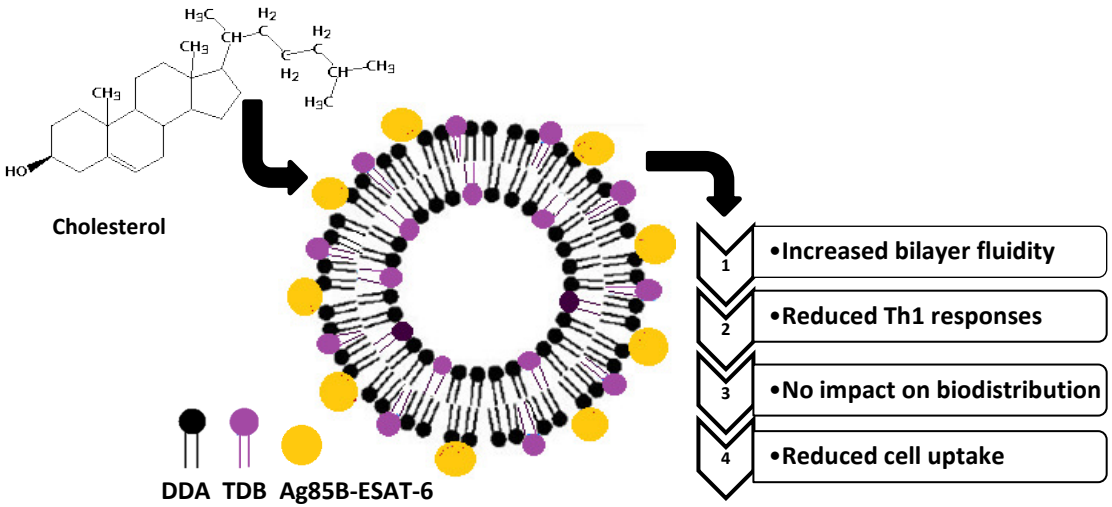
Graphical Abstract:



Title: The effect of incorporating cholesterol into DDA:TDB liposomal adjuvants on bilayer properties, biodistribution and immune responses.

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Date of manuscript: revised 1st Oct 2013

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Abstract

Cholesterol is an abundant component of mammalian cell membranes and has been extensively studied as an artificial membrane stabiliser in a wide range of phospholipid liposome systems. In this study, the aim was to investigate the role of cholesterol in cationic liposomal adjuvant system based on dimethyldioctadecylammonium (DDA) and trehalose 6,6'-dibehenate (TDB) which has been shown as a strong adjuvant system for vaccines against a wide range of diseases. Packaging of cholesterol within DDA:TDB liposomes was investigated using differential scanning calorimetery and surface pressure-area isotherms of lipid monolayers; incorporation of cholesterol into liposomal membranes promoted the formation of a liquid-condensed monolayer and removed the main phase transition temperature of the system, resulting in an increased bilayer fluidity and reduced antigen retention *in vitro*. *In vivo* biodistribution studies found that this increase in membrane fluidity did not alter deposition of liposomes and antigen at the site of injection. In terms of immune responses, early (12 days after immunisation) IgG responses were reduced by inclusion of cholesterol, thereafter there were no differences in antibody (IgG, IgG1, IgG2b) responses promoted by DDA:TDB liposomes with and without cholesterol. However, significantly higher levels of IFN-gamma were induced by DDA:TDB liposomes and liposome-uptake by macrophages *in vitro* was also shown to be higher for DDA:TDB liposomes compared to their cholesterol-containing counterparts, suggesting small changes in bilayer mechanics can impact both on cellular interactions and immune responses.

Keywords Vaccine, Adjuvant, Cholesterol, Biodistribution, DDA, bilayer fluidity, liposomes, subunit antigen.

1. Introduction

Liposomes composed of dimethyldioctadecylammonium (DDA) combined with an immunostimulatory component of the mycobacterial cell wall, trehalose 6,6-dibehenate (TDB) have been described as having immunostimulatory properties in numerous studies [e.g. 1-5]. TDB is a synthetic analogue of trehalose 6,6 α-dimycolate (TDM) often referred to as cord factor. Liposomes made of DDA and TDB, have been subject to stabilising and sterilisation methods [6] with GMP production already successfully established [7]. An intrinsic property of the DDA:TDB formulation is its ability to form a strong liposome-antigen depot at the site of injection after administration via the subcutaneous (s.c.) or intramuscular (i.m.) route [8], and this has been linked to the formulations ability to induce a powerful Th1 response as well as humoural immune responses [4, 5, 9]. In contrast, injection of antigen alone, neutral liposomes, or PEGylated cationic liposomes all result in low levels of antigen and adjuvant retention at the injection site and subsequently induce lower Th1 responses [8, 10, 11]. However, whilst all the formulations that promoted a depot effect were shown to produce higher immune responses, direct targeting of liposomes to the lymphatics can stimulate higher immune responses in some instances. One such study [12] investigated the role of liposome-adjuvant delivery by comparing immune responses of mice immunised via the intramuscular route and mice immunised via direct injection of the formulation into the lymph node. Direct injection of DDA or DDA:TDB liposomes intralymphatically made no notable difference to IgG1 responses in mice compared to those immunised intramuscularly. However, IgG2a responses in mice were higher after intralymphatical administration of DDA liposomes, but were not notably different when TDB was incorporated within the formulation [12]. Similarly, the route of administration was shown to play a critical role in IFN-gamma responses, with animals immunised with either DDA or DDA:TDB formulations directly into the lymph node giving significantly higher responses compared to those immunised via the intramuscular route. Based on these findings, it was important to consider various formulation strategies that that facilitate the cationic lipid DDA to be used within the adjuvant formulation, yet which would facilitate enhanced drainage to the lymphatics.

Cholesterol is an abundant component of mammalian cell membranes and has been extensively studied in phospholipid liposomal systems as a membrane stabiliser [13 -15]. The incorporation of cholesterol into liposomal membranes has been shown to lead to improved lipid packing consequently reducing or even eliminating the main phase transition temperature [7,16,17]. The resulting lower gel to liquid phase transition temperature leads to increased bilayer fluidity and liposomes show improved stability both *in vitro* and *in vivo* [13]. Whilst cholesterol is not inherently immunogenic, numerous studies have shown that

incorporation of cholesterol into liposomes leads to more favourable liposome properties such as increased transfection rates [18] and improved immunogenicity [19].

Therefore within his present study, we have considered the impact of including cholesterol within the DDA:TDB liposome adjuvant delivery system, with consideration of its ability to modulate bilayer fluidity and potentially alter the pharmacokinetic profile of the liposomal-adjuvant system. The packaging of cholesterol within the DDA:TDB bilayer was investigated using surface pressure-area isotherms and differential scanning calorimetry. *In vitro* studies investigating the ability of human macrophage-like cells to interact with liposomes containing cholesterol at varying molar ratios were compared with *in vivo* performance, to investigate *in vitro-in vivo* correlations. In addition, the ability of liposomes to form an antigen depot at the site of injection, to present antigen to the immune system, and to generate an immune response towards the co-administered antigen were investigated.

2. Materials and Methods

2.1 Materials

Dimethyldioctadecylammonium bromide (DDA) and trehalose 6,6'-dibehenate (TDB) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). Ag85B-ESAT-6 was kindly supplied by Statens Serum Institute, Denmark. Cholesterol, hydrogen peroxide, Sephadex™ G-75 and bicinchoninic acid protein assay (BCA) components were purchased from Sigma Aldrich (Dorset, UK). THP-1 cells were obtained from the American Type Culture Collection (via LGC Standards, Middlesex, UK). Foetal calf serum (FCS) was from Biosera, UK. RPMI was purchased from PAA (Yeovil, UK). Penicillin-streptomycin-glutamine (100X) was from Invitrogen, Paisley, UK. For radiolabelling, l-3-phosphatidyl[*N-methyl*-^{3}H]choline, 1,2-dipalmitoyl (^{3}H-DPPC) was obtained from GE Healthcare (Amersham, UK), Pierce pre-coated 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, Ohio) 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 characterisation of liposomes

Multilamellar vesicles (MLV) were prepared using the previously described lipid-film hydration method [25]. Briefly, weighed amounts of DDA, TDB and cholesterol were dissolved in chloroform/methanol (9:1, by volume) and the organic solvent was removed by

rotary evaporation followed by flushing with N_2 to form a thin lipid film which was hydrated in 10 mM Tris-buffer at pH 7.4 for 20 minutes at 60 °C, to a final concentration of 1.98 mM DDA, 0.25 mM TDB and concentrations of cholesterol of 0, 18 or 31 mol% (8:1, 8:2:1 or 8:4:1 DDA:Chol:TDB molar ratio respectively). Ag85B-ESAT-6 antigen was added to preformed vesicles to a final concentration of 10 µg/mL.

Physical characterisation of liposomes included vesicle size measurements (using dynamic light scattering) and zeta potential analysis (using particle electrophoresis); both techniques used a Brookhaven ZetaPlus (Brookhaven Instruments, Worcs, UK) to which 100 µL of liposomes were resuspended in 3 ml Tris buffer (1 mM, pH 7.4).

2.3 Langmuir-Blodgett Isotherms

An automated controlled film balance apparatus (KSV Langmuir Mini-trough, KSV Instruments Ltd., Helsinki, Finland) equipped with a platinum Wilhemy plate and placed on a vibration-free table was used to collect the surface pressure-area isotherms as previously reported [26]. The size of the trough was 24,225.0 mm^2 enclosing a total volume of approximately 220 mL; the subphase was composed of filtered double-distilled water. Lipids (at a fixed total concentration of 0.5 mg/mL^{-1}) were dissolved in chloroform and 20 µL of each solution was spread onto the air/water interface with a Hamilton microsyringe, (precision of ± 0.2 µL). After spreading, the monolayers were left for 10 minutes to allow the chloroform to evaporate. Thereafter, the molecules underwent constant compression (10 mm/s^{-1}) until the required surface pressure of less than 0.2 mN/m was attained. The spread monolayer was then compressed or expanded symmetrically with the two barriers until the desired surface pressure was reached with accuracy within 0.1 mN/m. The experiment was performed three times using monolayers prepared from different solutions, and with each monolayer being compressed only once. KSV software (KSV Instruments Ltd) was used for data analysis.

2.4 Differential scanning calorimetry

The gel-to-liquid phase transition temperatures were attained for the liposomal dispersions via DSC and thermograms were acquired using a Pyris Diamond DSC (Perkin Elmer Instruments LLC, USA). In this study, a scan rate of 10 °C/min was applied, over the range of 25 °C to 80 °C. All scans were carried out in triplicate. Suspensions were contained in air tight pans which were sealed immediately upon loading to reduce the effect of evaporation, with a sample load weight of approximately 10 mg. A reference pan filled with an equal volume of Tris buffer was used as a reference. This yielded an improved baseline,

achievable through a comparable thermal composition with the sample. Pyris software, version 5.00.02 (Perkin Elmer Instruments LLC, USA) was used for all data analysis.

2.5 Quantification of antigen loading and retention

In order to measure antigen loading and to trace its distribution *in vivo,* Ag85B-ESAT-6 antigen was radio-labelled with ^{125}I using Pierce pre-coated iodination tubes containing iodination reagent (Pierce Biotechnology, Rockford, IL) and a G-75 Sephadex chromatography column for separation of ^{125}I-antigen from ^{125}I [8]. Antigen loading to the various formulations was calculated by measuring radioactivity in supernatant and pellet fractions after ultracentrifugation. To aid liposome sedimentation during centrifugation, liposomes were placed in a solution of OVA (1 mg/mL) causing them to form a clear pellet, and subsequently centrifuged twice (125,000 ×*g*, 4 °C, 1 hour) to ensure removal of all non-associated antigen as previously reported [8]. Antigen release from liposomes stored in simulated *in vivo* conditions was determined using liposomes adsorbing and entrapping I125-labelled Ag85B-ESAT-6. Aliquots of each formulation were diluted (1:5) using 50 % v/v FCS in Tris buffer and incubated in a shaking water bath at 37 °C for 96 h. At various time intervals, samples were centrifuged and Ag85B-ESAT-6 release from liposomes was calculated by recording the proportion of radioactivity recovered in the supernatant as a percentage of the total radioactivity added.

2.6 Immunisation procedures

Five groups of five female C57BL/6 mice (6-10 weeks of age) received doses of liposome vaccine formulations containing 2 µg of Ag85B-ESAT-6 in a 50 µL volume. One group also received a non-liposome formulation containing 2 µg of Ag85B-ESAT-6 suspended in 50 µL PBS. Naïve groups received 50 µL of PBS. Vaccine formulations were administered intramuscularly, and each mouse received three doses at intervals of two weeks. Serum samples were taken 12 days after the first injection and at two week intervals thereafter.

2.6.1 Analysis of Ag85B-ESAT-6 specific antibody isotypes

Serum samples were analysed for the presence of anti-Ag85B-ESAT-6 IgG, IgG1 and IgG2b antibodies by enzyme-linked immunosorbent assay (ELISA). ELISA plates (flat bottom, high binding) were coated with 50 µl Ag85B-ESAT-6 per well (3 µg/well) in PBS and incubated at 4 ºC overnight. Plates were washed with phosphate buffered saline/tween buffer (PBST; 40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g Na_2HPO_4, ($2H_20$) per 5 litres of ddH_20, incorporating ~0.4 ml of Tween 20) and blotted firmly onto paper towel. Plates were blocked with 100 µL per well of 4 % w/v dried semi-skimmed milk powder in PBS. After 1 hour, plates were washed again and serially-diluted serum samples added. Plates were incubated for 1 hour at

37 ºC, followed by further washing and detection of anti-Ag85B-ESAT-6 antibodies using horseradish peroxidase conjugated anti-mouse isotype specific immunoglobulins (goat anti-mouse IgG1 and IgG2b), and subsequent addition of substrate solution, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) in citrate buffer incorporating 5 µL of 30 % H_2O_2/50 ml. After 20 minutes, the absorbance was measured at 405 nm using a plate reader (Bio-Rad, Herts, UK).

2.6.2 Proliferation of splenocytes *ex vivo*

To test cells for their ability to respond to antigen in vitro, splenocytes were restimulated with various concentrations of antigen (0.05, 0.5, 5 µg/mL) and their proliferation, determined by 3H-thymidine uptake, measured. On day 54 mice, (five groups of five mice, i.e. DDA:TDB, DDA:Chol:TDB 8:2:1 and 8:4:1 molar ratio, antigen and naive) were terminated by cervical dislocation and their spleens harvested and placed in a 7 mL bijoux containing 5 ml ice cold PBS. Each spleen was treated individually and kept on ice until processing. Spleens were gently grinded on a fine wire screen. After allowing the cell suspension to settle for approximately 5 minutes the liquid was transferred to sterile 20 mL falcon tubes, without disturbing the cellular debris at the bottom. The cell suspension was centrifuged at 1200 rpm, 15 ºC for 10 minutes. After centrifugation the supernatant was removed, the cell pellet re-suspended in 5 mL RPMI and a cell count performed. The cell number was adjusted to between 8 x 10^4 cells/ml.

For study of antigen specific proliferative responses, serial dilutions of Ag85B-ESAT-6 (0.05, 0.5 and 5 µg/ml) in RPMI were made and 100 µL added per well of a 96-well culture plate. Wells containing medium only or 3 µg/mL of concanavalin A (ConA) were included in all experiments as negative and positive controls respectively. Splenocytes (100 µL, 8 x 10^4 cells/mL) were added to each well making a final well volume of 200 µl. Cultures were incubated at 37 °C, 5 % CO2, 95 % humidity for 72 h following which 18.5 kBq (0.5 µCi) ^{3}H-thymidine (40 µL in RPMI/well) was added. After a further 24 h incubation under the same conditions, cells were harvested using a cell harvester (Titertek). For harvesting, media and cells from each well was aspirated onto a quartz filter mat. Each mat was placed into a plastic scintillation vial and 5 ml Ultima Gold™ scintillation fluid added/sample. All samples were counted using a standard ^{3}H scintillation counting protocol.

2.6.3 Analysis of cytokine production

Splenocytes isolated from mice were plated into 96-well plates (as described previously in section 2.8.1). Cells were incubated for 40 hours at 37 ºC, in a humid 5 % CO_2 environment, after which supernatants were removed and stored at -70 ºC for later analysis. Cytokine

levels of IL2, IL-5 and IFN-γ in the cell culture supernatants were quantified using the DuoSet® capture ELISA kits), purchased from R&D systems, Abingdon, UK) according to the manufacturer's instructions. Briefly, ELISA plates were first coated with capture antibody, followed by washing and blocking. Samples of cell culture supernatants were then added and cytokines quantified by addition of a biotinylated-detection antibody, detected by an enzyme marker (Streptavidin-HRP) and substrate solution following repeated incubation and washing steps. Absorbance was measured at 405 nm (Bio-Rad, Herts, UK).

2.7 Biodistribution studies

Inbred female BALB/c mice (6-10 weeks of age) were housed in cages within a laminar flow safety enclosure and provided with irradiated food and filtered drinking water *ad libitum*. All experiments adhered to the 1986 Scientific Procedures Act (UK) and were carried out in a designated establishment. Four to six days prior to injection, two groups of mice were injected subcutaneously with 200 µL pontamine blue (0.5 % w/v in PBS). Pontamine blue is an azo dye that has been described as being taken up by macrophages *in vivo* therefore allowing for the identification of lymphoid tissue such as lymph nodes. Although pontamine blue was primarily employed as a lymph node identification marker, it also served as a marker for identification of infiltrating macrophages to the site of injection. Liposomes containing the tracer molecule ^{3}H-DPPC were produced as described previously [8]. To obtain isotonicity, trehalose was added to the hydrating buffer to a final concentration of 10 % w/v. Mice were injected with Ag85B-ESAT-6 (radiolabelled with^{125}I) adsorbing liposome (radiolabelled with ^{3}H) formulations (50 µL/dose, i.m injection). At 1, 4 and 14 days post injection (p.i) mice were terminated by cervical dislocation and tissue from the site of injection (SOI), local draining lymph node (LN) and spleen removed for analysis of liposome (^{3}H) and antigen (^{125}I) presence using methods previously described elsewhere [8].

2.8 Macrophage studies

In vitro studies were performed using the human monocyte cell line THP-1 as previously described [22, 23]. Briefly, THP-1 cells were resuspended in fresh medium (RPMI 1640 + 10 % v/v FCS) at a density of 5 x 10^{5} cells/mL and stimulated for 48 h with 250 nM dihydroxyvitamin D3 (Enzo Life Sciences, Exeter UK) to differentiate cells. Prior to use, cells were resuspended at 2 x 10^{6} cells/mL in fresh RPMI with 10 % v/v FCS. Liposomes (1 mg/mL) were labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate) (DilC) (0.1 mol%) by inclusion of the lipid (dissolved in solvent) in the solvent evaporation stage of liposome production (as described in section 2.2). To ensure that all formulations incorporated the DilC fluorophore equally, the fluorescence was measured using a fluorimeter. Fluorescently labelled liposomes were diluted to a concentration of 10

μg/mL in RPMI, mixed with cells (1:1) in 6 well tissue culture plates and cocultured at 37 °C in 5 % CO2. At various time-points, 500 μL of co-culture were removed and mixed with ice-cold RPMI prior to immediate analysis. Association of fluorescent liposomes with THP-1 macrophages was analysed using non-fixed cells via flow cytometry using a Beckman-Coulter FC500 cytometer (High Wycombe, UK). For each sample a minimum of 20,000 events were analysed.

2.9 Statistical analyses

Data was analysed using analysis of variance (ANOVA) followed by the Tukey test to compare the mean values of different groups. Differences were considered significant when the *p* value was less than 0.05.

3. Results and discussion

3.1 The role of cholesterol in lipid packing.

Cholesterol is a common component in liposomal formulations and its beneficial role as a stabilising agent in liposomal bilayers is well recognised. Early studies investigating the effect of liposome composition on drug retention [13] demonstrated that inclusion of 50 mol % cholesterol within a liposome formulation increased the stability and reduced the permeability of liposomal bilayers. At molar percentages between 20 - 50 % (depending on the nature of the phospholipids), cholesterol can dissolve within the lipid bilayer, whereas at higher concentrations cholesterol can form crystal habits [24].

To understand the effect of the incorporation of cholesterol on the spatial orientation of the alkyl chains and the packing ability of DDA:TDB, initially we employed Langmuir studies [17] and thermal analysis (Figure 1). Figure 1A shows the surface area and pressure isotherm data of the various lipid combinations. The surface pressure/area isotherm of pure cholesterol was typical for the structural characteristics of a sterol; up to a mean molecular area of approximately 38 $Å^2$/molecule, the spread molecules show little interaction. After this point, the molecules compact to form a condensed monolayer, with the molecules tightly packed together (Figure 1A). Continued compression of this monolayer results in a collapse of the monolayer at 45.4 ± 0.4 mN/m and 32.5 ± 1.1 $Å^2$/molecule. In contrast, DDA shows a surface plot typical of a cationic lipid, where electrostatic repulsion between the head-groups deters close proximity of the lipids. Hence the plot shows the transition of the monolayer as compression is applied, initially starting as a gaseous monolayer, where the lipids are large distance apart, through the expanded monolayer state to a condensed monolayer prior to collapse (Figure 1A). In line with previous studies [21], the addition of TDB to DDA liposomes aids packing of the monolayer, by presumably slotting between the cationic DDA

and reducing electrostatic repulsion. Upon addition of increasing amounts of cholesterol to the DDA:TDB monolayer, the liquid-expanded phase transition seen with DDA:TDB was removed and there was a direct transition from gaseous to liquid-condensed, with the overall surface-pressure plot being more akin to the cholesterol plot at high cholesterol concentrations (DDA:Chol:TDB 8:4:1 molar ratio; Figure 1A). This trend is supported by a study on cholesterol inclusion within phosphatidylcholine (PC) systems, where Li et al [25] report that ordered states can be formed faster with fewer packaging defects with PC/cholesterol mixtures compared to PC alone [25]. Other studies [26] have also found that cholesterol is able to generate a liquid-ordered phase in PC membranes containing more than 25 mol % of cholesterol.

Differential scanning calorimetry (DSC) is a widely used method of thermal analysis that has been applied to investigate and characterise a range of pharmaceutical systems [32]. DDA lipid bilayers undergo a main phase transition at a characteristic temperature (T_c), with the lipid chains transferring from a lower temperature gel-phase dominated by ordered alkyl chain conformations, to a high-temperature fluid-phase characterised by disordered alkyl chain conformations [28, 29]. From Figure 1B, the phase transition temperature of DDA:TDB liposome (8:1 molar ratio) was 44.3 ± 0.15°C. Upon addition of cholesterol (at an 8:2:1 molar ratio; DDA:Chol:TDB molar ratio) there was a reduction in transition temperature to 42.7 ± 0.13°C (Figure 1B). The observation shows that cholesterol not only lowers the melting temperature, but also the energy required, as the enthalpy required for the transition to occur for DDA:TDB is 0.10 ± 0.01 J/g compared to the DDA:Chol:TDB (8:2:1 molar ratio) is 0.05 ± 0.01 J/g (Figure 1B). The hydrocarbon chains of lipids within DDA:TDB liposomes crystallise into the rigid crystalline phase hence producing a T_c at 44.3 ± 0.15°C. However, when cholesterol is added at DDA:Chol:TDB 8:4:1 molar ratio, there is complete removal of the transition temperature (Figure 1B) as the cholesterol prevents crystallisation of the hydrocarbon chains. A similar study [17] has shown that the inclusion of cholesterol at 33 - 50 molar ratio % to liposomes formed of the lipid DSPC also removed the transition [17].

This removal of the gel-liquid crystalline phase transition of liposome vesicles may facilitate enhanced fluidity of the system. Indeed, this was demonstrated by Coderch et al [30] who showed that bilayer fluidity (and in their studies, skin penetration) was increased by the addition of cholesterol to liposomes formulated from lipids with transition temperatures above the environment they were being used in. Thus, in the case of DDA:TDB, which has a transition temperature above body temperature, the addition of cholesterol to the liposomes will increase their fluidity and therefore could impact on the biodistribution of the vesicles after intramuscular injection.

3.2 The effect of cholesterol on DDA:TDB liposome characteristics.

From Figure 1 it was established that all three formulations tested gave high antigen loading; due to their cationic nature these systems are able to electrostatically bind the anionic antigen as previously reported [e.g. 7-11]. Inclusion of cholesterol into liposomes at a molar ratio of 8:4:1 DDA:Chol:TDB was sufficient to remove the phase transition of the bilayer, therefore a series of liposome formulations were prepared to consider the impact the addition of cholesterol had on the liposome phyisco-chemical characteristics. From Table 1, it can be seen that the incorporation of low levels of cholesterol to DDA:TDB liposomes (8:2:1 molar ratio) did not make a significant difference to the vesicle size, but did reduce antigen loading to a small extent (from 97% to 91%; Table 1). However, increasing cholesterol content to a molar ratio of 8:4:1 DDA:Chol:TDB in the liposome formulation resulted in a small increase in vesicle size and again a minor reduction in antigen loading (Table 1). These small changes in size and antigen loading ability are most probably due to the dilution of the overall cationic content of the liposomes as the cholesterol concentration is increased. However, given these were only minor changes in the physico-chemical characteristics, these would not be expected to have a notable impact on vaccine performance, therefore using these formulations we then evaluated the impact of cholesterol modified bilayer fluidity on liposomal adjuvant action, both with regards to the biodistribution and the ability of the liposome to deliver and present antigen successfully.

3.3 The impact of cholesterol induced fluidity on adjuvant function.

Immunological analyses were undertaken to determine the efficacy of the three liposome formulations (outlined in Table 1) in terms of antigen delivery and subsequent initiation of detectable immune responses. Quantification of antigen specific IgG, IgG1, and IgG2b antibody production, splenocyte proliferation and subsequent cytokine secretion were analysed. Figure 2 shows the IgG (A), IgG1 and IgG2b (B) responses over time. As expected, very little antibody production was noted when antigen was administered without an adjuvant, whereas all liposome formulations were able to induce measurable levels (Figure 2). When comparing between the formulations, only at day 12 were significant differences noted with IgG responses from mice immunised with DDA:TDB being significantly higher ($p<0.05$) that DDA;Chol:TDB (8:4:1 molar ratio; Figure 2A). At all time-points thereafter, there were no significant differences in antibody responses between the formulations.

Splenocytes from immunised mice were cultured in the presence of Ag85B-ESAT-6 and their proliferative abilities and cytokine production (cytokines IFN-γ, IL-2 and IL-5) measured. Upon restimulation, mice which had been immunised with DDA:TDB liposomes adsorbing

Ag85B-ESAT-6 showed the highest levels of splenocyte proliferation, in line with previous studies [13]. With increasing cholesterol content there was a trend, although not significant, of reduced proliferation in response to secondary exposure to antigen (Fig. 3). In correlation with previous reports [4,5] highlighting the strong Th1 mediating effects of DDA:TDB liposomes, high levels of IFN-γ were noted when Ag85B-ESAT-6 was co-delivered with DDA:TDB liposomes (Figure 4A). Whilst no significant differences between DDA:TDB and DDA:Chol:TDB (8:2:1 molar ratio) liposomes was noted, inclusion of cholesterol at the higher molar ratio of 8:4:1 resulted in significantly lower levels of IFN-γ and IL-2 ($p<0.05$) compared to non-cholesterol containing DDA:TDB liposomes (Fig 4A,B).

IFN-γ is an important correlate of protective immunity and numerous TB vaccine studies have shown that IFN-γ production is important for TB vaccine efficacy [6, 31, 32]. IL-2 is another essential signal in directing cell mediated immunity [38], whilst also playing a role in the humoural response. The production of IL-5 was also investigated as a signal of Th2 polarising abilities; in line with previous studies investing DDA:TDB liposomes, significantly higher levels of IL-5 were produced compared to delivery of free antigen; however, no differences were noted between liposomal groups (results not shown). Recent studies [34] investigating the effect of membrane fluidity compared the immune responses of DDA:TDB liposomes with liposomes composed of its unsaturated analog dimethyldioleoylammonium (DODA), the latter of which forms fluid disordered phase liposomes. These studies found that DDA-based liposomes induced a significantly higher immune response compared to that obtained with the fluid DODA-based liposomes. This is comparable to the finding in this study; as the cholesterol content within the liposome formulation is increased, the fluidity of the bilayer decreases and a reduction in Th1-biased responses are reduced.

3.4 The impact of cholesterol incorporation within DDA:TDB liposomes on their clearance from the injection site.

The role of cholesterol in eliciting a liposome or antigen depot-effect at the site of injection, and the subsequent presence of either component in the local lymph nodes, was investigated. As cholesterol inclusion at a 8:2:1 molar ratio had no effect in the aforementioned immunisation studies, only the higher proportion of cholesterol (8:4:1 molar ratio) was compared against the non-cholesterol containing counterpart. Figure 5 shows the presence of liposome and Ag85B-ESAT-6 antigen at the SOI after i.m. injection of antigen adsorbing formulations. DDA:TDB and DDA:Chol:TDB liposomes (Figure 5A) and their associated antigen (Figure 5B) showed no significant difference in clearance rates from the injection site, with between 40 - 50 % of the original liposome dose being recovered 2 weeks p.i. (Figure 5A). With regards to movement of vaccine components to the local draining

lymph node, the data suggests the DDA:TDB liposomes accumulate more rapidly at the draining lymph nodes over the first 4 days, with accumulation normalising by day 14 between the groups (Figure 5C), yet drainage of the antigen was not influenced by the presence of cholesterol in the bilayer formulation (Figure 5D). When considering the movement of infiltrating monocytes to the site of injection [8, 11], there was no notable difference in the intensity of monocyte recruitment to the injection site when cholesterol was included into DDA:TDB liposomes (Figure 5E).

From Figure 5 it can be seen that whilst initially high-levels of both liposomes and antigen are retained at the site of injection (~80 %; Figure 5 A and B). However by day 4, liposome levels remain at similar levels, yet antigen levels drop to ~20 % (Figure A and B), suggesting that the liposomes may not be able to retain high levels of antigen over a longer period. To consider the ability of these liposome systems to retain antigen, an antigen retention study was conducted in simulated *in vivo* conditions (Figure 5F). These results show that whilst the liposomes +/- cholesterol have similar zeta potential and antigen loading in the suspension buffer prior to injection; however, when in the presence of other proteins (such as might be found at the injection site, promoting liposome aggregation depot formation), competition for electrostatic binding to the cationic liposomes may occur resulting in antigen loss. Further, the ability of liposomes to retain antigen may also be influenced by cholesterol content (Figure 5E).

In a previous study where the movement of vaccine components from the SOI was studied, more rapid draining of the liposome component was observed when DDA was substituted for its unsaturated counterpart 'DODA' (which results in more fluid liposomes) [34]. However, whilst here we see that cholesterol inclusion alters the fluidity of DDA:TDB liposomes (when included at a 8:4:1 molar ratio), this does not translate into altered draining from the injection site, nor to the draining lymph node after 14 days. However with both formulations, antigen loss from the depot at the injection site was noted. The reduction in vaccine efficacy seen in DDA:Chol:TDB (8:4:1 molar ratio) liposomes must therefore be due to a separate factor; looking at other studies [34, 35] which have analysed the presence of adjuvant and antigen in APCs, an important correlation between co-localization of both components and successful responses was seen. Indeed recent studies by Kamath et al. [35] have shown that synchronisation of dendritic cell activation and antigen exposure is required for the induction of Th1 responses, but only a small population of DCs require such activation to promote strong Th1 responses [35]. In these studies the authors demonstrated that mice immunised with antigen and DDA/TDB liposomes separately but to the same injection site, induced similar Th2 responses, but weaker Th1 responses than mice immunised with

457 DDA/TDB with adsorbed antigen [34]. Similarly in another study [36], antigen administered
458 alone, one day prior to a standard vaccine was shown to be detrimental for the immune
459 response, due to the early exposure of APCs to free antigen. Overall, these studies [35, 36]
460 demonstrate that whilst the depot-effect is important, more important and detrimental to the
461 ensuing immune response is the pre-exposure of dendritic cells to antigen alone which can
462 mediate temporary anergy. Therefore inclusion of cholesterol within the formulation may
463 result in more rapid loss of antigen initially which subsequently normalises between the
464 formulations, but which could result in increased pre-exposure of DC to antigen alone,
465 reducing Th1 responses.
466
467 *3.5 Interaction of cholesterol containing DDA:TDB liposomes with phagocytes*
468 Given that the difference in bilayer packaging and transition temperature of the DDA:TDB
469 liposomes formulated with or without cholesterol had no impact on biodistribution but did
470 impact on Th1 responses, this would suggest that cellular uptake and activation may be
471 playing a key role in promoting the different immune responses noted. Therefore to quantify
472 liposome uptake by phagocytes, the human continuous cell line THP-1 was used.
473 Fluorescence-labelled DDA:TDB and DDA:Chol:TDB (8:2:1 and 8:4:1 molar ratio) liposomes
474 were co-cultured with THP-1-derived macrophages at a final lipid concentration of 5 μg/mL.
475 The proportion of macrophages associated with fluorescent liposomes, and the relative
476 amount of fluorescence associated, was quantified using flow cytometry. Figure 6 shows the
477 time-dependent uptake of DDA:TDB liposomes (±cholesterol) after application to THP-
478 derived macrophages at 37°C; with increased amounts of cholesterol, each cell associates
479 with fewer liposomes, as evidenced by less fluorescence (Figure 6B). For DDA:TDB
480 liposomes, around 75 % of the cells were associated with the liposomes (Figure 6C), which
481 is in line with previous studies [23]. However, with increasing cholesterol content the
482 percentage of cells associated with liposomes decreased to ~ 40 % (Figure 6C). Although
483 the data presented suggests uptake of DDA:TDB liposomes by macrophage in vitro was
484 higher compared to cholesterol-containing counterparts it is possible that the activity of
485 DDA:TDB liposomes in the presence/absence of cholesterol may be different in vivo,
486 particularly given the in vivo biodistribution suggests the liposomes and antigen are present
487 at the injection site for several days.

488 From these studies it can be seen that the cholesterol content within the liposomes reduces
489 liposomal uptake by phagocytes and this may be the contributing factor in the reduced Th1
490 responses noted in Figure 3 and 4. As found in a number of studies [8, 11], upon injection
491 with a vaccine antigen, DDA-based liposomes form a vaccine depot at the injection site that
492 results in a continuous attraction of antigen-presenting cells that engulf a high amount of

adjuvant. These cells are subsequently efficiently activated, as measured by an elevated expression of the co-stimulatory molecules CD40 and CD86 [34]. Furthermore, a study by Korsholm et al (2007) [36] proposed that cationic DDA liposomes promote uptake via endocytosis prior to disruption or fusion with internal cellular membranes, and that the delivery of associated antigen to cells occurs upon instant contact with the cell surface through electrostatic interactions prior to active antigen uptake and presentation, which is a key mechanism behind the adjuvant properties of cationic DDA liposomes. In addition, inclusion of DSPC into DDA demonstrated that antigen acquisition by APCs was dependent upon DDA concentration [36].

4. Conclusion

In this study, the aim was to investigate the role of cholesterol in the DDA:TDB adjuvant delivery system. Inclusion of ~30 mol% of cholesterol was able to abolish the transition temperature of the liposome formulation without notable impact of the vesicle size, zeta potential and antigen loading. However, whilst inclusion of cholesterol within the DDA:TDB liposomal adjuvant system could enhance the fluidity of the system, this did not translate to increased movement to the local lymphatics, nor impact on the recruitment of monocytes to the injection site, yet it resulted in a reduction in Th1-based immune responses. This would suggest that the reduction in immune responses is most likely a result of reduced APC uptake.

The effect of membrane fluidity has been investigated previously with the use of cationic lipid DODA exhibiting a low phase transition temperature. In accordance with the results presented here, these ‘fluid’ liposomes also showed a decreased ability to stimulate splenocytes to produce the Th1 biased cytokine IFN-γ. It is possible that the increased liposome rigidity noted with DDA:TDB liposomes, as compared to those including cholesterol, results in increased exposure of immune cells to the co-presented antigen. Supporting this theory, the flow cytometry results presented show a clear increase in liposome uptake of non-cholesterol containing DDA:TDB liposomes.

The results presented in this study show that a balance between physico-chemical properties and desired immunological outcome must be considered. Although cholesterol is widely considered a suitable liposome stabilising compound, we have shown that the strong Th1 cytokine polarising nature of TDB containing DDA liposomes is forfeited by addition of cholesterol to levels of ~30 mol% to this liposome formulation.

Acknowledgements

This work was partly funded by NewTBVAC (contract no.HEALTHF3-2009-241745). NewTBVAC has been made possible by contributions from the European Commission.

Supporting Information Available

This information is available free of charge via the Internet at http://pubs.acs.org/.

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633 different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity
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668 **Tables and Figures**

669

670 **Table 1. Characteristics of cationic liposomes**.

671

Formulation	Molar ratio	Size ± SD (nm)	Zeta-potential ± SD (mV)	Antigen loading (%)
DDA:TDB	8:1	586 ± 78	52 ± 5	97 ± 2.1
DDA:Chol:TDB	8:2:1	663 ± 66	47 ± 6	91 ± 1.1*
DDA:Chol:TDB	8:4:1	737 ± 53*	45 ± 3	87 ± 3.6*

672 Vesicle size, zeta-potential and antigen loading of DDA:TDB formulations with and without
673 the addition of cholesterol. Size and zeta-potential were measured in Tris buffer (1 mM)
674 using a Brookhaven ZetaPlus instrument. Antigen loading was measured using radiolabelled
675 antigen. Results represent mean ± SD of triplicate experiments. *denotes $p<0.01$ or greater
676 in comparison to DDA:TDB.

677

678 Figure 1. The effect of cholesterol on lipid packing. A) Compression isotherm studies of the
679 pure and mixture of lipid monolayers of DDA, cholesterol, DDA:TDB with the addition of
680 cholesterol at two different molar ratios 2 and 4 in deionised water at 20 °C. Results are
681 expressed as the means of three experiments. SD has not been shown for clarity. B) DSC
682 thermograms of the gel-to-liquid phase transition of DDA:TDB formulations with the addition
683 of cholesterol at two different molar ratios 2 and 4. Liposomes were produced via lipid
684 hydration in 10 mM Tris buffer (pH 7.4). DSC Thermograms were made at 10 °C/minute (600
685 °C/h) over the tested temperature range of 25-80 °C One of three thermograms for each
686 vesicle system is shown with the results representative of three independent experiments.

687 Figure 2. Ag85B-ESAT-6 specific antibody titres. Groups of five female C57Bl/6,
688 approximately six weeks old, received doses of liposome formulations containing 2 µg of
689 Ag85B-ESAT-6 in a 50 µL volume. Vaccine formulations were administered intramuscularly,
690 and each mouse received three doses at intervals of two weeks. Serum samples were taken
691 at 12 days after the first administration and at two week intervals thereafter. Anti-Ag85B-
692 ESAT-6 IgG responses (A) the IgG1/IgG2a balance over the period of the study (B) are
693 shown; anti-Ag85B-ESAT-6 IgG1 and IgG2b were measured by enzyme-linked
694 immunosorbent assay (ELISA). Results represent mean ± SD reciprocal end point serum
695 dilution (log_{10}) from five mice. ***Denotes significantly increased antibody titres ($p< 0.05$)

696

697 Figure 3. Spleen cell proliferation in response to Ag85B-ESAT-6 antigen. Cell proliferation
698 was measured by incorporation of ^{3}H-thymidine into cultured splenocytes derived from mice
699 immunised with Ag85B-ESAT-6 antigen containing formulations. Results represent mean ±
700 SD of five mice.

701

Figure 4. Ag85B-ESAT-6 specific cytokine production. Cytokines were detected using DuoSet® capture ELISA kits (mouse IFN-γ (A), IL-2 (B), purchased from R&D systems, Abingdon, UK) according to the manufacturer's instructions. Results shown mean+/- SD, n=5. * denotes significantly increased levels in comparison to naïve controls (n=5, $p<0.05$).

Figure 5. Presence of liposome and antigen at the site of injection following intramuscular injection of Ag85B-ESAT-6 antigen adsorbing to DDA:TDB with and without cholesterol. Tissue was collected on days 1, 4 and 14 p.i and assayed for the presence of ^{3}H and ^{125}I relating to A) liposome and B) antigen at the site of injection, C) liposome and D) antigen at the popliteal lymph node respectively. Results represent mean ± SD of four mice. E) shows monocyte infiltration (tracked by pontamine blue) at the SOI (quadriceps) after 14 days injection (i.m.) of DDA:TDB and DDA:Chol:TDB (8:4:1 molar ratio) liposomes with adsorbed Ag85B-ESAT-6 antigen. F) Ag85B-ESAT-6 antigen release profile of DDA:TDB, 8:2:1 and 8:4:1 liposomes using 125I-labelled Ag85B-ESAT-6 when stored under simulated *in vivo* conditions (50 % FCS, 37 °C). Results represent mean ± SD of triplicate experiments.

Figure 6. The presence of cholesterol in liposomes reduces their interaction with THP-1 cells. THP-1-derived macrophages were co-cultured with dilC-fluorescently labeled liposomes with the addition of cholesterol at two different molar ratios 2 and 4. At the indicated timepoints, a sample of cells was removed and immediately analysed using flow cytometry. Data from a minimum of 20,000 cells are shown: (A) frequency histograms for fluorescence associated (surface-bound or internalised) with macrophages at the indicated times, from a representative assay. (B) Mean fluorescence intensity of macrophages cocultured with the indicated liposomes. (C) Percentage of macrophages positive for fluorescence. Data shown for parts (B) and (C) are mean ± SEM from three independent experiments. Statistical analyses used ANOVA with Dunnett's post test. *$P<0.05$ compared to DDA:TDB (8:1 molar ratio) at each time point.

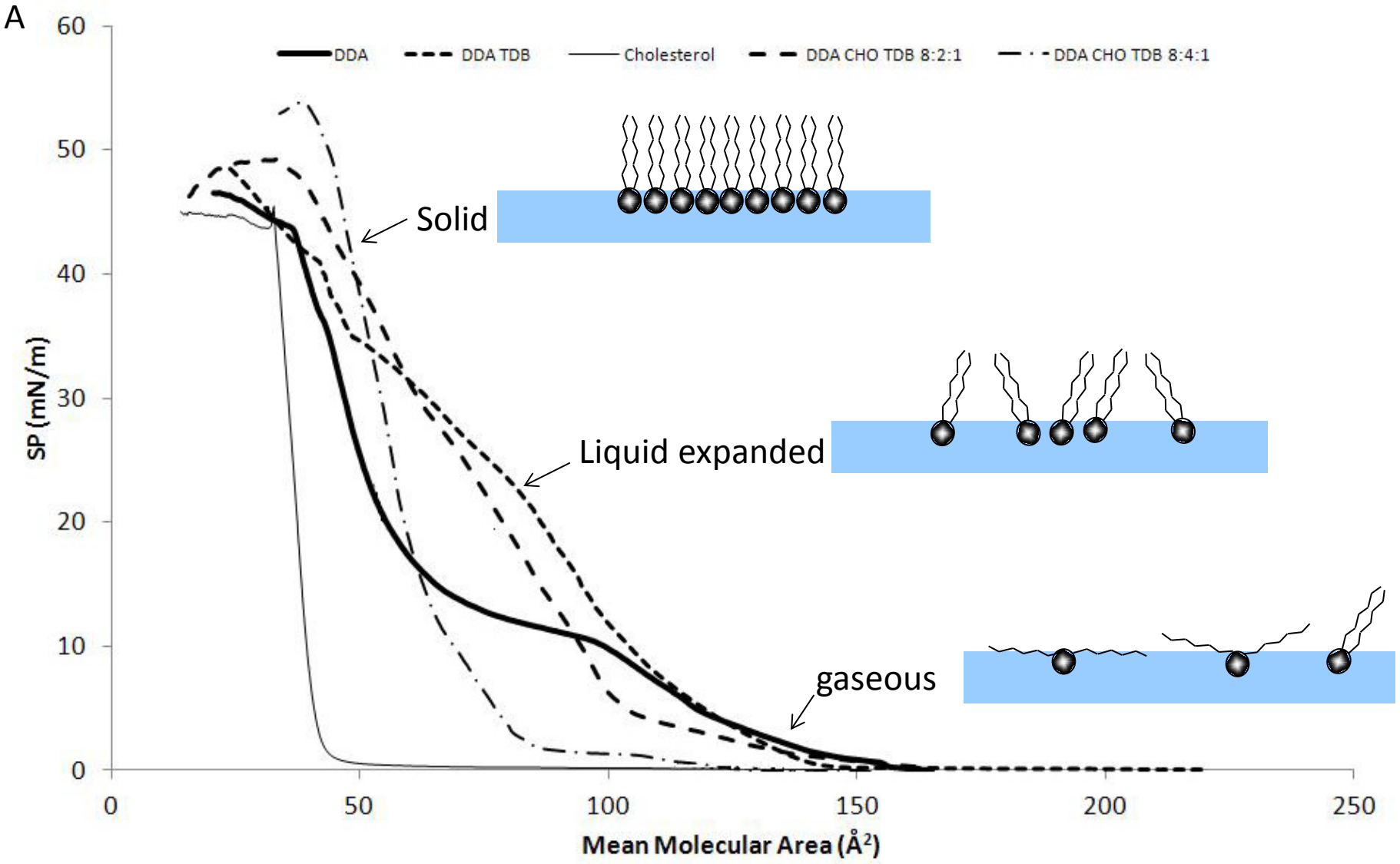

A
DDA
DDA TDB
Cholesterol
DDA CHO TDB 8:2:1
DDA CHO TDB 8:4:1
Solid
Liquid expanded
gaseous
SP (mN/m)
Mean Molecular Area (Å²)
0
10
20
30
40
50
60
0
50
100
150
200
250

Figure 1A

B

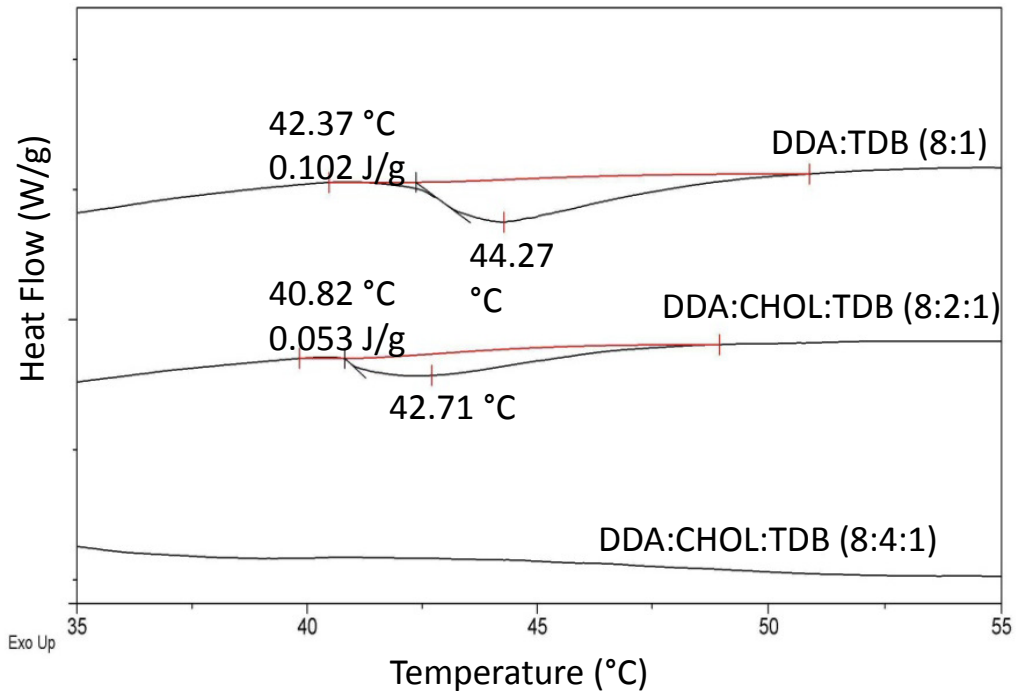
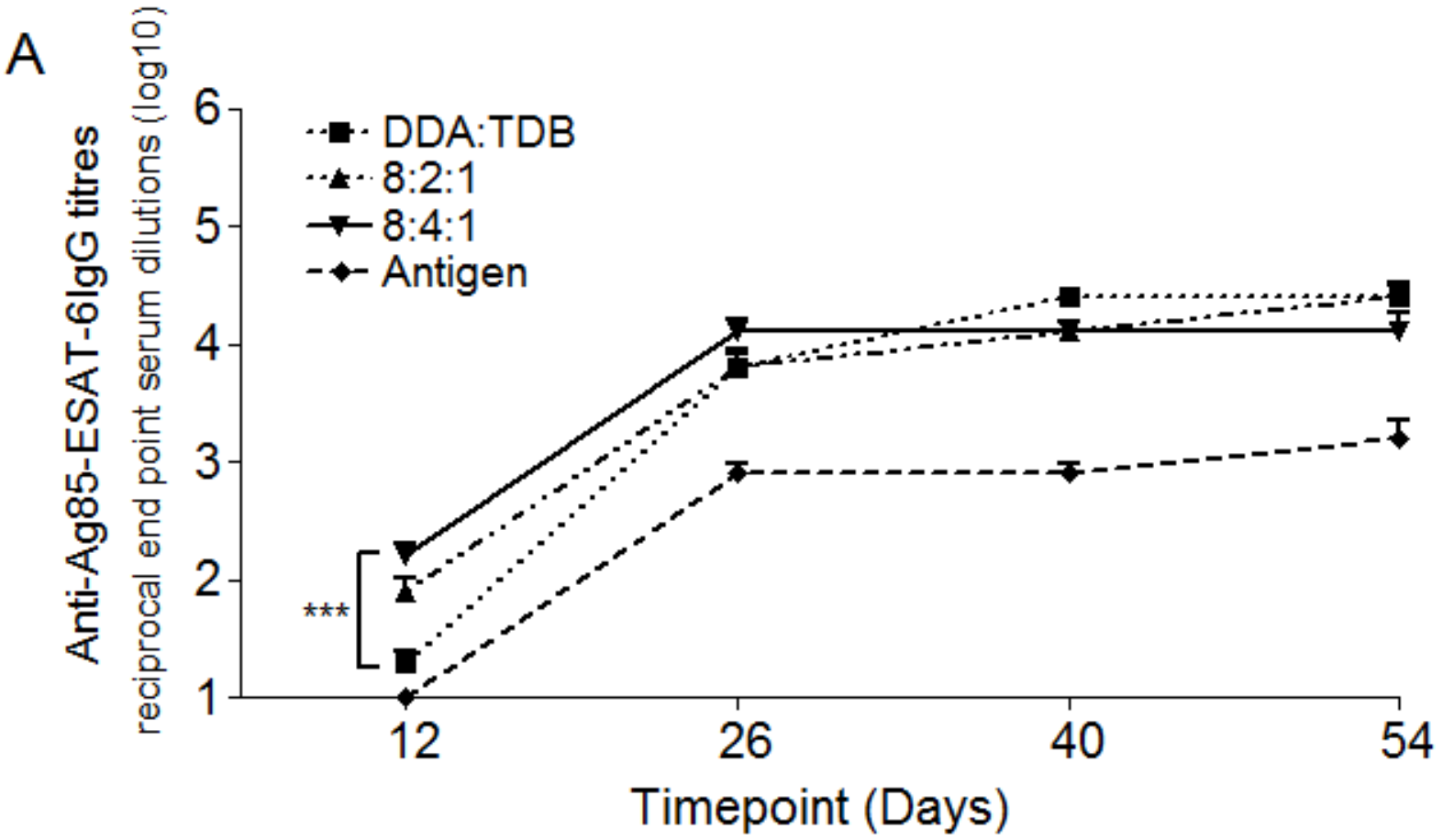

42.37 °C
0.102 J/g
DDA:TDB (8:1)
44.27
°C
40.82 °C
0.053 J/g
DDA:CHOL:TDB (8:2:1)
42.71 °C
DDA:CHOL:TDB (8:4:1)
Heat Flow (W/g)
Exo Up
35
40
45
50
55
Temperature (°C)

Figure 1B


A
Anti-Ag85-ESAT-6IgG titres
reciprocal end point serum dilutions (log10)
6
5
4
3
2
1
DDA:TDB
8:2:1
8:4:1
Antigen
***
12
26
40
54
Timepoint (Days)

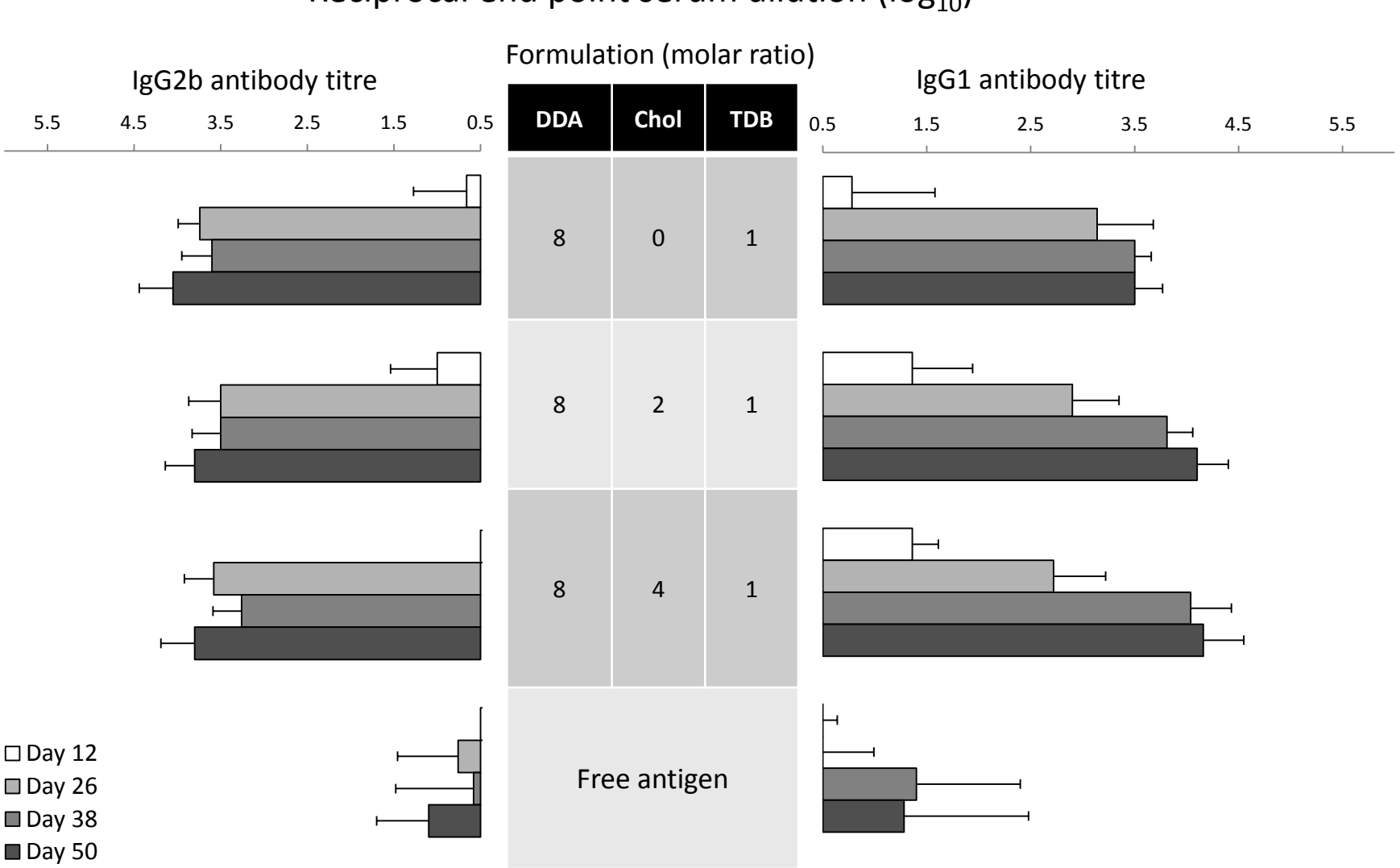

Reciprocal end point serum dilution (log_{10})
Formulation (molar ratio)
IgG2b antibody titre
IgG1 antibody titre
DDA
Chol
TDB
5.5
4.5
3.5
2.5
1.5
0.5
0.5
1.5
2.5
3.5
4.5
5.5
8
0
1
8
2
1
8
4
1
Free antigen
Day 12
Day 26
Day 38
Day 50

Figure 2B

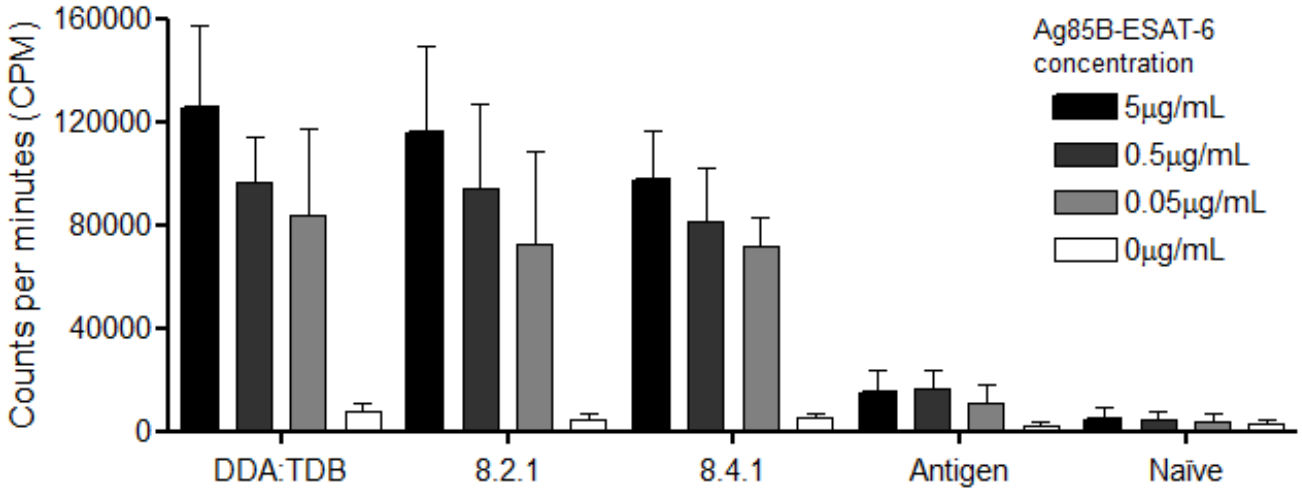

Counts per minutes (CPM)
160000
120000
80000
40000
0
DDA:TDB
8.2.1
8.4.1
Antigen
Naïve
Ag85B-ESAT-6
concentration
5µg/mL
0.5µg/mL
0.05µg/mL
0µg/mL

Figure 3.

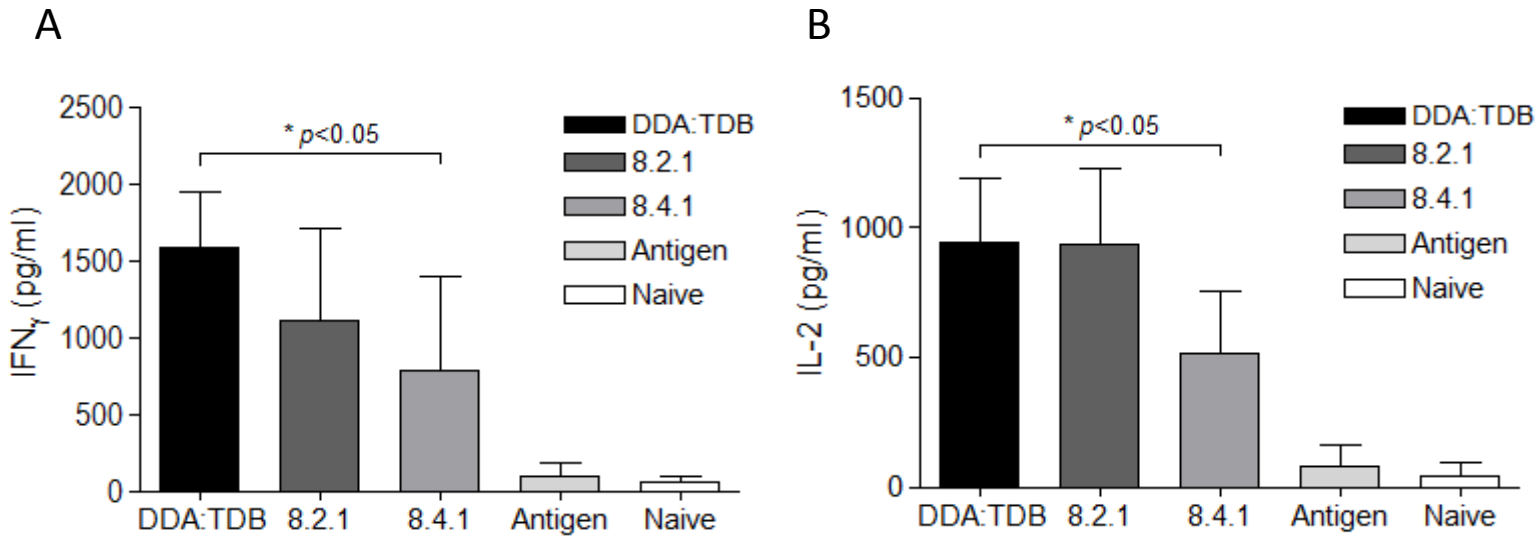

A
B
2500
2000
1500
1000
500
0
IFNγ (pg/ml)
* p<0.05
DDA:TDB
8.2.1
8.4.1
Antigen
Naive
1500
1000
500
0
IL-2 (pg/ml)
* p<0.05
DDA:TDB
8.2.1
8.4.1
Antigen
Naive

Figure 4.

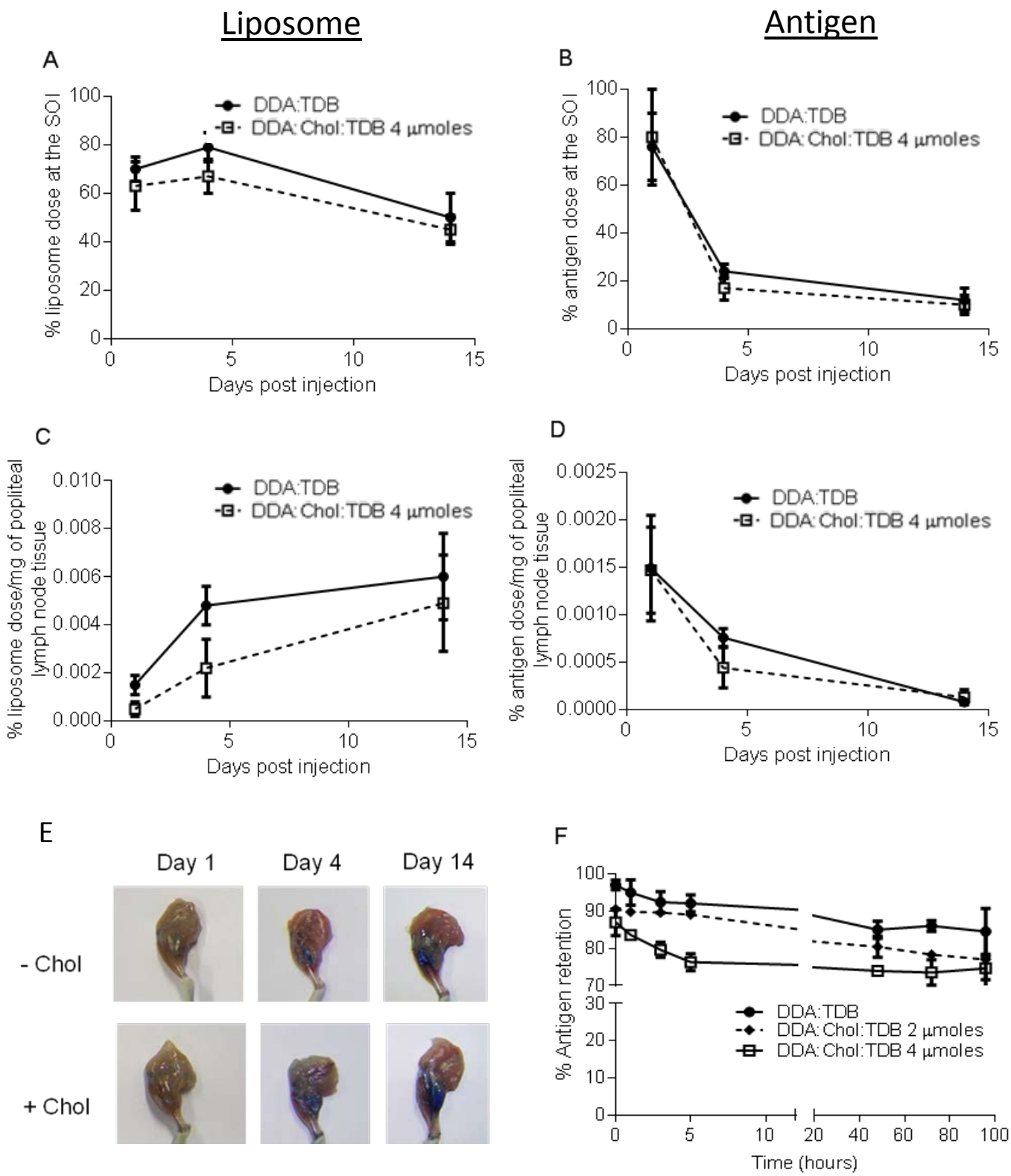

Liposome
Antigen
A
% liposome dose at the SOI
DDA:TDB
DDA:Chol:TDB 4 µmoles
Days post injection
B
% antigen dose at the SOI
DDA:TDB
DDA:Chol:TDB 4 µmoles
Days post injection
C
% liposome dose/mg of popliteal lymph node tissue
DDA:TDB
DDA:Chol:TDB 4 µmoles
Days post injection
D
% antigen dose/mg of popliteal lymph node tissue
DDA:TDB
DDA:Chol:TDB 4 µmoles
Days post injection
E
Day 1
Day 4
Day 14
- Chol
+ Chol
F
% Antigen retention
DDA:TDB
DDA:Chol:TDB 2 µmoles
DDA:Chol:TDB 4 µmoles
Time (hours)

Figure 5.

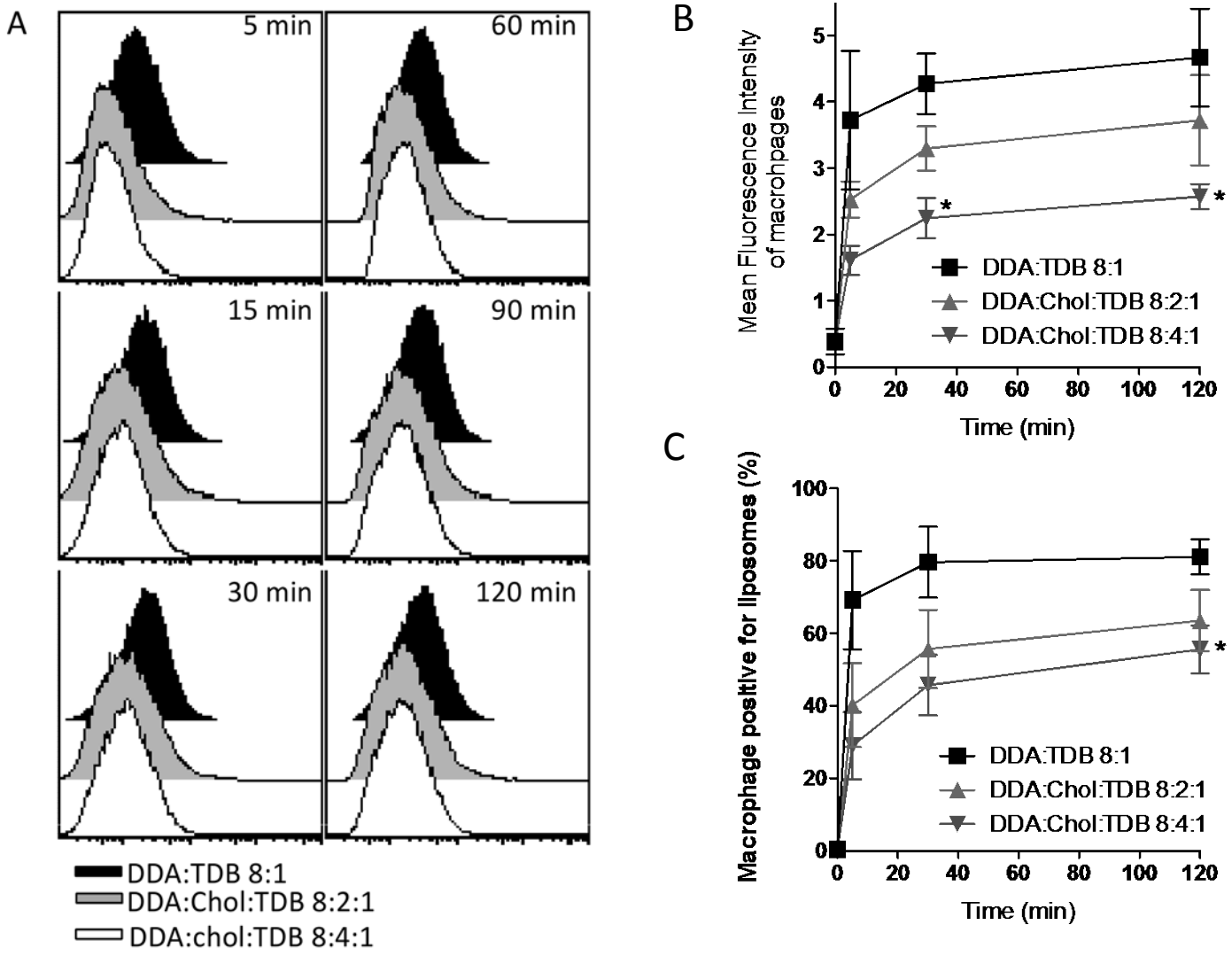

A
5 min
60 min
15 min
90 min
30 min
120 min
DDA:TDB 8:1
DDA:Chol:TDB 8:2:1
DDA:chol:TDB 8:4:1
B
Mean Fluorescence Intensity of macrohpages
0
1
2
3
4
5
0
20
40
60
80
100
120
Time (min)
*
*
DDA:TDB 8:1
DDA:Chol:TDB 8:2:1
DDA:Chol:TDB 8:4:1
C
Macrophage positive for liposomes (%)
0
20
40
60
80
100
0
20
40
60
80
100
120
Time (min)
*
DDA:TDB 8:1
DDA:Chol:TDB 8:2:1
DDA:Chol:TDB 8:4:1

Figure 6.