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Immunogenicity of diphtheria toxoid and poly(I:C) loaded cationic liposomes after hollow microneedle-mediated intradermal injection in mice

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ABSTRACT

In this study, we aimed to investigate the immunogenicity of cationic liposomes loaded with diphtheria toxoid (DT) and poly(I:C) after hollow microneedle-mediated intradermal vaccination in mice. The following liposomal formulations were studied: DT loaded liposomes, a mixture of free DT and poly(I:C)-loaded liposomes, a mixture of DT-loaded liposomes and free poly(I:C), and liposomal formulations with DT and poly(I:C) either individually or co-encapsulated in the liposomes. Reference groups were DT solution adjuvanted with or without poly(I:C) (DT/poly(I:C)). The liposomal formulations were characterized in terms of particle size, zeta potential, loading and release of DT and poly(I:C). After intradermal injection of BALB/c mice with the formulations through a hollow microneedle, the immunogenicity was assessed by DT-specific ELISAs. All formulations induced similar total IgG and IgG1 titers. However, all the liposomal groups containing both DT and poly(I:C) showed enhanced IgG2a titers compared to DT/poly(I:C) solution, indicating that the immune response was skewed towards a Th1 direction. This enhancement was similar for all liposomal groups that contain both DT and poly(I:C) in the formulations. Our results reveal that a mixture of DT encapsulated liposomes and poly(I:C) encapsulated liposomes have a similar effect on the antibody responses as DT and poly(I:C) co-encapsulated liposomes. These findings may have implications for future design of liposomal vaccine delivery systems.

1. Introduction

Vaccination has become the most effective method for preventing infectious diseases, having led to the eradication of smallpox and severe restriction of other devastating diseases such as polio and measles (Jiang et al., 2017; Peek et al., 2008). However, there is still a need for new and better vaccines against emerging infectious diseases (Rappuoli et al., 2014). Nowadays, vaccination gains increasing attention also for therapeutic use against established diseases such as cancer and chronic auto-immune disorders (Melief et al., 2015). Most vaccines are delivered by intramuscular or subcutaneous injection. However, these injections need special training and can cause pain (Kim et al., 2012). To avoid the drawbacks of the hypodermic needles, microneedles have been developed. Microneedles are micro-sized needle structures with a length shorter than 1 mm and can be used to penetrate skin barrier in a non-invasive and pain-free way (Larraneta et al., 2016; Tuan-Mahmood et al., 2013; van der Maaden et al., 2012). Owing to the large number of antigen presenting cells in viable dermis and epidermis, dose-sparing may be achieved (Li et al., 2011).

Traditional vaccines are derived from attenuated organisms or inactivated pathogens and toxins. Attenuated vaccines have safety concerns as they may revert back to their virulent form (Reed et al., 2013). Inactivated vaccines like subunit antigens are safer but they are generally less immunogenic (Peek et al., 2008; Reed et al., 2013). To enhance and modify the immune response, immune modulators or nanoparticle delivery systems can be used (Zhao et al., 2014).

Ligands for toll-like receptors (TLRs) can be used as immune modulators to enhance the immune response against antigens by acting as a danger signal to the antigen-presenting cells. Among different types of TLR ligands, poly(I:C), which is a virus-associated double-stranded RNA, has been extensively investigated (Ammi et al., 2015). Poly(I:C) is a ligand for TLR3, which is located in the membrane of the endosomal compartments of dendritic cells. Previously, it was shown that compared to other TLR agonists, poly(I:C) induced a more effective IFN-γ secretion, which is an important linker of innate and adaptive immunity (Longhi et al., 2009). Furthermore, poly(I:C) has been shown to enhance anti-tumor immune responses and facilitate tumor elimination (Ammi et al., 2015).

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Nanoparticles have been shown to improve the immunogenicity of antigens by protecting the antigens from degradation, increasing their uptake by antigen-presenting cells and co-delivering antigens and immune modulators (Fan and Moon, 2015). Among different types of nanoparticle delivery systems, liposomes have been studied frequently because of their excellent biocompatibility and biodegradability (Giddam et al., 2012). Studies have shown that co-formulating antigen and TLR ligands in liposomes can enhance Th1 and $CD8^+$ T cell responses compared to mixture of antigen and adjuvant after intradermal vaccination. Some studies have shown that co-encapsulation of OVA with poly(I:C) or CpG in cationic liposomes significantly increased the IgG2a response (Th1 type) and the $CD8^+$ T cell response compared to OVA and adjuvant solutions (Bal et al., 2011; Du et al., 2017; Guo et al., 2013). Other studies have shown that peptide and poly(I:C) loaded cationic liposomes induced potent Th1 and $CD8^+$ T cell responses needed for tumor vaccination (Varypataki et al., 2017; Varypataki et al., 2015). These results are noteworthy, as nowadays there is an increasing need for potent cellular immune responses, e.g., for immunotherapy of cancer (Ammi et al., 2015; Fan and Moon, 2015; Hamdy et al., 2008) and intracellular pathogens (Chong et al., 2005; Zaric et al., 2013). However, it is not yet well understood whether the antigen and immune modulator need to be co-encapsulated in liposomes, or they can similarly modulate the immune response when encapsulated individually in liposomes. Therefore, the aim of this study was to examine whether co-encapsulation of antigen and adjuvant is required for enhancing and modulating the immune response.

In the present study, we chose diphtheria toxoid (DT) as a model antigen and studied the effect of encapsulation of DT and poly(I:C) in liposomes on immune responses in mice after hollow microneedle mediated intradermal immunization. DT and poly(I:C) were either individually encapsulated or co-encapsulated in 1,2-dioleoyl-3-trimethylammonium-propane chloride (DOTAP) based cationic liposomes. To investigate the modulation of immune responses by the liposomal formulations, IgG1 and IgG2a titers, which are indications of a Th2 and a Th1 type immune response, respectively (Maassen et al., 2003), were determined.

2. Materials and methods

2.1. Materials

DT (batch 04–44, 1 μg equal to 0.3 Lf) and diphtheria toxin were provided by Intravacc (Bilthoven, The Netherlands). Aluminum phosphate was purchased from Brenntag (Ballerup, Denmark). Egg phosphatidylcholine (EggPC), DOTAP and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) were ordered from Avanti Polar Lipids (Alabaster, AL). Polyinosinic-polycytidylic acid (poly(I:C)) (low molecular weight) and its rhodamine-labeled version were purchased from Invivogen (Toulouse, France). Foetal bovine serum (FBS), M199 medium (with Hanks' salts and L-glutamin), bovine serum albumin (BSA) and hydrofluoric acid ≥48% were ordered from Sigma-Aldrich (Zwijndrecht, The Netherlands). Glucose solution, L-Glutamine (200 mM), penicillin–streptomycin (10,000 U/ml) and 1-step™ ultra 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from ThermoFisher Scientific (Waltham, MA). HRP-conjugated goat anti-mouse total IgG, IgG1 and IgG2a were purchased from Southern Biotech (Birmingham, AL). Sulfuric acid (95–98%) was obtained from JT Baker (Deventer, The Netherlands). VivaSpin 2 and 500 centrifugal concentrators (PES membrane, MWCO 1000 kDa) were obtained from Sartorius Stedim (Nieuwegein, The Netherlands). Sterile phosphate buffered saline (PBS, 163.9 mM Na^+, 140.3 mM Cl^-, 8.7 mM HPO_4^{2-}, 1.8 mM H_2PO^{4-}, pH 7.4) was obtained from Braun (Oss, The Netherlands). 10 mM PB (7.7 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, pH 7.4) was prepared in the laboratory. All the other chemicals used were of analytical grade and Milli-Q water (18 MΩ/cm, Millipore Co.) was used for the preparation of all solutions.

2.2. Preparation of liposomes

Liposomes were prepared by thin-film hydration followed by extrusion, as reported earlier (Varypataki et al., 2016). EggPC (25 mg/ml), DOPE (25 mg/ml) and DOTAP (25 mg/ml) in chloroform were mixed in a molar ratio of 9:1:2.5 in a round bottom flask. The organic solvent was evaporated by using a rotary evaporator (Buchi rotavapor R210, Flawil, Switzerland) for 1 h at 40 °C and 120 rpm. To prepare DT encapsulated liposomes (Lipo-DT), the lipid film was hydrated with 0.25 mg/ml DT dissolved in 10 mM PB (pH 7.4) by vortexing for 10 s, resulting in a 12.5 mg/ml lipid suspension. To prepare poly(I:C) encapsulated liposomes (Lipo-PIC), the lipid film was hydrated with 0.25 mg/ml poly(I:C) solution (containing 0.5% (w/w) rhodamine-labeled poly(I:C)). To prepare DT and poly(I:C) co-encapsulated liposomes (Lipo-DT-PIC), after lipid film hydration with DT solution, 0.25 mg/ml poly(I:C) (containing 0.5% (w/w) rhodamine-labeled poly (I:C)) dissolved in 10 mM PB (pH 7.4) was added slowly (2 μl/min) into the lipid suspension by using a syringe pump (NE-300, Prosense, Oosterhout, The Netherlands). Next, the lipid vesicles were extruded (LIPEX™ extruder, Northern Lipids, Burnaby, Canada) four times through a carbonate filter with a pore size of 400 nm and another four times through a filter with a pore size of 200 nm (Nucleopore Millipore, Amsterdam, The Netherlands). To remove the DT/poly(I:C) not associated with liposomes, the suspension was transferred into VivaSpin 2 centrifugal concentrators (1000 kDa MWCO) and centrifuged (Allegra X-12R, Beckman Coulter, Indianapolis, IN) for 6 h (350*g*, 22 °C). Finally, the liposomes were washed with 10 mM PB and kept at 4 °C in the refrigerator prior to use. The filtrates, containing the free DT/poly(I:C), were collected for determination of loading efficiency of DT and poly (I:C).

2.3. Characterization of liposomal formulations

2.3.1. Particle size and zeta potential measurements

The particle size of the liposomes was measured by dynamic light scattering by using a Nano ZS® zetasizer (Malvern Instruments, Worcestershire, U.K.). The zeta potential of liposomes was measured by the same instrument by using laser Doppler velocimetry. The liposomes were diluted with 10 mM PB (pH 7.4) to a concentration of 25 μg/ml for the measurements. The samples were measured 3 times with 10 runs for each measurement.

2.3.2. Determination of encapsulation efficiency (EE) and loading capacity (LC) of DT/poly(I:C) in liposomes

To determine the EE and LC of DT and poly(I:C), the intrinsic fluorescence intensity of DT (λ_{ex} 280 nm/λ_{em} 320 nm) and fluorescence intensity of rhodamine labeled poly(I:C) (λ_{ex} 545 nm/λ_{em} 576 nm) in the purification filtrates were measured by using a Tecan M1000 plate reader (Männedorf, Switzerland). The EE and LC were calculated by using Eqs. (1) and (2) as below:

$$EE = \frac{M_{loadedDT/poly(I:C)}}{M_{totalDT/poly(I:C)}} \times 100\% \quad (1)$$

$$LC = \frac{M_{loadedDT/poly(I:C)}}{M_{Liposomes+DT+poly(I:C)}} \times 100\% \quad (2)$$

where $M_{loaded\ DT/poly(I:C)}$ represents the mass of encapsulated DT or poly (I:C), $M_{total\ DT/poly(I:C)}$ is the total amount of DT or poly(I:C) added to the formulations and $M_{liposomes+DT+poly(I:C)}$ is the total weight of liposomes, DT and poly(I:C).

2.3.3. In vitro release of DT and poly(I:C) from liposomes

To study the *in vitro* release of DT and poly(I:C) from Lipo-DT, Lipo-PIC and Lipo-DT-PIC, the liposomes (containing about 80 μg/ml DT with or without 80 μg/ml poly(I:C)) were dispersed in PBS and shaken with a speed of 550 rpm at 37 °C by using an Eppendorf thermomixer

(Nijmegen, The Netherlands). At predetermined time points, 300 µl liposomes were transferred into the VivaSpin 500 concentrators and centrifuged for 30 min with a speed of 350*g*. After the centrifugation, fresh PBS with the same volume as the filtrates was added back to the liposomes. The concentration of DT and poly(I:C) in the filtrates was determined by measuring the intrinsic fluorescence intensity of DT (λ_{ex} 280 nm/λ_{em} 320 nm) and fluorescence intensity of rhodamine labeled poly(I:C) (λ_{ex} 545 nm/λ_{em} 576 nm), respectively, by using a Tecan M1000 plate reader.

2.3.4. Adsorption of free DT or poly(I:C) on liposomes loaded with the other active ingredient

To investigate the adsorption of free DT to Lipo-PIC, DT was mixed with Lipo-PIC in PBS, resulting in a concentration of 31 µg/ml for both DT and poly(I:C). The samples were incubated in the Eppendorf thermomixer (Nijmegen, The Netherlands) at 37 °C with a speed of 300 rpm. To investigate the adsorption of free poly(I:C) to Lipo-DT, poly (I:C) was mixed with Lipo-DT in PBS, resulting in a concentration of 31 µg/ml for both DT and poly(I:C). After 4 or 24 h, the samples were transferred to VivaSpin 500 centrifugal concentrators (PES membrane, 1000 kDa MWCO) and centrifuged for 30 min with a speed of 350*g*. The DT or poly(I:C) in the filtrates was quantified by measuring the intrinsic fluorescence intensity of DT (λ_{ex} 280 nm/λ_{em} 320 nm) or fluorescence intensity of rhodamine labeled poly(I:C) (λ_{ex} 545 nm/λ_{em} 576 nm), respectively. The adsorption efficiency of DT or poly(I:C) was calculated according to Eq. (3) as follow:

$$\text{Adsorption efficiency } \% = \left(1 - \frac{M_{DT/poly(I:C)\ in\ filtrates}}{M_{DT/poly(I:C)\ total}}\right) \times 100\% \quad (3)$$

where $M_{DT/poly(I:C)\ in\ filtrates}$ represents the mass of DT or poly(I:C) in filtrates after centrifugation, and $M_{DT/poly(I:C)\ total}$ is the total mass of DT or poly(I:C) added.

2.4. Hollow microneedles and applicator

The hollow microneedles were prepared by hydrofluoric acid etching of fused silica capillaries (Schipper et al., 2016; van der Maaden et al., 2014). Briefly, silica capillaries (Polymicro, Phoenix AZ, 375 µm outer diameter, 50 µm inner diameter) were cut into 4-cm pieces and filled with silicone oil in a vacuum oven (100 °C) overnight. The silicone oil-filled capillaries were etched into hollow microneedles by immersing their ends in ≥48% hydrofluoric acid for 4 h at room temperature. Finally, the polyimide coating on the microneedles was removed by dipping the microneedles into hot sulfuric acid (250 °C) for 5 min.

To reproducibly insert hollow microneedles into mouse skin, a hollow microneedle applicator developed in our lab was used (Schipper et al., 2016; van der Maaden et al., 2014). The system consists of a syringe pump (NE-300, Prosense, Oosterhout, The Netherlands) and an injector, which were used to accurately control the injection rate (10 µl/min), the injection volume (10 µl) and the injection depth (120 µm). The pump, injector and hollow microneedles were connected by using high-pressure resistant CapTite™ connectors and silica capillaries.

2.5. Immunization study

Female BALB/c ($H2^d$) mice were ordered from Charles Rivers (Maastricht, The Netherlands) and accommodated under standardized conditions in the animal facility of Leiden Academic Centre for Drug Research, Leiden University. The immunization study was approved by the ethical committee on animal experiments of Leiden University (Licence number 14166).

The mice were 7–8 weeks old at the beginning of the experiments. The mice were anesthetized by intraperitoneal injection of ketamine (60 mg/kg) and xylanize (4 mg/kg) before shaving of the injection site. The mice were then injected with 10 µl of the formulations, containing 0.31 µg DT with or without 0.31 µg poly(I:C), into abdomen skin by using the hollow microneedle and the applicator, as described above. The following liposomal formulations were used: DT encapsulated liposomes (Lipo-DT), a mixture of DT-encapsulated liposomes and free poly(I:C) (Lipo-DT + PIC), a mixture of free DT and poly(I:C)-encapsulated liposomes (DT + Lipo-PIC), a mixture of DT-encapsulated liposomes and poly(I:C)-encapsulated liposomes (Lipo-DT + Lipo-PIC), and DT and poly(I:C) co-encapsulated liposomes (Lipo-DT-PIC). Control groups were injected with 0.31 µg DT with or without 0.31 µg poly(I:C) solution (DT/poly(I:C)). All formulations were freshly prepared and mixed prior to the immunization study. Subcutaneously injected 5 µg DT and 150 µg aluminum phosphate (DT-Alum) was used as a positive control. The mice were immunized on day 0, 21, 42 and sacrificed on day 56. Serum was withdrawn from the tail vein of mice on day 0, 21 and 42 and the sacrifice serum was taken from the femoral artery on day 56.

2.6. Determination of DT-specific IgG antibody titers

DT-specific antibodies were measured by using a sandwich enzyme-linked immunosorbent assay (ELISA) as described earlier (Slutter et al., 2011). The wells of 96-well plates were coated with 140 ng DT and incubated overnight at 4 °C. The plates were blocked with 1% BSA at 37 °C for 1 h. After blocking, appropriate three-fold serial dilutions of mouse sera were transferred into the plates and incubated for 2 h at 37 °C. The plates were then incubated with horseradish peroxidase-conjugated goat antibodies against total IgG, IgG1 or IgG2a (1:5000 dilution) for 1.5 h at 37 °C. Subsequently, the plates were incubated with TMB and 2 M sulfuric acid was used to stop the reaction. The absorbance was measured at 450 nm by using a Tecan M1000 plate reader. The antibody titers were expressed as log10 value of the mid-point of S-shaped dilution-absorbance curve of the diluted serum level.

2.7. Determination of DT-neutralizing antibodies

The functionality of the antibody response was determined by measuring diphtheria toxin-neutralizing antibodies in a Vero cell test (Ding et al., 2009). The serum samples were first diluted by M199 medium supplemented with 5% FBS, 0.5% glucose, 0.8% L-glutamine and 1% penicillin–streptomycin. Appropriate two-fold serial dilutions of the serum were applied to 96-well plates. Next, 5×10^{-5} Lf diphtheria toxin was added to each well. Subsequently, 1.25×10^4 Vero cells were added to each well and incubated for 6 days at 37 °C in 5% CO_2. Finally, the neutralizing antibodies were expressed as the log2 value of the highest serum dilution that protected the Vero cells.

2.8. Statistics analysis

All the data of antibody titers were analyzed by one way ANOVA with Newman-Keuls Multiple post-test by using GraphPad Prism software (version 5.02). The level of significance was set at $p < 0.05$.

3. Results

3.1. Physicochemical characteristics of the liposomes and in vitro release of DT and poly(I:C)

Lipo-DT, Lipo-PIC and Lipo-DT-PIC were first characterized in terms of particle size, poly dispersity index (PDI) and zeta potential. As shown in Table 1, Lipo-DT and Lipo-PIC had a similar average size below 200 nm with a low PDI, while Lipo-DT-PIC showed a slightly larger size and PDI. All the liposomes had a positive zeta potential above +35 mV. Both DT and poly(I:C) were efficiently encapsulated into the liposomes, with a EE higher than 96%. Furthermore, DT and poly(I:C) had a

Table 1
Physicochemical characteristics of DT/poly(I:C) encapsulated liposomes (n = 3).

Liposomes	Size[a] (nm)	PDI[b]	ZP[c] (mV)	EE[d] (%)		LC[e] (%)	
				DT	Poly(I:C)	DT	Poly(I:C)
Lipo-DT	182 ± 8	0.195 ± 0.012	+37 ± 1	96.5 ± 2.1	–	1.6 ± 0.1	–
Lipo-PIC	184 ± 6	0.153 ± 0.010	+37 ± 1	–	98.5 ± 0.8	–	1.7 ± 0.0
Lipo-DT-PIC	238 ± 11	0.243 ± 0.003	+35 ± 1	98.0 ± 0.8	98.9 ± 0.5	1.7 ± 0.0	1.7 ± 0.0

Data are average ± SEM of 3 independent batches.
[a] Size: Z-average diameter.
[b] PDI: poly dispersity index.
[c] ZP: zeta potential.
[d] EE: encapsulation efficiency.
[e] LC: loading capacity.

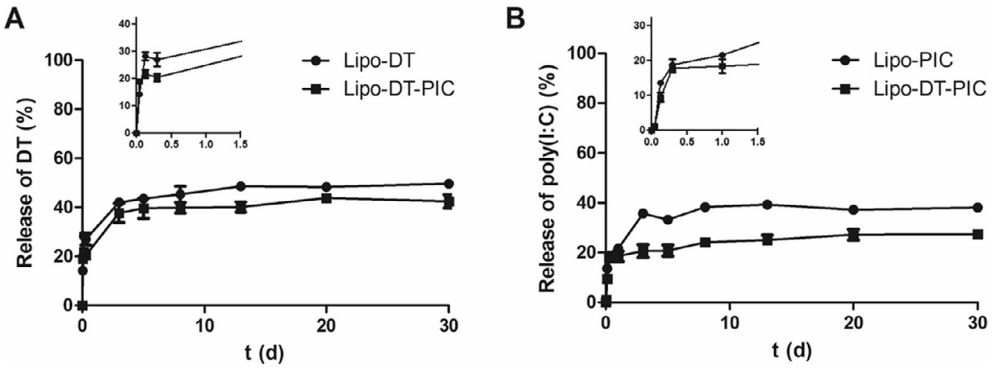

Fig. 1. *In vitro* release of DT and poly(I:C) from liposomes. Lipo-DT, Lipo-PIC and Lipo-DT-PIC were suspended in PBS at 37 °C and shaken with a speed of 550 rpm. The release sample was collected during one month at different predetermined time points to determine the released amounts of DT and poly(I:C). A: Release of DT from Lipo-DT (spheres) and Lipo-DT-PIC (squares). Insertion: release over a period of 1.5 days. B: Release of poly(I:C) from Lipo-PIC (spheres) and Lipo-DT-PIC (squares). Insertion: release over a period of 1.5 days. Bars represent mean ± SEM, n = 3.

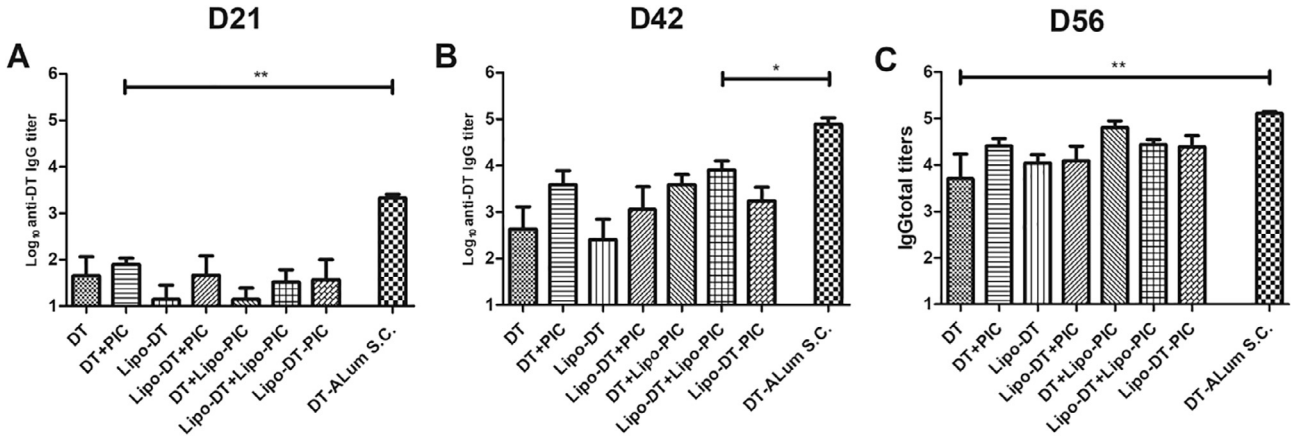

Fig. 2. DT-specific total IgG titers on day 21 (A), 42 (B) and 56 (C). Mice were immunized on day 0 (prime), 21 (first boost), 42 (second boost) and sacrificed on day 56. Blood serum was withdrawn from the tail vein of mice before each immunization and sacrifice. On day 0 there was no detectable response (data not shown). The total IgG titers were measured by ELISA and expressed as log10 values of the mid-point of S-shaped dilution-absorbance curve of the diluted serum level. Bars represent mean ± SEM, n = 8. $^{*}p < 0.05$, $^{**}p < 0.01$.

similar LC% of about 1.7% (Table 1).

To study the *in vitro* release of DT and poly(I:C), the prepared liposomes were incubated in PBS for one month. As shown in Fig. 1A, there was a burst release of DT of about 25% from both Lipo-DT and Lipo-DT-PIC within the first day. After 3 days, almost no additional DT was released. On day 30, the total release of DT from Lipo-DT and Lipo-DT-PIC was about 50% and 40%, respectively. Similarly, about 20% of the poly(I:C) was quickly released from Lipo-PIC and Lipo-DT-PIC within the first day and after day 3 almost no additional release was detected (Fig. 1B). On day 30, about 40% and 25% of the loaded poly (I:C) were released from Lipo-PIC and Lipo-DT-PIC, respectively. In summary, after incubation in PBS for one month, less than half of the loaded DT or poly(I:C) was released from the liposomes.

In order to study the interaction between free DT or poly(I:C) and the positively charged liposomes loaded with the other active ingredient after mixing in PBS, the adsorption of DT or poly(I:C) on the liposomes were determined. There were 75.9 ± 3.8% (mean ± SEM, n = 3) and 77.4 ± 0.8% (mean ± SEM, n = 3) of DT adsorbing on the surface of Lipo-PIC at 4 h or 24 h after mixing, respectively. In case of poly(I:C), 95.9 ± 2.7% (mean ± SEM, n = 3) and 95.6 ± 1.0% (mean ± SEM, n = 3) were adsorbed on the surface of Lipo-DT at 4 or 24 h after mixing, respectively. In summary, most of free DT or poly (I:C) was adsorbed on the surface of liposomes after mixing.

3.2. Intradermal vaccination study

The formulations were intradermally delivered into mice by using hollow microneedles with a DT dose of 0.31 μg with or without 0.31 μg

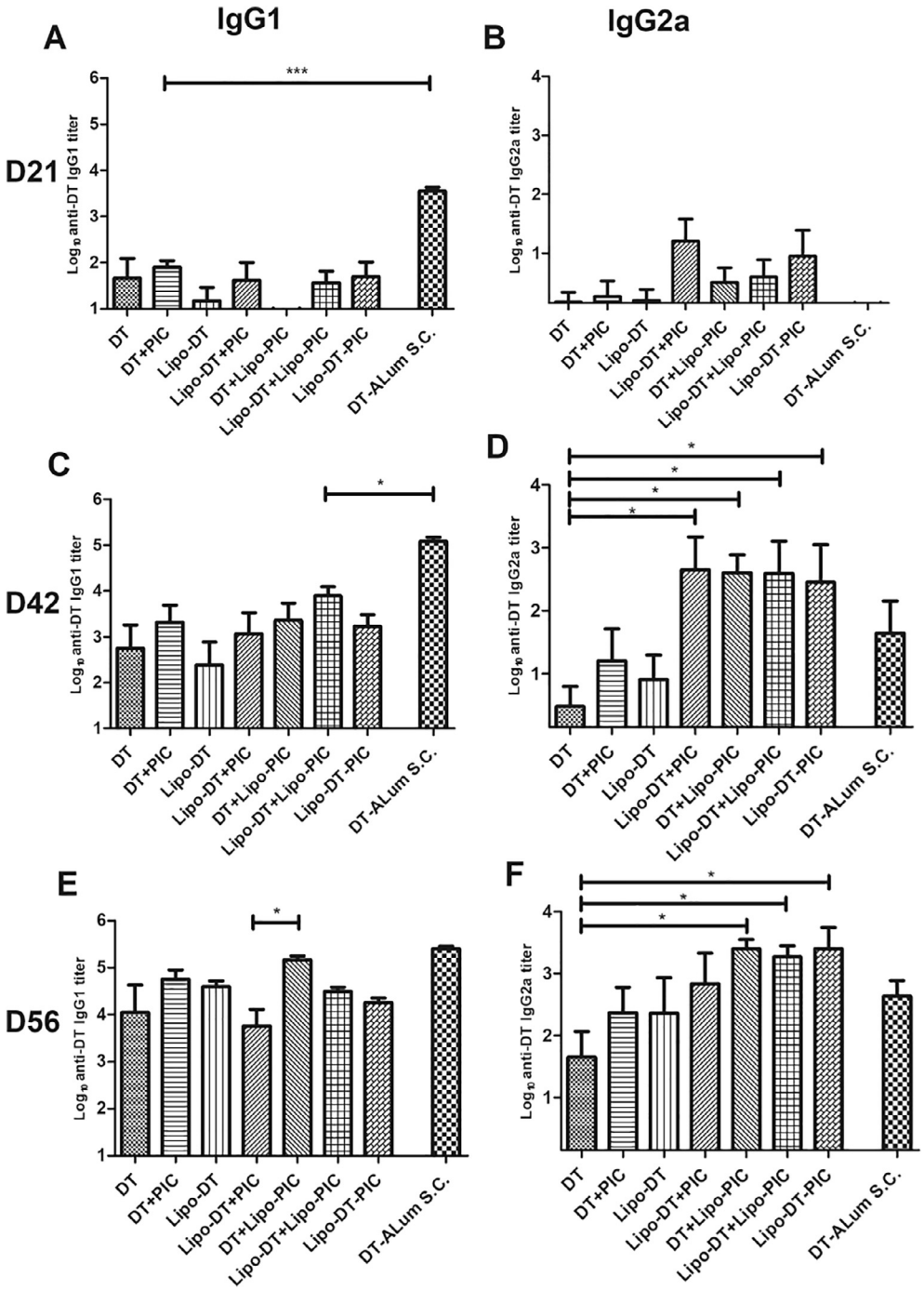

Fig. 3. DT-specific IgG1 (A, C, E) and IgG2a (B, D, F) titers on day 21 (A, B), 42 (C, D) and 56 (E, F). Mice were immunized on day 0 (prime), 21 (first boost), 42 (second boost) and sacrificed on day 56. Blood serum was withdrawn from the tail vein of mice before each immunization and sacrifice. On day 0 there was no detectable response (data not shown). The IgG titers were measured by ELISA and expressed as log10 values of the mid-point of S-shaped dilution-absorbance curve of the diluted serum level. Bars represent mean ± SEM, n = 8. $^{*}p < 0.05$, $^{***}p < 0.001$.

poly(I:C), based on our previous dose response study (Schipper et al., 2017). Subcutaneously injected DT-Alum with a much higher dose (5 μg DT and 150 μg Alum) was used as a positive control. During the injection, there was no visible leakage and successful injection was indicated by the formation of the bleb on the abdomen area of mouse skin.

DT-specific total IgG and subtype titers (IgG1 and IgG2a) are presented in Figs. 2 and 3, respectively. As shown in Fig. 2, total IgG titers increased after each immunization. As expected, DT-Alum induced significantly higher total IgG responses than all other formulations on day 21 and 42 ($p < 0.05$) (Fig. 2A and B). However, on day 56 the response of the intradermal groups increased to similar levels to that induced by DT-Alum (except DT), despite the 15-fold lower dose administered (Fig. 2C). The encapsulation of DT/poly(I:C) in liposomes did not increase the total IgG response compared to DT/poly(I:C) solutions ($p > 0.05$). Furthermore, the addition of poly(I:C) did not change the total IgG response.

Next, the IgG1 and IgG2a titers were measured. As shown in Fig. 3, the IgG1 response followed the total IgG response: liposomal formulation groups induced equally strong IgG1 responses compared to DT/poly(I:C) solutions ($p > 0.05$). However, when focusing on IgG2a response, clear differences were observed among the groups. On day 21

all groups except DT-Alum developed a detectable IgG2a response (Fig. 3B). After the 1st boost (day 42), all groups showed an IgG2a response (Fig. 3D). Moreover, liposomal groups that contained both DT and poly(I:C), i.e., Lipo-DT + PIC, DT + Lipo-PIC, Lipo-DT + Lipo-PIC and Lipo-DT-PIC, induced a similar IgG2a response that was higher than the response induced by DT solution ($p < 0.05$) and the DT and poly(I:C) mixture (Fig. 3D), although compared with the latter the difference is not significant ($p > 0.05$). After the 2nd boost (day 56), the IgG2a response of all groups increased to a higher level, but still the liposomal groups containing both DT and poly(I:C) induced a distinctly higher IgG2a response than DT solution (Fig. 3F). In summary, the results showed that the IgG2a response was enhanced when DT/poly(I:C) was loaded in liposomes, no matter whether only one ingredient or both of them were encapsulated in liposomes. Furthermore, DT and poly(I:C) individually encapsulated in liposomes induced a similar IgG2a response compared to DT and poly(I:C) co-encapsulated in liposomes. In contrast, DT encapsulated liposomes (Lipo-DT) did not enhance the IgG2a response compared to DT solution. Additionally, the DT-Alum group did not improve the IgG2a response in spite of a much higher dose.

In order to study functionality of the antibody response, the neutralizing antibody titers in serum on day 56 were determined by a Vero cell assay. The sera of the DT-Alum group contained higher levels of toxin-neutralizing antibodies than all other groups ($p < 0.05$) (Fig. 4). The liposomal groups containing both DT and poly(I:C) showed similar neutralizing titers compared to DT/poly(I:C) solutions. The Lipo-DT group seemed to have the lowest titers among the intradermal groups, but the difference is not significant. In summary, the encapsulation of DT/poly(I:C) in liposomes did not improve the protective immunity against diphtheria toxin.

4. Discussion

Liposome-based delivery system for vaccination has been extensively investigated (Giddam et al., 2012; Perrie et al., 2016). When focusing on intradermal vaccination, several studies have shown that co-encapsulation of antigen and adjuvant in liposomes can increase IgG2a and $CD8^+$ T cell responses (Bal et al., 2011; Boks et al., 2015; Du et al., 2017; Guo et al., 2013; Varypataki et al., 2015). In one of our previous studies, we used hollow microneedles to examine the effect of nano-encapsulation of OVA and poly(I:C) on the immune response in mice after intradermal vaccination. In that study, four types of nanoparticles were compared and the results indicated that OVA- and poly(I:C)-containing cationic liposomes were more potent than the other nanoparticles and significantly increased the IgG2a response compared to OVA and poly(I:C) solutions (Du et al., 2017). In the present study, we used the same liposome composition to study whether co-encapsulation of antigen and immune modulator is the essential factor for increased IgG2a levels. To investigate this, we co-encapsulated DT and poly(I:C) or encapsulated them individually in liposomes and studied the IgG (subtype) response following hollow microneedle-mediated intradermal vaccination. The results showed that liposomal formulations containing both DT and poly(I:C), induced higher IgG2a titers than those induced by DT/poly(I:C) solutions, no matter whether only one ingredient or both of them were encapsulated in liposomes. Furthermore, DT and poly(I:C) that were both individually encapsulated in liposomes induced similar IgG2a titers compared to DT and poly(I:C) co-encapsulated in liposomes.

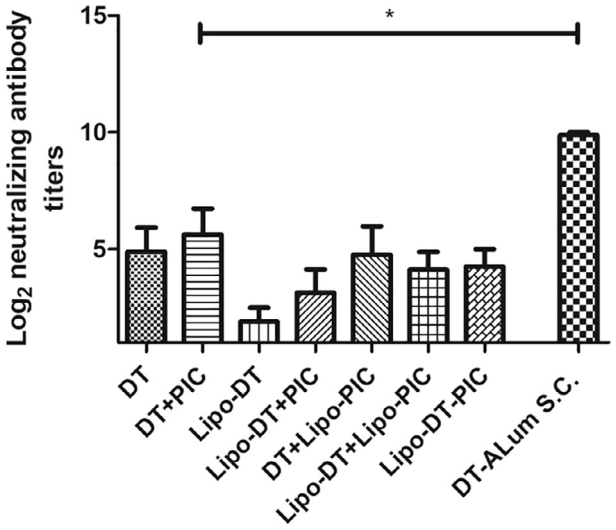

Fig. 4. DT-neutralizing antibody titers of mice. Results are shown for serum collected before sacrifice (day 56) from the tail vein of mice. The neutralizing antibody titers were measured by Vero-cell assay and the titers were expressed as the log2 values of the highest serum dilution that protected the Vero cells against diphtheria toxin. Bars represent mean ± SEM, n = 8. $^*p < 0.05$.

The results of Lipo-DT-PIC are well in accordance with previously reported results (Bal et al., 2011; Varypataki et al., 2016), showing that the co-encapsulation of antigen and immune modulator in liposomes can increase IgG2a responses and favor Th1 type immune responses after intradermal delivery by using a hypodermic needle. This may be caused by a liposome induced increase in the uptake of antigen and adjuvant by antigen presenting cells, as the size of liposomes (smaller than 200 nm) is favorable for uptake by dendritic cells (Benne et al., 2016; Manolova et al., 2008; Oyewumi et al., 2010). Burke et al. additionally showed that liposomes may facilitate the access of poly(I:C) to cellular cytoplasm and up-regulate TLR signalling molecules and NLRP3 inflammasome pathway (Burke et al., 2014). Furthermore, cationic liposomes may have a stronger interaction with the negatively charged cell membrane due to the attractive electrostatic interaction compared to negatively charged particles, which allows longer retention time on the cell surface and subsequently sustained release of antigen and adjuvant (Foged et al., 2004; Giddam et al., 2012; Ma et al., 2011). As shown in the release study, less than 50% of the encapsulated DT or poly(I:C) was released within one month, indicating that liposomes act as a reservoir and allow the sustained release of DT and poly(I:C). The slow release maybe due to strong electrostatic interactions between the antigen and adjuvant (negatively charged) and the liposomes (positively charged) and the stability of a large fraction of the liposomes during the study (as encapsulated DT and poly(I:C) cannot pass intact phospholipid bilayers). One limiting factor of using cationic liposome based formulations is that toxicity of cationic liposomes has been reported *in vitro* in salmonid cell (Romoren et al., 2004) and *in vivo* studies after intravenous delivery (Knudsen et al., 2015). Further studies are needed to investigate the safety of cationic liposomes after intradermal delivery. Finally, several studies have indicated that the co-processing of antigen and adjuvant by antigen presenting cells after being taken up may be the reason for a higher IgG2a response (Bal et al., 2011; Boks et al., 2015; Du et al., 2017). The delivery of antigen and the triggering of TLR in the same dendritic cell may synergistically induce a superior antigen presentation to T cells (Boks et al., 2015; Schulz et al., 2005).

The results of Lipo-DT + PIC and DT + Lipo-PIC are in line with our previous study by Varypataki et al, who found that the mixture of antigen-loaded liposomes and free poly(I:C) solution induced a similar IFN-γ-producing $CD4^+$ T cell response (Th1) as antigen and adjuvant co-encapsulated liposomes after hypodermic needle-mediated intradermal delivery (Varypataki et al., 2017). One explanation may be that the negatively charged antigen and adjuvant mixed with the liposome formulation adhered to the surface of positively charged liposomes after intradermal injection due to the electrostatic interaction. As a result, most of the antigen and adjuvant were co-delivered into the antigen presenting cells although they were not co-encapsulated in the liposomes. The adsorption study supported our hypothesis, as most of

the DT or poly(I:C) was found to adsorb on the surface of liposomes after mixing in PBS. Therefore, the association of DT and poly(I:C) is probably needed for the modulation of immune responses by liposomes.

In case of the Lipo-DT + Lipo-PIC formulation, we expect that these two liposomes would repel each other due to their strong surface charge. Indeed, the particle size and zeta potential of Lipo-DT + Lipo-PIC were found to remain the same within 24 h after mixing in PBS (data not shown). Nevertheless, Lipo-DT + Lipo-PIC induced similar IgG2a responses as Lipo-DT-PIC. These results indicate that the individually encapsulated DT and poly(I:C) are as efficient as the DT and poly(I:C) co-encapsulated in the liposomes for activation of immune system when administered intradermally. This may be explained by an efficient uptake of both Lipo-DT and Lipo-PIC by the same antigen presenting cells. One possible approach to further investigate this is to use confocal microscopy to study the fate of fluorescently labeled liposomes and antigen/adjuvant following administration *in vivo*.

In order to produce liposomes loaded with the optimal ratio of antigen/adjuvant with a high loading efficiency, optimization work needs to be done. Our results clearly suggest that co-encapsulation of antigen and adjuvant in liposomes may not be necessary for the use in intradermal delivery. This might simplify the work for the development of formulations. This may even be particularly beneficial for developing formulations that must contain multiple antigens and adjuvants, e.g., for personalized therapies of cancer patients (Grabbe et al., 2016). Such formulations can be prepared by mixing different liposomes loaded with only antigen or only adjuvant. Furthermore, it may be interesting to test whether our current findings also hold true for other nanoparticulate vaccine delivery systems.

Finally, the results of neutralizing antibody assay indicate that high IgG2a titers did not contribute to the immunity against diphtheria, which is in line with a previous study (Ding et al., 2009). The high IgG2a titers may be more suitable for anti-viral immune responses where a Th1 response is more desired (Cenna et al., 2008). Based on the obtained results, it would be of interest to examine the T cell responses (such as antigen specific T-cell proliferation and secretion of cytokines, such as IFN-γ). This will provide more detailed information about the mechanisms involved in the effect of individual versus co-encapsulation of antigen and poly(I:C) in liposomes on the immune responses.

5. Conclusion

Our results show that DT and poly(I:C) can be successfully encapsulated into cationic liposomes with a high loading efficiency. After hollow microneedle-mediated intradermal vaccination, the antigen and adjuvant encapsulated liposomes evoked a potent immune response and shifted the IgG1/IgG2a balance more to the IgG2a direction. The combination of DT-encapsulated and poly(I:C)-encapsulated liposomes are able to simulate an equally strong IgG2a response compared to DT and poly(I:C) co-encapsulated liposomes. These findings may have implications for future design of liposomal formulations aiming for modification of immune response after intradermal delivery.

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