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Comparison of the Depot Effect and Immunogenicity of Liposomes Based on Dimethyldioctadecylammonium (DDA), 3β-[*N*-(*N*′,*N*′-Dimethylaminoethane)carbomyl] Cholesterol (DC-Chol), and 1,2-Dioleoyl-3-trimethylammonium Propane (DOTAP): Prolonged Liposome Retention Mediates Stronger Th1 Responses

Malou Henriksen-Lacey,[†] Dennis Christensen,[‡] Vincent W. Bramwell,[†] Thomas Lindenstrøm,[‡] Else Marie Agger,[‡] Peter Andersen,[‡] and Yvonne Perrie*,[†]

School of Life and Health Sciences, Aston University, Birmingham, United Kingdom, and Statens Serum Institut, Copenhagen, Denmark

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Abstract: The immunostimulatory capacities of cationic liposomes are well-documented and are attributed both to inherent immunogenicity of the cationic lipid and more physical capacities such as the formation of antigen depots and antigen delivery. Very few studies have however been conducted comparing the immunostimulatory capacities of different cationic lipids. In the present study we therefore chose to investigate three of the most well-known cationic liposome-forming lipids as potential adjuvants for protein subunit vaccines. The ability of 3β-[*N*-(*N*′,*N*′-dimethylaminoethane)carbomyl] cholesterol (DC-Chol), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and dimethyldioctadecylammonium (DDA) liposomes incorporating immunomodulating trehalose dibehenate (TDB) to form an antigen depot at the site of injection (SOI) and to induce immunological recall responses against coadministered tuberculosis vaccine antigen Ag85B-ESAT-6 are reported. Furthermore, physical characterization of the liposomes is presented. Our results suggest that liposome composition plays an important role in vaccine retention at the SOI and the ability to enable the immune system to induce a vaccine specific recall response. While all three cationic liposomes facilitated increased antigen presentation by antigen presenting cells, the monocyte infiltration to the SOI and the production of IFN-γ upon antigen recall was markedly higher for DDA and DC-Chol based liposomes which exhibited a longer retention profile at the SOI. A long-term retention and slow release of liposome and vaccine antigen from the injection site hence appears to favor a stronger Th1 immune response.

Keywords: DDA; DC-Chol; DOTAP; TDB; vaccine; adjuvant; depot effect

1. Introduction

The use of cationic liposomes as antigen delivery vesicles in vaccines is a well-documented method to increase the immune recognition against otherwise inert or poorly immunogenic subunit proteins.[1] Some of the most thoroughly investigated liposome-forming lipids with such properties are dimethyldioctadecylammonium (DDA), 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), and 3β-[*N*-(*N*′,*N*′-dimethylaminoethane) carbomyl] cholesterol (DC-Chol). DDA has been extensively studied in combination with immunomodulators such as trehalose 6,6′-dibehenate (TDB).[2] The combination DDA:TDB, also designated CAF01, has

* Corresponding author. Professor Yvonne Perrie, Aston University, School of Life and Health Sciences, Aston Triangle, Birmingham, B4 7ET, United Kingdom. Tel.: +44 121 204 3991. Fax: +44(0) 121 204 4187. E-mail address: y.perrie@aston.ac.uk.

[†] Aston University.

[‡] Statens Serum Institut.

(1) Christensen, D.; Korsholm, K. S.; Rosenkrands, I.; Linderstrøm, T.; Andersen, P.; Agger, E. M. Cationic liposomes as vaccine adjuvants. *Expert Rev. Vaccines* **2007**, *6* (5), 785–796.

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been proven to be effective at inducing protective immune responses against pathogens like chlamydia and blood-stage malaria,[3] influenza (C. Martel; in preparation), and tuberculosis (TB).[4] DDA:TDB is currently in phase I clinical trials in combination with the TB vaccine candidate Ag85B-ESAT-6. It was recently shown that DDA:TDB liposomes increase the deposition of antigen at the injection site and prolong the presence of soluble antigen in the draining lymph nodes,[5] resulting in sustained antigen uptake and activation by dendritic cells (DCs).[6] Replacement of the cationic DDA lipid component with the neutral lipid DSPC, thereby reducing Ag85B-ESAT-6 adsorption to the liposomal carrier due to weaker electrostatic interactions between the liposome and the antigen (pI 4.6), led to a reduced deposition of antigen at the injection site, its presentation on the major histocompatibility complex (MHC), and the ability of the vaccine to induce a cell-mediated immune (CMI) response.[7,8]

DOTAP and DC-Chol are commonly cited as transfection agents[9−11] and vaccine delivery systems for both DNA-encoded[12,13] and protein[14,15] antigens. Structurally the only similarity is the presence of an ammonium ion headgroup conferring a net cationic charge. Their hydrophobic "tail" regions are considerably different; DOTAP comprises two unsaturated hydrocarbon chains of equal length to DDA (C18) and a main phase transition temperature of −12 °C,[16] resulting in a fluid lipid bilayer at physiological temperatures; in contrast, the cholesterol backbone of DC-Chol composed of numerous planar rings forms a more rigid bilayered structure. The structural similarity between cholesterol and DC-Chol has led to its more recent use as a cholesterol replacement in other lipid based constructs such as the ISCOM structures Posintro[17] and PLUSCOMs.[18,19] Only very few studies have, however, been conducted comparing the immunostimulatory capacities of different cationic lipids. In the present study we characterized liposomes composed of DDA, DOTAP, or DC-Chol. TDB was incorporated into all three formulations to obtain comparable immunomodulation. Vaccines composed of these liposomes in association with Ag85B-ESAT-6 antigen were compared for their abilities to form an antigen depot at the site of injection (SOI), to present Ag85B-ESAT-6 to the immune system, and to generate an immune response toward coadministered Ag85B-ESAT-6.

(2) Rosenkrands, I.; Agger, E. M.; Olsen, A. W.; Korsholm, K. S.; Andersen, C. S.; Jensen, K. T.; Andersen, P. Cationic liposomes containing mycobacterial lipids: a new powerful Th1 adjuvant system. *Infect. Immun.* **2005**, *73* (9), 5817–5826.

(3) Agger, E. M.; Rosenkrands, I.; Hansen, J.; Brahimi, K.; Vandahl, B. S.; Aagaard, C.; Werninghaus, K.; Kirschning, C.; Lang, R.; Christensen, D.; Theisen, M.; Follmann, F.; Andersen, P. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements. *PloS ONE* **2008**, *3* (9), e3116.

(4) Holten-Andersen, L.; Doherty, T. M.; Korsholm, K. S.; Andersen, P. Combination of the cationic surfactant dimethyldioctadecyl ammonium bromide and synthetic mycobacterial cord factor as an efficient adjuvant for tuberculosis subunit vaccines. *Infect. Immun.* **2004**, *72:3*, 1608–1617.

(5) Henriksen-Lacey, M.; Bramwell, V. W.; Christensen, D.; Agger, E. M.; Andersen, P.; Perrie, Y. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. *J. Controlled Release* **2009**, *142* (2), 180–186.

(6) Kamath, A. T.; Rochat, A.-F.; Christensen, D.; Agger, E. M.; Andersen, P.; Lambert, P.-H.; Siegrist, C.-A. A liposome-based mycobacterial vaccine induces potent adult and neonatal multifunctional T cells through the exquisite targeting of dendritic cells. *PLoS ONE* **2009**, *4* (6), e5771.

(7) Henriksen-Lacey, M.; Christensen, D.; Bramwell, V. W.; Lindenstrøm, T.; Agger, E. M.; Andersen, P.; Perrie, Y. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and ability of the vaccine to induce a CMI response. *J. Controlled Release* **2010**, *145*, 102–108.

(8) Christensen, D.; Korsholm, K. S.; Wood, G. K.; Mohammed, A. R.; Bramwell, V. W.; Andersen, P.; Agger, E. M.; Perrie, Y. Liposomes in adjuvant systems for parenteral delivery of vaccines. In *Delivery Technologies for Biopharmaceuticals*; John Wiley & Sons Ltd.: New York, 2009; pp 357−376.

(9) Fletcher, S.; Ahmad, A.; Perouzel, E.; Heron, A.; Miller, A. D.; Jorgensen, M. R. *In vivo* studies of dialkynoyl of DOTAP demonstrate improved gene transfer efficiency of cationic liposomes in mouse lung. *J. Med. Chem.* **2006**, *49* (1), 349–357.

(10) Gao, X.; Huang, L. A novel cationic liposome reagent for efficient transfection of mammalian cells. *Biochem. Biophys. Res. Commun.* **1991**, *179* (1), 280–285.

(11) Ramezani, M.; Khoshhamdam, M.; Dehshahri, A.; Malaekeh-Nikouei, B. The influence of size, lipid composition and bilayer fluidity of cationic liposomes on the transfection efficiency of nanolipoplexes. *Colloids Surf., B* **2009**, *72*, 1–5.

(12) Perrie, Y.; Frederik, P. M.; Gregoriadis, G. Liposome-mediated DNA vaccination: the effect of vesicle composition. *Vaccine* **2001**, *19*, 3301–3310.

(13) Perrie, Y.; McNeil, S.; Vangala, A. Liposome-mediated DNA Immunisation via the Subcutaneous Route. *J. Drug Targeting* **2003**, *11* (8−10), 555–563.

(14) Walker, C.; Selby, M.; Erickson, A.; Cataldo, D.; Valensi, J.-P.; Nest, G. V. Cationic lipids direct a viral glycoprotein into the class I major histocompatibility complex antigen-presentation pathway. *Proc. Natl. Acad. Sci. U.S.A.* **1992**, *89*, 7915–7918.

(15) Bei, R.; Guptill, V.; Masuelli, L.; Kashmiri, S. V. S.; Muraro, R.; Frati, L.; Schlom, J.; Kantor, J. The use of a cationic liposome formulation (DOTAP) mixed with a recombinant tumor-associated antigen to induce immune responses and protective immunity in mice. *J. Immunother.* **1998**, *21* (3), 159–169.

(16) Zuidam, N. J.; Hirsch-Lerner, D.; Margulies, S.; Barenholz, Y. Lamellarity of cationic liposomes and mode of preparation of lipoplexes affect transfection efficiency. *Biochim. Biophys. Acta* **1999**, *1419* (2), 207–220.

(17) Madsen, H. B.; Ifversen, P.; Madsen, F.; Brodin, B.; Hausser, I.; Nielsen, H. M. *In Vitro* cutaneous application of ISCOMs on human skin enhances delivery of hydrophobic model compounds through the stratum corneum. *AAPS J.* **2009**, *11* (4), 728–739.

(18) Lendemans, D. G.; Egert, A. M.; Hook, S.; Rades, T. Cage-like complexes formed by DOTAP, Quil-A and cholesterol. *Int. J. Pharm.* **2007**, *332*, 192–195.

(19) McBurney, W. T.; Lendemans, D. G.; Myschik, J.; Hennessy, T.; Rades, T.; Hook, S. *In Vivo* activity of cationic immune stimulating complexes (PLUSCOMS). *Vaccine* **2008**, *26*, 4549–4556.

2. Materials and Methods

2.1. Materials. DDA, DOTAP, DC-Chol, and TDB were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ag85B-ESAT-6 (produced as previously described[20]) was obtained from Statens Serum Institute, Denmark. Hydrogen peroxide, Sephadex G-75, and bicinchoninic acid protein assay (BCA) components were purchased from Sigma Aldrich (St. Louis, MO). Foetal calf serum (FCS) was from Biosera, UK. For radiolabeling, L-3-phosphatidyl[*N*-methyl-^{3}H]choline, 1,2-dipalmitoyl (^{3}H-DPPC) was obtained from GE Healthcare (Amersham, UK), IODO-GEN precoated iodination tubes from Pierce Biotechnology (Rockford, IL), and ^{125}I (NaI in NaOH solution), SOLVABLE, and Ultima Gold scintillation fluid were purchased from Perkin-Elmer (Waltham, MA). Methanol and chloroform (both HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). Tris-base, obtained from IDN Biomedical, Inc. (Aurora, OH), was used to make Tris buffer and adjusted to pH 7.4 using HCl; unless stated otherwise Tris buffer was used at 10 mM, pH 7.4.

2.2. Preparation and Characterization of Liposomes. Liposomes composed of DDA, DOTAP, or DC-Chol, in combination with TDB (8:1 molar ratio) were produced using the lipid-film hydration method[21] which has been described in detail previously.[5] Briefly, a lipid film, formed using rotoevaporation, was hydrated above the main phase transition temperature of the lipid with trehalose containing Tris buffer. The solution was frequently vortexed to encourage lipid mixing and vesicle formation. Ag85B-ESAT-6 was added to liposomes post lipid-film hydration to a final concentration of 10 μg/mL, equivalent to the experimental dose (2 μg/dose). Physical characterization of liposomes including vesicle size, polydispersity, and ζ-potential measurements were undertaken using a Brookhaven *ZetaPlus* as described previously.[5] The stability of liposomes over 56 days with Ag85B-ESAT-6 (10 μg/mL) was conducted at both 4 °C and room temperature (25 °C).

2.3. Radiolabeling of Ag85B-ESAT-6 and Characterizing Adsorption and Release Kinetics. Ag85B-ESAT-6 was radiolabeled with ^{125}I and used immediately for characterization of antigen adsorption and release from DDA:TDB, DOTAP:TDB, and DC-Chol:TDB liposomes.[5,7,22] Briefly, ^{125}I-Ag85B-ESAT-6 was added to liposomes and allowed to adsorb for 1 h prior to being centrifuged to separate bound and unbound antigen present in the pellet and supernatants, respectively. Antigen adsorption was measured based on the total ^{125}I recovered. Antigen release was undertaken in simulated *in vivo* conditions (50% FCS solution, 37 °C) over a 96 h period with samples processed as above at periodic intervals.

2.4. Biodistribution of DDA:TDB, DOTAP:TDB, and DC-Chol:TDB Liposomes Adsorbing Ag85B-ESAT-6 Antigen. Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. Groups of five female 6−8 week old BALB/c mice were housed appropriately and given a standard mouse diet ad-libitum. Four to six days prior to each vaccination, mice were injected subcutaneously (s.c.) with 200 μL of pontamine blue (Sigma Aldrich, 0.5% w/v). Pontamine blue is phagocytosed by monocytes[5,23] and is therefore a suitable marker for aiding the location of lymph nodes during dissection. Liposomes composed of DDA, DOTAP, or DC-Chol in combination with TDB and the radiolabeled tracer lipid ^{3}H-DPPC (Perkin-Elmer) were produced as described in Section 2.2 and outlined in detail previously.[5,7,22] ^{3}H-DPPC is commercially available and has been employed as a tracer for liposomes in numerous studies (e.g., refs 24 and 25). Furthermore, the concentration of ^{3}H-DPPC used (25 ng/mL) was sufficiently low to not favor micelle formation should DPPC leave the liposomal bilayer nor to alter the physicochemical characteristics of the liposomes. Trehalose (10% w/v) was added to the hydrating Tris buffer for isotonicity. ^{125}I-radiolabeled Ag85B-ESAT-6 (see Section 2.3) adsorption to liposomes was conducted by simple mixing of equal volumes of both components. Each immunization dose contained 0.4 μmol of lipid (DDA, DOTAP, or DC-Chol), 0.05 μmol of TDB, and 2 μg of Ag85B-ESAT-6. Mice were injected intramuscularly (i.m.; 50 μL) into the left quadriceps. At time points one day, four days, and 14 days post-injection (p.i.), the mice were terminated by cervical dislocation. Tissue from the injected muscle site and local draining popliteal lymph nodes (PLN) from both noninjected and injected legs was removed and processed as described previously[22] to determine the proportion of ^{3}H (liposome) and ^{125}I (antigen) in the tissues.

2.5. Assessment of *in Vivo* Cycling of Ag-Specific T-cells. Spleen cells from Ag85B241-255 TCR transgenic mice (kindly donated by Jan Pravsgaard Christensen) were harvested and labeled with carboxyfluorescein succinimidyl ester (CFSE). Briefly, spleen cell suspensions were prepared and red blood cells removed with ammonium chloride solution. Spleen cells were resuspended in PBS (1×10^7/mL), and an equal volume of CFSE (100 μM in PBS; final conc. 50 μM) was added. After 10 min at 37 °C, an equal

(20) Olsen, A. W.; van Pinxteren, L. A. H.; Okkels, L. M.; Rasmussen, P. B.; Andersen, P. Protection of mice with a tuberculosis subunit vaccine based on a fusion protein of antigen 85B and ESAT-6. *Infect. Immun.* **2001**, *69* (5), 2773–2778.

(21) Bangham, A. D.; Standish, M. M.; Watkins, J. C. Diffusion of univalent ions across the lamellae of swollen phospholipids. *J. Mol. Biol.* **1965**, *13* (1), 238–252.

(22) Henriksen-Lacey, M.; Bramwell, V. W.; Perrie, Y. Radiolabelling of antigen and liposomes for vaccine biodistribution studies. *Pharmaceutics* **2010**, *2* (2), 91–104.

(23) Tilney, N. L. Patterns of lymphtic drainage in the adult laboratory rat. *J. Anat.* **1971**, *109* (3), 369–383.

(24) Vermehren, C.; Hansen, H. S.; Clausen-Beck, B.; Frøkjæ, S. In vitro and in vivo aspects of N-acyl-phosphatidylethanolamine-containing liposomes. *Int. J. Pharm.* **2003**, *254* (1), 49–53.

(25) Verkade, H. J.; Derksen, J. T.; Gerding, A.; Scherphof, G. L.; Vonk, R. J.; Kuipers, F. Differential hepatic processing and biliary secretion of head-group and acyl chains of liposomal phosphatidylcholines. *Biochem. J.* **1991**, *275*, 139–144.

volume of FCS was added for quenching. After 5 min at 4 °C, cells were washed with RPMI + 10% FCS and finally resuspended in Hank's buffered salt solution (HBSS). CFSE labeled (2×10^7) cells were injected intravenously into recipient C57BL/6 mice (Harlan Scandinavia, Allerod, Denmark) immunized once i.m. in each quadriceps at day −3 or −14. Four or 14 days later, recipient mice were euthanized, and the numbers of donor origin (P63/P25+) T-cells that were cycling (based on reduction in CFSE intensity) were determined by flow cytometry.

2.6. Immunogenicity of DDA:TDB, DOTAP:TDB, and DC-Chol:TDB Liposomes Associated with Ag85B-ESAT-6. All experiments were conducted in accordance with the regulations of the Danish Ministry of Justice and animal protection committees and in compliance with European Community Directive 86/609. Female 6−12 week old C57BL/6 mice were obtained from Harlan Scandinavia (Allerod, Denmark). All mice were immunized with 2 μg of the vaccine antigen Ag85B-ESAT-6 mixed with DDA:TDB, DC-Chol:TDB, or DOTAP:TDB liposomes (produced as described in Section 2.2) in a total volume of 100 μL. The mice were immunized three times i.m. with 50 μL in each quadriceps with a two week interval between each immunization. Peripheral blood mononuclear cells (PBMCs) were restimulated with 0.05 μg, 0.5 μg, or 5 μg Ag85B-ESAT-6, and the production of IFN-γ and IL-5 was quantified by an enzyme-linked immunosorbent assay (ELISA) as previously described.[20,26]

2.7. Statistics. Statistical analysis of data was tested by one- or two-way analysis of variance (ANOVA). When significant differences were indicated, differences between means were determined by Bonferroni's multiple comparison tests. All statistical analyses were performed in GraphPad Prism version 5 (GraphPad Software Inc., La Jolla, CA).

3. Results and Discussion

3.1. Physical Characterization of Liposomes with and without Antigen. The addition of 10 μg/mL Ag85B-ESAT-6 to DDA:TDB, DOTAP:TDB, or DC-Chol:TDB liposomes did not have any significant effect on the mean vesicle size, polydispersity, or ζ-potential of the liposomes. DDA:TDB liposomes with or without Ag85B-ESAT-6 displayed characteristics similar to previous observations with a mean vesicle size of ~400 nm observed.[5,26] DC-Chol:TDB liposomes were approximately half of the size of DDA:TDB liposomes with an average vesicle size of 220 nm, while DOTAP:TDB liposomes were the largest and most heterogeneous liposomes with a mean size of 760 nm and polydispersity between 0.32 and 0.35. These findings are in correlation with previous reports showing that the average vesicle size for multilamellar DOTAP liposomes is between 500 and 1000 nm[16] and the mean particle size of DC-Chol suspensions in Tris buffer is 218 nm.[27] All formulations had a ζ-potential of between +45 to +58 mV irrespective of the presence of Ag85B-ESAT-6.

The stability of vaccine components is an important issue for the further development of experimental vaccines. Thus, we examined the stability of DDA:TDB, DOTAP:TDB, and DC-Chol:TDB liposomes over a 56 day period by measuring the vesicle size and ζ-potential of liposomes adsorbing Ag85B-ESAT-6 (Figure 1). The vesicle size of all formulations, regardless of the storage temperature, remained stable over the initial 28 day period; differences in vesicle size compared to day one post formulation became significant by the latter time point, day 56 (Figure 1A−C). Significant changes in the ζ-potential occurred as early as 14 (DDA:TDB) or 28 (DC-Chol:TDB and DOTAP:TDB) days post formulation. The storage temperature had little effect on the vesicle size or ζ-potential of DDA:TDB or DC-Chol:TDB liposomes. In contrast, a much more significant effect on the stability of DOTAP:TDB liposomes was noted for liposomes stored at 25 °C as opposed to 4 °C: significant decreases in vesicle size ($p < 0.01$) and ζ-potential ($p < 0.001$) occurred on days 56 and 28, respectively (Figure 1C,*c*).

Our results suggest that the addition of Ag85B-ESAT-6 (10 μg/mL) to DDA:TDB, DC-Chol:TDB, or DOTAP:TDB liposomes does not have a detrimental effect to their stability, at least over the initial 14 days. After 14 days the most significant changes in these physical properties are noted for DOTAP:TDB liposomes. The steep decrease in vesicle size and ζ-potential observed after storage of DOTAP:TDB liposomes at 25 °C (Figure 1, *c*) could be explained by breakdown of the lipid components. The hydrolysis of lipids containing ester bonds (such as phospholipids and DOTAP) has been reported to affect the physical stability of liposomal systems[10,28−31] resulting in a decrease in vesicle size and enhanced permeability of the bilayer, which is also dependent

(26) Davidsen, J.; Rosenkrands, I.; Christensen, D.; Vangala, A.; Kirby, D.; Perrie, Y.; Agger, E. M.; Andersen, P. Characterisation of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6′-dibehenate) - A novel adjuvant inducing both strong CMI and antibody responses. *Biochim. Biophys. Acta* **2005**, *1718*, 22–31.

(27) Guy, B.; Pascal, N.; Françon, A.; Bonnin, A.; Gimenez, S.; Lafay-Vialon, E.; Trannoy, E.; Haensler, J. Design, characterization and preclinical efficacy of a cationic lipid adjuvant for influenza split vaccine. *Vaccine* **2001**, *19*, 1794–1805.

(28) Rabinovich-Guilatt, L.; Dubernet, C.; Gaudin, K.; Lambert, G.; Couvreur, P.; Chaminade, P. Phospholipid hydrolysis in a pharmaceutical emulsion assessed by physiochemical parameters and a new analytical method. *Eur. J. Pharm. Biopharm.* **2005**, *61*, 69–76.

(29) Vernooij, E. A. A. M.; Kettenes-van den Bosch, J. J.; Underberg, W. J. M.; Crommelin, D. J. A. Chemical hydrolysis of DOTAP and DOPE in a liposomal environment. *J. Controlled Release* **2002**, *79*, 299–303.

(30) Zhong, Z.; Ji, Q.; Zhang, A. Analysis of cationic liposomes by reversed-phase HPLC with evaporative light-scattering detection. *J. Pharm. Biomed. Anal.* **2010**, *51*, 947–951.

(31) Koeber, R.; Pappas, A.; Michaelis, U.; Schulze, B. Extraction and quantification of the cationic lipid 1,2-dioleoyl-3-trimethylammonium propane from human plasma. *Anal. Biochem.* **2007**, *363*, 157–159.

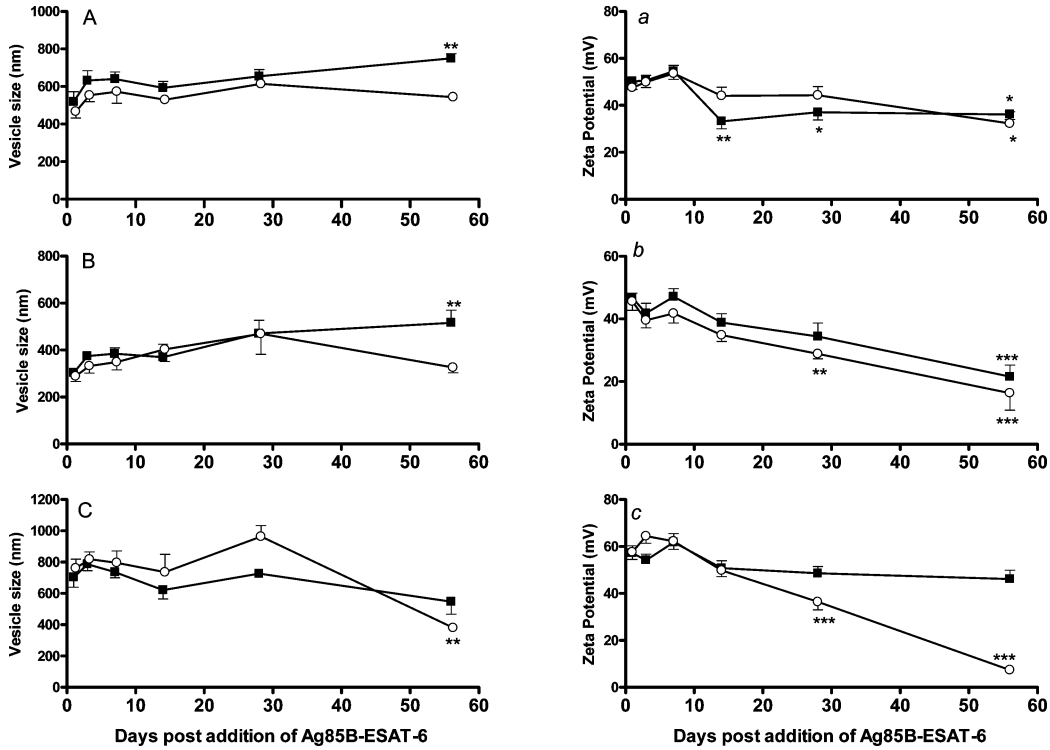

Figure 1. Stability of liposomes over a 56 day period as measured by changes in vesicle size (A, B, C) and ζ-potential (*a*, *b*, *c*) after the addition of Ag85B-ESAT-6. The liposome formulations DDA:TDB (A, *a*), DC-Chol:TDB (B, *b*), and DOTAP:TDB (C, *c*) were all produced at a final lipid:TDB concentration of 1.98:0.25 mM and stored at 4 °C (■) or 25 °C (○). Significant differences in vesicle size and ζ-potential over time (compared to day one) are shown. Results represent mean ± SEM of triplicate samples.

on the T_m of the lipid.[32] Cleavage of the positively charged headgroup of DOTAP[31] would result in a decrease in the net surface charge of the liposome (Figure 1), which is known to decrease the stability of the system as the charge repulsion between adjacent liposomes is weaker.[33] It is however possible that the significant decrease in vesicle size observed for DOTAP:TDB when stored at 25 °C and not at 4 °C is due to hydrolysis of ester linkages and unsaturated carbons present in the hydrocarbon chains which occurs when the liposome exists in its fluid phase. A further possibility is oxidation of the unsaturated bonds leading to instabilities in the bilayer; however, the role this would play in the surface charge of the vesicle is unclear.

3.2. Antigen Release from Liposomes in Simulated in Vivo Conditions. A short-term (96 h) study was conducted to measure the adsorption and release kinetics of Ag85B-ESAT-6 from DDA:TDB, DOTAP:TDB, and DC-Chol:TDB liposomes placed in conditions simulating the *in vivo* environment (Figure 2). The initial level of Ag85B-ESAT-6 adsorption was between 95−98% for all formulations which corresponds to ∼10 μg Ag85B-ESAT-6 per mL liposomes (1.98 mM). Upon placement of the liposomes in a 50% FCS solution there was significant ($p < 0.001$) loss of Ag85B-ESAT-6 from DOTAP:TDB and DC-Chol:TDB liposomes but not from DDA:TDB liposomes (Figure 2). After four hours in FCS the loss of Ag85B-ESAT-6 from all formulations stabilized, and no further significant changes in Ag85B-ESAT-6 adsorption were observed. The results for Ag85B-ESAT-6 adsorption and release from DDA:TDB liposomes are in correlation with previous findings.[5,7] We also measured the vesicle size and ζ-potential of the cationic

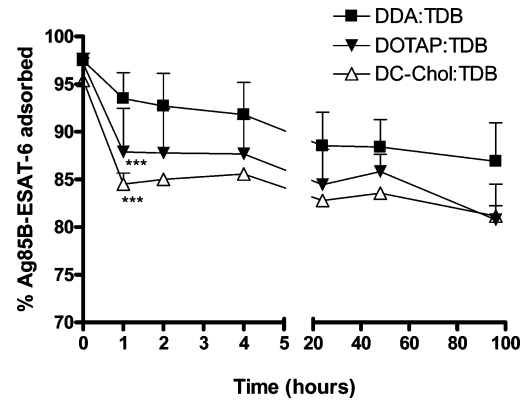

Figure 2. Using ^{125}I-labeled Ag85B-ESAT-6, the percentage antigen adsorption to DDA:TDB, DOTAP:TDB, and DC-Chol:TDB and the antigen release kinetics followed after addition to a 50% FCS solution at 37 °C. Results represent mean ± SD ($n = 3$).

(32) Zuidam, N. J.; Gouw, H. K. M. E.; Barenholz, Y.; Crommelin, D. J. A. Physical (in) stability of liposomes upon chemical hydrolysis: the role of lysophospholipids and fatty acids. *Biochim. Biophys. Acta* **1995**, *1240*, 101–110.

(33) Martin, F. Pharmaceutical manufacturing of liposomes. In *Specialized Drug Delivery Systems: Manufacturing and Production Technology*; Marcel Dekker Inc.: New York, 1990; pp 267−316.

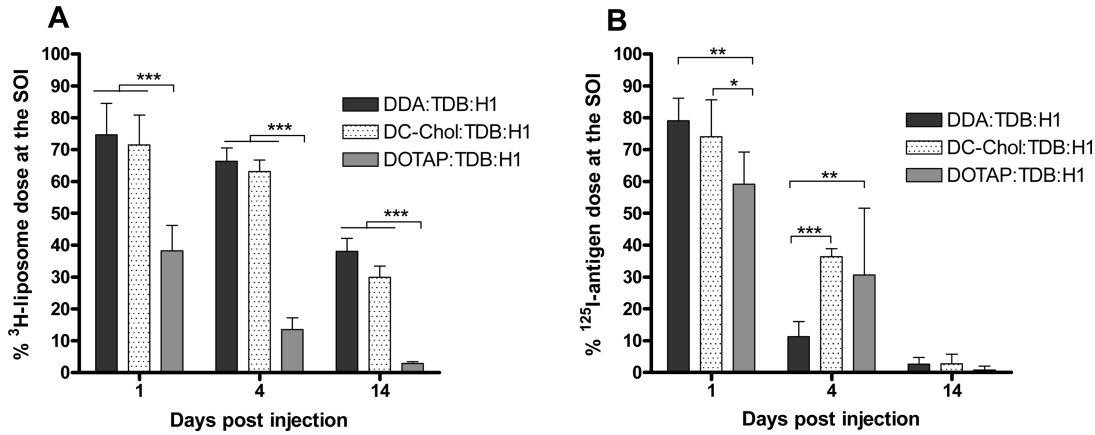

Figure 3. Presence of liposome (A) and Ag85B-ESAT-6 (B) at the SOI after injection (i.m.) of vaccine formulations DDA:TDB, DC-Chol:TDB, or DOTAP:TDB, all adsorbing Ag85B-ESAT-6. The proportion of each radionucleotide as a percentage of the initial dose was calculated; results represent the mean ± SD of five mice. H1; Ag85B-ESAT-6.

liposome formulations upon exposure to FCS; as noted previously[5] an immediate increase in vesicle size and decrease in ζ-potential to ∼ −10 mV was observed, highlighting the serum protein-mediated aggregation noted *in vivo*.

3.3. Biodistribution of Cationic Liposomes Adsorbing Ag85B-ESAT-6. The biodistribution of radiolabeled liposomes and antigen was studied at days one, four, and 14 p.i. Only tissue from the SOI and PLN was investigated since previous studies have shown negligible presence of radiolabeled components in the lung, liver, kidney, heart, brain, and small intestine. Figure 4 shows the percentage of the injected liposome (A) and antigen (B) dose that was recovered from the SOI. In accordance to previous studies,[5,7] DDA:TDB liposomes were well-retained at the SOI with nearly 40% of the original dose still detectable 14 days p.i. (Figure 3A). While DC-Chol:TDB liposomes followed very similar kinetics to DDA:TDB liposomes, the draining of DOTAP:TDB liposomes from the SOI was significantly faster ($p < 0.001$) on all days p.i. By day 14 p.i., the presence of DOTAP:TDB liposomes was between 10 and 14-fold less than DDA:TDB and DC-Chol:TDB liposomes. With regards to the depot effect of Ag85B-ESAT-6 at the SOI, all formulations were able to cause antigen retention with between 59 and 79% of the antigen dose recovered one day p.i. (Figure 3B). The detection of Ag85B-ESAT-6 after delivery with DDA:TDB liposomes correlates with our previous findings whereby approximately 10−15% of the dose was recovered four days p.i., and by two weeks p.i. there remained just 2% of the dose.[5,7] Interestingly, both DC-Chol:TDB and DOTAP:TDB liposomes were able to retain more of the antigen at the SOI than DDA:TDB liposomes at the later time points: by day four p.i., the level of Ag85B-ESAT-6 at the SOI was significantly higher for both DC-Chol:TDB ($p < 0.001$) and DOTAP:TDB ($p < 0.01$) liposomes. The unusual finding that the proportion of antigen retained at the SOI when delivered with DOTAP:TDB liposomes remains higher than the liposome itself suggests instabilities in the bilayer of DOTAP:TDB liposomes and/or dissociation of Ag85B-ESAT-6 from DOTAP:TDB liposomes *in vivo*. This observation is in contradiction to the *in vitro* studies showing no significant release between days one and four (Figure 2). It is important to note that there are factors which occur *in vivo* that we did not replicate in our simulated studies described in Section 3.2. These include the constant flow of interstitial fluid draining to the lymphatics, tissue infrastructure, and the presence of complement able to bind antigens or pathogenic substances. Therefore, while we attempted to simulate the *in vivo* setting, this is ultimately very difficult to achieve, and it is conceivable that APCs take up antigen at the SOI, whereas DOTAP:TDB liposomes passively drain from the SOI due to the high fluidity of their bilayer at 37 °C. This hypothesis supports findings by our laboratory in which liposomes composed of an unsaturated analogue to DDA drain faster from the SOI than their coadministered antigen (manuscript in preparation).

3.4. Draining of Liposomes Adsorbing Ag85B-ESAT-6 to the Local Lymph Nodes. The popliteal lymph node (PLN) is the local lymphoid tissue to which antigen detected in the quadriceps (the SOI) drains. We therefore investigated the presence of liposomes and Ag85B-ESAT-6 antigen in the PLN. Liposomal draining to the PLN followed two distinct patterns: while the presence of both DDA:TDB and DC-Chol:TDB liposomes continually increased over the time points studied, detection of DOTAP:TDB peaked at day four p.i. and then decreased (Figure 4A). At both one and four days p.i. the presence of DOTAP:TDB liposomes was significantly ($p < 0.001$) higher than DDA:TDB or DC-Chol:TDB liposomes. By day 14 p.i. the presence of DOTAP:TDB dropped significantly ($p < 0.001$), whereas the influx of DDA:TDB and DC-Chol:TDB liposomes continued to increase. In accordance with previous experiments,[5,7] the levels of observed antigen were very low, and it was difficult to measure any significant differences between formulations and time points (Figure 4B). The exception to this was upon coadministration of DOTAP:TDB liposomes which at day one p.i. resulted in a significantly ($p < 0.001$) higher presence of antigen than after coadministration with either DDA:TDB or DC-Chol:TDB liposomes.

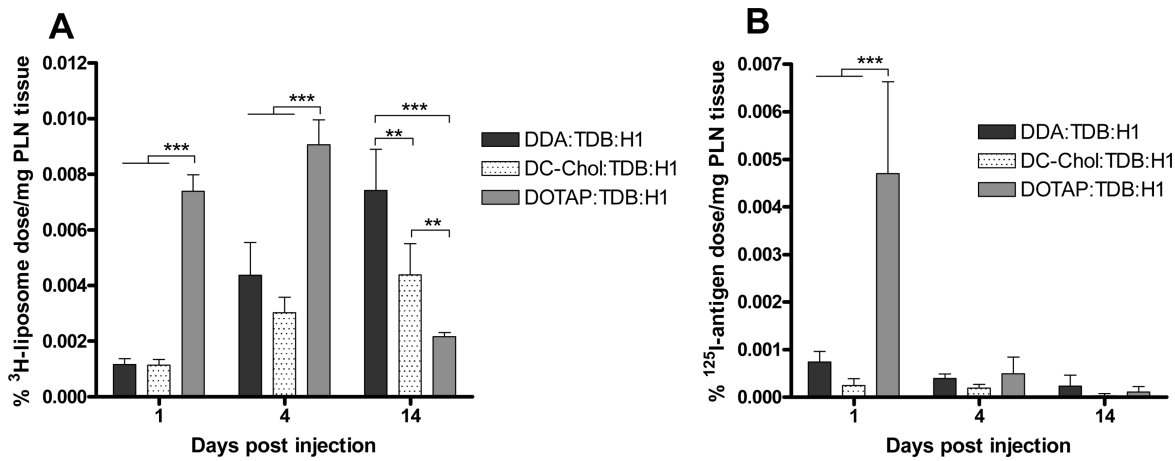

Figure 4. Draining of liposomes (A) and Ag85B-ESAT-6 (B) to the PLN after i.m. injection of DDA:TDB, DOTAP:TDB, or DC-Chol:TDB liposomes adsorbing Ag85B-ESAT-6. The proportion of each radionucleotide as a percentage of the initial dose and divided by the mass of the tissue (mg) was calculated; results represent the mean ± SD of five mice. H1; Ag85B-ESAT-6.

The peak in DOTAP:TDB liposome observed in the PLN at day four p.i. (Figure 4A) suggests that DOTAP:TDB drains rapidly from the SOI (Figure 3A) to the PLN. In contrast, both DDA:TDB and DC-Chol:TDB liposomes are better retained at the SOI (Figure 3A) resulting in a slower and prolonged draining of these liposomes to the PLN (Figure 4A). The high level of Ag85B-ESAT-6 detected in the PLN one day p.i with DOTAP:TDB liposomes was similar to the antigen draining observed after Ag85B-ESAT-6 delivery with neutral DSPC:TDB liposomes[7] and suggests that antigen does not remain adsorbed to DOTAP:TDB liposomes *in vivo* as efficiently as expected from the *in vitro* studies (Figure 2).

3.5. Monocyte Influx to the SOI as Determined by Pontamine Blue Staining. In accordance with our previous studies, pontamine blue dye was used as a marker for infiltrating monocytes to the SOI.[5,7,23] All vaccines induced monocyte infiltration; however, the kinetics and intensity were varied. DC-Chol:TDB liposomes induced the slowest monocyte influx to the SOI with little blue staining being apparent on day 4 p.i. (Figure 5). It is interesting to note however that on day 4 p.i. the presence of DC-Chol:TDB liposomes at the SOI was equal to that of DDA:TDB liposomes (Figure 3A). In contrast, the presence of DOTAP:TDB liposomes was 5-fold less than that of DDA:TDB or DC-Chol:TDB liposomes at the same time point; yet, relatively similar levels of blue staining are observed for DOTAP:TDB and DDA:TDB liposomes (Figure 5). Korsholm et al. found that intraperitoneal injection of DDA-based liposomes resulted in a significant recruitment of neutrophils, monocytes, mature macrophages, and activated natural killer cells[34] correlating nicely with the large monocyte infiltration observed in this and previous studies.[5,7] We do not know of any studies on DC-Chol or DOTAP liposomes in which the cellular infiltrate at the SOI has been investigated; however, it is well-reported that liposomal cationic charge is linked to inflammation.

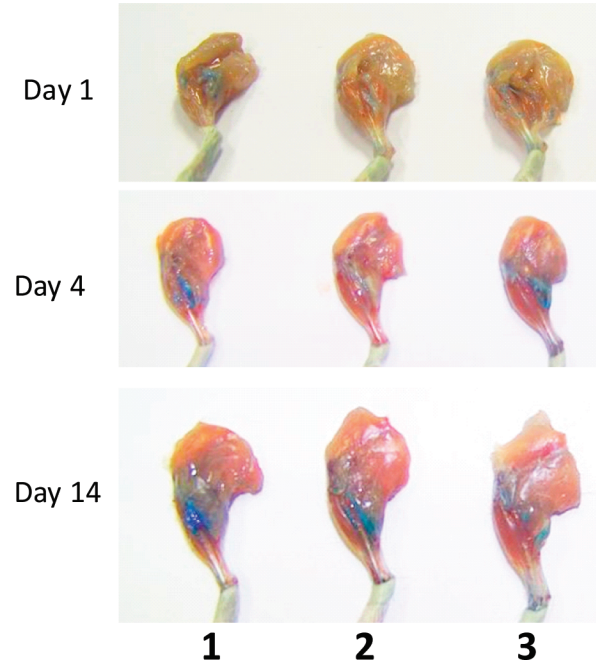

Figure 5. Figure showing pontamine blue staining at the SOI (quadriceps) after injection (i.m.) of DDA:TDB (1), DC-Chol:TDB (2), or DOTAP:TDB (3) liposomes, all adsorbing Ag85B-ESAT-6 antigen.

3.6. In Vivo Antigen Presentation Induced by the Three Different Cationic Liposomes Systems Was Not Significantly Different. The differences in draining kinetics to the lymph nodes (Figure 4) and monocyte influx to the SOI (Figure 5) could suggest that the different cationic liposomes also mediated diverse uptake by APCs. We therefore investigated the ability of the different cationic liposomes to increase

(34) Korsholm, K. S.; Petersen, R. V.; Agger, E. M.; Andersen, P. T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. *Immunology* **2010**, *129* (1), 75–86.

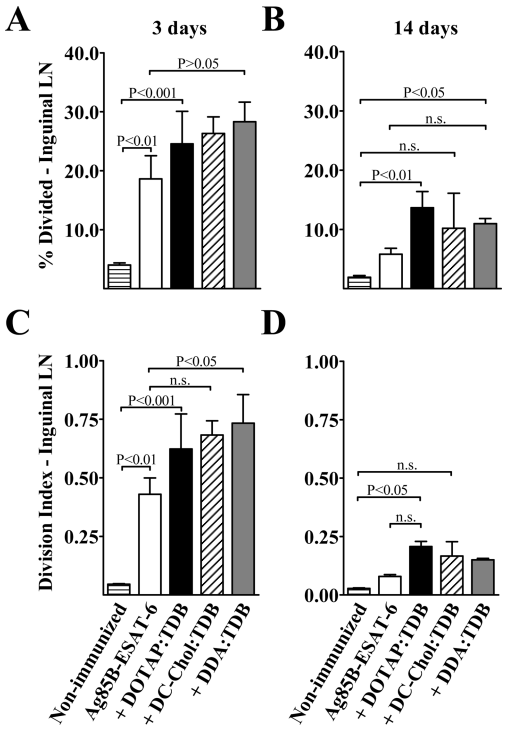

Figure 6. Percentage dividing of (A, B) and division index for (C, D) the initial amount of $Ag85B_{241-255}$ specific T-cells four days after transfer into mice immunized three days (A, C) or 14 days (B, D) previously with Ag85B-ESAT-6 alone (white) or in combination with DOTAP:TDB (black), DC-Chol:TDB (dashed), or DDA:TDB (gray) liposomes.

the antigen specific T-cell proliferation in an *in vivo* mouse model using Ag85B-ESAT-6 as the model antigen. Naive CFSE labeled spleen cells from $Ag85B_{241-255}$ TCR transgenic mice were injected into C57BL/6 mice immunized three or 14 days earlier with Ag85B-ESAT-6 in combination with DDA:TDB, DC-Chol:TDB, or DOTAP:TDB liposomes, and the proliferation of antigen specific inguinal lymph node derived CD4+ T-cells was tracked four days later based on CFSE intensity reduction. Significant cell division was observed three days after cell transfer in all groups receiving the Ag85B-ESAT-6 antigen. Of the groups receiving cationic liposomes together with the antigen, only a DDA:TDB induced division of a significantly higher proportion of the total CFSE labeled population as compared to the group receiving the antigen alone (Figure 6A,C). After two weeks there was no significant difference in the division of antigen specific T-cells between any of the groups receiving antigen (Figure 6B,D). The same pattern was observed in both the PLN and the spleen although the levels were lower (data not shown). These data suggest that there was no difference in the ability of the cationic liposomes to ensure presentation of the antigen by the APCs.

3.7. Induction of CMI Responses by DDA:TDB, DC-Chol:TDB, and DOTAP:TDB Were Both Qualitatively and Quantitatively Different. The transgenic T-cells used in the previous study are already activated and hence do not need costimulatory signals to start proliferating. Therefore this study cannot predict the ability of the three cationic liposomes to induce strong immune responses. We therefore decided to investigate the immune responses generated by DDA:TDB, DC-Chol:TDB, and DOTAP:TDB liposomes. The three types of cationic liposomes have all been associated with the ability to induce both Th1 and Th2 responses. We investigated the ability of the formulations to induce IFN-γ and IL-5, characteristic of Th1 and Th2 responses, respectively. The results illustrated in Figure 7 indicate that the induction of the Th1 IFN-γ type responses were predominantly induced by liposomes composed of either DDA or DC-Chol and that DDA:TDB induced significantly higher amounts of IFN-γ than DC-Chol:TDB one week after the last immunization, whereas DOTAP only induced a weak IFN-γ response (Figure 7A). Surprisingly the IFN-γ response induced by DC-Chol:TDB had almost disappeared three weeks after the last immunization, whereas the responses induced by DDA:TDB persisted (Figure 7). This observation could be explained by DC-Chol:TDB primarily inducing an effector response, whereas DDA:TDB, as previously published,[35] induces a memory response. This will be investigated more thoroughly in future studies. IL-5 production was not significantly altered with the different cationic liposomes suggesting that the Th2 response is less influenced than the Th1 response by the differences in *in vivo* kinetics of the different formulations. The finding that DOTAP only induces a weak Th1 response is in disagreement with previously published results suggesting that DOTAP predominantly induces a Th1 immune response.[36] The divergence could however be explained by the lack of a strong Th1 inducer as a comparison in the mentioned study.

The presented results suggest that the obtained immune response is highly dependent on the choice of cationic lipid and that a long-term retention and slow release of liposome and vaccine antigen from the injection site appears favorable for a stronger Th1 immune response. It should be noted however that there may be other explanations to the different immune responses observed. One such alternative explanation could be different uptake mechanisms as both DDA- and DOTAP-based liposomes have been previously reported to increase the internalization of antigen by APCs;[37−39] however, no similar studies have been conducted using DC-Chol-based liposomes. Another explanation may involve different intracellular stimulation pathways. DDA based liposomes did not, in an in vitro setting, induce maturation of murine bone-marrow-derived dendritic cells (BM-DCs) as measured by the surface expression of MHC Cl II, CD40, CD80, and CD86.[39] In contrast, DOTAP liposomes were reported to activate DCs with up-regulation of the expression of costimulatory molecules CD80 and CD86 via an NF-κB-independent pathway,[40,41] reactive oxygen species production,[42] and chemokine expression through the ERK

(35) Linderstrøm, T.; Agger, E. M.; Korsholm, K. S.; Darrah, P. A.; Aagaard, C.; Seder, R. A.; Rosenkrands, I.; Andersen, P. Tuberculosis subunit vaccination provides long-term protective immunity characterized by multifunctional CD4 memory T cells. *J. Immunol.* **2009**, *182*, 8047–8055.

(36) Yan, W.; Chen, W.; Huang, L. Mechanism of adjuvant activity of cationic liposome: Phosphorylation of a MAP kinase, ERk and induction of chemokines. *Mol. Immunol.* **2007**, *44*, 3672–3681.

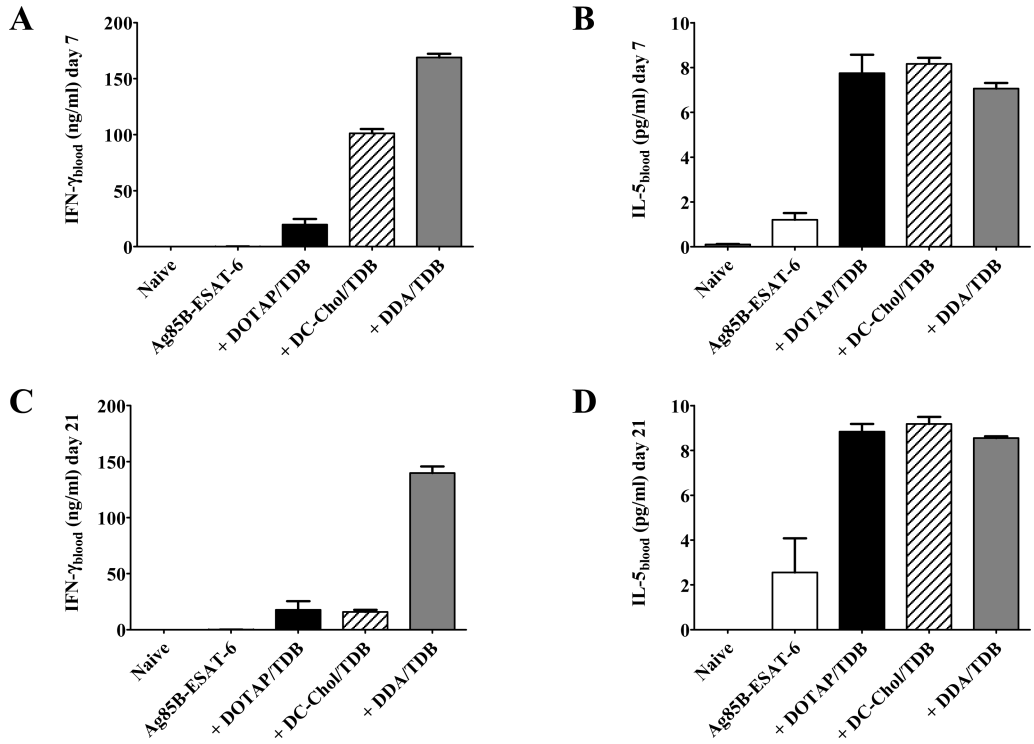

Figure 7. IFN-γ (A, C) and IL-5 (B, D) responses in mice (pooled PBMCs) one (A, B) and three (C, D) weeks after the last of three immunizations with 2 μg of Ag85B-ESAT-6 antigen alone (white) or combined with DOTAP:TDB (black), DC-Chol:TDB (dashed), or DDA:TDB (gray) liposomes.

pathway.[36] DC-Chol liposomes on the other hand have been shown to stimulate the secretion of the migratory chemokines CCL20 from epithelial cells via an NF-κB-dependent pathway without expression of CD80 or CD86.[43]

4. Conclusion

In this study we investigated the physical, pharmacokinetic, and immunological characteristics of three different cationic lipids with documented immunostimulatory capacity. The data presented here suggest that a positive surface charge is not the only physicochemical property that determines the immunological properties of the liposomes. Other properties such as membrane fluidity and headgroup structure can also have an effect on the deposition of liposomes and antigen at the SOI and the ensuing immune response. Even though markedly different *in vivo* kinetics were observed, there were no significant differences in the ability of the different cationic liposomes to induce antigen presentation on MHC molecules. The resulting immune response was however significantly different for the three cationic liposome systems with IFN-γ production, typical of a Th1 response, following the order DDA:TDB > DC-Chol:TDB $\gg$ DOTAP:TDB. Interestingly, the Th2 cytokine IL-5 response was comparable for the three different liposome systems. Our results reiterate the complexity of liposomes used as adjuvants in subunit protein vaccination. It is most likely that multiple parameters, such as the depot effect, improved antigen uptake and presentation, costimulatory signaling, and so forth, play a role in the immunological outcome. Experiments are underway to further elucidate the adjuvant function attributed to these liposomes.

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(37) Zuhorn, I. S.; Hoekstra, D. On the mechanism of cationic amphiphile-mediated transfection. To fuse or not to fuse: is that the question? *J. Membr. Biol.* **2002**, *189*, 167–179.

(38) Rejman, J.; Conese, M.; Hoekstra, D. Gene transfer by means of lipo- and polyplexes: role of clathrin and caveolae-mediated endocytosis. *J. Liposome Res.* **2006**, *16:3*, 237–247.

(39) Korsholm, K. S.; Agger, E. M.; Foged, C.; Christensen, D.; Dietrich, J.; Andersen, C. S.; Geisler, C.; Andersen, P. The adjuvant mechanism of cationic dimethyldioctadecylammonium liposomes. *Immunology* **2006**, *121*, 216–226.

(40) Vangasseri, D. P.; Cui, Z.; Chen, W.; Hokey, D. A.; Falo, L. D., Jr.; Huang, L. Immunostimulation of dendritic cells by cationic liposomes. *Mol. Membr. Biol.* **2006**, *23* (5), 385–395.

(41) Cui, Z.; Han, S. J.; Vangasseri, D. P.; Huang, L. Immunostimulation mechanism of LPD nanoparticle as a vaccine carrier. *Mol. Pharm.* **2005**, *2*, 22–28.

(42) Yan, W.; Chen, W.; Huang, L. Reactive oxygen species play a central role in the activity of cationic liposome based cancer vaccine. *J. Controlled Release* **2008**, *13*, 22–28.

(43) Cremel, M.; Hamzeh-Cognasse, H.; Genin, C.; Delézay, O. Female genital tract immunization: Evaluation of candidate immunoadjuvants on epithelial cell secretion of CCL20 and dendritic/Langerhans cell maturation. *Vaccine* **2006**, *24*, 5744–5754.