



Co-encapsulation of antigen and Toll-like receptor ligand in cationic liposomes affects the quality of the immune response in mice after intradermal vaccination

Suzanne M. Bal, Sander Hortensius, Zhi Ding, Wim Jiskoot, Joke A. Bouwstra*

Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research, Leiden University, The Netherlands

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ABSTRACT

Enhanced immunogenicity of subunit antigens can be achieved by antigen encapsulation in liposomes and the addition of immune potentiators. In this study we co-encapsulated ovalbumin (OVA) and a Toll-like receptor (TLR) ligand (PAM₃CSK₄ (PAM) or CpG) in cationic liposomes and investigated the effect of the formulations on dendritic cell (DC) maturation *in vitro* and on the immune response in mice after intradermal immunisation. Co-encapsulation of PAM did not affect the OVA content of the liposomes, but co-encapsulation of CpG led to a decrease in OVA content by 25%. After liposomal encapsulation, both ligands retained the ability to activate TLR-transfected HEK cells, though PAM only induced activation at elevated concentrations. DC maturation induced by liposome-based adjuvant formulations was superior compared to the free adjuvants. Encapsulation of PAM and CpG in liposomes did not influence the total IgG titres compared to the antigen/adjuvant solution, but OVA/CpG liposomes shifted the IgG1/IgG2a balance more to the direction of IgG2a compared to non-encapsulated CpG. Moreover, only this formulation resulted in IFN- γ production by restimulated splenocytes from immunised mice. These data show that co-encapsulation of antigen and immune potentiator in cationic liposomes, can affect the type of immune response generated after intradermal immunisation.

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1. Introduction

Vaccines should be capable of eliciting a strong and protective immune response, but are also required to be safe. Subunit antigens are regarded safer than live-attenuated and inactivated pathogens, but lack strong immunogenicity. Optimising the formulation of subunit vaccines could be instrumental in improving the immunogenicity and therefore in the development of safe and effective vaccines [1]. Approaches to achieve a higher efficacy include optimising the delivery to and interaction with dendritic cells (DCs) and the addition of immune potentiators to improve the activation of these DCs.

Lessons to improve the interaction with DCs can be learned from nature, as all pathogens are particulates. Particles are better taken up by DCs and may provide an additional benefit by offering prolonged antigen delivery due to slow antigen release [2]. Liposomes are elegant and flexible nanoparticulates that have been used for a long time as drug delivery systems. Actually, when they were used for the first time in the pharmaceutical field in 1974, it was for the delivery of vaccines [3]. Since then they have been used successfully for the delivery of protein antigens [4–6] and DNA vaccines [7,8]. By

changing the lipid composition of liposomes, their characteristics can be varied. The usage of positively charged lipids, for instance, creates cationic liposomes. It has become clear that cationic liposomes are one of the most effective liposomal delivery systems for antigens to antigen presenting cells [9–12].

Liposomes themselves may function as an adjuvant by improving the uptake of antigens by DCs, but generally lack intrinsic immune-stimulatory effects [11,13]. By co-encapsulation of an immune potentiator, the immunogenicity of liposomes can be improved. As classified by Schijns [14], immune potentiators (i) interact with pattern recognition receptors (PRRs) (Signal 0) [15,16]; (ii) are co-stimulatory molecules necessary for activating naïve T cells (Signal 2) or (iii) act as a 'danger-signal' [17]. Pathogens express specific pathogen-associated molecular patterns (PAMPs) that are recognised by PRRs, of which the Toll-like receptors (TLRs) are an important subclass. All cells, but mainly antigen presenting cells such as DCs, have TLRs that recognise specific ligands. In humans 11 different TLRs have been identified, the majority of them being specific for microbial products. Most TLRs are present on the cell surface, but TLRs that recognise nucleic acids (TLR3, 7, 8 and 9) are located intracellularly [18].

In this study we co-encapsulated a model antigen, ovalbumin (OVA) and two TLR ligands in cationic liposomes. The selected TLR ligands are Pam₃CSK₄, a synthetic lipoprotein consisting of a tri-palmitoyl-S-glycerol cysteine lipopeptide with a pentapeptide SKKKK (PAM), and unmethylated CpG oligonucleotide (CpG). PAM

* Corresponding author at: Division of Drug Delivery Technology, Leiden/Amsterdam Center for Drug Research, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA Leiden, The Netherlands. Tel.: +31 71 527 4208.

is recognised by TLR2 in association with TLR1, both cell surface expressed receptors. CpG is a TLR9 ligand, which is expressed intracellularly. By co-encapsulation in liposomes it is ensured that both the antigen and the immune potentiator are co-delivered to the DCs, which is considered essential for induction of a strong immune response [19–21]. To examine the effect of co-encapsulation, a comparison was made to solutions of OVA mixed with the respective TLR ligands. The formulations were tested *in vitro* for their DC-stimulating properties and their immunogenicity was studied *in vivo* by intradermal (ID) injection, an immunisation route which has regained interest in recent years due to the dose-sparing potential compared to intramuscular immunisation [22–25].

2. Materials and methods

2.1. Materials

Soybean phosphatidylcholine (PC), 1,2-dioleoyl-3-trimethylammonium-propane chloride salt (DOTAP) and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were kindly provided by Lipoid GmbH (Ludwigshafen, Germany). Ovalbumin grade VII was obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). FITC-labelled ovalbumin (OVA_{FITC}) was purchased from Invitrogen (Breda, The Netherlands). PAM, rhodamine-labelled PAM, CpG 2006 and 1826 and their FITC-labelled analogues were purchased from Invivogen (Toulouse, France). Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (γ chain specific), IgG1 (γ 1 chain specific) and IgG2a (γ 2a chain specific) were purchased from Southern Biotech (Birmingham, USA). Chromogen 3,3',5,5'-tetramethylbenzidine (TMB) and the substrate buffer were purchased from Invitrogen. All cell culture media, including serum and trypsin were purchased from Gibco (Invitrogen). 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 the injection fluid (0.9% NaCl) were obtained from a local pharmacy. Phosphate buffered saline (PBS) pH 7 was obtained from Braun (Oss, The Netherlands). All other chemicals were of analytical grade.

2.2. Animals

Female BALB/c mice (H2d), 8-weeks old at the start of the vaccination study were purchased from Charles River (Maastricht, The Netherlands), and maintained under standardised conditions in the animal facility of the Leiden/Amsterdam Center for Drug Research, Leiden University. The study was carried out under the guidelines compiled by the Animal Ethic Committee of the Netherlands.

2.3. Liposome preparation and characterization

Liposomes with a lipid:OVA:TLR ligand ratio of 50:1:2 (w/w) were prepared using the film hydration method [26] followed by extrusion. Soy-derived phosphatidyl choline (PC), dioleoyl trimethyl ammonium propane (DOTAP) and dioleoyl phosphatidyl ethanolamine (DOPE), dissolved in chloroform, were mixed in a 9:1:1 molar ratio in a flask. A thin lipid film was formed at the bottom of this flask using a rotary evaporator. The residual organic solvent was removed by nitrogen flow. The film was rehydrated in a 10 mM phosphate buffer pH 7.4 (7.7 mM Na₂HPO₄ and 2.3 mM NaH₂PO₄) containing 1 mg/ml OVA. The final concentration of lipids was 5% (w/v). The dispersion was shaken in the presence of glass beads at 200 rpm for 2 h at room temperature. To obtain monodisperse liposomes, the dispersion 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 co-encapsulation of a TLR ligand, after hydration either PAM or CpG was added to a final concentration of 2 mg/ml. The dispersions were dehydrated by freeze-drying and subsequently rehydrated in the same buffer solution to encapsulate the TLR ligands [27]. Extrusion was performed as described above.

The size and zeta potential of the liposomes were determined by dynamic light scattering and laser Doppler velocimetry, respectively, using a Zetasizer[®] Nano ZS (Malvern Instruments, UK). The amount of OVA, PAM and CpG present in the liposomes was determined by using their fluorescently labelled analogues (10% of used OVA, PAM or CpG were labelled). The free antigen and TLR ligand were separated from the liposomes by filtration using a Vivaspin 2 centrifugal concentrator (PES membrane, MWCO 300 kDa, Sartorius Stedim, Nieuwegein, The Netherlands) and quantified using a FS920 fluorimeter (Edinburgh Instruments, Campus Livingston, UK). The stability of the OVA-loaded liposomes and OVA release from the liposomes was determined in PBS pH 7.4. Liposomes containing OVA_{FITC} were diluted to a 0.5% lipid concentration and stored at 37 °C under constant stirring. Samples were taken at selected time intervals and the size of the liposomes and antigen encapsulation were measured after filtration.

2.4. Activity of TLR ligands

HEK293 cells, stably transfected with human CD14/TLR2 or TLR9 and a NF- κ B inducible IL-8 (TLR2) or luciferase (TLR9) plasmid [28,29], were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal calf serum (FCS), 1 mM sodium pyruvate and 10 μ g/ml ciprofloxacin. To the HEK293-CD14/TLR2 cells 5 μ g/ml puromycin and to the HEK293/TLR9 cells 700 μ g/ml Geneticin (G418) was added as a selection marker. For stimulation experiments, both cell types were seeded at a density of 4.0×10^4 cells/well in 96-well flat bottom plates and stimulated the next day. The cells were stimulated with the formulations containing different concentrations of PAM (maximum 450 ng/ml) or CpG (maximum 10 μ g/ml). Medium was used as a negative control. TLR2 stimulation was measured by determining the IL-8 production in supernatants after 24 h using a commercial kit (Sanquin, Amsterdam, The Netherlands), following the manufacturer's recommendations. The HEK-293/TLR9 cells were stimulated for 6 h with the formulations. The luciferase expression was determined with a luciferase assay kit (Promega, Leiden, The Netherlands) according to the manufacturer's manual, using a DLReady Berthold Centro XS luminometer (Berthold Detection Systems, Germany).

2.5. DC activation

Monocytes were isolated from human donor blood before each experiment by Ficoll and Percoll density centrifugation and depletion of platelets was performed by surface adherence of the monocytes in 24-well plates (Corning, Schiphol, The Netherlands) as described previously [30]. The monocytes were cultured for 6 days at 37 °C and 5% CO₂ after seeding at a density of 0.5×10^6 cells/well in RPMI 1640, supplemented with 10% (v/v) FCS, 2 mM glutamine, 1 mM sodium pyruvate and 500 U/l penicillin/streptomycin. To differentiate monocytes into immature DCs 250 U/ml granulocyte macrophage-colony stimulating factor (GM-CSF) and 100 U/ml IL-4 (Invitrogen) was added. Medium was refreshed after 3 days.

DC were incubated for 48 h at 37 °C in RPMI 1640 containing 500 U/ml GM-CSF with OVA (highest concentration 5 μ g/ml), either free or encapsulated into liposomes with and without PAM or CpG (highest concentration 10 μ g/ml), keeping the lipid:OVA:TLR ligand ratio 50:2:1 (w/w). OVA, OVA liposomes and mixtures of OVA with PAM or OVA with CpG were used as controls and LPS

Table 1
Characteristics of liposomal formulations. All data are averages \pm SD of at least 3 different batches.

	Size [nm]	PDI	ZP [mV]	LE OVA [%]	LE TLR ligand [%]
OVA liposomes	130 \pm 10	0.19 \pm 0.03	23 \pm 1	98 \pm 2	–
OVA/PAM liposomes	128 \pm 9	0.25 \pm 0.01	20 \pm 2	96 \pm 3	85 \pm 4
OVA/CpG liposomes	263 \pm 22	0.30 \pm 0.09	18 \pm 2	72 \pm 5	61 \pm 6

PDI = polydispersity index, ZP = zeta potential, LE = loading efficiency.

(100 ng/ml, Invivogen) was added as a positive control. Cells were washed 3 times with PBS containing 1% (w/v) bovine serum albumin and 2% (v/v) FCS and incubated for 30 min with a mixture of 20 \times diluted anti-HLADR-FITC, anti-CD83-PE and anti-CD86-APC (Becton Dickinson) in the dark at 4 °C. Cells were washed and the expression of MHCII, CD83 and CD86 was quantified using flow cytometry (FACSCanto II, Becton Dickinson) relative to LPS, assuming 100% maturation for LPS-treated DC. Live cells were gated based on forward and side scatter.

2.6. Intradermal immunisation

Groups of 8 mice were immunised with the OVA-loaded liposomes with and without PAM or CpG by ID injection into the abdominal skin as described previously [30]. Besides the liposomes, solutions of OVA or OVA with PAM or CpG in PBS were injected and subcutaneous (SC) injection of OVA served as a control. The mice were vaccinated twice with three weeks intervals with a dose of 5 μ g OVA and 10 μ g PAM or CpG in a total volume of 30 μ l. To maintain this ratio between antigen and immune potentiator, liposomes used for the immunisation study were not filtered to remove free antigen and TLR ligand. Blood samples were collected from the tail vein 1 day before each immunisation. Three weeks after the last vaccination the mice were sacrificed. Just before euthanasia total blood was collected from the femoral artery. Afterwards the spleens were removed. Blood samples were collected in MiniCollect[®] tubes (Greiner Bio-one, Alphen a/d Rijn, The Netherlands) till clot formation and centrifuged 10 min at 10,000 \times g to obtain cell-free sera. The sera were stored at -80° C until further use.

2.7. Detection of IgG, IgG1 and IgG2a

OVA specific antibodies (IgG, IgG1 and IgG2a) in the sera were determined by sandwich ELISA as described previously [30]. Briefly, plates were coated overnight with 100 ng OVA/well. After blocking, two-fold serial dilutions of sera from individual mice were applied to the plates. HRP-conjugated antibodies against IgG, IgG1 or IgG2a were added and detected by TMB. Antibody titres were expressed as the reciprocal of the sample dilution that corresponds to half of the maximum absorbance at 450 nm of a complete s-shaped absorbance-log dilution curve.

2.8. T cell activation

The spleens from immunised mice were maintained in RPMI with 10% FCS, 50 μ M β -mercaptoethanol, 2 mM glutamine, 1 mM sodium pyruvate and 500 U/l penicillin/streptomycin. Cell suspensions were obtained using a cell strainer (70 μ m, Becton Dickinson). Cells were washed and cultured in 96-well flat bottom plates at a density of 2.0×10^5 cells/well in triplicate and restimulated with 40 μ g/ml OVA. ConA (Sigma-Aldrich) 5 μ g/ml was used as a positive control. After 3 days the supernatants were collected and stored at -80° C until further use. The amount of IFN- γ in the supernatant was determined by ELISA using a commercial kit (Becton Dickinson) according to the manufacturer's instructions.

2.9. Statistics

Statistical analysis was performed with Prism 5 for Windows (Graphpad, San Diego, USA). Statistical significance was determined either by a one way or a two way analysis of variance (ANOVA) with a Bonferroni post-test, depending on the experiment set-up.

3. Results

3.1. Liposome characteristics

With the film hydration method and subsequent extrusion, OVA-containing liposomes with an average size of 130 nm and a positive zeta potential could be prepared in a reproducible manner (Table 1). Ultrafiltration showed that nearly 100% OVA was associated with the liposomes. PAM could be easily incorporated into the liposomes (\sim 85%) and the incorporation did not affect the (measured) liposome characteristics. The addition of CpG did influence the liposome characteristics as the size augmented by two-fold. Furthermore, CpG reduced OVA association with the liposomes, probably due to competition between the antigen and the TLR ligand as both compounds bear a negative charge.

The stability and release of the OVA liposomes was studied over time in PBS at 37 °C. Dilution in PBS had an initial effect on the size of the liposomes as their size decreased from 130 nm to 90 nm, due to the influence of PBS on the hydrodynamic diameter of the liposomes [31]. After this initial size decrease, the size remained stable during the following 8 days (Fig. 1). During this period OVA was released from the liposomes. An initial burst release of 25% was observed and after 5 h already 50% of the OVA was no longer associated with the liposomes. During the following 8 days the remaining OVA was slowly released.

3.2. Preservation of TLR-activation of liposome encapsulated PAM and CpG

PAM and CpG are two TLR ligands. The effect of ligand encapsulation in OVA liposomes on their interactions with the TLRs was studied on HEK293 cells transfected with either TLR2 (receptor for

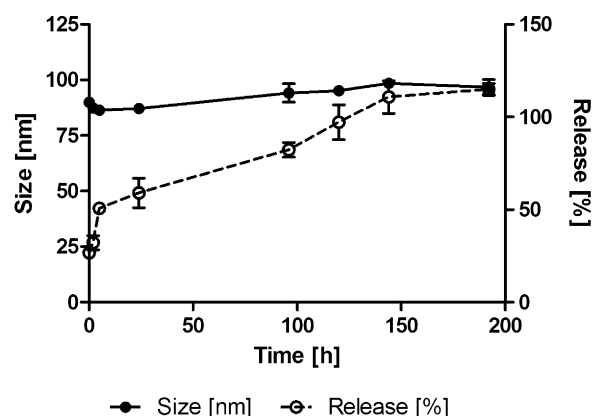


Fig. 1. Size and OVA release of OVA liposomes over time in PBS (pH 7.4) at 37 °C. Mean \pm SEM of 3 individual batches.

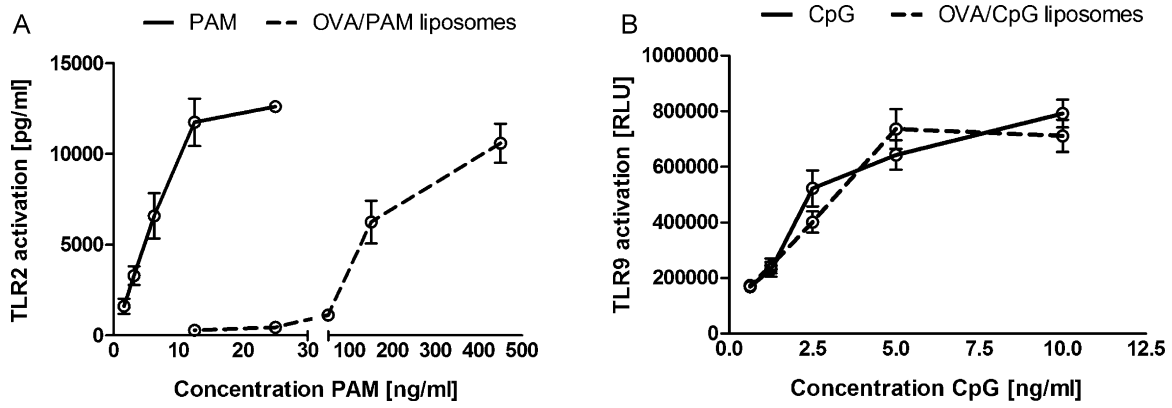


Fig. 2. Preservation of TLR-activating capacity of encapsulated TLR ligands tested on HEK293 cells transfected with TLR2 or TLR9. (A) Ability of OVA/PAM liposomes and a PAM solution to activate TLR2-transfected HEK-293 cells; (B) ability of OVA/CpG liposomes and a CpG solution to activate TLR9-transfected HEK-293 cells. Data are mean \pm SEM of 3 different experiments.

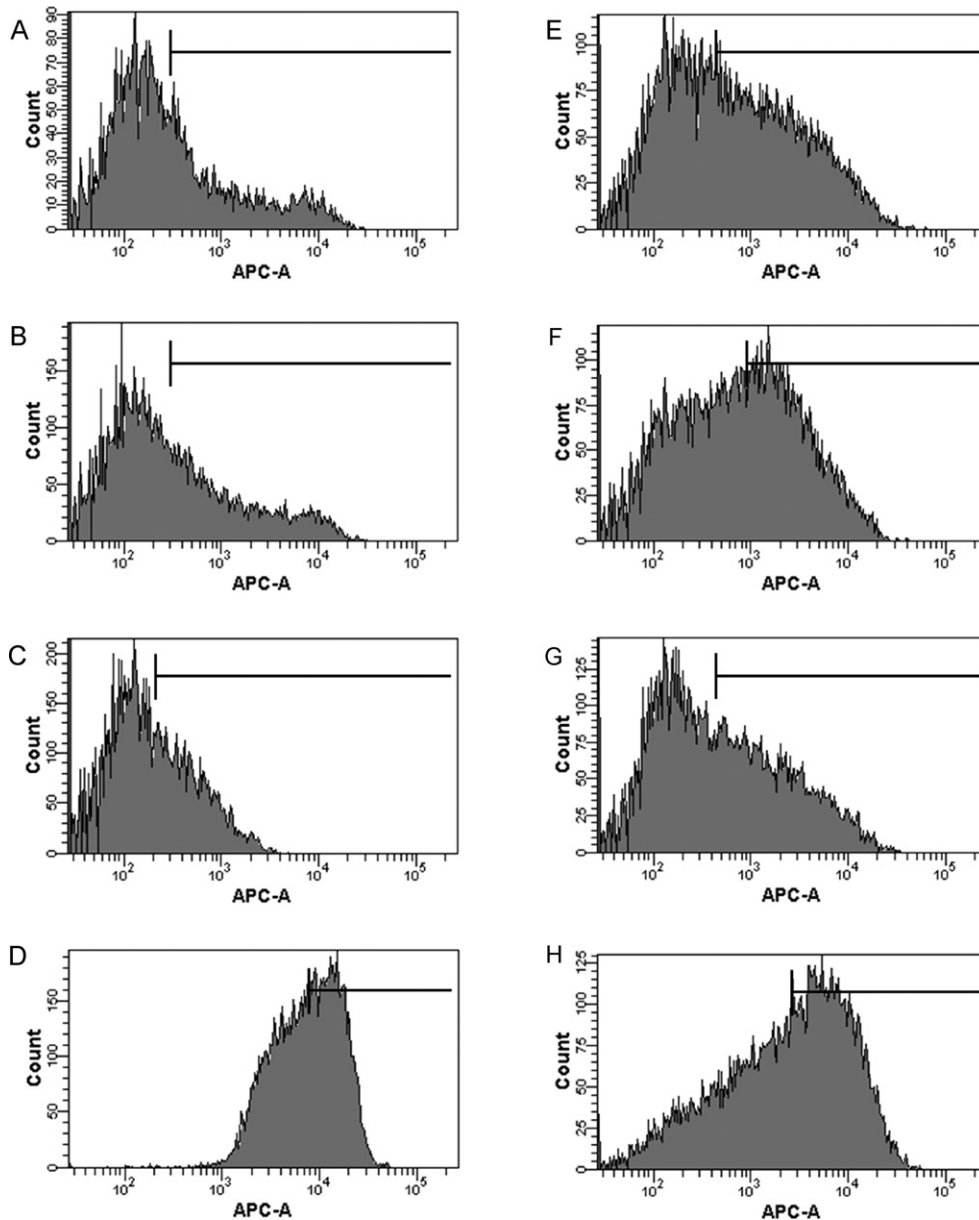


Fig. 3. Representative examples of FACS plots of the expression of CD86 on DCs after incubation with medium (A), OVA (B), OVA liposomes (C), LPS (D), OVA + PAM (E), OVA/PAM liposomes (F), OVA + CpG (G) and OVA/CpG liposomes (H). Horizontal bars represent mean fluorescence intensity.

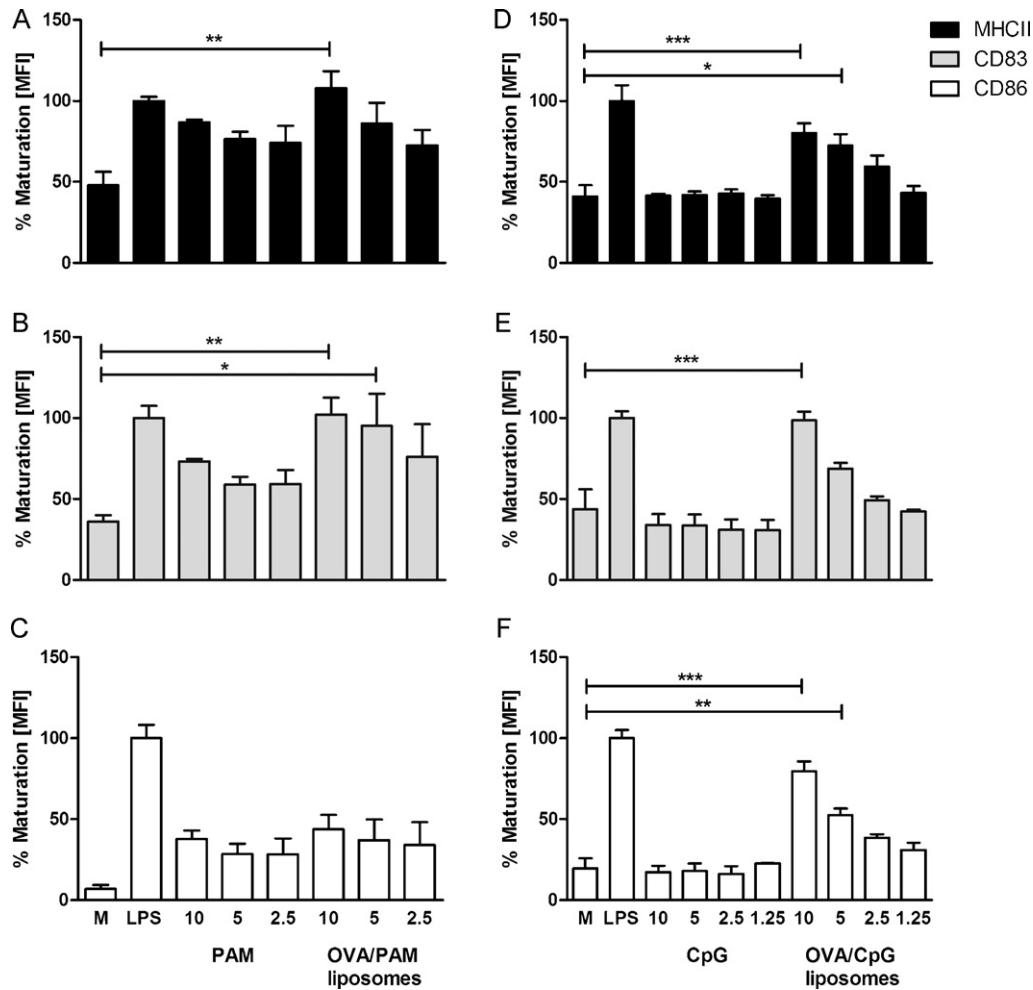


Fig. 4. Upregulation of DC maturation markers by OVA/PAM liposomes (A–C) and OVA/CpG liposomes (D–F). The liposomes had a lipid:OVA:TLR ligand ratio of 50:1:2 (w/w). M = medium and the concentrations are expressed in $\mu\text{g/ml}$. The values are expressed as mean fluorescence intensity (MFI) \pm SEM relative to LPS ($n = 6$, two separate experiments). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

PAM) or TLR9 (receptor for CpG). Non-adjuvanted liposomes and a solution of OVA did not induce TLR2 or TLR9 activation (data not shown). PAM in solution was a stronger TLR2 activator compared to the liposome encapsulated PAM (Fig. 2A). A 15-fold higher dose of PAM was necessary to obtain the same level of IL-8 production from the HEK293-CD14/TLR2 cells. Both PAM in solution and OVA/PAM liposomes activated the cells in a concentration dependent manner.

CpG activated TLR9-transfected HEK cells in a concentration dependent way as well. Encapsulation of CpG in liposomes did not affect its ability to activate TLR9, as no difference in activation between a solution of CpG and OVA/CpG liposomes was observed (Fig. 2B).

3.3. Liposomes with encapsulated TLR ligand activate DCs

DCs express TLRs which upon stimulation with TLR ligands induces the expression of maturation markers on the DC's surface as shown for CD86 in Fig. 3. Whereas application of OVA and OVA liposomes (maximum OVA concentration 5 $\mu\text{g/ml}$) did not stimulate the DCs, encapsulation of both TLR ligands had a clear effect on the DC activation. Application of 10 $\mu\text{g/ml}$ PAM encapsulated in OVA-containing liposomes (OVA concentration 5 $\mu\text{g/ml}$) significantly elevated the MHCII and CD83 expression ($p < 0.01$) compared to untreated cells and this activation proved to be concentration dependent (Fig. 4A and B). Moreover, a similar pattern was observed for the CD86 levels. After application of a

PAM solution also a trend of elevated MHCII and CD83 levels was observed, but these levels were not significantly higher compared to untreated DCs. PAM had a minor effect on the CD86 expression (Fig. 4C).

The effect of CpG encapsulation was more pronounced. Whereas a CpG solution did not activate the DCs at all, encapsulation of CpG in liposomes induced increased MHCII, CD83 and CD86 expression (Fig. 4D–F). The level of expression obtained with the highest CpG concentration was comparable to that induced by LPS, the positive control.

3.4. Intradermal vaccination with liposomal formulations

To investigate whether the improved DC activation ability *in vitro* correlated with the immunogenicity in mice, an immunisation study was performed. The liposomal formulations and physical mixtures of OVA with CpG or PAM were applied ID. Both the OVA-specific total serum IgG titres (Fig. 5A) and the antibody subclass (IgG1 and IgG2a, Fig. 5B) were measured.

The addition of either PAM or CpG into liposomes significantly increased the immunogenicity of OVA-loaded liposomes ($p < 0.05$), which did not enhance the immune response compared to an OVA solution. Incorporation of the TLR ligands in OVA-containing liposomes induced similar IgG titres as compared to the physical mixtures of OVA and the TLR ligand. However, the liposomes did influence the IgG1/IgG2a balance of the immune

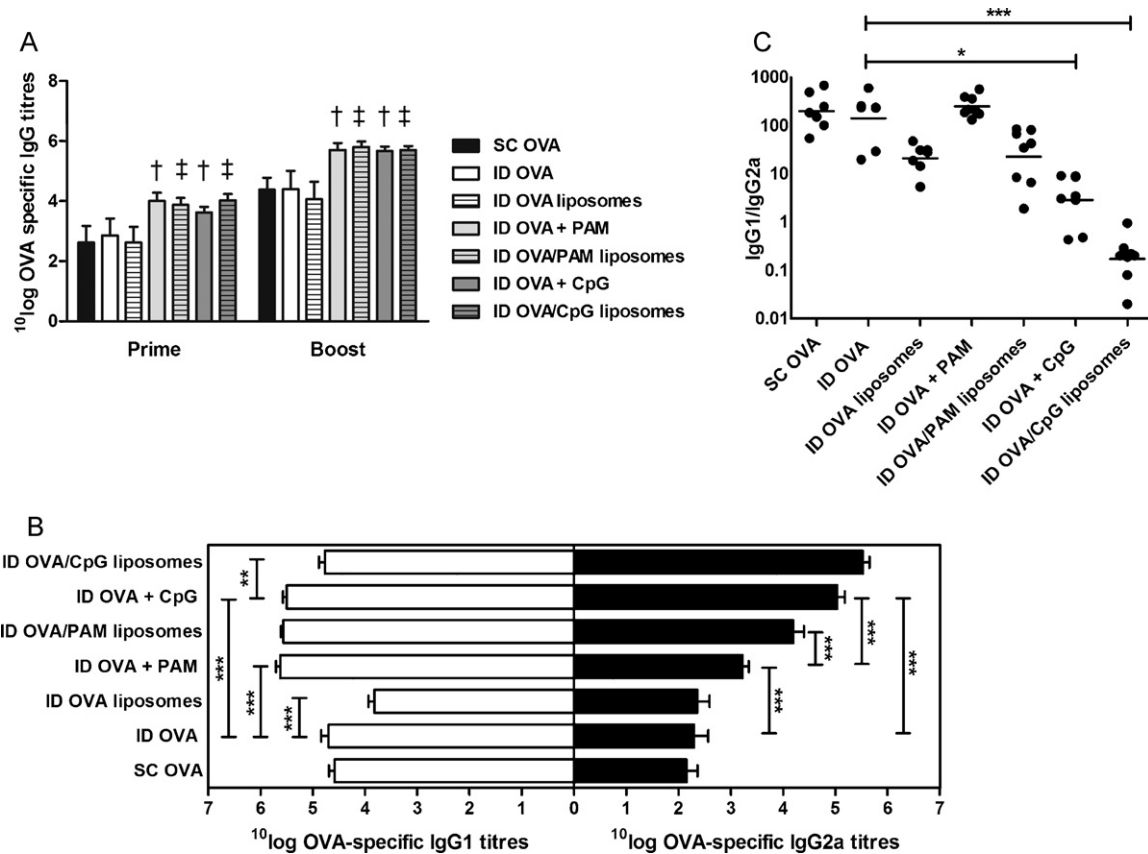


Fig. 5. OVA specific IgG titres in serum after a prime and subsequent booster immunisation (A) and IgG1 (white bars) and IgG2a (black bars) after the second boost (B). Mean \pm SD of 8 mice. (C) Corresponding IgG1/IgG2a ratios of individual mice. Non-responders for IgG1 or IgG2a were excluded. Bar represents geometric mean. †Significantly higher than ID OVA; ‡significantly higher than ID OVA liposomes; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

response (Fig. 5B/C). The main IgG subtype induced by plain OVA was IgG1. The addition of PAM resulted in equally elevated IgG1 and IgG2a levels upon ID immunisation. Encapsulation of OVA alone in liposomes and co-encapsulation of OVA and PAM resulted in a tendency of altering the balance more towards IgG2a (Fig. 5B/C). Co-administration of CpG with OVA significantly shifted the IgG1/IgG2a balance towards IgG2a ($p < 0.05$). This alteration was even more pronounced when OVA and CpG were co-encapsulated in liposomes ($p < 0.001$).

Besides the humoral immune response, the effect of the different formulations on the cellular immunity was investigated by measuring the IFN- γ production by restimulated splenocytes. Th1 cells produce IFN- γ which is reported to induce isotype switching and IgG2a production [32,33]. In agreement with the antibody subclass titres, only formulations containing CpG, which resulted in the highest IgG2a titres, induced the production of measurable IFN- γ levels and these levels were the highest for mice receiving OVA/CpG liposomes ($p < 0.05$, Fig. 6).

4. Discussion

Liposomes are an attractive delivery system for vaccines as they protect the antigen from degradation, opsonise the uptake of the encapsulated antigen by DCs and provide controlled release of the antigen over time. Moreover, it is a versatile system that permits the inclusion of various immune potentiators. This is reflected by the fact that high encapsulation efficiencies of both PAM and CpG were achieved, whereas both TLR ligands have very different physical chemical characteristics. This is an important feature, as in line with other reports [11,13], this study shows that cationic liposomes

themselves are not that immunogenic; OVA loaded liposomes did not enhance the antibody response compared to free OVA. The inclusion of immune potentiators into liposome-based formulations will therefore be necessary to improve their application in vaccination strategies.

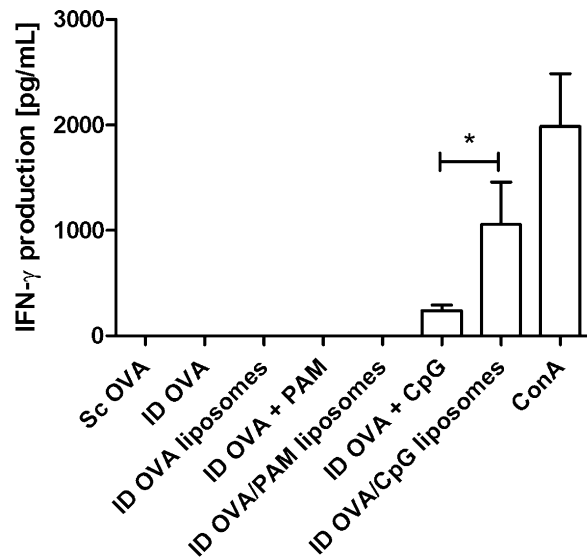


Fig. 6. IFN- γ production by splenocytes after restimulation with OVA. Mean + SEM of 5 mice are shown. * $p < 0.05$.

Here we showed that co-encapsulation of antigens and TLR ligands in liposomes can enhance antigen delivery *in vitro* and combine this with potent stimulation of the innate immune response as can be concluded from the vaccination study with PAM- or CpG-containing liposomes. The anti-OVA serum IgG titres after the prime and booster vaccinations with these adjuvanted formulations were significantly higher than those obtained with plain liposomes or OVA. Interestingly, the IgG titres elicited in mice vaccinated with a physical mixture of OVA and PAM or CpG, were comparable with those elicited by those that were immunised with PAM- or CpG-adjuvanted liposomes. This is in accordance with previous studies by us and other groups, where no additional effect of liposomes on the IgG titres was observed after vaccination via different routes [11,13,34]. It not only holds true for liposomes, but also for antigen-loaded N-trimethyl chitosan nanoparticles [30]. This raises questions regarding the usefulness of nanoparticles for ID immunisation. However, IgG titres not necessarily correlate with protection and are therefore not the only parameter to express the extent or quality of an immune response. A cellular response, which can be measured by the production of IgG2a antibodies and IFN- γ production by T-cells, can sometimes be more predictive [35]. The present study shows that liposomes did influence the quality of the immune response. A trend of higher IgG2a levels compared to antigen and TLR ligand solutions was observed for all three liposomal formulations. Similar results were also reported by Brgles et al. after SC immunisation; OVA-containing liposomes were able to modulate the immune response towards a Th1/CD8⁺ cytotoxic T lymphocyte (CTL) direction, without influencing the overall intensity of the immune response [13].

How liposomes modify the quality of the response remains to be clarified. The *in vitro* DC study clearly demonstrates that CpG, and to a lesser extent also PAM, needs to be encapsulated to activate the DCs. This is in accordance with a study by Fernandes et al. who showed that the liposomal incorporation of two other triacylated lipopeptides enhanced the proliferation of murine splenocytes [36], which could be attributed to improved adjuvant uptake by the DCs [20,21]. The prominent advantage of liposomal encapsulation of CpG correlates excellently with the cellular localisation of the PAM and CpG receptors. Whilst TLR2 is expressed on the cell surface, TLR9 is present in the endosomal compartment. Conceivably, CpG profits more from liposomal delivery than PAM. For PAM this is illustrated *in vitro* as liposome encapsulation decreases its ability to stimulate HEK293-CD14/TLR2 cells, probably due to reduced interaction with the receptor. It is known that liposomal incorporation can have a profound influence on the immunomodulatory properties of lipoproteins [37]. PAM's functionality is dependent on different structural components. The peptide segment linked to the carboxyl terminus of the palmitoyl lipopeptide, the SKKKK sequence, was shown to elevate the adjuvant activity compared to other peptide sequences [38]. Changes to the lipopeptide fatty acid chains, the O-linked fatty acids in particular, appear to have a substantial effect on the signalling through TLRs. The palmitoyl groups (C16) provide better adjuvant activity than longer and shorter fatty acids [39,40]. If the interaction of either of these moieties with the TLR2 is disturbed, the adjuvant activity will be diminished. Liposomal encapsulation can also have a positive effect on the adjuvant activity as it improves the solubility of PAM [41] and the DC uptake of OVA, which may improve DC maturation. However, probably due to loss of interaction with the TLR2, this did not enhance the immune response *in vivo*.

For CpG, improved DC uptake of OVA/CpG liposomes facilitates the interaction with the endosomal TLR9 [18,42], thereby inducing DC maturation. The *in vivo* situation is more complicated. Even though the DCs will preferentially take up the liposomes, the speed and duration of antigen and immune potentiator exposure will differ between the solution and the liposomal formulations. CpG and

OVA in solution will probably reach the lymph nodes faster than the liposomes, but only liposomes ensure uptake of CpG and OVA by the same DC, which was reported to influence the type of immune response generated [21]. Indeed, the enhanced DC uptake does result in a more Th1-biased response, which is most pronounced for the CpG-containing liposomes. Similar results were reported by Gursel et al., who showed that co-encapsulation of OVA and CpG in cationic liposomes induced elevated IgG2a titres and IFN- γ secretion compared to free CpG after intraperitoneal injection [43]. It has to be noted that liposome size also affects the Th1/Th2 bias; larger liposomes tend to induce a Th1 shift [44,45]. As OVA/CpG liposomes are larger this may further shift the immune response towards Th1.

Finally the bias towards a more cellular response by the liposomes could also be attributed to the presence of DOPE in the liposomes. DOPE, a neutral pH-sensitive lipid, is capable of improving delivery of CpG into the cytosol following APC uptake [46]. Endosomal escape is crucial for MHC I presentation of the antigen and the induction of CTL responses. It has been reported that liposomes complexed with antigen and either CpG or poly(I:C), which binds to TLR3 that is also expressed intracellularly, are capable of cross priming CD8⁺ T cells [47]. Whether this is also the case after ID immunisation with our liposomes requires further investigation, but the elevated IFN- γ production is a first indication that a CTL response could be induced [48].

In conclusion, the advantage of co-encapsulation of antigen and TLR ligand in cationic liposomes is their potency to steer the immune bias. This depends on the type of TLR ligand used, as CpG, binding to the intracellular TLR9, induced the production of IgG2a antibodies and a potent cellular immune response after ID immunisation, whereas PAM, ligand of extracellular TLR2, did not.

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