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RESEARCH PAPER

Incorporation of the TLR4 Agonist Monophosphoryl Lipid A Into the Bilayer of DDA/TDB Liposomes: Physico-Chemical Characterization and Induction of $CD8^+$ T-Cell Responses *In Vivo*

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ABSTRACT

Purpose The combination of delivery systems like cationic liposomes and immunopotentiators such as Toll-like receptor (TLR) ligands is a promising approach for rational vaccine adjuvant design. The purpose of this study was to investigate how the incorporation of the poorly soluble TLR4 agonist monophosphoryl lipid A (MPL) into cationic liposomes based on dimethyldioctadecylammonium (DDA) and trehalose 6,6′-dibehenate (TDB) influenced the physicochemical and immunological properties of the liposomes.

Methods The DDA/TDB/MPL liposomes were characterized with regard to particle size, poly dispersity, surface charge, stability and thermodynamic properties. The adjuvant formulations were tested *in vivo* in mice using ovalbumin (OVA) as model antigen.

Results Integration of MPL into the bilayer structure of DDA/TDB liposomes was evident from a decreased phase transition temperature, an improved membrane packing, and a reduction in surface charge. The particle size and favorable liposome storage stability were not affected by MPL. In mice, DDA/TDB/MPL liposomes induced an antigen-specific $CD8^+$ T-cell response and a humoral response.

Conclusions Enhancing the solubility of MPL by inclusion into the bilayer of DDA/TDB liposomes changes the membrane characteristics of the adjuvant system and provides the liposomes with $CD8^+$ T-cell inducing properties without compromising humoral responses.

KEY WORDS dimethyldioctadecylammonium (DDA) · liposome · monophosphoryl lipid A (MPL) · trehalose 6,6-dibehenate (TDB) · vaccine

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INTRODUCTION

Next generation vaccine candidates include highly purified and well-defined antigens, which represent a significant safety improvement for modern vaccines. However, as they are poorly immunogenic, the co-administration of efficient and safe adjuvants is necessary (1,2). Very few adjuvants are currently marketed, and, besides Alum, these include MF59 from Novartis as well as AS03 and AS04 from GlaxoSmithKline (GSK). An unmet medical need therefore exists for new adjuvants, in particular adjuvants that can induce a cellular immune response (1,3–5), which is important for the prevention of challenging vaccine targets such as human immunodeficiency virus and tuberculosis (4,5).

Generally, adjuvants can be sub-divided into two classes: (i) vaccine delivery systems such as emulsions, liposomes, immune-stimulating complexes (ISCOMs) and mineral salts; and (ii) immunopotentiators like the Toll-like receptor (TLR) agonists (3,5,6). However, some delivery systems may also possess intrinsic immunopotentiating activity (2) e.g. ISCOMs and mineral salts. Whereas delivery systems ensure the delivery of antigens to the antigen-presenting cells (APCs), immunopotentiators activate immune cells through specific receptors and/or pathways. Cationic liposomes are promising delivery systems, since they interact efficiently with and are taken up by the APCs (7).

Furthermore, *in vivo* studies have shown a superior and prolonged adjuvant effect of cationic liposomes compared to anionic or neutral liposomes (8), suggesting that they do not merely function as efficient delivery systems but also mediate formation of an antigen depot at the injection site (9). In addition, the versatility of the liposome structure allows for the combination with additional components, such as immunopotentiators, either by surface interaction, encapsulation, or membrane intercalation.

Monophosphoryl lipid A (MPL, Fig. 1c) is a well-known immunopotentiator which is recognized by the pattern recognition receptor TLR4 (10–12). Various TLR agonists have been shown to induce cross-priming (13,14), which is the stimulation of naive cytotoxic $CD8^+$ T-cells upon APC internalization of exogenous antigen and antigen presentation via major histocompatibility complex (MHC) class I. The TLR4 ligands are among these TLR agonists (14), and MPL has been shown to induce $CD8^+$ T-cell responses *in vivo* (15,16). MPL is derived from lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria such as *Salmonella minnesota* (17). Even though the adjuvant activity of MPL is comparable to LPS (18), MPL is a safe alternative by being more than 1,000-fold less toxic than the parent endotoxin or diphosphoryl lipid A (19). Therefore, the efficacy and safety of MPL is currently exploited in clinical trials, and a few non-liposomal vaccines containing MPL are already marketed, including the human papilloma virus (HPV) vaccine Cervarix™ and the hepatitis B virus (HBV) vaccine Fendrix™ (both from GSK).

MPL is a poorly soluble compound, which is difficult to disperse in an aqueous solution. Different solubility-enhancing approaches are therefore used, which include formulation in emulsions (20–23), formulation in aqueous dispersions containing low amounts of surfactants or helper lipids (20,22,24,25), or inclusion into liposomes (16,23,26). In the present study, MPL is incorporated into an adjuvant system based on cationic liposomes (designated CAF01), consisting of the synthetic surfactant dimethyldioctadecylammonium (DDA, Fig. 1a) and the immunopotentiator trehalose 6,6′-dibehenate (TDB, Fig. 1b), which has shown promising results for vaccination against a wide range of diseases (15). The immunostimulatory activity of DDA/TDB liposomes is characterized by the induction of a strong cell-mediated immune response as well as a humoral response (27), which is desirable for a number of vaccine targets. Whereas protein-based antigens adjuvanted with DDA/TDB liposomes induce a strong $CD4^+$ T-cell response, the stimulation of $CD8^+$ T-cell responses is limited (15). Thus, incorporating MPL into DDA/TDB liposomes would provide for a solubility-enhancing effect as well as an attractive strategy for expanding the applicability of DDA/TDB liposomes towards $CD8^+$ T-cell dependent vaccination targets.

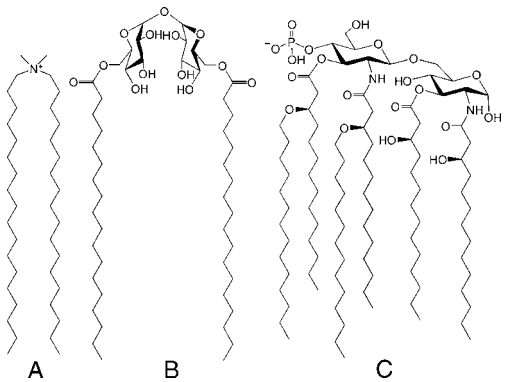

Fig. 1 Structure of (**A**) dimethyldioctadecylammonium (DDA), (**B**) trehalose 6,6′-dibehenate (TDB) and (**C**) monophosphoryl lipid A (MPL).

MATERIALS AND METHODS

Materials

DDA, TDB and synthetic MPL were acquired from Avanti Polar Lipids (Alabaster, AL, USA). All other chemicals and reagents were obtained commercially at analytical grade.

Preparation of Adjuvant Formulations

The adjuvants were prepared by the thin film method as described previously (27). Briefly, weighed amounts of lipids were dissolved in chloroform/methanol (9:1, v/v), and the organic solvent was evaporated using a gentle stream of N_2. The lipid film was dried overnight at room temperature to remove trace amounts of the organic solvent, followed by rehydration with Tris buffer (10 mM Tris, pH 7.4) at 60°C for 1 h with vortex mixing every tenth minute. The final lipid concentrations were 2.5 mg/ml DDA, 0.5 mg/ml TDB and 0 to 1.0 mg/ml MPL.

Size, Polydispersity and Zeta-Potential

The particle size distribution and polydispersity index (PDI) of the liposomes were determined by dynamic light scattering using the photon correlation spectroscopy (PCS) technique. The surface charge of the particles was estimated by analysis of the zeta-potential (laser-Doppler electrophoresis). For determination of the size distribution and PDI, six measurements were performed on triplicate samples diluted 10-fold in Tris buffer. For evaluation of the zeta-potential, three measurements were performed on the triplicate samples diluted 300-fold in Tris buffer. Both types of measurements were performed at 25°C using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK) equipped with a 633-nm laser and 173° detection optics.

Malvern DTS v.5.10 software (Malvern Instruments) was used for data acquisition and analysis. For viscosity and refractive index, the values of pure water were used. The particle size distribution is reflected in the PDI, which ranges from 0 for a monodisperse to 1.0 for an entirely heterodisperse dispersion.

Cryo-TEM

Morphological analysis was carried out by cryo-Transmission Electron Microscopy (cryo-TEM) using a Philips CM120 BioTWIN transmission electron microscope (Philips, Eindhoven, Holland). Samples for cryo-TEM were prepared under controlled temperature and humidity conditions within an environmental verification system. A small droplet (5 μl) of sample was deposited on a Pelco Lacey carbon-filmed grid. After carefully spreading the drop, excess liquid was removed with a filter paper, forming a thin (10–500 nm) sample film, and the sample was immediately plunged into liquid ethane and kept at −180°C. The vitrified sample was then transferred in liquid nitrogen to an Oxford CT3500 cryo holder connected to the electron microscope. The sample temperature was kept below −180°C. All observations were made in the bright field mode at an acceleration voltage of 120 kV. Digital pictures were recorded with a Gatan Imaging Filter 100 CCD camera (Gatan, Pleasanton, CA, USA).

Differential Scanning Calorimetry

The gel-to-liquid phase transition temperature of the undiluted vesicle suspensions (2.5 mg/ml DDA, 0.5 mg/ml TDB and 0 to 1.0 mg/ml MPL) was determined using differential scanning calorimetry (DSC). Thermograms were obtained with a MicroCal VP-DSC MicroCalorimeter (MicroCal LLC, Northamton, MA, USA) at a scanning rate of 30°C/h from 25°C to 60°C. VPViewer 2000 and Origin® 7 scientific plotting software were used for data analysis. The first of three scans of each sample ($n=3$) was used for data analysis.

Liposome Stability

For liposome stability studies, the liposome dispersions were kept in Tris buffer with 1 mM NaN_3 at 4°C for up to 3 months. The concentrations of the components in the liposomal formulations were 2.5 mg/ml DDA, 0.5 mg/ml TDB and 0–1.0 mg/ml MPL. The particle size was measured as described above.

Langmuir-Blodgett Isotherms

Monolayers were formed at room temperature by spreading 27.7 nmol of lipid mixture in chloroform:methanol (9:1, v/v) onto an aqueous sub-phase consisting of 10 mM Tris buffer (pH=7.4) in a KSV minitrough 1 (KSV instruments Ltd., Helsinki, Finland) using a Hamilton microsyringe. The monolayer compression was initiated 10 min after spreading lipids to allow the organic solvent to evaporate. The monolayer was compressed with a barrier speed of 10 mm/min, and the surface pressure/area isotherm was detected using a Wilhelmy platinum plate (KSV instruments Ltd.). Each sample ($n=3$) was compressed once. KSV software (KSV instruments Ltd.) was used for data analysis.

Immunization of Mice

Female C57BL/6 mice, 6 to 12 weeks old, were obtained from Harlan Laboratories (Venray, The Netherlands). The handling of mice was conducted in accordance with the regulations of the Danish Ministry of Justice and animal protection committees and in compliance with the European Community Directive 86/609. The vaccines were prepared by mixing the adjuvants with the model antigen ovalbumin (OVA, Grade V, Sigma, St. Louis, MO, USA). For comparative studies, MPL was dispersed in Tris buffer containing 0.2% triethylamine to enhance the solubility of the compound. As controls, mice immunized with OVA alone and naive mice were included. The final OVA concentration was 25 μg/ml, and the final concentration of DDA and TDB was 1.25 and 0.25 mg/ml, respectively, for all formulations, while the final concentrations of MPL were 0.25 or 0.50 mg/ml. The isotonicity of the vaccines was adjusted with 9% (w/v) trehalose. Mice were immunized intraperitoneally (i.p.) three times with a 2-week interval (0.2 ml/dose). Spleens were harvested 1 week after the last immunization. Splenocyte cultures ($n=3$–4) were obtained by passage of spleens through a metal mesh followed by two washings with RPMI 1640 (Gibco Invitrogen, Carlsbad, CA, USA). The cells were dispersed in RPMI 1640 supplemented with 5×10^{-5} M 2-mercaptoethanol, 1 mM glutamine, 1% (v/v) pyruvate, 1% (v/v) penicillin-streptomycin, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10% (v/v) fetal calf serum (FCS) (all from Invitrogen).

Detection of Vaccine-Specific $CD8^+$ T-Cells

Isolated lymphocytes were stained with phycoerythrin (PE)-H-2Kb SIINFEKL MHC pentamer (ProImmune, Oxford, UK) for 10 min at room temperature and washed in phosphate-buffered saline (PBS) supplemented with 0.1% (w/v) bovine serum albumin (BSA). The SIINFEKL pentamers enable detection of antigen-specific $CD8^+$ T-cells directed against the SIINFEKL epitope from OVA. Subsequently, the cells were stained for 30 min at 4°C for surface markers with mAbs using 1:200 dilutions of

anti-CD4, anti-CD8, and anti-CD44 (all BD Pharmingen, San Diego, CA, USA). After washing, the cells were re-suspended in PBS containing 0.1% (w/v) sodium azide and 1% (v/v) FCS (FACS buffer) and analyzed on a six-colour BD FACS Canto flow cytometer (BD Biosciences, San Jose, CA, USA). Responses were analysed with FlowJo software V.7.2.2 (Tree Star Inc., Ashland, OR, USA).

Detection of Vaccine-Specific Interferon (IFN)-γ Cytokines by Enzyme-Linked Immunosorbent Assay (ELISA)

Isolated splenocytes were cultured in duplicate in round-bottomed microtiter wells containing 2×10^5 cells/well in 200 µl RPMI 1640 supplemented as described above. Cells were re-stimulated with OVA and the CD8 peptide epitope of OVA (OVA 257–264, SIINFEKL, Schafer-N, Copenhagen, Denmark) in concentrations ranging from 0.5 to 50 µg/ml. Medium with or without 5 µg/ml of concanavalin A (Sigma-Aldrich, St. Louis, MO, USA) served as positive and negative controls, respectively. Culture supernatants were harvested after 72 h of incubation with antigen, and the amount of IFN-γ was determined by ELISA as described elsewhere (28).

Intracellular Flow Cytometry Analysis

Isolated splenocytes (1×10^7 cells/well) were stimulated for 1 h with 5 µg/ml OVA or 5 µg/ml of SIINFEKL in the presence of 1 µg/ml anti-CD28 and anti-CD49d (both BD Pharmingen, San Diego, CA, USA) in 200 µl supplemented RPMI 1640. Cells were subsequently incubated for 5–6 h at 37°C after addition of 10 µg/ml Brefeldin A (Sigma-Aldrich, Brøndby, Denmark) and 0.7 µl/ml Monensin/Golgi-stop (BD Pharmingen). Following overnight storage at 4°C, the cells were washed in FACS buffer and stained for surface markers for 30 min at 4°C with mAbs using 1:200 dilutions of anti-CD4, anti-CD8 and anti-CD44 (all BD Pharmingen) in FACS buffer. Cells were then washed in FACS buffer, permeabilized using the Cyto-fix/Cyto-perm kit (BD Pharmingen) according to the manufacturer's instructions, and stained intracellularly for 30 min at 4°C in dilutions of 1:200 using anti-IFN-γ mAb (eBioscience, San Diego, CA, USA) in Perm wash buffer. After washing, cells were re-suspended in FACS buffer and analyzed by flow cytometry as described above.

Detection of Vaccine-Specific Antibodies by ELISA

Micro titers plates (Nunc Maxisorp, Roskilde, Denmark) were coated with OVA (1.0 µg/ml) in carbonate buffer overnight at 4°C. Free binding sites were blocked with 2% BSA in PBS. Individual mouse sera were analyzed in 5-fold dilutions at least 11 times in PBS with BSA starting with a 10-fold dilution. Horseradish peroxidase (HRP)-conjugated secondary antibodies (rabbit antimouse immunoglobulin (Ig) G1 (AH Diagnostics, Aarhus, Denmark) and goat antimouse IgG2c (Southern Biotech, Birmingham, Alabama, USA)) diluted 1:5,000 in PBS with 1% BSA was added. After 1 h, antigen-specific antibodies were detected using the substrate TMB (Kem-En-Tec, Copenhagen, Denmark). The reaction was stopped with 0.2 M H_2SO_4, and the absorbance (optical density) was read at 450 nm. The absorbance values were plotted as a function of the reciprocal dilution of serum samples. The highest dilution of sera that gave an absorbance value, which was at least two times higher than the background absorbance, was used as endpoint titers.

Statistics

Statistical calculations were performed using SigmaPlot v. 11.0 (Systat Software Inc., San Jose, CA, USA) by a one-way analysis of variance (ANOVA) at a 0.05 significance level and means comparison by a Tukey's test. For statistical analysis of the particle size and *in vivo* data, the test for normality failed, and the data was thus analyzed by the non-parametric Kruskal-Wallis test and means comparison by Tukey's test.

RESULTS

Incorporation of MPL Into the Bilayer of DDA/TDB Liposomes Reduces the PDI and the Surface Charge

The dispersion of MPL in an aqueous solution is dependent on the use of a surfactant such as triethylamine or a helper lipid. Dispersing MPL in an aqueous suspension by sonication and rehydration of a lipid film resulted in vesicles around 100 nm as well as aggregates in the visible size range (results not shown). Thus, in order to fully solubilize MPL, MPL was incorporated into the bilayer of DDA/TDB liposomes at two different concentrations (6 and 11 mol%), corresponding to DDA:TDB:MPL weight ratios of 5:1:1 and 5:1:2, respectively. Inclusion of MPL into the DDA/TDB liposomes did not significantly affect the average particle size, which was around 500 nm for all formulations (Table I). However, incorporation of MPL into the bilayer reduced the PDI (Table I), indicating a more narrow size distribution, and significantly reduced the surface charge in a concentration-dependent manner (Table I). The cryo-TEM micrograph confirmed the size range and the presence of liposomes, and unilamellar as well as multilamellar and multivesicular liposomes were observed (Fig. 2). In contrast to DDA/TDB liposomes, no

Table I Physico-Chemical Data Describing the Average Particle Diameter, PDI, Zeta Potential and Thermodynamic Parameters Characterizing the Gel-to-Liquid Phase Transition of DDA/TDB Liposomes with Varying Amounts of MPL

Mol% MPL	0	6	11
Average size (nm)	550 ± 49	544 ± 93	442 ± 76
PDI	0.48 ± 0.10	0.25 ± 0.12	0.25 ± 0.10
Zeta-potential (mV)	80.6 ± 0.4	66.6 ± 4.8**	51.9 ± 1.8***
T_m (°C)	42.9 ± 0.1	40.9 ± 0.3***	39.9 ± 0.1***
$T_{½}$ (°C)	2.3 ± 0.2	2.2 ± 0.1	4.7 ± 2.3
ΔH (kcal/mole)	10.9 ± 0.5	10.3 ± 0.1	10.0 ± 0.0*

Results denote mean ± SD from three batches of each formulation. Significant differences from 0 mol% MPL are indicated: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

giant vesicles in the micrometer range was observed (27), which correlates well with the reduced PDI upon MPL incorporation.

The Membrane Fluidity is Affected by Integration of MPL

The effect on the thermotropic phase behavior of including MPL into the bilayer of DDA/TDB liposomes was characterized by DSC (Fig. 3). The gel-to-liquid phase transition temperature, T_m, of DDA/TDB liposomes was observed at 42.9 ± 0.1°C, as reported in the literature (27, 29). Incorporation of 6 mol% MPL resulted in a thermogram profile similar to DDA/TDB but with a significant decrease in the main transition temperature to 40.9 ± 0.3°C (Fig. 3, Table I). The decrease in T_m suggests that the acyl groups of MPL are embedded into the lipid bilayer, affecting the packing of the membrane. The highest concentration of MPL (11 mol%) caused a broadening of the mean transition and the appearance of two interconnected peaks with the main phase transition peak at around 40°C and a smaller peak at approximately 45°C (Fig. 3), suggesting that the lipids were inhomogenously distributed in the bilayer, resulting in phase separation and the formation of local microstructures of the lipid bilayer enriched in one or more of the components. The heat capacities (ΔH) were similar for DDA/TDB liposomes with and without 6 mol% MPL, indicating that liposomes are formed when MPL is incorporated (Table I). The bilayer formation for DDA/TDB liposomes has been suggested to depend on DDA, whereas TDB only adds to the quality of the bilayer phase transition (30). The present results suggest a similar role for MPL since the heat capacity correlated to the concentration of DDA and not to that of MPL. Incorporation of 11 mol% MPL into the bilayer of DDA/TDB liposomes resulted in a slightly decreased heat capacity, suggesting formation of alternative lipid structures which do not contribute to the transition enthalpy. A very weak phase transition around 55°C was detectable for a crude aqueous suspension of MPL (results not shown), suggesting that MPL is incorporated into the lipid bilayer since no transition was detectable at that specific temperature for the DDA/TDB/MPL liposomes.

The Stability of DDA/TDB Liposomes is not Compromised by MPL

DDA liposomes tend to fuse or aggregate during storage, whereas DDA/TDB liposomes are physically stable for more than 3 months at 4°C (27). Thus, it was investigated whether incorporation of MPL into the DDA/TDB liposomes compromised the physical stability of the formulation. All formulations, except pure DDA liposomes, had a stable particle size for at least 3 months (Fig. 4), irrespective of the MPL content, indicating that incorporation of MPL does not compromise the colloidal stability of DDA/TDB liposomes. No major surface changes occurred, since the zeta-potential remained constant during the 3 months (results not shown).

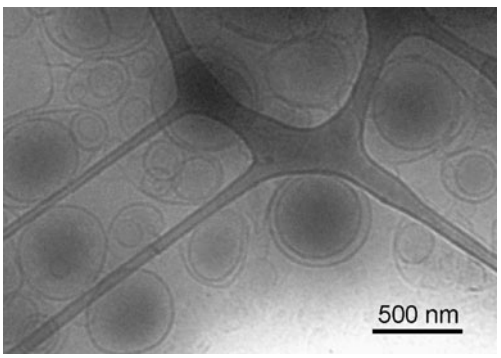

Fig. 2 Cryo-TEM micrograph of DDA/TDB liposomes with 6 mol% MPL. The long dark structures are the carbon grid the sample was deposited on before analysis.

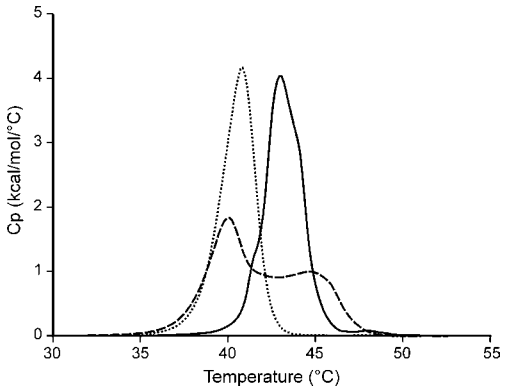

Fig. 3 Differential scanning heat capacity curves for DDA/TDB liposomes (*solid*) with 6 mol% MPL (*dot*) and 11 mol% MPL (*dash*). The curves have been normalized to the molar lipid content and represent averages of three experiments for each type of formulation.

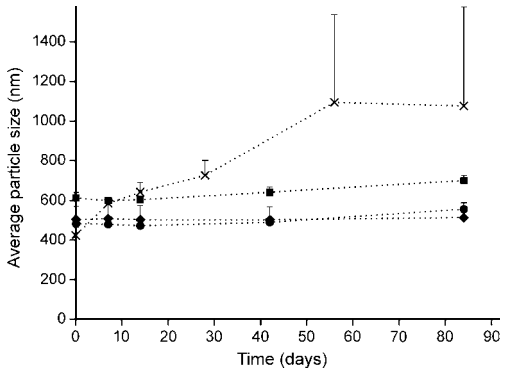

Fig. 4 Stability at 4°C of DDA liposomes (-x-) and DDA/TDB liposomes (-♦-) containing 6 mol% (-■-) and 11 mol% (-●-) MPL. Results denote mean + SD for three batches of each formulation.

MPL Increases the Surface Pressure and Provides an Improved Packing of DDA/TDB Monolayers

An increased hydration of the lipid bilayer has been suggested to account for the increased colloidal stability of DDA liposomes upon TDB incorporation (27). This was supported by studies of Langmuir-Blodgett monolayers of DDA, which resulted in an increased surface pressure in the presence of TDB, suggesting that the trehalose head group of TDB interacts with the water subphase (30). Similar to TDB, MPL also contains groups capable of interacting with the water subphase; thus, the effect of MPL on a Langmuir-Blodgett monolayer of DDA and TDB was evaluated in the current study (Fig. 5 and Table II). The collapse of the DDA/TDB monolayer was observed at a surface pressure around 47 mN as reported in the literature (30). Inclusion of 6 mol% MPL into DDA/TDB monolayers increased the surface pressure at the final collapse point to approximately 57 mN (Fig. 5 and Table II), indicating that inclusion of MPL increased the interaction with the water subphase. Addition of 6 mol% MPL shifted the π-*A*-isotherm towards a lower mean molecular area despite the large head group of MPL. This suggests that introduction of the negatively charged MPL reduces the repulsion between the positively charged ammonium head groups of the DDA molecules, and thereby provides a better packing of the lipid monolayer. Addition of 11 mol% MPL shifted the π-*A*-isotherm back towards the DDA/TDB isotherm, indicating that addition of 11 mol% MPL did not further improve the monolayer packing.

DDA/TDB/MPL Liposomes Enhance $CD8^+$ T-Cell Responses *In Vivo*

To investigate the $CD8^+$ T-cell inducing adjuvant effect of DDA/TDB/MPL liposomes, C57BL/6 mice were immunized using OVA as a model antigen. The OVA protein is a well-studied, commonly used, cheap model antigen with a well-characterized $CD8^+$ T-cell epitope making it suitable for comparative investigations of different adjuvant formulations. The OVA protein has a pI value around 4.5 and is thus negatively charged at pH 7.4. OVA is therefore expected to adsorp to the cationic liposomes, as demonstrated for DDA liposomes (31). The concentration of OVA used for the *in vivo* experiment was very low (0.025 mg/ml) compared to the lipid concentration (1.5–2.0 mg/ml), and addition of OVA did not affect the size, zeta-potential, or thermal profile of the DDA/TDB/MPL liposomes. However, the average particle size of DDA/TDB liposomes without MPL was slightly increased to around 700 nm, but neither the zeta-potential nor the thermal profile was affected (results not shown). Adjuvant formulations of DDA/TDB liposomes with 0, 6, or 11 mol % MPL incorporated into the bilayer were included in the *in vivo* experiment. The results indicated that inclusion of MPL into the DDA/TDB liposomes enhanced an antigen-specific $CD8^+$ T-cell response since approximately 1% SIINFEKL positive cells were observed as compared to DDA/TDB liposomes, which induced around 0.1% SIINFEKL positive cells (Fig. 6a). Furthermore, incorporation of either 6 or 11 mol% MPL significantly increased the IFN-γ release from splenocytes re-stimulated with the $CD8^+$ T-cell epitope *in vitro*, but there was no difference between the two MPL doses (Fig. 6b). Comparison of an MPL dispersion (containing 0.2% triethylamine to enhance the solubility of MPL) and DDA/TDB/MPL liposomes showed that the two formulations induced comparable IFN-γ responses from $CD8^+$ T-cells re-stimulated *in vitro* (Fig. 7a), confirming that MPL alone promotes $CD8^+$ T-cell responses. All groups receiving

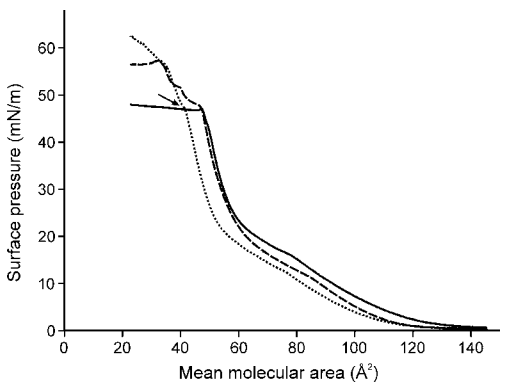

Fig. 5 Pressure/area isotherms of Langmuir-Blodgett monolayers composed of DDA/TDB (*solid*), DDA/TDB with 6 mol% MPL (*dot*) and DDA/TDB with 11 mol% MPL (*dash*) on 10 mM Tris buffer (pH 7.4) subphases. The total molar lipid concentration is identical for all experiments. The curves are representative for at least three experiments.

Table II Surface Pressures (π) and Mean Molecular Areas (A) at the Collapse Point for DDA/TDB Liposomes with Various Amounts of MPL

Mol% MPL	0	6	11
Surface Pressure (mN/m)	47.4 ± 0.5	56.5 ± 0.5***	57.3 ± 0.3***
Mean molecular area (Å^2)	46.3 ± 1.5	35.1 ± 0.9***	32.7 ± 0.6***

Results denote mean ± SD from three separate experiments. Significantly differences from 0 mol% MPL are indicated: *** $p < 0.001$

either a liposomal adjuvant formulation or MPL alone induced comparable levels of the antibodies IgG1 and IgG2c in sera (Fig. 7b+7c), indicating that inclusion of MPL does not compromise the humoral response induced by DDA/TDB liposomes.

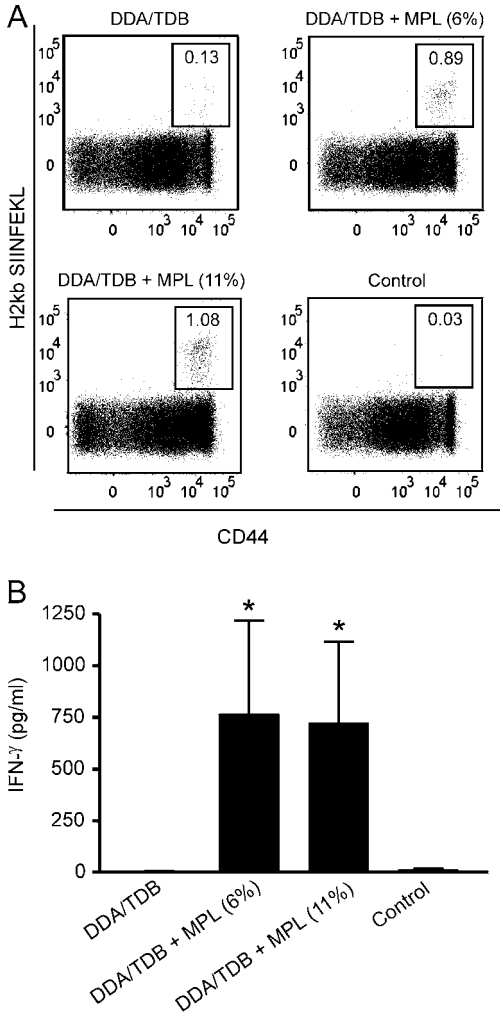

Fig. 6 CD8$^+$ T-cell responses after immunization with OVA and liposomal MPL formulations. **A** Representative dot plots of H2kb-SIINFEKL positive CD8$^+$ T-cells isolated from the blood one week after the third immunization with OVA and the indicated adjuvant formulations. Data shown are representative of two experiments. **B** IFN-γ responses after re-stimulation of isolated splenocytes with the CD8 epitope from OVA (25 μg/ml) as measured by ELISA. Significant differences from control mice are indicated: * $p < 0.05$.

DISCUSSION

MPL is a well-known immunopotentiating molecule which has been widely applied in adjuvant research and is an important component of a few already approved vaccines. MPL is also a key constituent of the AS01 and AS02 adjuvants from GSK currently in clinical development (32). AS01 and AS02 both contain MPL and the saponin QS21 in liposomes and in an oil-in-water (o/w) emulsion, respectively. Combining the same immunopotentiating molecule with different formulation principles has been shown to result in diverse types of immune responses (22, 33–35). For example, using MPL in an aqueous suspension of lipids elicited a Th2-biased response, whereas an emulsion-based MPL formulation induced a mixed Th1/Th2 type response (22). Thus, careful formulation optimization of an adjuvant system is of great importance to ensure proper presentation of not only the vaccine antigen but also the co-administered immunopotentiators to the cells of the immune system.

MPL is an acylated di-glucosamine with six hydrophobic acyl chains and a hydrophilic phosphate head group. The dispersion of MPL in an aqueous solvent is dependent on the use of excipients such as triethylamine or inclusion into a delivery system. In the present study, MPL was included into the bilayer of DDA/TDB liposomes, which act as a solubility enhancer. The negatively charged phosphate head group of MPL may interact with the cationic head group of DDA through electrostatic interaction, and since MPL is a larger and more complex molecule compared to DDA and TDB, it was thus investigated whether incorporation of MPL affected the physico-chemical properties of DDA/TDB liposomes. The cryo-TEM micrograph confirmed that liposomes were indeed formed in the presence of MPL, and, in general, MPL was incorporated into the DDA/TDB liposomes without compromising the particle size and the storage stability of the liposomes. The thermal analyses also indicated that lipid bilayers were formed and that phase separation was not induced by the presence of MPL, although some heterogeneity was observed for liposomes with 11 mol% MPL. The Langmuir-Blodgett studies showed that addition of 6 mol% MPL to a DDA/TDB monolayer resulted in a higher surface pressure when the monolayer was fully

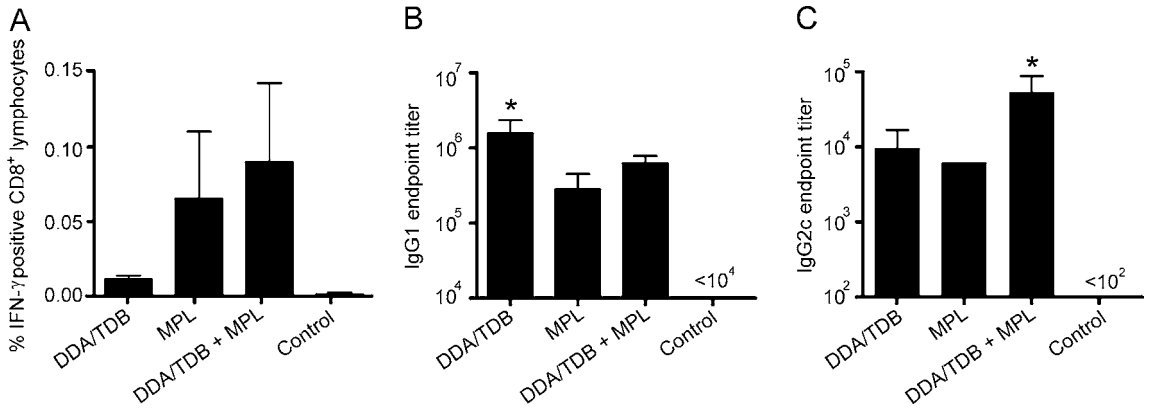

Fig. 7 $CD8^+$ T-cell and humoral responses after immunization with OVA and MPL in liposomal formulation (6 mol%, 50 μg MPL/dose) or in aqueous dispersion (50 μg MPL/dose). (**A**) IFN-γ responses from $CD8^+$ T-cells measured by intracellular flow cytometry after re-stimulation of isolated splenocytes with the CD8 epitope. (**B**) Sera were analyzed for the presence of OVA-specific IgG1 and (**C**) IgG2c antibodies by ELISA. Data shown are mean values for four mice ± SEM. Significant differences from control mice are indicated: * $p < 0.05$.

compressed and a shift in the π-A isotherm to a lower molecular area, suggesting an increased interaction with the aqueous subphase and a better packing of the lipid monolayer, probably due to improved monolayer hydration and reduced electrostatic repulsion between the DDA head groups. Previous studies of mixed monolayers of cationic DDA and an anionic surfactant also indicate a decreased molecular area due to a better packing of the monolayer by introduction of negative charges into the positively charged DDA monolayer (36,37). The π-A isotherms of the monolayers containing MPL exhibit a plateau at a surface pressure similar to the collapse point for DDA/TDB monolayers (arrow, Fig. 5). This behavior has previously been suggested to be caused by phase separation of the monolayer into a phase that remains at the water surface and a phase rich in DDA, which is then squeezed out of the monolayer (36,37).

The physico-chemical analyses further indicated that the surface charge of the liposomes is reduced in a concentration-dependent manner after incorporation of MPL. This is expected since MPLs possess a negatively charged phosphate head group. Likewise, it has been shown that addition of MPL to an o/w emulsion decreased the zeta-potential, suggesting that the acyl chains are buried in the oil droplet and that the phosphate groups are exposed towards the aqueous phase (22). Although the surface charge was reduced after incorporation of MPL, the liposomes remained cationic. The surface charge is of importance for the interaction with antigen-presenting cells (7,38), and cationic liposomes are more potent inducers of immune responses than neutral or negatively charged liposomes (8). Thus, incorporation of the negatively charged MPL into the cationic DDA/TDB liposomes might facilitate the delivery of MPL to APCs; however, this was not reflected in the current study, as the cationic liposomal formulation of MPL did not induce a superior immune response as compared to an aqueous MPL dispersion.

DDA/TDB/MPL liposomes mediated a $CD8^+$ T-cell response, indicating that MPL and the antigen are delivered to the same APC. The results suggest that the inclusion of MPL into the DDA/TDB liposomes did not significantly affect the humoral response. No significant differences were observed in the immune response induced by the MPL dispersion and the DDA/TDB/MPL liposomes, but the combination of DDA/TDB liposomes and MPL might still be synergistic and favorable from more than a pharmaceutical point of view, since DDA/TDB liposomes activate the immune system through other signaling pathways than MPL. The adjuvant effect of DDA/TDB liposomes is suggested to occur through TLR2, −3, −4, or −7 independent pathways, but is partly dependent on the MyD88-signaling pathway (15), and TDB mediates signaling through the TLR-independent Syk-Card9-dependent pathway (39). In contrast, MPL is recognized by TLR4 on APCs and shows weak signaling through the common MyD88-dependent signaling pathway but is biased towards the MyD88-independent TRIF pathway (17,40). Thus, the combination of the TLR ligand MPL and DDA/TDB liposomes might lead to induction of additional signaling pathways, currently under investigation, that may broaden the applicability of the adjuvant system.

CONCLUSIONS

In conclusion, the present study provides a thorough pharmaceutical characterization of the incorporation of MPL into the bilayer of DDA/TDB liposomes to

improve MPL solubility. Inclusion of MPL affected the physico-chemical properties of DDA/TDB liposomes, since the surface charge and the phase transition temperature were significantly reduced. However, MPL did not compromise the storage stability of the liposomes. The DDA/TDB/MPL liposomes mediated an antigen-specific $CD8^+$ T-cell response after immunization with OVA in mice, which did not compromise the humoral response.

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