

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

4
5 Authors: M. Jubair Hussain^a, Alexander Wilkinson^a, Vincent W Bramwell^a, Dennis
6 Christensen^b, and Yvonne Perrie^a

7 ^aSchool of Life and Health Sciences, Aston University, Birmingham, UK.

8 ^bStatens Serum Institute, Copenhagen, Denmark.

9
10 **Key Words:** Cationic liposomes, vaccine adjuvant, dimethyldioctadecylammonium,
11 distearoyl-sn-glycero-3-phosphocholine, Th1 responses.

12
13 **Corresponding author:**

14 **Professor Yvonne Perrie**
15 **School of Life and Health Sciences,**
16 **Aston University,**
17 **Aston Triangle,**
18 **Birmingham,**
19 **B4 7ET.**
20 **Tel: +44 121 204 3991**
21 **Email address: y.perrie@aston.ac.uk**

22
23 **Date of Manuscript: October 2013**

24

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 completely 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

47 **Introduction**

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

75

76 Although DDA-TDB is a well characterised vaccine delivery system, key
77 characteristics that dictate the adjuvant properties of the liposomes are still not fully
78 understood. To further develop this, in this study, cationic DDA content was gradually
79 replaced with an alternative non-cationic lipid, the neutral lipid distearoyl-sn-glycero-
80 3-phosphocholine (DSPC), in order to further consider the role of DDA within DDA-
81 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 [³H] 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 N₂ 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

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

122

123 *Radiolabelling of H56 antigen*

124 The protein antigen, H56 was radiolabelled with ¹²⁵I using IODOGEN® pre-coated
125 iodination tubes (Pierce Biotechnology, Rockford, IL). Separation of labelled protein
126 from free ¹²⁵I was carried out using a Sephadex G-75 gel column, pre-soaked in
127 ddH₂O and equilibrated with Tris buffer (10 mM; pH 7.4). This method was then
128 carried out as described previously [2].

129

130 *Quantification of H56 antigen adsorption*

131 Radiolabelled (¹²⁵I) H56 antigen was added to each liposome formulation at an *in vivo*
132 concentration of 5 µg per dose (0.1 mg/ml), and left to surface adsorb to the liposome
133 for 45 minutes with intermittent swirling. Surface-adsorbed and non-adsorbed protein
134 antigen within the liposomal suspensions were separated by diluting the suspension to
135 1 ml using Tris buffer (10 mM; pH 7.4), followed by centrifugation using an Optima
136 Max-XP Ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA). The quantity of
137 radiolabelled antigen (¹²⁵I-H56) prior to centrifugation and within subsequent
138 fractions (pellet and supernatant) was measured using a Cobra™ CPM Auto-
139 Gamma® counter (Packard Instruments Company Inc., Downers Grove, IL). The
140 total recovery of protein antigen was then determined by calculating the %
141 radioactivity in the liposome pellet fraction.

142

143 *Vaccine study: Immunisation of mice*

144 All experiments were undertaken in accordance with the 1986 Scientific Procedures
145 Act (UK). Female C57BL/6 mice, 6-8 weeks old (Charles River, UK) were split into
146 11 groups of 5. Vaccine preparations were made with the liposomes adsorbing H56)
147 antigen to a final concentration of 0.1 mg/mL (5 µg/vaccine dose). All mice, with the
148 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% CO₂, and upon
164 72 hours incubation, 40 µL of [³H] 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 [³H] 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 KH₂PO₄, 7.2 g Na₂HPO₄, (2H₂O) per
178 5 litres of ddH₂O, 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 peroxidase (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_2HPO_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 peroxidase (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

220 *Statistical analysis*

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

224

225 **Results**

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

228 In order to investigate the role of the cationic DDA component in the DDA:TDB
229 liposomal adjuvant system previously shown to be an effective adjuvant [e.g. 2,
230 15,16], DDA was gradually replaced with DSPC (also previously been used in a range
231 of vaccine formulations [e.g. 17, 18]) whilst the amount of TDB remained fixed
232 within the formulation. These vesicles were then mixed with H56 antigen (0.1 mg/ml)
233 and their size, zeta potential and antigen loading measured (Figure 1). The
234 DDA/TDB vesicles were 650 – 750 nm in size, with a highly cationic zeta potential
235 (~70 mV) which promoted strong antigen loading efficiency (~85 %, 0.1 mg/ml;
236 Figure 1). As DDA was replaced with DSPC within the formulation, the cationic
237 nature of the vesicles decreases which subsequently reduces their ability to
238 electrostatically bind the anionic H56 antigen (from 84% down to 15% when DDA is
239 replaced with DSPC; Figure 1). However, in terms of vesicle size, it was only when
240 DDA was completely replaced with DSPC that vesicle size significantly ($p < 0.001$)
241 increased (~ 1.4 μm ; Figure 1).

242

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

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

253 between the IgG1 responses stimulated by the various liposome formulations. In
254 contrast, for IgG2b responses, only mice immunised with the formulations containing
255 250 or 150 µg DDA/dose gave significantly higher immune responses than those mice
256 immunised with free antigen (Figure 2). This would suggest the higher level of DDA
257 within the formulation potentiates a stronger Th1-type antibody profile.

258

259 *Spleen cell proliferation levels increased with increased DDA concentration*

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

268

269 *Cell mediated immune responses correlate DDA concentration with Th1 responses*

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

278

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

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

289

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

297

298 *Addition of DSPC to cationic liposomes reduces Th1 responses*

299 To consider if these changes in adjuvant performance were a result of the replacement
300 of DDA with DSPC, or a result of reducing DDA concentration within the
301 formulation alone, an additional vaccine formulation was considered where the DDA
302 content was reduced to 100 µg/dose but no DSPC was added to the formulation
303 (therefore DDA: DSPC: TDB 100/150/50 µg vs. 100/0/50 µg /dose; Table 1). In terms
304 of physico-chemical attributes, the vesicles were comparable in size and zeta potential
305 (Table 1). Similarly in terms of immune response profiles, IgG1 and IgG2b responses
306 were not significantly different over the period of the study yet spleen cell
307 proliferation levels were significantly higher (approximately 2 fold higher) for the
308 formulation without the addition of DSPC (Table 1). In terms of the cytokine profile
309 promoted by the liposomal adjuvants, the addition of DSPC to the DDA: TDB
310 formulation make no significant different to IL-5 and IL-10 responses; however, the
311 addition of DSPC within the formulation significantly ($p < 0.05$) reduced IFN- γ and IL-
312 2 responses (Table 1). This would suggest that at equivalent DDA concentrations, the
313 inclusion of DSPC within the liposomal adjuvant formulation reduces the Th1 types
314 responses.

315

316 **Discussion**

317 The mechanism of DDA: TDB has been investigated and through a range of studies;
318 the key attributes of the system has been identified as the combination of the ability
319 to co-deliver antigen and immunomodulators to antigen presenting cells (which may
320 result from these systems forming a depot at the injection site), and the ability for the
321 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.

370

371 **Acknowledgements**

372 This work was partly funded by newTBVAC (contract no. Health-F3-2009-241745).
373 NewTBVAC has been made possible by contributions from the European
374 Commission.

375

376 **References**

- 377 [1] Cox JC and Coulter AR. Adjuvants- a classification and review of their modes
378 of action. *Vaccine* 1997; 15: 248-256.
- 379 [2] Henriksen-Lacey M et al. Liposomes based on dimethyldioctadecylammonium
380 promote a depot effect and enhance immunogenicity of soluble antigen. *J.*
381 *Controlled Release* 2010; 142: 180-186.
- 382 [3] Korsholm KS et al. T-helper 1 and T-helper 2 adjuvants induce distinct
383 differences in the magnitude, quality and kinetics of the early inflammatory
384 response at the site of injection. *Immunology* 2009; 129: 75-86.
- 385 [4] Henriksen-Lacey M et al. Comparison of the depot effect and immunogenicity
386 of liposomes based on DDA, DC-Chol and DOTAP: Prolonged liposome
387 retention mediates stronger Th1 responses. *Mol. Pharmaceutics* 2011; 8: 153-
388 161.
- 389 [5] Holten-Andersen L et al. Combination of the Cationic Surfactant Dimethyl
390 Dioctadecyl Ammonium Bromide and Synthetic Mycobacterial Cord Factor as
391 an Efficient Adjuvant for Tuberculosis Subunit Vaccines. *Infect. Immun.*
392 2004; 72: 1608-1617.
- 393 [6] Davidsen J et al. Characterization of cationic liposomes based on
394 dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis*
395 (trehalose 6,6'-dibehenate)-a novel adjuvant inducing both strong CMI and
396 antibody responses. *Biochim. Biophys. Acta.* 2005; 1718: 22-31.
- 397 [7] Werninghaus K et al. Adjuvant activity of a synthetic cord factor analogue for
398 subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-
399 Card9-dependent innate immune activation. *J. Exp. Med.* 2009; 206: 89-97.
- 400 [8] Henriksen-Lacey M et al. Liposomal cationic charge and antigen adsorption
401 are important properties for the efficient deposition of antigen at the injection
402 site and immunogenicity of the vaccine *J Controlled Release* 2010; 145: 102-
403 108.
- 404 [9] Henriksen-Lacey M et al. The vesicle size of DDA:TDB liposomal adjuvants
405 plays a role in the cell-mediated immune response but has no significant effect
406 on antibody production. *J Control Release* 2011; 154: 131-137.
- 407 [10] Senior J, Gregoriadis G. Stability of small unilamellar liposomes in serum and
408 clearance from the circulation: the effect of the phospholipid and cholesterol
409 components. *Life Sciences.* 1982; 30: 2123-2136.
- 410 [11] Aagaard C et al. A multistage tuberculosis vaccine that confers efficient
411 protection before and after exposure. *Nat. Med.* 2011; 17: 189-194
- 412 [12] Bangham AD et al. Diffusion of univalent ions across the lamellae of swollen
413 phospholipids. *J. Mol. Biol.* 1965; 13: 238-252.
- 414 [13] Christensen D et al. Trehalose preserves DDA/TDB liposomes and their
415 adjuvant effect during freeze-drying. *Biochim. Biophys. Acta.* 2007; 1768:
416 2120-2129.
- 417 [14] Ali S et al. A differential scanning calorimetry study of phosphocholines
418 mixed with paclitaxel and its bromoacylated taxanes. *Biophys. J.* 2000; 78:
419 246-256.
- 420 [15] Agger EM et al. Cationic liposomes formulated with synthetic mycobacterial
421 cordfactor (CAF01): a versatile adjuvant for vaccines with different
422 immunological requirements *PloS One* 2008; 3: 1-10.
- 423 [16] Christensen D et al. CAF01 liposomes as a mucosal vaccine adjuvant: In vitro
424 and in vivo investigations. *Int. J. Pharm.* 2010; 390: 19-24.

- 425 [17] Mazumdar T et al. A mixed Th1/Th2 response elicited by a liposomal
426 formulation of Leishmania vaccine instructs Th1 responses and resistance to
427 Leishmania donovani in susceptible BALB/c mice. *Vaccine*. 2004; 1162-1171.
- 428 [18] McNeil et al. Subunit vaccines: distearoylphosphatidylcholine-based
429 liposomes entrapping antigen offer a neutral alternative to
430 dimethyldioctadecylammonium-based cationic liposomes as an adjuvant
431 delivery system. *J Pharm Sci* 2011; 1856-1865.
- 432 [19] Sable SB et al. Tuberculosis subunit vaccine design: the conflict of
433 antigenicity and immunogenicity. *Clin. Immunol*. 2007; 122: 239–251.
- 434 [20] Vangala, A et al. Comparison of vesicle based antigen delivery systems for
435 delivery of hepatitis B surface antigen. *J Control Release* 2007; 119: 102-110.
- 436 [21] Korsholm KS et al. The adjuvant mechanism of cationic
437 dimethyldioctadecylammonium liposomes. *Immunology* 2007; 121: 216-226.
- 438 [22] Christensen D et al. A cationic vaccine adjuvant based on a saturated
439 quaternary ammonium lipid have different in vivo distribution kinetics and
440 display a distinct CD4 T cell-inducing capacity compared to its unsaturated
441 analog. *J Control Release* 2012; 160: 468-476.
- 442 [23] Perrie Y et al. A case-study investigating the physicochemical characteristics
443 that dictate the function of a liposomal adjuvant. *Hum Vaccin Immunother*.
444 2013; 12. In press.
- 445 [24] Noe SM et al. Mechanism of immunopotentiality by aluminium-containing
446 adjuvants elucidated by the relationship between antigen retention at the
447 inoculation site and the immune response, *Vaccine* 2010; 28: 3588-94.
- 448 [25] Romero Mendez IZ et al. Potentiation of the immune response to non-
449 adsorbed antigens by aluminium-containing adjuvants. *Vaccine* 2007; 25: 825-
450 33.
- 451 [26] Hutchison S, et al. Antigen depot is not required for alum adjuvant activity.
452 *FASEB J* 2012; 26: 1272-9.
- 453 [27] Kamath AT et al. Synchronization of dendritic cell activation and antigen
454 exposure is required for the induction of Th1/Th17 responses. *J Immunol*
455 2012; 188: 4828-37.
- 456
- 457

458
459
460
461

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

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
<u>IgG1 (serial end point dilution, log₁₀)</u>			
Day 24	4.30 ± 0.00	4.36 ± 0.70	n/s
Day 37	4.60 ± 0.30	4.78 ± 0.16	n/s
Day 49	4.54 ± 0.25	4.54 ± 0.39	n/s
<u>IgG2b (serial end point dilution, log₁₀)</u>			
Day 24	3.70 ± 0.30	3.82 ± 0.33	n/s
Day 37	4.12 ± 0.45	4.54 ± 0.33	n/s
Day 49	4.06 ± 0.39	4.42 ± 0.45	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
463
464
465
466

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.

467 **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 \pm 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 $\mu\text{g}/\text{dose}$, with sera
477 collected before the first immunisation and on days 9, 24, 37 and 49 thereafter, and
478 analysed for anti-H56 antibodies by ELISA. Results signify the reciprocal end point
479 dilution (\log_{10}) compared with untreated control sera ($n=5 \pm \text{SD}$). 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 $\mu\text{g}/\text{dose}$, 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 $\mu\text{g}/\text{dose}$ respectively). H56 antigen
486 specific splenocyte proliferation was indicated by the level of [^3H]Thymidine
487 incorporation into cultured splenocytes at antigen concentrations of 0-25 $\mu\text{g}/\text{ml}$. ConA
488 was used as a positive control at 2 $\mu\text{g}/\text{mL}$ 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 $\mu\text{g}/\text{mL}$.

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 $\mu\text{g}/\text{ml}$). Cytokines were measured from splenocyte using sandwich ELISAs.

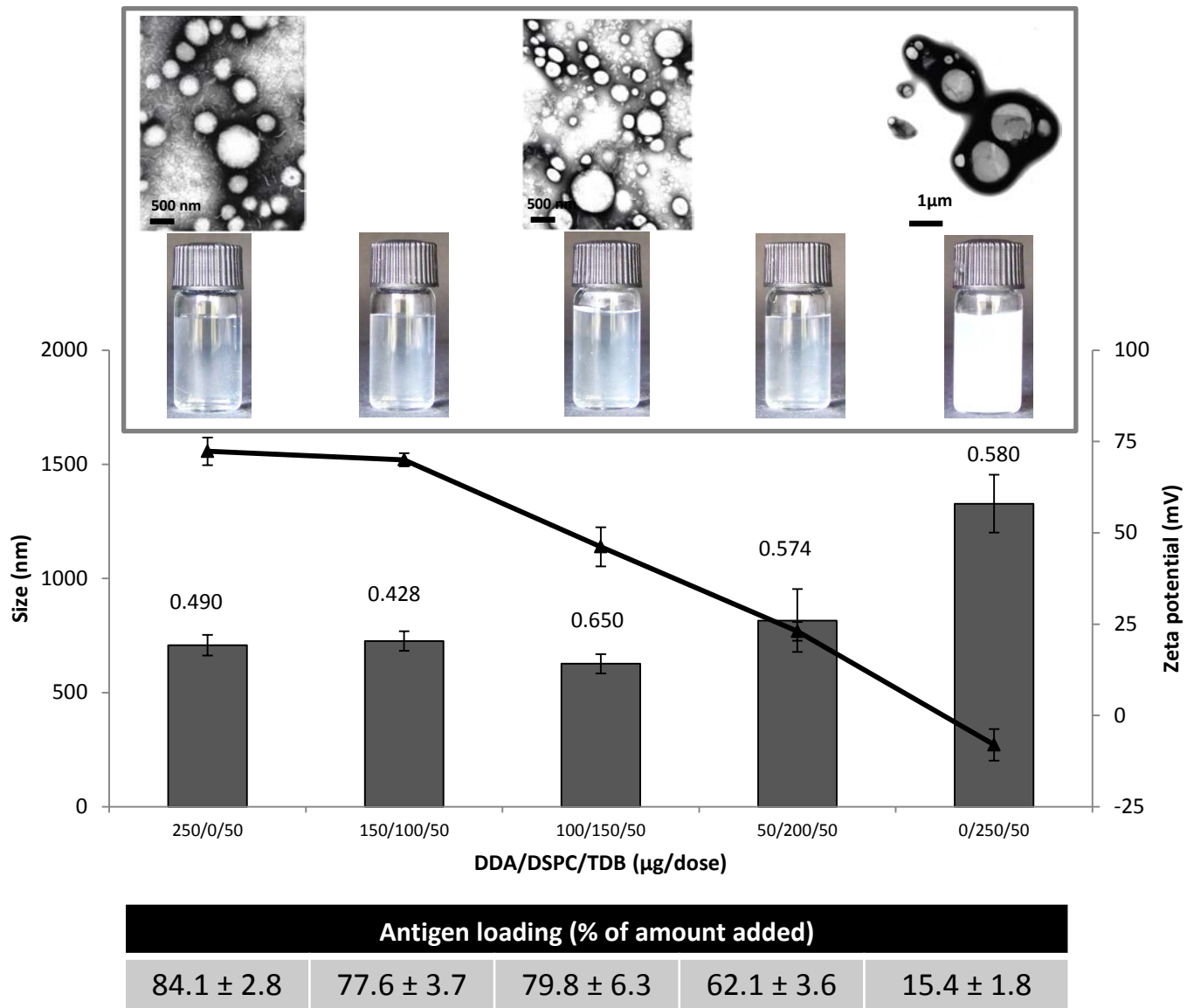


Figure 1

Reciprocal end point serum dilution (\log_{10})

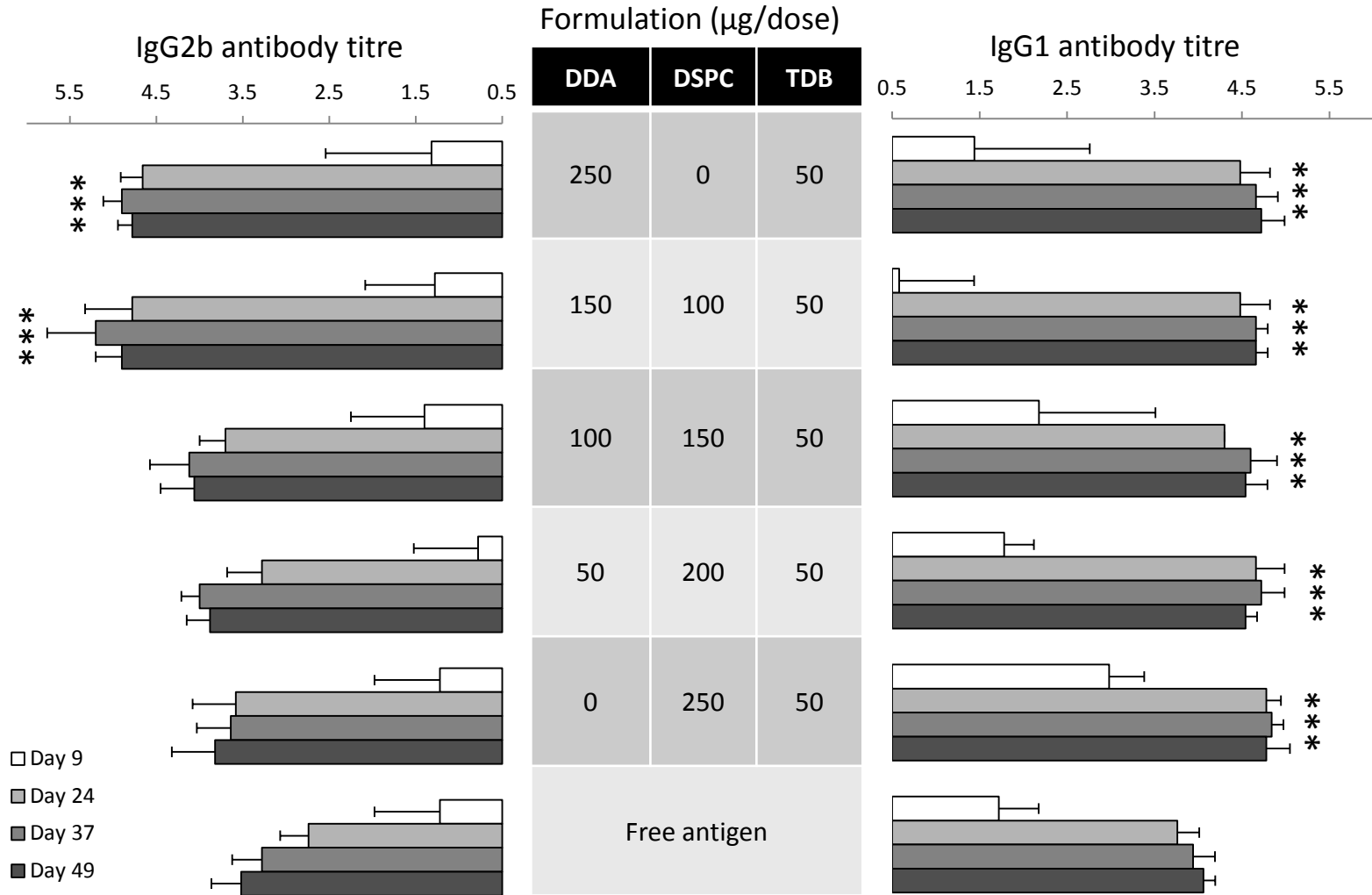


Figure 2

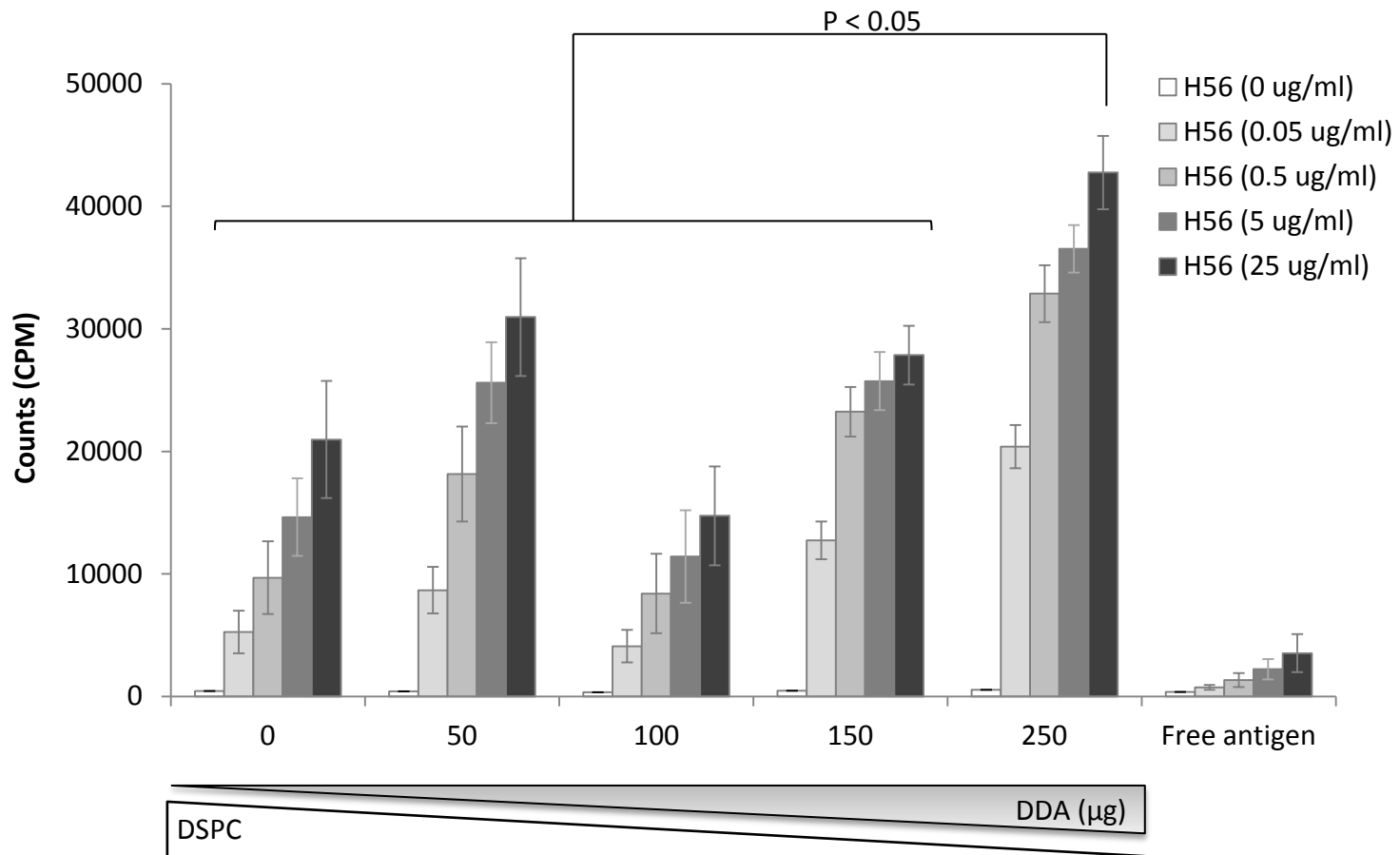


Figure 3

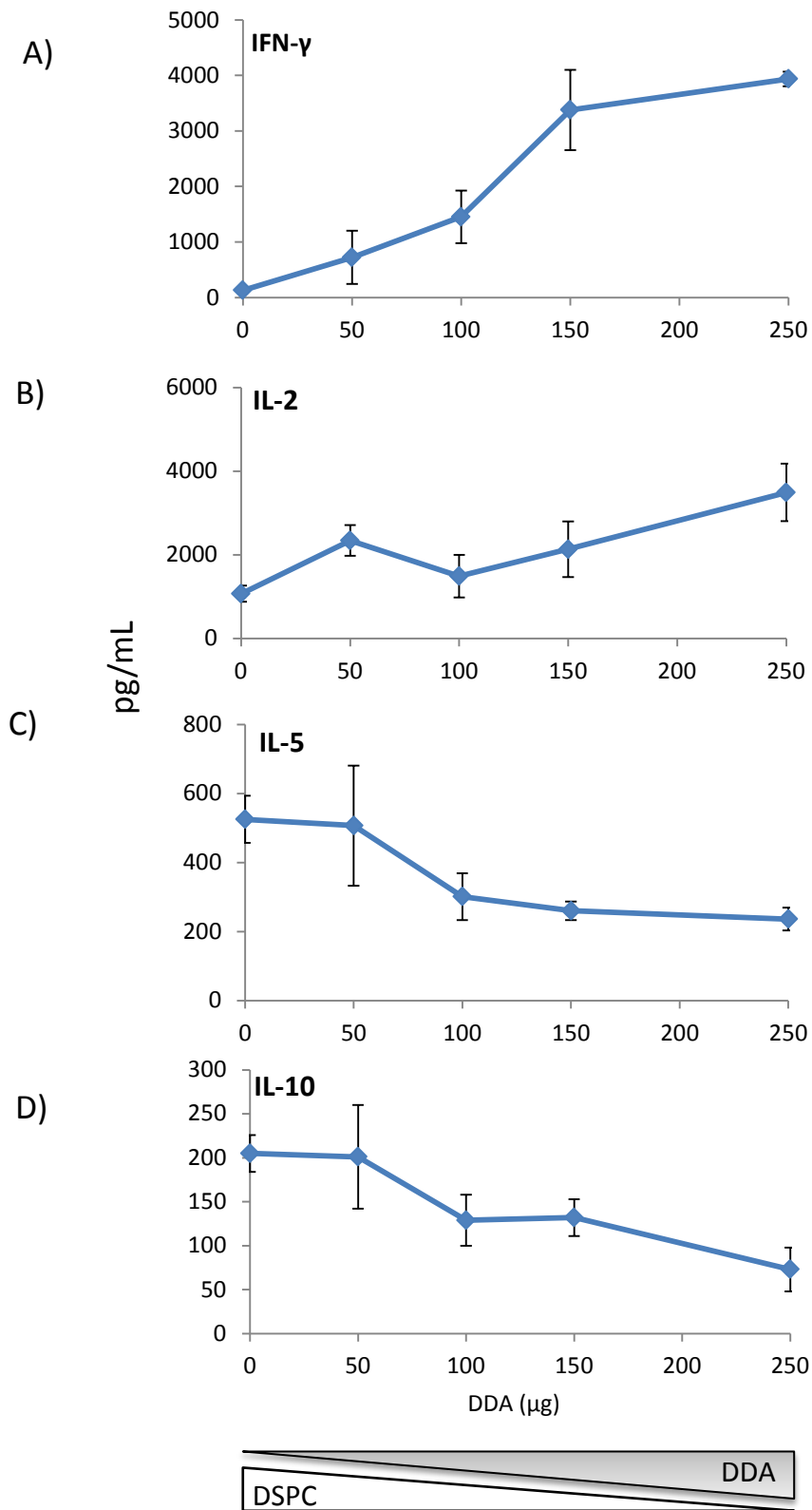


Figure 4