1 Title: Th1 immune responses can be modulated by varying
2 dimethyldioctadecylammonium and distearoyl-sn-glycero-3-phosphocholine content
3 in liposomal adjuvants.

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10 **Key Words:** Cationic liposomes, vaccine adjuvant, dimethyldioctadecylammonium,
11 distearoyl-sn-glycero-3-phosphocholine, Th1 responses.

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25   **Abstract**

26   **Objectives** Cationic liposomes of dimethyldioctadecylammonium bromide (DDA)

27   combined with trehalose 6,6-dibehenate (TDB) elicit strong cell mediated and

28   antibody immune responses; DDA facilitates antigen adsorption and presentation,

29   whilst TDB potentiates the immune response. To further investigate the role of DDA,

30   DDA was replaced with the neutral lipid of distearoyl-sn-glycero-3-phosphocholine

31   (DSPC) over a series of concentrations and these systems investigated as adjuvants

32   for the delivery of Ag85B–ESAT-6-Rv2660c, a multistage tuberculosis vaccine.

33   **Methods** Liposomal were prepared at a 5:1 DDA-TDB weight ratio and DDA content

34   incrementally replaced with DSPC. The physicochemical characteristics were

35   assessed (vesicle size, zeta potential and antigen loading) and the ability of these

36   systems to act as adjuvants was considered.

37   **Key findings** As DDA was replaced with DSPC within the liposomal formulation, the

38   cationic nature of the vesicles decreases as does electrostatically binding of the

39   anionic H56 antigen; however, only when DDA was completed replaced with DSPC

40   did vesicle size increase significantly. Th1 type cell-mediated immune responses

41   reduced. This reduction in responses was attributed to the replacement of DDA with

42   DSPC rather than the reduction in DDA dose concentration within the formulation.

43

44   **Conclusion** These results suggest Th1 responses can be controlled by tailoring the

45   DDA/DSPC ratio within the liposomal adjuvant system.

46

Introduction

The development of novel vaccines against pathogens like tuberculosis and HIV, where a strong CMI response is required, has been hampered by the lack of suitable adjuvants. Producing an adjuvant capable of eliciting strong Th1 type responses remains a key challenge. However in recent years, research has resulted in a number of potential lead candidates, including liposome systems. Liposomes as adjuvants have been investigated in a range preclinical models and have been shown to effectively deliver associated vaccine antigen to antigen presenting cells (APCs), providing antigen specific immunity [1]. Previous studies suggest that cationic liposomes are more locally reactive than neutral liposomes, causing deposition of antigen and infiltration of monocytes to the site of injection [2], whilst also producing high local levels of pro-inflammatory cytokines [3]. The choice of the cationic lipid also plays an important role, influencing the immunostimulatory capacity of the system. Indeed liposomal adjuvants based on the synthetic amphiphile dimethyldioctadecylammonium bromide (DDA), have been found to generate stronger Th1 responses compared to other cationic systems, characterised by their levels of interferon-γ (IFN-γ) production [4]. Furthermore, the combination of DDA with the synthetic analogue to mycobacterial cord-factor, trehalose dibehenate (TDB), can enhance Th1 responses [5]. The incorporation of the TDB glycolipids has been shown to stabilise DDA vesicles and enhance the CMI response of DDA whilst at the same time inducing high levels of antigen specific antibodies [6]. The TDB glycolipid has been shown to activate macrophages and dendritic cells (DCs) through the FcRγ-Syk-Card9 pathway, stimulating an innate immune activation program which mediates protective Th1 and Th17 type responses [7]. The cationic charge of DDA-TDB results in higher antigen retention and an enhanced infiltration of monocytes at the site of injection compared to neutrally charged counterparts [8] and whilst the surface charge is recognised as a crucial factor driving cellular immunity [8], its adjuvant effect appears to be less influenced by liposomal size [9].

Although DDA-TDB is a well characterised vaccine delivery system, key characteristics that dictate the adjuvant properties of the liposomes are still not fully understood. To further develop this, in this study, cationic DDA content was gradually replaced with an alternative non-cationic lipid, the neutral lipid distearoyl-sn-glycero-3-phosphocholine (DSPC), in order to further consider the role of DDA within DDA-TDB in modulating Th1 response profiles. DSPC was selected to replace DDA due to

82   the similarity in alkyl chain lengths and phase transition temperature to that of DDA,

83   factors shown to impact on liposome stability in vivo [10]. Therefore a range of

84   formulations where DSPC systematically replaced DDA within the liposomal system

85   were investigated in combination with the subunit TB vaccine candidate, H56 [11].

86

87

88   **Materials and Methods**

89   *Materials*

90   Dimethyldioctadecylammonium (DDA), trehalose 6,6-dibehenate (TDB) and 1,2-

91   distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar

92   Lipids (Alabaster, Alabama, USA). The purity of all the compounds used was > 99%,

93   determined by HPLC. The fusion protein Ag85B-ESAT-6-Rv2660c (H56 antigen)

94   obtained from the Statens Serum Institut (SSI, Copenhagen, Denmark). Tris (Ultra

95   Pure) was purchased from ICN Biomedicals (Aurora, OH). Phosphate buffered saline

96   (PBS) tablets were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Methanol

97   and chloroform (extra pure) were purchased from Fisher (UK). Ultima Gold

98   scintillation fluid and [^{3}H] thymidine were obtained from Perkin Elmer (Waltham,

99   MA). Double distilled water was used in preparation of all solutions.

100

101   *Liposome preparation: lipid hydration*

102   Liposome formulations were prepared by the previously established method of lipid

103   hydration [12]. Briefly, lipids were dissolved in a chloroform:methanol mixture (9:1

104   v/v), with DDA and TDB set to concentrations of 1.25 mg and 0.25 mg TDB per mL

105   respectively, representing a 5:1 DDA-TDB weight ratio. The level of DDA within the

106   formulation was incrementally replaced with increasing levels of DSPC, with levels

107   of TDB remaining fixed. Lipid mixtures were added to a round bottomed flask and

108   upon solvent extraction via rotary evaporation and N_2 flushing, a dry film was

109   produced. The lipid film was hydrated in Tris-buffer (10 mM, pH 7.4) for 20 min at

110   10 °C above the main gel-to-liquid phase transition of DDA at ~47 °C [7, 13] or

111   DSPC at ~55 °C [14] to completely hydrate the film and form liposomes.

112

113   *Characterisation of liposomes for particle size and zeta potential*

114

The intensity mean diameter of all liposome formulations were measured using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcs., UK) via dynamic light scattering. The measurement of vesicle size took place at 25 ˚C in Tris buffer (1/10 dilution; 1 mM, pH 7.4). The indirect measurement of liposome surface charge was determined by assessing the zeta potential, using the same Malvern Zetasizer Nano-ZS instrument, in Tris buffer (1/300 dilution; 1 mM, pH 7.4). All characterisation was undertaken in triplicate.

Radiolabelling of H56 antigen

The protein antigen, H56 was radiolabelled with ^{125}I using IODOGEN® pre-coated iodination tubes (Pierce Biotechnology, Rockford, IL). Separation of labelled protein from free ^{125}I was carried out using a Sephadex G-75 gel column, pre-soaked in ddH$_2$0 and equilibrated with Tris buffer (10 mM; pH 7.4). This method was then carried out as described previously [2].

Quantification of H56 antigen adsorption

Radiolabelled (^{125}I) H56 antigen was added to each liposome formulation at an *in vivo* concentration of 5 µg per dose (0.1 mg/ml), and left to surface adsorb to the liposome for 45 minutes with intermittent swirling. Surface-adsorbed and non-adsorbed protein antigen within the liposomal suspensions were separated by diluting the suspension to 1 ml using Tris buffer (10 mM; pH 7.4), followed by centrifugation using an Optima Max-XP Ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA). The quantity of radiolabelled antigen (^{125}I-H56) prior to centrifugation and within subsequent fractions (pellet and supernatant) was measured using a Cobra™ CPM Auto-Gamma® counter (Packard Instruments Company Inc., Downers Grove, IL). The total recovery of protein antigen was then determined by calculating the % radioactivity in the liposome pellet fraction.

Vaccine study: Immunisation of mice

All experiments were undertaken in accordance with the 1986 Scientific Procedures Act (UK). Female C57BL/6 mice, 6-8 weeks old (Charles River, UK) were split into 11 groups of 5. Vaccine preparations were made with the liposomes adsorbing H56) antigen to a final concentration of 0.1 mg/mL (5 µg/vaccine dose). All mice, with the exception of the naive group, were immunised intramuscularly with the proposed

149   vaccine (0.05 mL/dose) three times, with two week intervals between each

150   immunisation. At scheduled time points, blood samples were taken and stored at -20

151   °C for future analysis.

152

153   Upon experiment termination, mice were culled and the spleens were collected.

154   Spleen cell suspensions were produced upon light grinding through a fine wire mesh

155   into 10 ml RPMI 1640 cell culture medium (W/O Glutamine) supplemented with 10%

156   (v/v) FBS and 1% (v/v) PSG (BioSera, East Sussex, UK). Cell suspensions were

157   centrifuged at 1000 RPM for 10 min at 15 °C and upon supernatant removal, the

158   pellet was resuspended in 10 mL RPMI, before repeated centrifugation prior to pellet

159   resuspension in 5 mL RPMI. Single cell suspensions were used to evaluate splenocyte

160   proliferation and antigen specific cytokine responses. For assessment of splenocyte

161   proliferation, H56 was added to sterile 96 well cell culture plates (Greiner Bio-One

162   Ltd, Gloucestershire, UK) with a positive control of concanavalin A (2 μg/mL). 100

163   μL of spleen cell suspensions were added and incubated at 37 °C, 5% CO_2, and upon

164   72 hours incubation, 40 μL of [^{3}H] thymidine at 0.5 (μCi) in supplemented RPMI was

165   added per well and incubated for 24 hours. Well contents were harvested onto quartz

166   filter mats (Skatron/Molecular Devices, Berkshire, UK) using a cell harvester

167   (Titertek Instruments, Alabama, USA) and transferred to 20 mL scintillation vials

168   (Sarstedt, Leciester, UK) containing 5 mL scintillation cocktail (Ultima Gold,

169   PerkinElmer, Cambridgeshire, UK). Incorporation of [^{3}H] thymidine in cultured cells

170   was measured with a scintillation counter according to standard operating procedures.

171

172   *Evaluation of H56 specific antibody isotypes*

173   Serum samples were assessed for levels of IgG1 and IgG2b antibodies by the enzyme-

174   linked immunosorbent assay (ELISA). The ELISA plates (96 well, flat bottomed, high

175   binding, Greiner Bio-One Ltd, Gloucestershire, UK) were firstly coated with 3 µg/mL

176   H56 antigen prior to overnight incubation at 4 °C. All plates were washed three times

177   with PBST wash buffer (40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.2 g Na_2HPO_4, ($2H_2O$) per

178   5 litres of ddH_2O, incorporating ~0.4 mL of Tween 20) using a using a plate washer

179   (Microplate washer, MTX Lab Systems, INC., Virginia, USA) with subsequent

180   blotting to remove unbound antigen. Plates were blocked by coating each well with

181   100 µL of Marvel in PBS (dried skimmed milk powder, 4% W/V, Premier Foods,

182   Hertfordshire, UK) and incubated for one hour at 37 °C before washing three times

183   with PBST buffer. 140 µL of serum sample was serially diluted in PBS (70 µL

184   sequentially), added to the washed ELISA plates and incubated for one hour at 37 °C.

185   Known positive serum and pooled naïve mice sera were used as positive and negative

186   controls respectively. Plates were washed five times with PBST buffer before the

187   addition of 60 µL/well of horseradish peroxidise (HRP) conjugated anti-mouse

188   isotype specific immunoglobulins of IgG1 and IgG2b (AbD serotec, Oxfordshire,

189   UK), to identify anti-H56 antibodies. Plates were washed a further five times with

190   PBST buffer before adding 60 µL/well substrate solution (colouring agent: 6x 10 mg

191   tablets of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma,

192   Dorset, UK) in citrate buffer (0.92g Citric Acid + 1.956g NA_2 HPO_4 per 100 ml)

193   incorporating 10 µL of hydrogen peroxide (30% H_2O_2/100 ml) and incubation for 30

194   min at 37 °C. Absorbance was read at 405 nm using a microplate reader (Bio-Rad

195   Laboratories, model 680, Hertfordshire, UK).

196

197   *Quantification of cytokines via the sandwich ELISA*

198   Isolation of splenocyte cell suspensions and plating onto 96 well cell culture plates

199   was conducted as summarised in section 2.6.1. The cells were subsequently incubated

200   for 48 hours at 37 °C, prior to supernatant removal and storage at -70 °C for future

201   analysis. Quantification of the cytokines, IL-2, IL-5, IL-10 and IFN-γ within cell

202   culture supernatants took place using each specific DuoSet ELISA development kit

203   (R&D Systems, Oxfordshire, UK). The plates were firstly coated with 100 µL capture

204   antibody per well and incubated at room temperature overnight. The plates were then

205   washed three times with PBST buffer before blocking and incubation at room

206   temperature for one hour before washing a further three times. 100 µL/well of

207   samples/standards was then added to each well and incubated for two hours at room

208   temperature. The plates were washed three times before adding 100 µL of cytokine

209   specific detection antibody per well and incubating for two hours at room

210   temperature, prior to washing three times and adding 100 µL of streptavidin-

211   horseradish peroxidise (HRP) per well (diluted 1/200). The plates were then covered

212   to avoid exposure to direct light and incubated at room temperature for 20 min. After

213   three washes, 100 µL of substrate solution was added per well (1:1 mixture of colour

214   reagent A and B: stabilised hydrogen peroxide and stabilised tetramethylbenzidine

215   (TMB) respectively) and the plates were covered and incubated at room temperature

216   for 20 min. The experimental reaction was stopped by adding 50 µL stop solution (2N

217   H_2SO_4) per well and the optical density was then determined using a microplate

218   reader at 450 nm (Bio-Rad Laboratories, model 680, Hertfordshire, UK).

219

Statistical analysis

Data was tested by one-way analysis of variance (ANOVA) followed by the Tukey test in order to compare the mean values of different groups. Differences were considered to be statistically significant at *p* < 0.05.

Results

DDA presence within the liposomal system provides a cationic zeta potential for adsorption of H56 antigen

In order to investigate the role of the cationic DDA component in the DDA:TDB liposomal adjuvant system previously shown to be an effective adjuvant [e.g. 2, 15,16], DDA was gradually replaced with DSPC (also previously been used in a range of vaccine formulations [e.g. 17, 18]) whilst the amount of TDB remained fixed within the formulation. These vesicles were then mixed with H56 antigen (0.1 mg/ml) and their size, zeta potential and antigen loading measured (Figure 1). The DDA/TDB vesicles were 650 – 750 nm in size, with a highly cationic zeta potential (~70 mV) which promoted strong antigen loading efficiency (~85 %, 0.1 mg/ml; Figure 1). As DDA was replaced with DSPC within the formulation, the cationic nature of the vesicles decreases which subsequently reduces their ability to electrostatically bind the anionic H56 antigen (from 84% down to 15% when DDA is replaced with DSPC; Figure 1). However, in terms of vesicle size, it was only when DDA was completed replaced with DSPC that vesicle size significantly (p < 0.001) increased (~ 1.4 µm; Figure 1).

An increased cationic DDA content generates a Th1-skewed antibody profile

Given the differences in the formulations shown in figure 1, we further investigated the impact of DDA concentration on the ability of these systems to act as adjuvants. Female C57BL/6 mice in groups of 5 were immunised with the various liposome/antigen formulations (outlined in Figure 1) and mice received a 5 µg antigen dose intramuscularly three times, with two week intervals between each immunisation. Figure 2 shows IgG1 and IgG2b responses (as reciprocal end point serum dilution) over the period of the study. By day 24, all formulations produced significantly (p<0.05) higher IgG1 antibody responses compared to responses in mice immunised with free antigen (Figure 2); however, there was no significant difference

between the IgG1 responses stimulated by the various liposome formulations. In contrast, for IgG2b responses, only mice immunised with the formulations containing 250 or 150 µg DDA/dose gave significantly higher immune responses that those mice immunised with free antigen (Figure 2). This would suggest the higher level of DDA within the formulation potentiates a stronger Th1-type antibody profile.

Spleen cell proliferation levels increased with increased DDA concentration

To further consider the impact of DDA/DSPC content in liposomal adjuvants, antigen specific spleen cell proliferation upon re-stimulation with H56 antigen at the concentrations of 0, 0.05, 0.5, 5 and 25 µg/mL. From the formulations tested there was a general DDA dose dependent trend, with DDA-TDB (250/50 µg/dose) inducing the peak of proliferation, indicated by elevated levels of [^{3}H]Thymidine (Figure 3) with significantly ($p < 0.05$) higher splenocyte proliferation, upon re-stimulation with H56 antigen at 0.05-25 µg/ml, compared to the other formulations with lower DDA contents (Figure 3).

Cell mediated immune responses correlate DDA concentration with Th1 responses

T-cells harvested upon vaccination were tested for their ability to generate a range of cytokines after restimulation with H56. It is understood that cellular immunity and especially a Th1 response is vital to mediate protection against intracellular pathogens such as MTB. IFN-γ is the prime indicator of a Th1 type effector response [19] whereas IL-2 is generated by Th1 central memory cells and essential to T-cell proliferation [20]. Interleukins 5 and 10 are associated with Th2 type responses with IL-5 stimulating growth and differentiation of B cells and enhancing immunoglobulin secretion, whilst IL-10 down-regulates the expression of Th1 cytokines.

In terms of IFN-γ production, again there is a trend of increasing IFN-γ production with increasing DDA (and reducing DSPC) content within the liposome formulation with DDA-TDB (250/50 µg) stimulated the highest levels (Fig. 4A). Indeed complete replacement of DDA with DSPC resulted in IFN-γ levels in line with the naive and non-adjuvanted H56 vaccine groups (data not shown). A similar DDA dose dependent effect was observed for the quantified levels of IL-2 (Fig. 4B), suggesting that increasing levels of DDA promotes a stronger Th1 bias response. For IL-5 and IL-10 production (Fig 4C and 4D respectively), a reversal of this trend is seen with higher

levels of DSPC (and reducing levels of DDA) promoting higher IL-5 and IL-10 production suggesting the Th2 bias responses increase as the Th1 responses decrease.

These outcomes correlate with the antibody isotypes trends of reducing DDA content within the liposomal formulations reducing the Th1 IgG2b responses (Figure 2). Indeed, the well-defined Th1-skewed antibody profile observed for DDA-TDB, previously shown to be most effective in a 5:1 weight ratio [6], has been associated with a strong Th1response [15]. More specifically, DDA is believed to promote accelerated antigen uptake by antigen presenting cells [21], whilst TDB enables a pro-inflammatory response to obtain a Th1 cytokine imprint [15].

Addition of DSPC to cationic liposomes reduces Th1 responses

To consider if these changes in adjuvant performance were a result of the replacement of DDA with DSPC, or a result of reducing DDA concentration within the formulation alone, an additional vaccine formulation was considered where the DDA content was reduced to 100 μg/dose but no DSPC was added to the formulation (therefore DDA:DSPC:TDB 100/150/50 μg vs. 100/0/50 μg /dose; Table 1). In terms of physico-chemical attributes, the vesicles were comparable in size and zeta potential (Table 1). Similarly in terms of immune response profiles, IgG1 and IgG2b responses were not significantly different over the period of the study yet spleen cell proliferation levels were significantly higher (approximately 2 fold higher) for the formulation without the addition of DSPC (Table 1). In terms of the cytokine profile promoted by the liposomal adjuvants, the addition of DSPC to the DDA:TDB formulation make no significant different to IL-5 and IL-10 responses; however, the addition of DSPC within the formulation significantly ($p<0.05$) reduced IFN-γ and IL-2 responses (Table 1). This would suggest that at equivalent DDA concentrations, the inclusion of DSPC within the liposomal adjuvant formulation reduces the Th1 types responses.

Discussion

The mechanism of DDA:TDB has been investigated and through a range of studies; the key attributes of the system has been identified as the combination of the ability to co-deliver antigen and immunomodulators to antigen presenting cells (which may result from these systems forming a depot at the injection site), and the ability for the system to stimulate these antigen presenting cells [22]. Physicochemical factors that

322 control and promote these attributes include the cationic nature of the vesicles and the
323 rigidity of the liposomal bilayer with vesicle size playing a less important role [23].
324 Indeed recent studies suggest that these physicochemical characteristics are key in
325 controlling the ability of the liposomes to promote the formation of an
326 adjuvant/antigen depot at the injection site, and that Th1 responses may be supported
327 by depot formation whilst Th2 responses are not reliant on a vaccine depot, as is the
328 case with Alum [25-26]. Indeed recent studies by Kamath et al. [22] have shown that
329 synchronisation of dendritic cell activation and antigen exposure is required for the
330 induction of Th1/ Th17 responses. By comparing responses from mice immunised
331 with antigen adsorbed to DDA/TDB with mice immunised with antigen and
332 DDA/TDB separately (but at the same site), the authors were able to show that both
333 immunisation strategies produced the same weak Th2 immune responses. However,
334 injection of vaccine and antigen separately, but to the same site, produced weaker Th1
335 responses than immunisation with DDA/TDB with adsorbed antigen [25]; by injecting
336 of antigen and adjuvant separately, early production of an Antigen+/Adjuvant-
337 dendritic cell (DC) population with a non-activated phenotype was promoted [22].
338 Furthermore the authors were able to demonstrate that such DCs could recruit
339 Antigen-specific T cells and trigger their initial proliferation, but this interfered with
340 Th1 induction in a dose dependent manner [27].

341

342 To consider the controlling role of charge in the above attributes of DDA:TDB,
343 previous studies [8] have considered the impact of complete replacement of DDA
344 with DSPC and demonstrated that cationic nature of the liposomes, induced by the
345 DDA content, promotes prolonged antigen presentation and inducing Th1 type
346 responses, and replacing DDA with DSPC removed the depot-forming action of the
347 vesicles and reduced Th1 responses. To this end, within this study we have explored
348 the impact of varying the ratio of cationic DDA to the 'neutral' DSPC lipid in
349 liposomal adjuvant formulations on Th1 control. Within this study we show a
350 concentration DDA/DSPC dependent Th1 immune response profile. Furthermore it
351 was shown that replacement of DDA with DSPC, rather than a reduction in DDA
352 content alone, was the controlling factor. This may be due to the DDA/DSPC
353 formulations offering reduced loading/retention of the antigen after administration,
354 resulting in loss of antigen and therefore loss of antigen/adjuvant synchronisation of
355 DC targeting, shown as a critical factor in determining Th1 responses [27].

356

357 **Conclusion**

358 This present study demonstrates the that Th1 responses generated from a liposomal
359 DDA adjuvant system are dose controlled with the ratio of the cationic lipid DDA, to
360 the 'neutral' DSPC lipid impacting on the Th1 responses. With the exception of full
361 DDA replacement with DSPC, the physicochemical findings demonstrated no major
362 differences in terms of particle size, but a general decrease in zeta potential as DDA
363 content reduced was noted. This change in cationic nature was also linked to the
364 immune response profile, with immune responses being modulated by the DDA to
365 DSPC ratio adopted for the proposed adjuvants. However given that replacement of
366 DDA with DSPC within the formulation had more of an impact on immunological
367 responses that merely reducing the DDA content alone, this would suggest that
368 consideration of the overall lipid content compared to DDA content within a
369 liposomal construct is an important parameter to consider.

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Table 1: Comparison of formulations of DDA/TDB (100/50 µg) with and without DSPC.

461

Factor	DDA/DSPC/TDB		Significance
	100/150/50 µg	100/0/50 µg	
z-average diameter (nm)	626 ± 46	693 ± 64	n/s
Zeta potential (mV)	46 ± 5	48 ± 6	n/s
IgG1 (serial end point dilution, $\log_{10}$) Day 24 Day 37 Day 49	4.30 ± 0.00 4.60 ± 0.30 4.54 ± 0.25	4.36 ± 0.70 4.78 ± 0.16 4.54 ± 0.39	n/s n/s n/s
IgG2b (serial end point dilution, $\log_{10}$) Day 24 Day 37 Day 49	3.70 ± 0.30 4.12 ± 0.45 4.06 ± 0.39	3.82 ± 0.33 4.54 ± 0.33 4.42 ± 0.45	n/s n/s n/s
Spleen cell proliferation (counts/CPM)	11416 ± 8441	28149 ± 6672	P < 0.05
IFN-γ (pg/mL)	1454 ± 474	2789 ± 662	P < 0.05
IL-2 (pg/mL)	1491 ± 509	2887 ± 585	P < 0.05
IL-5 (pg/mL)	301 ± 68	289 ± 60	n/s
IL-10 (pg/mL)	129 ± 29	125 ± 36	n/s

462
Results represent mean ± SD for n=3 for liposome characterisation and n=5 for in vivo responses. For further details on antibody responses, spleen cell proliferation and cytokine levels see Figures 2, 3 and 4 respectively.
466

Figure legends.

468 Figure 1. Physico-chemical characteristics of liposomes prepared with varying ratios

469 of DDA and DSPC combined with TDB. A) TEM and visual images of liposomes

470 prepared. B) Vesicle size, polydispersity and zeta potential together with C) H56

471 antigen loading. Vesicles were prepared via lipid hydration in Tris buffer (10 mM, pH

472 7.4), with systems surface adsorbed with 0.1 mg/ml H56 antigen and measured in 1

473 mM Tris buffer. Results represent the mean average ± standard deviation (n=3).

474

475 Figure 2. H56 specific antibody titres generated by DDA-TDB and its cationic

476 replacement with DSPC for IgG1 and IgG2b. Values represent µg/dose, with sera

477 collected before the first immunisation and on days 9, 24, 37 and 49 thereafter, and

478 analysised for anti-H56 antibodies by ELISA. Results signify the reciprocal end point

479 dilution (log10) compared with untreated control sera (n=5 ± SD). Significance is

480 illustrated as p<0.05 increase compared to H56 vaccination group.

481

482 Figure 3. Spleen cell proliferation in response to stimulation/re-stimulation with H56

483 antigen upon replacement of cationic content within DDA-TDB. Formulation values

484 represent µg/dose, with DDA-TDB and DSPC-TDB set to a 5:1 weight ratio

485 (DDA/DSPC/TDB at 250/0/50 and 0/250/50 µg/dose respectively). H56 antigen

486 specific splenocyte proliferation was indicated by the level of [^{3}H]Thymidine

487 incorporation into cultured splenocytes at antigen concentrations of 0-25 µg/ml. ConA

488 was used as a positive control at 2µg/mL with all counts in the region of 100,000

489 CPM. The results displayed denote the mean average for each group with associated

490 standard error at n=5. Significance is illustrated between the liposomal vaccination

491 groups, and comparisons shown against one another are upon re-stimulation with H56

492 vaccine antigen at 25 µg/mL.

493

494 Figure 4. IFN-γ, IL-2, -5, and -10 cytokine production from splenocytes (A–D)

495 derived from mice immunised with H56 combined with DDA/DSPC/TDB liposomes.

496 Mice received 3 injections with 2-week intervals; splenocytes were obtained 3 weeks

497 post the final immunisation. Splenocytes were restimulated for 48 h in the presence of

498 H56 (5 µg/ml). Cytokines were measured from splenocyte using sandwich ELISAs.

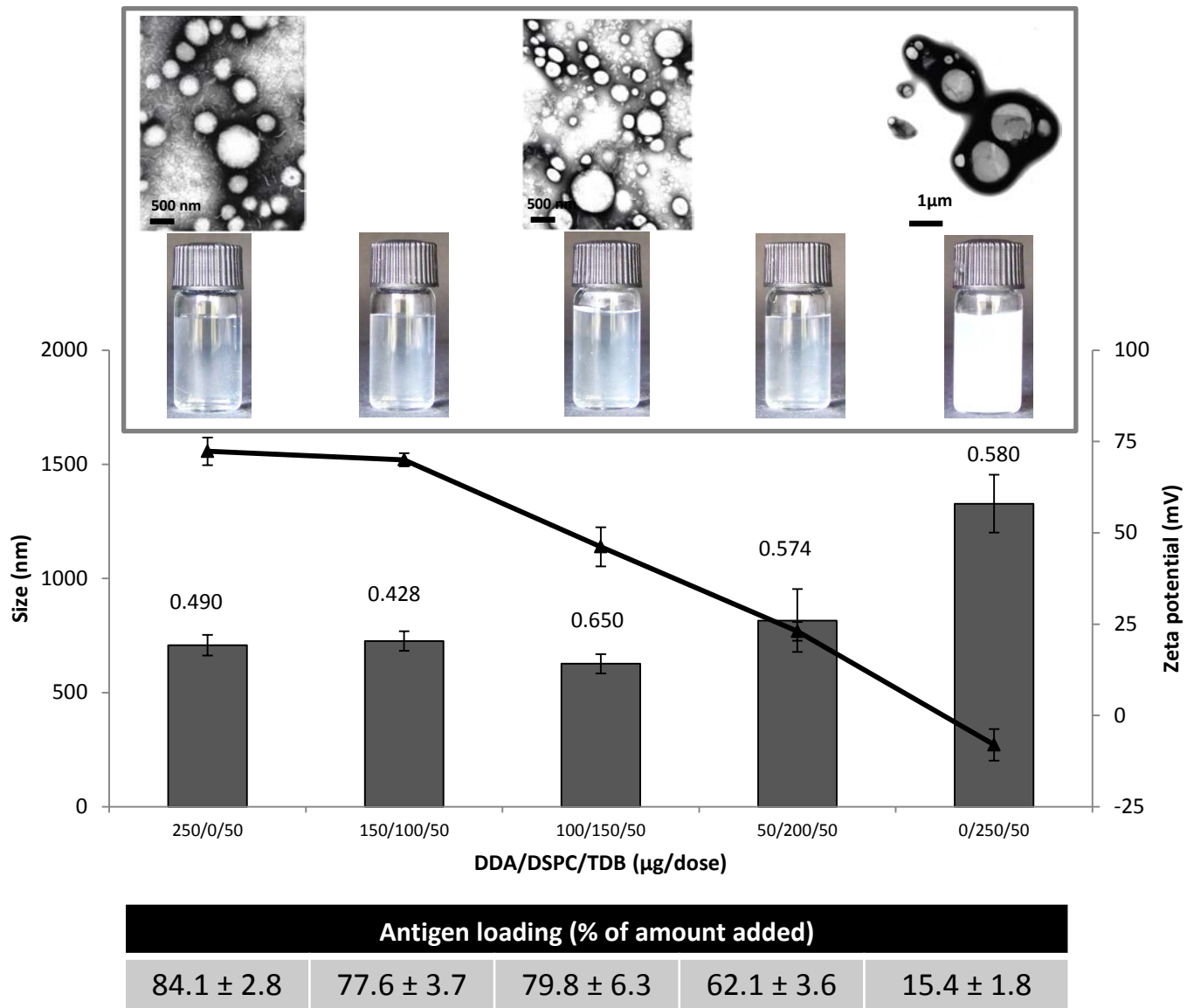| Antigen loading (% of amount added)				
84.1 ± 2.8	77.6 ± 3.7	79.8 ± 6.3	62.1 ± 3.6	15.4 ± 1.8

Figure 1

Reciprocal end point serum dilution (log_{10})

IgG2b antibody titre

5.5	4.5	3.5	2.5	1.5	0.5

Formulation (μg/dose)

DDA	DSPC	TDB
250	0	50
150	100	50
100	150	50
50	200	50
0	250	50
Free antigen		

IgG1 antibody titre

0.5	1.5	2.5	3.5	4.5	5.5

Legend:
- ☐ Day 9
- ☐ Day 24
- ☐ Day 37
- ☐ Day 49

Figure 2

Figure 3

Figure 4