect of either liposome encapsulation or 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 titers 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 (humoral) response and IgG2a titers, indicative of a Th1 type (cellular) response [34, 35], were quantified to investigate the quality of the immune response after the boost immunization (figure 4B). IgG1 titers appeared to be virtually in synchronicity with the IgG titers, 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 ($p<0.001$) as well as non encapsulated OVA + CpG ($p<0.001$). As such OVA/CpG liposomes caused a significant decrease in IgG1/IgG2a ratio, compared to all other formulations ($p<0.05$, figure 3C). This shows that intrinsically, OVA/CpG liposomes are a very immunogenic and effectively delivery system that can induce a mixed Th1/Th2 type immune response.

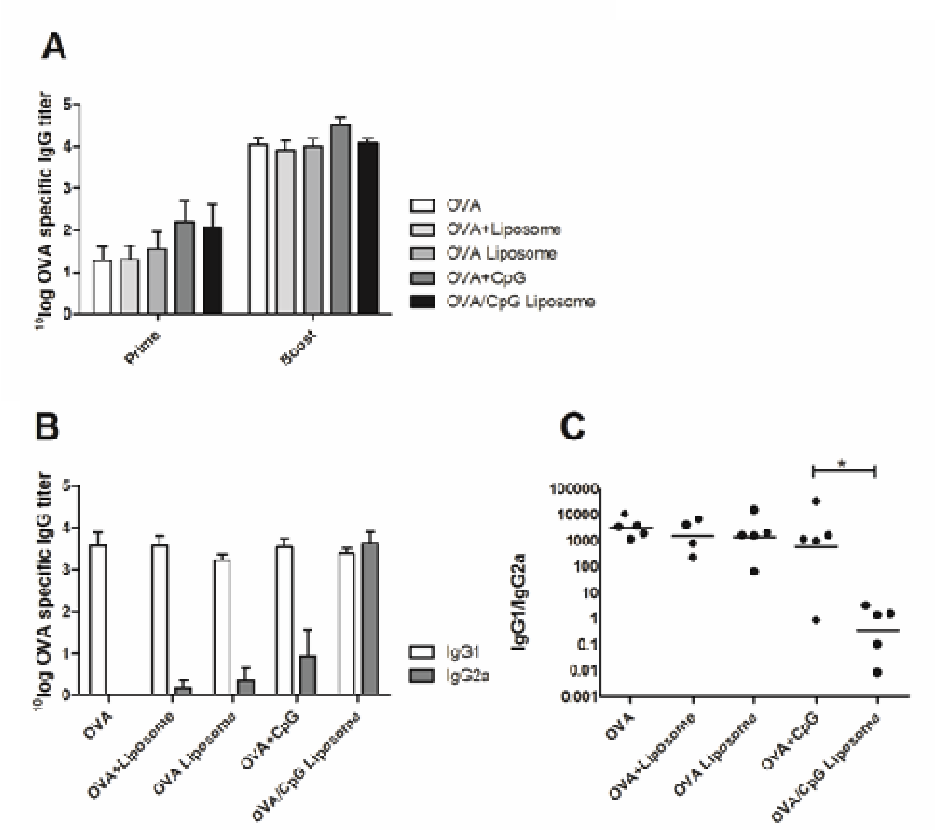

***Figure 4**: OVA-specific serum IgG, IgG1 and IgG2a titers after intranodal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=5 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05.*

Intradermal vaccination

Cationic liposomes reduce direct drainage 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 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 1). Intradermal injection of fluorescent OVA and CpG showed the presence of both these routes; already 4 h after administration, OVA^+ DCs could be detected in the lymph nodes, but these cells had not

taken up 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. These suggest both OVA and CpG were retained at the injection side. Indeed the formation of an antigen depot that was visible by eye at the injection site, even 24h after injection. 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.

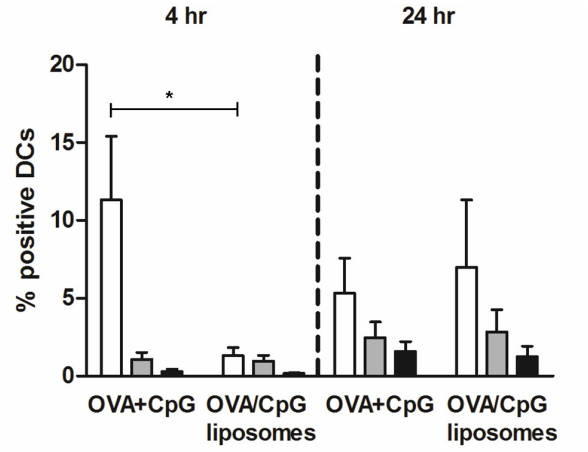

***Figure 5**: Quantification of OVA^+ (white bars), CpG^+ (grey bars) and $OVA/CpG^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after intradermal vaccination, Bars represent the mean n=3 + SEM. *p<0.05*

Cationic liposomes have an adjuvant effect and increase IgG2a levels significantly

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 clearly increased the antibody levels compared to OVA after the first ($p<0.001$), as well as the second immunization ($p<0.001$) 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 a solution of OVA and CpG.

After intradermal administration IgG1 titers mimicked the IgG titers and IgG2a levels were very low with the exception those of the mice immunized with OVA/CpG liposomes (figure 6B). Just like after intranodal vaccination, these mice had significantly higher IgG2a titers compared to those receiving plain OVA ($p<0.001$), resulting in a significant shift in the IgG1/IgG2a ratio (figure 6C). OVA + CpG did not provoke significantly higher IgG2a titers.

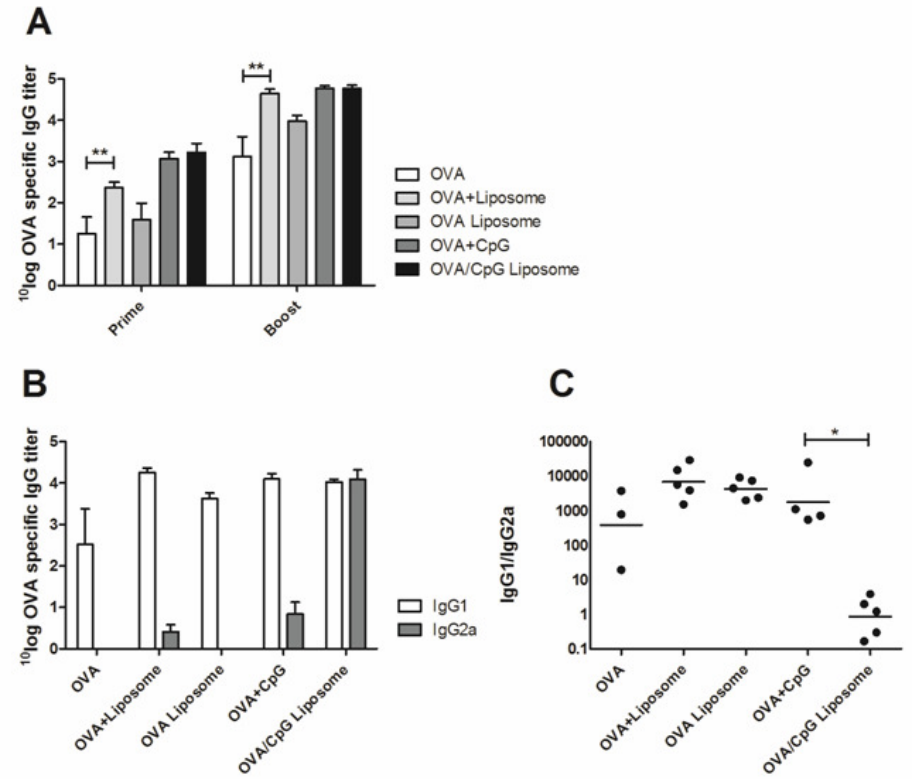

***Figure 6**: OVA-specific serum IgG, IgG1 and IgG2a titers after intradermal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=5 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05, ** p<0.01.*

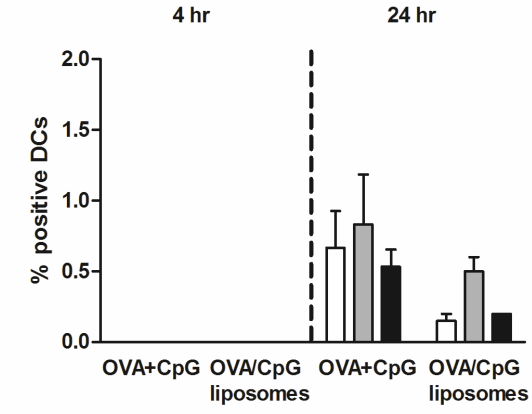

***Figure 7**: Quantification of OVA^{+} (white bars), CpG^{+} (grey bars) and OVA/CpG$^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after transcutaneous vaccination with microneedle pretreatment, Bars represent the mean n=3 + SEM.*

Transcutaneous immunization

Liposomes reduce transport of OVA and CpG through the skin

Transcutaneous administration involves an extra transport step across the epidermis compared to intranodal and intradermal injection (figure 1). Consequently, there was no trafficking to the lymph node resident DCs after 4 h. 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 non encapsulated OVA and CpG resulted in significantly higher numbers of OVA^+ and CpG^+ DCs than administration of OVA/CpG liposomes.

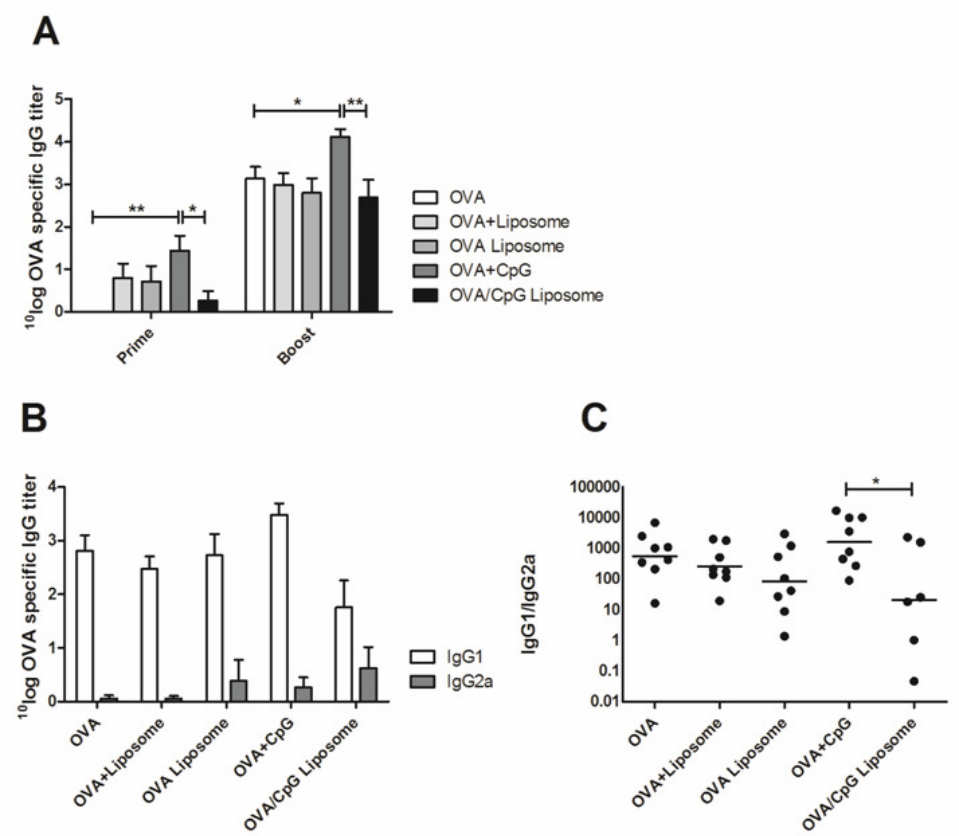

***Figure 8**: OVA-specific serum IgG, IgG1 and IgG2a titers after transcutaneous vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=8 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * $p<0.05$, ** $p<0.01$.*

Liposomal co-encapsulation of OVA and CpG reduce IgG titers, but enhance IgG2a levels

The immune enhancing effect of cationic liposome, as observed after intradermal administration was not apparent after transcutaneous administration. Regardless whether OVA was encapsulated or not encapsulated in liposomes no increased IgG titers compared to plain OVA after transcutaneous immunization on microneedle pre-treated skin were observed (figure 8A). Contrarily, administration of a OVA + CpG solution resulted in strongly enhanced IgG titers both after the prime and subsequent booster vaccination compared to an OVA solution ($p<0.05$). This effect was abolished by encapsulation of antigen and adjuvant into liposomes ($p<0.01$). As far as the subtiters are concerned, unlike after intranodal and intradermal vaccination, transcutaneous immunization with encapsulated and non-encapsulated CpG did not significantly elevate OVA-specific IgG2a titers (figure 8B), but encapsulated CpG reduced the IgG1 levels, thereby causing a significant decrease in the IgG1/IgG2a ratio ($p<0.05$).

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 1). 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 positive DCs in the draining (cervical) lymph node were reduced when a liposomal formulation was used.

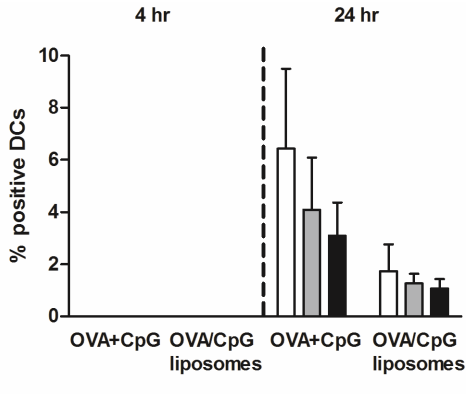

***Figure 9**: Quantification of OVA^+ (white bars), CpG^+ (grey bars) and $OVA/CpG^{+/+}$ (black bars) DCs in the draining lymph nodes 4 and 24 h after transcutaneous vaccination with microneedle pretreatment, Bars represent the mean n=3 + SEM.*

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

Mice receiving the formulations nasally showed a similar IgG pattern compared to transcutaneous vaccination (figure 10A): liposomes did not stimulate the anti-OVA IgG response and soluble OVA adjuvanted with CpG induced the strongest response already after a priming dose ($p<0.001$ compared to OVA). When CpG was co-encapsulated with OVA in liposomes the effect of CpG was reduced, altough the serum antibody levels were higher as compared to OVA alone. This was not observed in contrast to transcutaneous vaccination. Nasal co-administration of OVA + CpG, did significantly increase the IgG2a levels compared to OVA (figure 10B, $p<0.001$). Co-encapsulation of CpG and OVA in liposomes also increased the IgG2a titers compared to encapsulation of OVA alone ($p<0.01$), but did not result in a significant shift in the IgG1/IgG2a ratio compared to a solution of OVA and CpG (figure 9C), as observed for intradermally and intranodally vaccinated mice.

Finally, only nasal administration resulted in detectable levels of secretory IgA (sIgA) in the nasal washes of the mice. Nasal immunization with a both encapsulated as well free OVA and CpG induce significantly higher levels of sIgA than vaccination with OVA alone (figure 11).

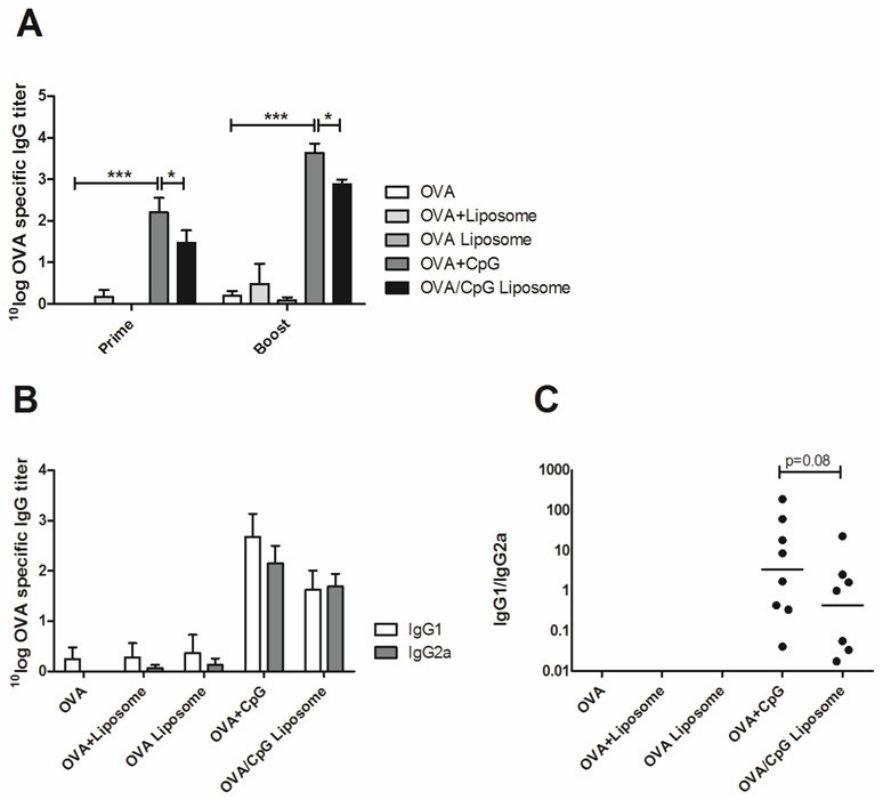

***Figure 10**: OVA-specific serum IgG, IgG1 and IgG2a titers after nasal vaccination. A: IgG titers after prime and boost. B: IgG1 and IgG2a titers after booster immunization. Bars represent SEM of n=8 (A and B). C: Corresponding IgG1/IgG2a ratio for each individual mouse. Mice non-respondent for either IgG1 or IgG2a were removed from the data set. Bar represents geomean * p<0.05, *** p<0.001.*

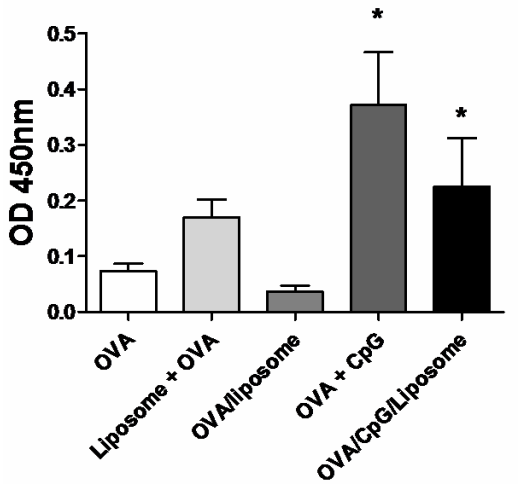

***Figure 11**: OVA-specific secretory IgA in nasal washes after nasal vaccination. Bars represent SEM of n=8 * p<0.05 compared to OVA.*

Discussion

Nasal and microneedle-based transcutaneous vaccination potentially provides a safe and patient friendly alternative to classical vaccine administration via the needle. However, vaccination via non-invasive routes is challenging as the antigen will first have to pass a barrier (nasal epithelium or the skin), which limits the amount of antigen that reaches the DCs. To provoke a strong immune response with a limited amount of antigen, high immunogenicity of vaccine is very important. The immunogenicity of subunit vaccines can be enhanced if the antigen is properly formulated. Therefore the use of cationic liposomes as a carrier system makes sense; they provide efficient antigen encapsulation and their particulate nature makes them a natural target for DCs, which can enhance the uptake of their cargo by DCs. Co-encapsulation of an adjuvant will result in the concomitant delivery of antigen and adjuvant to DCs, which has been described as crucial for a potent immune response [36, 37]. However, compared to solutions of antigens and adjuvants, liposomes might have difficulties passing the epithelial barriers.

Co-encapsulation of antigen and adjuvant into liposomes had a positive effect when the formulations were injected: intranodal or intradermal injection of OVA/CpG liposomes strongly boosted IgG2a titers (indicative for a Th1 response), whereas administration of non-

encapsulated OVA + CpG did not. This effect may be related to the site of action of the adjuvant used. CpG is a ligand for TLR9, which is localized 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. Here we show that *in vitro*, cationic liposomes can facilitate CpG uptake by DCs, adding to the increase in IgG2a titers after intradermal and intranodal injection. *In vivo* the DC uptake after intranodal injection corresponds well with the *in vitro* data, as in both cases a clear benefit of co-encapsulation on DC uptake was observed.

Interestingly, after intranodal administration OVA/CpG liposomes was the only formulation to have a beneficial effect on the IgG2a titer, whereas all formulations induced similar IgG and IgG1 titers via this route. It is likely that the injection itself will already induce a danger signal to the residing DCs, thereby inducing DC activation and maturation [38], sufficient to induce a humeral (IgG1) response [29]. The additional benefit of adjuvanted liposomes on the total immune response via this route, using this amount of antigen, is therefore negligible, but the effect on the immune bias is substantial. Since high IgG2a levels were obtained with the OVA/CpG liposomes, this indicates that liposomal co-encapsulation of antigen and adjuvant is essential for induction of a Th1 type immune response.

Intradermal administration of OVA with empty liposomes significantly increased antibody titers compared to administration of OVA alone. As empty liposomes do not activate immature DCs, the adjuvanticity of these vesicles can most probably be attributed to the antigen depot it forms upon injection, likely through interaction of liposomes and antigen with the extracellular matrix. According to Henriksen-Lacey et al. this depot could be detected up to 14 days post intramuscular or subcutaneous injection and promoted the immunogenicity of the antigen [10]. However, the current study also shows that depot formation prohibits rapid drainage of the antigen to the lymph node. Antigens in solution can directly drain to the lymph node and be taken up by a large population of immature lymph node resident DCs [39], as reflected in our study by the high number of OVA^+ DCs found in the lymph nodes 4 h after injection of OVA. Liposomal administration and the resulting antigen depot reduced the amount of antigen that directly drains to the lymph nodes, but induced prolonged OVA delivery compared to a mixture of OVA and CpG. These two processes can induce two distinct waves of antigen reaching the lymph nodes, and may be imperative for provoking a good (memory) immune response [40]. This could explain why intradermal application of OVA with empty liposomes induced a better response than OVA loaded liposomes. The latter does not allow direct drainage of the antigen to the lymph nodes, but only a prolonged release, whereas the first approach might have resulted in both direct and prolonged release.

Nasally and especially transcutaneously, liposomes were found to be a suboptimal vaccine delivery system. Although nasal administration of OVA/CpG loaded liposomes did show an increase in IgG titers 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 as a solution induced superior antibody titers. After transcutaneous vaccination with microneedles, encapsulation of CpG into liposomes even completely inhibited the positive effect of the adjuvant. We showed that liposomes dramatically decreased the amount of antigen and adjuvant reaching the DCs. Significantly less OVA^+ or CpG^+ DCs were detected in the draining lymph nodes 24 h after nasal or transcutaneous administration of OVA/CpG liposomes compared to administration of OVA + CpG. Possibly 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.

An interesting difference between the the nasal and the transcutaneous route was observed; whereas after transcutaneous immunization 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 titers. This may be related to the nasal epithelium being a rather tolerogenic immunization site [41], 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 playing a role in tissue homeostasis, but also having a strong pro-inflammatory function [42, 43]. 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 total humoral immune response did not benefit from co-encapsulation of OVA and CpG into liposomes, liposomal co-encapsulation may have a pronounced effect on the induction of a cellular response. Nasal and transcutaneous administration of OVA/CpG liposomes induced relatively more IgG2a compared to IgG1 than the administration of a physical mixture of these 2 components. Co-localization of antigen and adjuvant therefore still remains an important mechanism 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 [44, 45] 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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