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1 **Title:** Correlating liposomal adjuvant characteristics to in-vivo cell mediated  
2 immunity using a novel mycobacterium tuberculosis fusion protein: A  
3 multivariate analysis study.  
4

5

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10

11 **Key Words:** Liposome, subunit vaccine, multivariate analysis, in vivo correlation

12

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22

23

24 **Abstract**

25 **Objective:** In this study, we have used a chemometrics-based method to correlate key  
26 liposomal adjuvant attributes with in vivo immune responses based on multivariate  
27 analysis.

28 **Methods:** The liposomal adjuvant, composed of the cationic lipid  
29 dimethyldioctadecylammonium bromide and trehalose 6,6-dibehenate was modified  
30 with 1,2-distearoyl-sn-glycero-3-phosphocholine at a range of mol% ratios and the  
31 main liposomal characteristics (liposome size and zeta potential) was measured along  
32 with their immunological performance as an adjuvant for the novel, post exposure  
33 fusion tuberculosis vaccine, Ag85B–ESAT-6-Rv2660c (H56 vaccine). Partial least  
34 square regression analysis was applied to correlate and cluster liposomal adjuvants  
35 particle characteristics with in-vivo derived immunological performances (IgG, IgG1,  
36 IgG2b, spleen proliferation, IL-2, IL-5, IL-6, IL-10, INF- $\gamma$ ).

37 **Key Findings:** Whilst a range of factors varied in the formulations, decreasing the  
38 DSPC content (and subsequent zeta potential) together built the strongest variables in  
39 the model. Enhanced DDA and TDB content (and subsequent zeta potential)  
40 stimulated a response skewed towards a cell mediated immunity, with the model  
41 identifying correlations with INF- $\gamma$ , IL-2 and IL-6.

42 **Conclusion:** This study demonstrates the application of chemometrics-based  
43 correlations and clustering, which can inform liposomal adjuvant design.

44

45

46

## 47 **Introduction**

48 For a vaccine to be regarded as effective, it must stimulate an adequate immune  
49 response, sustain safe administration and be patient friendly [1, 2]. Subunit vaccines  
50 contain selected purified antigens and potentially reduce side effects, eradicate  
51 reversion to virulence and the need for culturing harmful pathogens, whilst eliciting  
52 specific immune responses, ultimately generating a safer, more immunologically  
53 defined form of vaccination [2, 3]. As purified recombinant proteins generally induce  
54 low immunogenicity when administered alone, a suitable immunostimulatory adjuvant  
55 is required to produce a more potent vaccine [4, 5]. Liposomes are one of few  
56 immunological adjuvants approved for human administration and have been shown to  
57 be competent stimulators of an immune response [6]. In recent studies, key factors  
58 that influence the efficacy of liposomal adjuvant activity include vesicle charge, size  
59 and bilayer fluidity, as these affect interactions with immune system components [7].  
60 For example, enhance antigen adsorption and retention, and an increased intensity in  
61 intracellular liposome presence, promoted by using cationic liposomal adjuvants is  
62 seen as a viable approach for effective vaccine delivery [[1, 8, 9, ].

63

64 Despite potentially curative pharmacotherapies being readily available for many  
65 decades, tuberculosis (TB) is still the primary cause of preventable deaths worldwide  
66 [10]. The necessity of a host to inhibit Mycobacterium tuberculosis (MTB) infection is  
67 dependent upon the stimulation of cellular Th1 type immunity. Liposomal composition  
68 is a key variable that can influence the potency of such adjuvant delivery systems for  
69 TB vaccines. Cationic liposomes of dimethyldioctadecylammonium bromide (DDA)  
70 with an optimised incorporation of the glycolipid trehalose 6,6-dibehenate (TDB) forms  
71 an adjuvant system (CAF01) capable of stimulating powerful cell-mediated immunity  
72 against MTB, upon successful delivery of the recombinant TB fusion protein, Ag85B–  
73 ESAT-6 (H1 vaccine) [11].

74

75 With modern and high throughput analytical equipment, researchers often accumulate  
76 a large quantity of data, which necessitates the use of appropriate analytical tools for  
77 extraction of valuable information. Analysing such large data sets requires time and is  
78 a particular challenge for extracting the most useful information out of that data set.  
79 Computer-based methodologies are incorporated into the analysis of large data sets,  
80 in order to extract features within a reasonable timeframe. Often, the analysis of only  
81 one variable at a time is not sufficient and the simultaneous analysis of several  
82 variables is highly desirable. Multivariate analysis (MVA) is a flexible and multipurpose  
83 tool for data analysis. MVA can be used to provide an overview in a data set, for

84 classification and comparison between groups of data and for regression modelling  
85 between two sets of data, often referred as variables (X) and responses (Y). Opposed  
86 to multiple linear regression tools, MVA handles many variables and many  
87 observations at a time and deals with dimensionality problems. Furthermore, it can  
88 extrapolate using limited data sets and is relatively robust to noise in the variables, as  
89 well as the responses [12]. Principal components (PC) are computed through the  
90 multidimensional space to approximate the best data fit. In order to model the  
91 systematic variation in the data set, usually at least two PC are computed, orthogonal  
92 to each other, which aim to approximate the data as much as possible.

93

94 Principal component analysis (PCA) is the basis in a multivariate analysis, where a  
95 simple overview of the information in a dataset is required. Here, a large data set is  
96 grouped and trends and outliers are identified [13, 14]. PCA produces a summary,  
97 which identifies correlation between observations or groups. Furthermore, trends or  
98 sudden shifts in the dataset can be identified. PCA is used for identification of the  
99 relationship between the X-variables only and reduces the dimensionality of a  
100 multivariate data table into a lower-dimensional plane. Partial least square (PLS)  
101 analysis additionally deals with the Y-variables, the responses in a particular system  
102 or measurement. Here, the aim is to predict Y from X. The application of PLS  
103 determines how the responses are influenced by the factors and variables in a process,  
104 as well as identifying response correlations. Furthermore, we can use PLS to identify  
105 controlling factors responsible in achieving a desired response [14-16].

106

107 The application of relatively simple statistical analysis on experimentally obtained data  
108 is common practice. The use of more advanced statistical tools like Design of  
109 Experiment (DoE) studies and MVA studies are becoming more commonplace.  
110 Nevertheless, the combination of such theoretical multivariate models with  
111 experimentally obtained data or offline analysis may result in powerful systems  
112 providing extra information and confidence in a given research application. In-vivo  
113 testing of new pharmaceutical or biopharmaceutical compounds is time and cost  
114 intensive and currently indispensable during the development of new pharmaceutically  
115 active compounds. Whereas offline analytics are relatively simple and cost-effective,  
116 and if effective would be beneficial in-vivo predictions. This necessitates that the critical  
117 quality parameters of a given system are known and identified.

118

119 The goal of this study was to correlate and cluster in-vivo adjuvant activity from  
120 characteristics of a set of liposomal adjuvants containing the cationic lipid  
121 dimethyldioctadecylammonium bromide (DDA) and trehalose 6,6-dibehenate (TDB).

122 Liposomes formulated from DDA:TDB were chosen as the initial formulation as we  
123 have investigated and characterised its activity as an adjuvant [e.g. 7-9]. To generate  
124 a set of formulations based on DDA:TDB, we incorporated increasing levels of the  
125 saturated phosphatidylcholine, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC),  
126 into DDA:TDB, where the ratio of DDA:TDB remained locked at a 8:1 molar ratio,  
127 resulting in 4 formulations with varying DDA,TDB and DSPC concentrations(Table 1).  
128 Using these formulations, we investigated the effect of liposomal composition and  
129 physical attributes on adjuvant action to identify key controlling features of the  
130 liposomes using MVA. MVA was used to both identify clusters of specific immune  
131 responses, and to verify a possible link to the physicochemical properties of an  
132 adjuvant, namely the size and zeta potential of the liposomes. A tuberculosis antigen  
133 vaccine candidate, known as H56, that combines the early secreted antigens of  
134 Ag85B–ESAT-6 with the latently expressed Rv2660c antigen, shown to provide  
135 protective immunity before and after exposure [17] was used in these studies. In this  
136 study, we combine the experimentally obtained data with a theoretical model that was  
137 based on PCA and PLS analysis in order to allow for prediction of liposomal adjuvant  
138 in-vivo performance.

139

## 140 **Materials and Methods**

### 141 **Materials**

142 Dimethyldioctadecylammonium (DDA), trehalose 6,6-dibehenate (TDB) and 1,2-  
143 distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar  
144 Lipids (Alabaster, Alabama, USA). The fusion protein Ag85B-ESAT-6-Rv2660c (H56  
145 antigen), synthesised to a final concentration of 0.7 mg/mL, was obtained from the  
146 Statens Serum Institut (SSI, Copenhagen, Denmark). Tris-base (Ultra Pure),  
147 purchased from ICN Biomedicals (Aurora, OH) was used to make Tris buffer (adjusted  
148 to pH 7.4 with HCl). Phosphate Buffered Saline (PBS) tablets were purchased from  
149 Sigma-Aldrich Co. Ltd. (Dorset, UK). Chloroform and methanol (extra pure) were  
150 purchased from Fisher (UK). Double distilled water was used in preparation of all  
151 solutions.

152

### 153 **Preparation of liposomes via lipid hydration**

154 Liposome formulations were prepared by the long established method of lipid hydration  
155 [18]. Lipids were dissolved in a chloroform:methanol mixture (9:1 v/v), with DDA and  
156 TDB set to a 5:1 DDA:TDB weight ratio/8:1 molar ratio. Additional liposomal  
157 formulations were prepared where this DDA:TDB remained locked at this ratio but  
158 DDA:TDB was substituted with DSPC at ratios of 25, 50 and 75 % (Table 1). These  
159 lipid mixtures were added to a round bottomed flask and upon solvent extraction via

160 rotary evaporation and N<sub>2</sub> flushing, a dry film was produced. The remaining film was  
161 hydrated in Tris buffer (10 mM, pH 7.4) for 20 minutes at 10 °C above the main gel-to-  
162 liquid phase transition of DDA at ~47 °C [11, 19] or DSPC at 55 °C to completely  
163 hydrate the film and form liposomes. Addition of H56 was performed after liposome  
164 formation at final concentrations of 0.1 mg/mL. Antigen adsorption to liposomes was  
165 promoted by incubation for 30 minutes at room temperature.

166

167 Determination of particle size and zeta potential by dynamic light scattering

168 The z-average diameter and zeta potential was measured using via dynamic light  
169 scattering (DLS) (Malvern Zetasizer Nano-ZS, Malvern Instruments, Worcs., UK).  
170 Measurements took place at 25 °C in (1/10 dilution; 1 mM TRIS, pH 7.4). All  
171 measurements were carried out on triplicate batches of formulations.

172

173 Immunisation study

174 Vaccination of mice

175 All experiments were undertaken in accordance with the 1986 Scientific Procedures  
176 Act (UK). All protocols have been subject to local ethical review and were carried out  
177 in a designated establishment under the project license number PPL 30/2743. Female  
178 C57BL/6 mice, 6-8 weeks old were obtained from Charles River, UK. Vaccine  
179 preparations were prepared with the liposomes (Table 1) with the addition of Ag85B-  
180 ESAT-6-Rv2660 (H56) antigen to a final concentration of 0.1 mg/mL (5 µg/vaccine  
181 dose). All mice, with the exception of the naive group, were immunised intramuscularly  
182 (i.m.) with the proposed vaccines (0.05 mL/dose) three times, with two week intervals  
183 between each immunisation.

184

185 Sera collection

186 Five scheduled bleeds took place over the seven-week immunisation study with blood  
187 samples taken at regular intervals prior to termination. Blood drawn from the tail vein  
188 (50 µL) with micropipette capillary tubes coated in heparin solution (0.1% w/v in PBS),  
189 was added to 450 µL PBS (giving a final dilution of 1/10) and centrifuged using a Micro  
190 Centaur centrifuge at 13,000 RPM for 5 minutes. The supernatants of each mouse  
191 sample was collected and stored at -20 °C for future analysis.

192

193 In-vitro spleen cell culture

194 Spleen cell suspensions were produced into 10 mL RPMI 1640 cell culture medium  
195 (w/o Glutamine) supplemented with 10% (v/v) FBS and 1% (v/v) PSG (BioSera, East  
196 Sussex, UK). Cell suspensions were then centrifuged at 1000 RPM for 10 min at 15  
197 °C and upon supernatant removal, the remaining pellet was resuspended in 10 mL

198 RPMI, before repeated centrifugation prior to pellet resuspension in 5 mL RPMI. Single  
199 cell suspensions were used to evaluate splenocyte proliferation and antigen specific  
200 cytokine responses. For splenocyte proliferation, H56 was added to sterile 96 well cell  
201 culture plates (Greiner Bio-One Ltd, Gloucestershire, UK) at various concentrations of  
202 0-25 µg/mL with a positive control of concanavalin A (2 µg/mL). 100 µL of spleen cell  
203 suspensions were added and incubated at 37 °C, 5% CO<sub>2</sub>, and upon 72 hours  
204 incubation, 40 µL of [<sup>3</sup>H] thymidine at 0.5 (µCi) in supplemented RPMI was added per  
205 well and incubated for 24 hours. Well contents were harvested onto quartz filter mats  
206 (Skatron/Molecular Devices, Berkshire, UK) using a cell harvester (Titertek  
207 Instruments, Alabama, USA) and transferred to 20 mL scintillation vials (Sarstedt,  
208 Leciester, UK) containing 5 mL scintillation cocktail (Ultima Gold, PerkinElmer,  
209 Cambridgeshire, UK). Incorporation of [<sup>3</sup>H] thymidine in cultured cells was measured  
210 with a scintillation counter.

211

#### 212 Assessment of H56 specific antibody isotype titres

213 Serum samples were assessed for levels of IgG, IgG1 and IgG2b antibodies by the  
214 enzyme-linked immunosorbent assay (ELISA). The ELISA plates (96 well, flat  
215 bottomed, high binding, Greiner Bio-One Ltd, Gloucestershire, UK) were firstly coated  
216 with 3 µg/mL H56 antigen prior to overnight incubation at 4 °C. All plates were washed  
217 three times with PBST wash buffer (40 g NaCl, 1 g KCl, 1 g KH<sub>2</sub>PO<sub>4</sub>, 7.2 g Na<sub>2</sub>HPO<sub>4</sub>,  
218 (2H<sub>2</sub>O) per 5 litres of ddH<sub>2</sub>O, incorporating ~0.4 mL of Tween 20) (Microplate washer,  
219 MTX Lab Systems, INC., Virginia, USA). Plates were then blocked by coating each  
220 well with 100 µl of Marvel in PBS (dried skimmed milk powder, 4% W/V, Premier Foods,  
221 Hertfordshire, UK) and incubated for one hour at 37 °C before washing three times  
222 with PBST buffer. 140 µL of serum sample was serially diluted in PBS (70 µL  
223 sequentially) in dilution plates, added to the washed ELISA plates and incubated for  
224 one hour at 37 °C. Plates were then washed five times with PBST buffer before the  
225 addition of 60 µL/well of horseradish peroxidase (HRP) conjugated anti-mouse isotype  
226 specific immunoglobulins of IgG, IgG1 and IgG2b (AbD serotec, Oxfordshire, UK)  
227 diluted to 1/750, 1/4000 and 1/4000 in PBS respectively, to identify anti-H56  
228 antibodies. Plates were washed a further five times with PBST buffer before adding 60  
229 µL/well substrate solution (colouring agent: 6x 10 mg tablets of 2,2'-azino-bis (3-  
230 ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Dorset, UK) in citrate buffer (0.92g  
231 Citric Acid + 1.956g NA<sub>2</sub> HPO<sub>4</sub> per 100 mL) incorporating 10 µL of hydrogen peroxide  
232 (30% H<sub>2</sub>O<sub>2</sub>/100 mL) and incubation for 30 min at 37 °C. Absorbance was read at 405  
233 nm using a microplate reader (Bio-Rad Laboratories, model 680, Hertfordshire, UK).  
234 Known positive serum and pooled naïve mice sera were used as positive and negative  
235 controls respectively.



236

### 237 Quantification of cytokine production by the sandwich ELISA

238 Isolation of splenocyte cell suspensions and plating onto 96 well cell culture plates was  
239 conducted as summarised above. The cells were subsequently incubated for 48 hours  
240 at 37 °C (5% CO<sub>2</sub>), prior to supernatant removal and storage at -70 °C for future  
241 analysis. Quantification of the cytokines, IL-2, IL-5, IL-6, IL-10 and IFN-γ within cell  
242 culture supernatants took place using each specific DuoSet ELISA development kit  
243 (R&D Systems, Oxfordshire, UK). The plates were firstly coated with 100 μL capture  
244 antibody per well and incubated at room temperature overnight. The plates were then  
245 washed three times with PBST buffer before blocking. The plates were subsequently  
246 incubated at room temperature for a minimum of one hour before washing a further  
247 three times. 100 μL/well of sample or standards was then added to each well and  
248 incubated for two hours at room temperature. The plates were washed three times  
249 before adding 100 μL of cytokine specific detection antibody per well and incubation  
250 for two hours at room temperature. Upon washing three times, 100 μL of Streptavidin-  
251 horseradish peroxidase (HRP) was added per well (diluted 1/200). The plates were then  
252 covered to avoid exposure to direct light and incubated at room temperature for 20  
253 minutes. After three more washes, 100 μL substrate solution was added to each well  
254 (1:1 mixture of colour reagent A and B: stabilised hydrogen peroxide and stabilised  
255 tetramethylbenzidine respectively). The plates were then covered and incubated at  
256 room temperature for 20 minutes. The experimental reaction was halted by adding 50  
257 μL stop solution (2N H<sub>2</sub>SO<sub>4</sub>) per well. The optical density was immediately determined  
258 using a microplate reader at 450 nm (Bio-Rad Laboratories, model 680, Hertfordshire,  
259 UK).

260

### 261 Statistical tests

262 Data was analysed by one-way analysis of variance (ANOVA) followed by the Tukey  
263 test to compare mean values of different groups. Differences were considered to be  
264 statistically significant at  $p < 0.05$ .

265

### 266 Multivariate Data Analysis

267 Principal Component Analysis (PCA) and Partial Least Square (PLS) regression  
268 analysis was performed (SIMCA version 13.0, Umetrics) in order to analyse more than  
269 one variable at a time. The relationship between the variables DDA concentration,  
270 liposome size and zeta potential and the immunological responses (IgG, IgG1, IgG2b,  
271 INF-γ, IL-2, IL-5, IL-6, IL-10, spleen proliferation) was displayed in a loading plot, using  
272 all experimentally obtained raw data in this study. Model fit was interpreted by  
273 goodness of fit ( $R^2$ ) and goodness of prediction ( $Q^2$ ) and regarded as good for  $R^2 > 0.5$

274 Weights were selected to maximize the correlation. The loading scatter plot was used  
275 for identifying relationships between the variables and the responses, as well as the  
276 relationships between the variables themselves and the responses themselves. For  
277 interpretation, a line from a selected variable was drawn through the origin of the loading  
278 scatter plot and X- and Y-variables were projected on the line. Variables opposite to  
279 each other were determined as negatively correlated, positive correlation was  
280 determined with variables adjacent to each other. The specific regression coefficients  
281 plots are used to evaluate the X-Y relations in the here computed PLS model.  
282 Correlated responses demonstrate similar coefficient profiles, whereas uncorrelated  
283 responses would show a different profile. The model was validated using a  
284 permutations plot with 40 permutations for each Y-response.

285

## 286 **Results and Discussion**

### 287 Liposomal adjuvants characteristics

288 Upon vesicle production, dynamic light scattering was used to determine the particle  
289 size, polydispersity and zeta potential of the liposomes before and after H56 antigen  
290 addition (0.1 mg/mL: 5 µg/vaccine dose). In the present study, DDA-TDB remained  
291 locked at a molar ratio of 8:1, as previous studies found this ratio to be most beneficial  
292 in immunological performance [11]. This formulation was modified by the incorporation  
293 of DSPC in substitution for DDA-TDB at various molar % ratios, therefore the  
294 concentrations of DSPC, DDA and TDB were each varied but the DDA and TDB  
295 concentrations were linked (Table 1). From the results, it can be seen that varying the  
296 composition of the liposomes resulted in changes in both vesicle size and zeta  
297 potential (Table 2). The particle size of DDA-TDB liposomes in Tris buffer prior to  
298 substitution was ~500 nm, with a polydispersity of 0.3 and a strong cationic surface  
299 charge of ~50 mV (Table 2), in accordance with previous results [11, 19, 20].  
300 Incorporation of DSPC generated significantly larger vesicles ( $P < 0.05$ ) but with no  
301 clear trend of DSPC concentration to vesicle size and all remained in a sub micrometer  
302 size range of 650-850 nm. In contrast the zeta potential decreasing with increasing  
303 DSPC, as would be expected (Table 2). Upon surface adsorption of H56 antigen the  
304 particle size of all formulations increased significantly ( $P < 0.05$ ) to 850 -1300 nm  
305 depending on the formulation, whilst cationic zeta potential decreased (Table 2). For  
306 all 4 formulations tested antigen loading was  $> 85\%$  (results not shown), with no  
307 significant difference, presumably due to the high cationic lipid content/anionic antigen  
308 content even with the 75 % DSPC formulation. For MVA analysis the liposome  
309 characteristics post-addition of antigen were used.

310

311 Immunological characterization for H56 specific antibody isotypes

312 When considering the antibody responses in mice immunised, by day 37 all four of the  
313 liposome formulations induced significantly higher ( $P < 0.05$ ) IgG immune responses  
314 in mice compared to mice immunised with antigen alone, with no significant difference  
315 between the formulations (Fig. 1A). A similar trend was noted with IgG1 responses in  
316 the vaccinated mice (Fig. 1B). In the case of IgG2b (Fig. 1C), liposomal adjuvants  
317 composed of 75 mol% DSPC generated significantly lower ( $P < 0.05$ ) levels of antibody  
318 titres at all time points tested compared to DDA-TDB, and IgG2b responses were not  
319 significantly different to responses in mice immunised with non-adjuvanted H56 (Fig.  
320 1C). This suggests that up to 50 % DSPC within the liposome formulation did not  
321 compromise the immunogenic effect of the DDA-TDB adjuvant, which is capable of  
322 inducing protective cellular immunity against TB when administered with a model  
323 vaccine antigen [21]. This data is in line with previous studies conducted within our  
324 group, where DDA was directly replaced with DSPC but the TDB concentrations were  
325 not changed (and hence the 8:1 molar of DDA-TDB was not maintained) [22]. This  
326 suggests that IgG1 antibody responses remain high over a wider range of DDA and  
327 TDB concentrations and liposome characteristics whilst IgG2b decreased with  
328 decreasing DDA content, irrespective of the DDA-TDB ratio.

329

330 Immunological characterization for H56 specific spleen proliferation rates

331 Antigen specific splenocyte proliferation in mice previously vaccinated with the  
332 liposomal systems and upon re-stimulation with H56 vaccine at increasing  
333 concentrations from 0-25  $\mu\text{g}/\text{mL}$  was assessed. DDA-TDB liposomal adjuvants  
334 generated the strongest cell proliferation (Fig. 2). However, cell proliferation was seen  
335 to be dependent on DDA-TDB concentration as there is a notable trend of decreasing  
336 responses from cells harvested from mice immunised with liposomes containing  
337 increasing DSPC levels (and corresponding decreasing levels of DDA-TDB) within the  
338 liposome formulation (Fig. 2). Indeed, liposomal adjuvants containing 75 mol% DSPC  
339 were consistently low even upon re-stimulation at higher H56 concentrations (Fig. 2).

340

341 Spleen cell cytokine responses

342 Spleen cell cytokine responses from mice immunised with the various liposomal  
343 formulations show variable correlation to the DSPC content (Fig. 3). In general, IFN- $\gamma$ ,  
344 IL-2 and IL-6 levels were shown to decrease with increasing DSPC content (Fig. 3A,  
345 B and D). Whilst IL-5 production was low for all groups (Fig. 3C), with mice which  
346 received antigen alone having similar levels to those mice which received liposomal  
347 adjuvants. In contrast, the presence of DSPC in the liposomal adjuvant tended to  
348 increase IL-10 responses (Fig 3E).

349

350 With increasing replacement of DDA-TDB with DSPC in the formulation, the zeta  
351 potential decreases and the strength of immune response tends to skew towards a  
352 Th2 type response, even with the small decreases in zeta potential noted in these  
353 formulations (Table 1). The effect of liposomal charge has been studied previously for  
354 the quality of immunity stimulated with Ag85B-EAST-6 antigen [20] in which it was  
355 noted that production of IFN- $\gamma$  was strongly dependent upon the liposomal adjuvants  
356 being positively charged. In contrast, DDA-TDB substituted with 75 mol% DSPC  
357 displayed a weak cellular immune response. The resultant Th2 type immune response  
358 observed can be considered to be independent of the surface charge of the system,  
359 corresponding with previous studies [20] stating that a Th2 type elicited response was  
360 not significantly affected by liposomal adjuvant charge.

361

362 Multivariate analysis for clustering Th1 and Th2 type immune responses to adjuvant  
363 characteristics

364 Multivariate model evaluation

365 Whilst the above in-vivo results are in line with previous studies, it is difficult to  
366 investigate the multifactorial changes in liposome attributes that occur when the lipid  
367 composition is modified, therefore the principle aim of this work was to analyse this in-  
368 vivo data set using MVA. Initially, the correlation of two fitted principal components  
369 (PC1 and PC2) for the overall model fit was determined as loadings and weights. The  
370 model type was PLS with 12 observations. Initially we selected the liposome size and  
371 the DDA concentration as x-variables, (2 X-variables and 10 Y-variables). This data  
372 was chosen in order to assess whether the size of the liposome or the DDA  
373 concentration (which was linked to the TDB concentration) is the most contributing  
374 factor in the vaccine immune response. The fraction of the X-variation modelled in PC1  
375 was 62 % (eigenvalue 1.24) and 100 % in PC2 (eigenvalue 0.764). The fraction of the  
376 Y-variation modelled with the first PC was 46 %, and 13 % in the second PC. The  
377 cumulative goodness of fit was 0.59 and the cumulative goodness of prediction was  
378 0.37.

379

380 In the second analysis study, we selected the liposome size, zeta potential and DDA  
381 concentration as variables (3 X-variables and 9 Y-variables). Obviously, given that zeta  
382 potential measurements are be directly linked to the amount and type of lipid used (as  
383 well as the aqueous media the liposomes are suspended in), the zeta potential  
384 represents a response towards lipid composition. However, this set allowed us to verify  
385 how the model predicted the influence of zeta potential on immune responses in-vivo.  
386 Here, the cumulative goodness of fit was 0.97 and the goodness of prediction was  
387 0.52, with two PC fitted (PC1 with 64% of the fraction in the X-variation modelled

388 (eigenvalue 1.92), 97% respectively in the PC2 (eigenvalue of 0.98); Y-variation  
389 modelled in first PC was 44%, 53% in PC2). Unfortunately, including the TDB  
390 concentration as a variable resulted in a non-statistically valid model.

391 PC1 and PC2 in both model setups were regarded to comprise satisfactory information  
392 to construct a predictive model on the data set. Furthermore, we analysed the  
393 cumulated  $R^2$  and  $Q^2$  values for each Y-variable (Fig. 4), in both model setups.  $R^2$   
394 represents a goodness of the model fit and describes how well the variation of the  
395 respective variable is explained;  $Q^2$  indicates how well the respective variable can be  
396 predicted. A threshold value for  $R^2 > 0.5$  was chosen for valid models; values below 0.5  
397 indicated noise present. IgG and IgG1 responses were shown to be insignificant in  
398 both designs chosen, due to negative  $Q^2$  value (Fig. 4 A and B). Spleen proliferation,  
399 INF- $\gamma$ , IL-2, and IL-6 showed good model fit above 0.5, with respective good prediction  
400 power indicated by a relatively low level of noise in the data set (Fig. 4 A and B).  
401 Goodness of prediction for the responses IgG2b, IL-5 and IL-10 was at or below 0.5,  
402 indicating a higher amount of noise present for these responses.

403 PLS regression to cluster H56 specific antibody isotypes

404 Modelling of the data revealed no strong or moderate outliers present (evaluated in the  
405 PCA analysis; data not shown). Due to insignificance in the model for the antibody  
406 subtypes IgG and IgG1, these were removed from further analysis, with IgG2b  
407 remaining, but at a low confidence level. This is in line with the basic statistical analysis  
408 in Figure 1 that revealed no significant difference between the formulations for IgG and  
409 IgG1, confirming that these antibody subtypes are not an ideal measure for vaccine  
410 efficacy in these systems, indicated by statistical insignificance in the PLS analysis.

411

412 PLS regression to analyse specific spleen proliferation rates

413 The liposomal adjuvants were shown to promote splenocyte proliferation upon  
414 restimulation with H56 antigen, demonstrated by the strong correlation between the  
415 variables DDA (Fig. 4C) (and zeta potential; Fig. 4D) to splenocyte proliferation in the  
416 coefficient plot, with size not shown to correlate with responses. DDA concentration is  
417 the most influential variable for the response spleen proliferation, visible by the high  
418 coefficient value (close to 1) as well as a small confidence interval. The loading scatter  
419 plots shows a close correlation of splenocyte proliferation response to the variable  
420 DDA (Fig. 4E) and zeta potential (Fig. 4F), identifying their strong correlation. This  
421 confirms that the biggest effect to spleen proliferation rates is the increase in DDA  
422 content, which is strongly linked to the zeta potential of a vaccine. As indicated in  
423 Figure 3, the peak of proliferation correlates with DDA-TDB liposomes, which have the  
424 strongest zeta potential (Table 2).

425

426 PLS regression to cluster cytokines responses

427 The PLS analysis revealed a statistical significance for the responses INF- $\gamma$ , IL-2, IL-6  
428 and IL-10 for the variables DDA (Fig. 4 C), as well as for the variable zeta potential  
429 (Fig. 4D) again as would be expected due to their link. Overall, the DDA content as  
430 well as the zeta potential showed a positive correlation to INF- $\gamma$ , IL-2 and IL-6, and an  
431 inverse correlation to the response IL-10. The increase in DDA in a vaccine adjuvant  
432 formulation gave no notable correlation in size but does result in a higher zeta potential,  
433 which is predicted to increase the specific INF- $\gamma$ , IL-2 and IL-6 production in-vivo. The  
434 corresponding peak in INF- $\gamma$  production (Fig. 3A) was detected for the DDA-TDB  
435 liposomes, which also provided the strongest cationic zeta potential (Table 2). Here,  
436 the model predictions are in line with the previous reported results that showed  
437 increasing cationic charge (but with constant TDB concentrations across the  
438 formulations) enhanced INF-  $\gamma$  as well as IL-6 [22]. However in addition to this, the  
439 model suggests no impact of DDA and zeta potential content on IL-5, but an inverse  
440 correlation between the response IL-5 and the liposome size (Fig. 4C and D), indicating  
441 that a smaller liposome size is predicted to increase the specific IL-5 production.  
442 Nevertheless, initial model evaluation of the response IL-5 indicated a level of noise  
443 present in the data set, which should be considered in any predictions made until model  
444 validation is verified.

445

446 The specific regression coefficients (Fig. 4E and F) represent the X-Y relations in the  
447 computed PLS model; which simplifies the model overview. Correlated responses  
448 demonstrate similar coefficient profiles. Similar coefficient profiles for the responses  
449 INF- $\gamma$  , IL-2 and IL-6 for the variables DDA and zeta potential suggesting a grouping  
450 and relation between those cell mediated responses, which can be clustered together  
451 as Th1-specific immune responses driven by the DDA content. This cluster is visible  
452 in both loading scatter plots (Fig. 4E and F) and not influenced by zeta potential being  
453 included in the model as a variable or a response, with a strong cluster of the  
454 responses INF- $\gamma$ , IL-2, IL-6 and IgG2b, all which are linked with Th1 specific immune  
455 responses. When several Y-variables need to be modelled and analysed together, PLS  
456 offers the ability to generate a simpler depiction of data sets, rather than generating  
457 separate models for each response. It is recommended to analyse strongly correlated  
458 Y-variables together and group them, as their correlation stabilizes the model [12].  
459 However, this only applies for dependent responses that measure and incorporate  
460 similar measurements.

461

462 Model summary

463 We see that the DDA (and the linked TDB) concentration in a vaccine formulation is a  
464 crucial variable and most importantly more influential to the immunological response  
465 than the actual liposome size (across the range considered). Generally, a strong link  
466 between the DDA concentration and zeta potential could be identified; for selecting the  
467 zeta potential as a Y-response (Fig. 4E) as well as a X-variable (Fig. 4F), its close link  
468 to DDA as a variable confirms the significance of the zeta potential to initiating a Th1  
469 mediated immune response in-vivo. Overall, the model developed was statistically  
470 valid for the variables, DDA and zeta potential (spleen proliferation, IFN-  $\gamma$ , IL-2, IL-6,  
471 IL-10, IgG2b), and to limited extent liposome size (in the case of IL-5), as summarized  
472 by the importance of the x-variables. The variable influence on projection plot (VIP)  
473 (Fig. 5), which summarizes all components and y-variables [23], indicated that the  
474 variable DDA content (Fig. 5A) and zeta potential (Fig. 5A&B) were ranked as the  
475 variables with the highest impact in the PLS models. However, whilst the zeta potential  
476 is shown to strongly influence the immune responses in-vivo and thus could be taken  
477 as a controlling factor, it is directly linked to the DDA content. Furthermore, we have  
478 previously shown that liposomes of the same DDA content, and hence same zeta  
479 potential, gave different immunological profiles depending on the TDB content [11].  
480 This demonstrates that controlling factors between the formulation and the physico-  
481 chemical characteristics must be identified when applying MVA to avoid incorrect  
482 interpretation.

483

#### 484 Model validation

485 To assess the validity of the predictions made by the PLS analysis, the model was  
486 validated using respective permutations plots for each specific Y-response (Fig. 6).  
487 The permutation plots helped to assess the validity of the PLS model by assessing the  
488 risk of invalidity and verifying that the model does not only fit the current data set, but  
489 also predicts Y from new observations.

490

491 Model validation is a crucial diagnostic function of MVA. Here, the X-data is left  
492 unmodified, whilst the Y-data is permuted and arranged in a different order after which  
493 a PLS model is fitted to the permuted data set. The derived models are cross-validated  
494 by computing  $R^2$  and  $Q^2$ . This random shuffling of the Y-data allows comparing the  
495 permuted values with the real  $R^2$  and  $Q^2$  values of the model. This permutation  
496 procedure is repeated for a certain number, mostly between 25 and 100, (here, we  
497 chose 40), which leads to the generation of parallel PLS models thus establishing  
498 reference distributions based on random data. These references are used to assess  
499 the statistical significance in the initial PLS model [24].

500

501 Here, the goodness of fit and prediction ( $R^2$  and  $Q^2$ ) of the current model were  
502 compared with the  $R^2$  and  $Q^2$  of randomly permuted Y-observations while the X-  
503 variables were maintained constant. For each Y-variable, 40 permutations were  
504 selected. The  $R^2$  and  $Q^2$  values from the original model were shown on the far right  
505 end of the respective graphs, whereas the Y-permuted models were shown on the left  
506 side. The correlation between permuted Y-vector to the original X-vector was depicted  
507 by the horizontal correlation axis. The criteria for model validity have been selected as  
508 the intercept of the  $Q^2$  regression line at or below zero. Furthermore, the validity was  
509 assessed by depiction of all permuted  $R^2$  values below the  $R^2$  of the original model.

510

511 The initial model that evaluated the zeta potential as a response, showed an excellent  
512 model validity with its respective permutation plot (Fig. 6A), confirming that the  
513 response zeta potential can be modelled and described by PLS methods. Models for  
514 the responses spleen proliferation, INF- $\gamma$ , IL-2 and IL-6 showed excellent permutation  
515 plots (Fig. 6 B, C, D, E), confirming the validity of the PLS model and predictions made  
516 from selected responses. Validation for the variables IgG, IgG1, IgG2b, IL-5 and IL10  
517 failed (plots not shown), confirming the previous invalidity of the models as already  
518 seen in initial model evaluation (Figure 4 A and B). Furthermore, this confirms that the  
519 initially detected higher level of noise present for IL-5, IL-10 and IgG2b resulted in a  
520 non-valid model, exemplifying that any predictions made using MVA depend on  
521 verifying the validity of the models by the permutation testing.

522

523 Nevertheless, interpretations should be made in consideration of the assay accuracy,  
524 which might lead to a higher level of noise in the data set, as seen for the variables  
525 IgG2b, IL-5 and IL-10. Although clear trends and clusters were visible, interpretation  
526 always depends on the accuracy of the assay. Furthermore, wider formulation profiling  
527 is required to challenge this use of MVA in more complex vaccine adjuvant studies.  
528 However, results here emphasize the use of multivariate analysis as a new tool for in-  
529 vivo vaccine efficacy correlations and cluster analysis for Th1 specific immune  
530 responses.

531

532 This study shows that useful clustering, trends and predictions can be made using  
533 MVA tools when a range of factors are varied (in this case DDA, TDB and DSPC  
534 content which results in variations in vesicle size and zeta potential). Correlating in-  
535 vivo data may be a cost effective way for initial information about vaccine efficiency.  
536 Information extracted from MVA may speed up the drug and process development  
537 process, as desired in-vivo immune response targets might be predicted and are  
538 dictated by the characteristics of the adjuvant or delivery system. From the present



539 study evaluations, the extraction of information from in-vivo data by partial least square  
540 regression models gives a powerful tool to further characterize a vaccine formulation.  
541 It can be used for initial clustering of in-vivo specific immune responses and help to  
542 allow for future predictions of vaccine efficiency; overall, a new and useful method to  
543 speed up the development process of a vaccine candidate.

544

545 MVA is a useful tool for not only summarizing and visualizing data sets, it also allows  
546 for classification and identification of quantitative relationships between variables [12].  
547 Matrices can be of alterable amounts of variables and observations, allowing for  
548 flexibility in generating the data set. The application of those mathematical and  
549 statistical tools is highly applicable for determination of relationships between various  
550 measurements derived from a system or process [25]. We define the relationship  
551 between two properties, where the effect of one property that can easily be measured  
552 in the laboratory is related to the second property, which is more difficult to measure.  
553 Initially, data of both property measurements are obtained, which are then built into a  
554 model using multivariate regression, linking the dependent and independent variables.

555

556 The most significant advantage of using multivariate tools is the ability to analyse  
557 multiple variables simultaneously, along with the reduction of the dimensionality of the  
558 data set by projecting the data into a lower dimension thus improving data  
559 interpretation and presentation [26]. Visualization and simplification of complex  
560 pharmaceutical data is one of the main advantages of using MVA tools, and it is highly  
561 applicable in pharmaceutical research and process or product development [27]. MVA  
562 is furthermore often applied in diagnostics tools, where the identification of the major  
563 contributing variables leads to the isolation of the deviation, frequently applied in  
564 industrial processes for product quality control [26].

565

## 566 **Conclusion**

567 In conclusion, models were developed to cluster and predict Th1 immune responses  
568 to the vaccine formulation dependent on liposomal adjuvant characteristics.  
569 Substitution of DDA:TDB with DSPC reduced the cationic zeta potential and resulted  
570 in variations in vesicle size. The extent of DSPC incorporation correlated to polarised  
571 immune responses with a combination of cellular and humoral immunity. We have  
572 shown that the use of multivariate tools allows for clustering and predictions from key  
573 liposome characteristics to specific in-vivo immune responses. The reliability of derived  
574 PLS models suggests its general usefulness for predicting in-vivo specific immune  
575 responses from offline measurements. Such multivariate approaches may be useful in  
576 correlating key characteristics to critical quality attributes of a vaccine formulation.

577 Specific variable-dependences and independences support the selection of key  
578 variables that need to be further optimized in a development process. Such methods  
579 may be particularly useful for screening many variables at a time, especially in early  
580 stage development processes.

581

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586

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659  
660  
661

662 **Tables.**

663

664 **Table 1:** Incorporation of DSPC into DDA-TDB formulations at 25, 50 and 75 mol%.

Formulation (mol%)	Weight $\mu\text{g}$ per dose		
	DDA	TDB	DSPC
DDA:TDB	250	50	0
+ 25% DSPC	188	36	88
+ 50% DSPC	125	25	175
+ 75% DSPC	63	14	264

665 Values of weight and  $\mu\text{moles}$  in the various liposome formulations where DDA:TDB  
666 was locked at a 5:1 wt ratio/8:1 molar ratio and increasingly replaced with DSPC in a  
667 50  $\mu\text{L}$  dose.

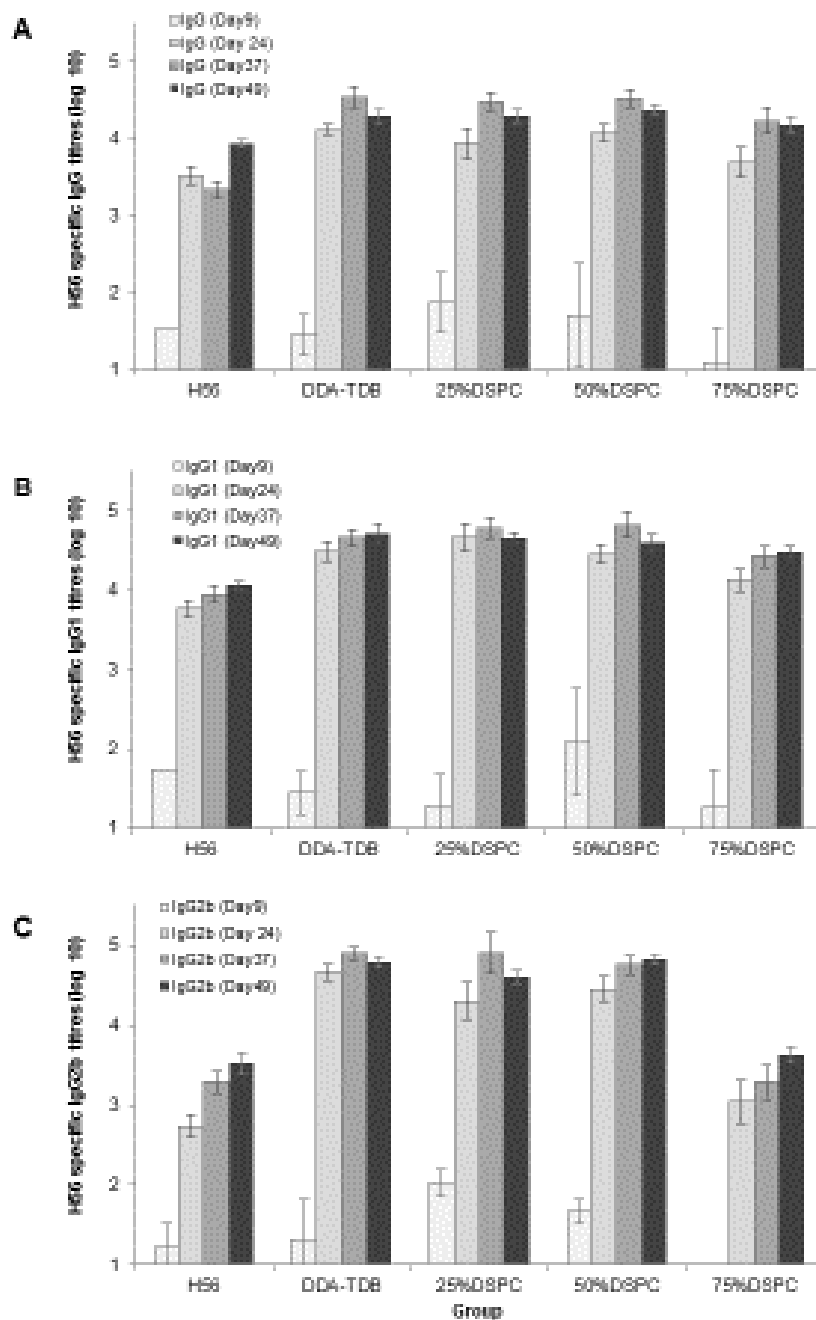
668

669 **Table 2:** Particle size, polydispersity and zeta potential liposomal adjuvants prior to  
670 and post H56 antigen adsorption.

Formulation	Antigen	DDA/TDB	25% DSPC	50% DSPC	75% DSPC
Vesicle size (nm)		517 $\pm$ 29	640 $\pm$ 24	856 $\pm$ 114	734 $\pm$ 67
	+ H56	981 $\pm$ 198	1266 $\pm$ 151	1036 $\pm$ 92	852 $\pm$ 52
Polydispersity		0.32 $\pm$ 0.01	0.34 $\pm$ 0.01	0.32 $\pm$ 0.01	0.33 $\pm$ 0.02
	+ H56	0.42 $\pm$ 0.02	0.46 $\pm$ 0.06	0.54 $\pm$ 0.14	0.42 $\pm$ 0.1
ZP (mV)		45.7 $\pm$ 0.7	42.7 $\pm$ 1.9	35.4 $\pm$ 3.6	33.2 $\pm$ 0.5
	+H56	47.4 $\pm$ 6.1	41.4 $\pm$ 3.7	31.7 $\pm$ 6.4	28.7 $\pm$ 5.3

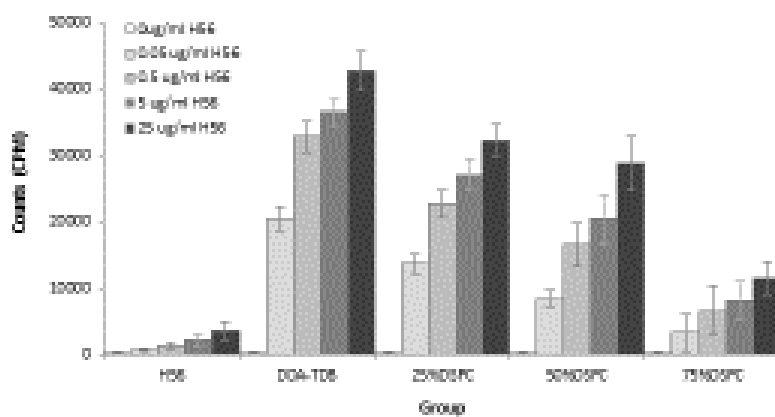
671 The liposomes were produced by lipid hydration in Tris buffer (10 mM, pH 7.4) and  
672 with H56 vaccine antigen added at 0.1 mg/mL. Characterisation used a Malvern  
673 Nanosizer ZS. Results denote the mean  $\pm$  s.d. for three independent experiments.

674

**Figure 1**

677

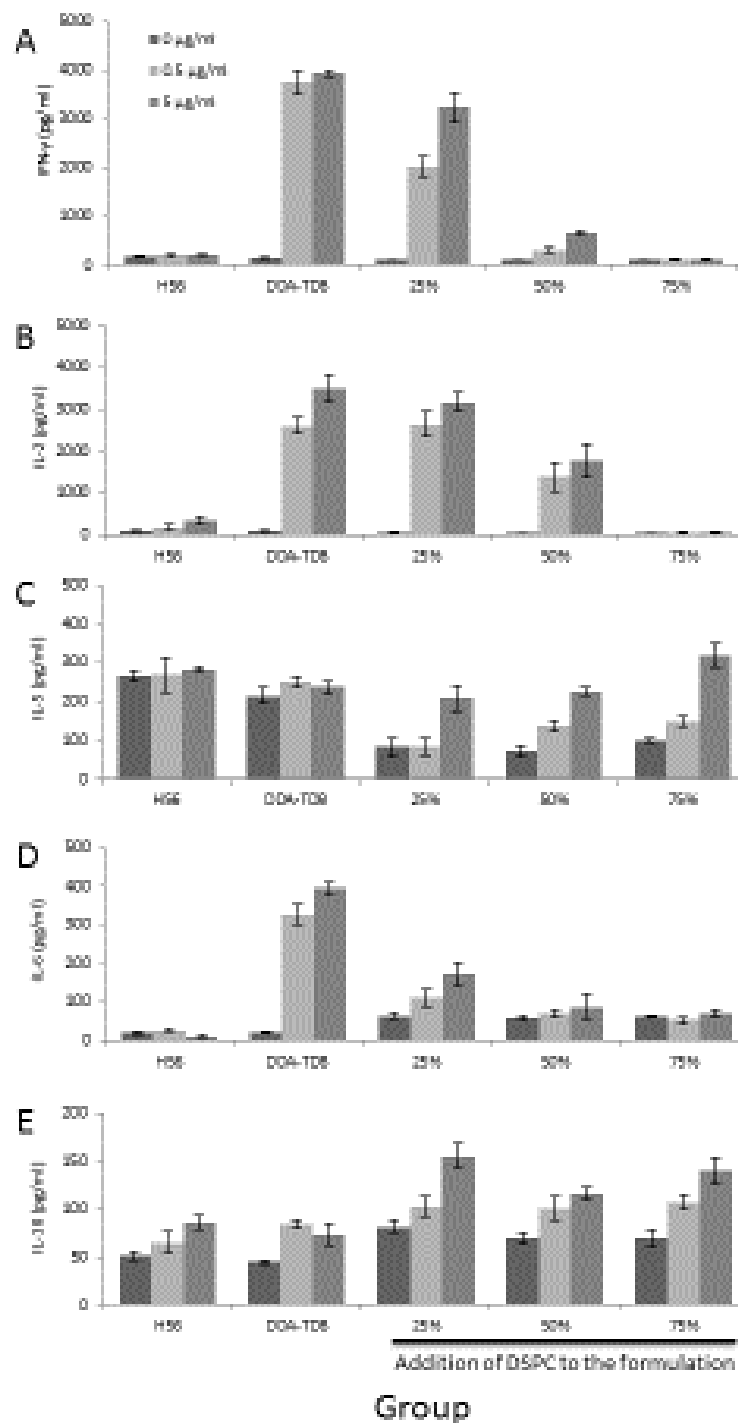
678 **Figure 1:** Mean serum H56 specific antibody isotype titres stimulated by DDA-TDB  
 679 and substitution with 25-75 mol% DSPC (n=5, +/- standard error) for A: IgG, B: IgG1  
 680 and C: IgG2b subsets. Values display the positive reciprocal end point dilution  
 681 (log<sub>10</sub>). Sera was collected prior to the first immunisation and on days 9, 24, 37 and  
 682 49 respectively thereafter. Serum samples obtained across various time intervals  
 683 upon immunisation were analysed for the presence of anti-H56 specific antibodies by  
 684 the enzyme-linked immunosorbent assay (ELISA).



685

686 **Figure 2.** Spleen cell proliferation stimulated by H56 vaccine antigen (at 0, 0.05, 0.5,  
 687 5 and 25 µg/mL; n=5, mean of replicates ± standard error) for DDA-TDB and  
 688 substitution with 25-75 mol% DSPC. The level of H56 antigen specific splenocyte  
 689 proliferation was indicated by the extent of [<sup>3</sup>H] labelled Thymidine incorporation into  
 690 cultured splenocytes.

691



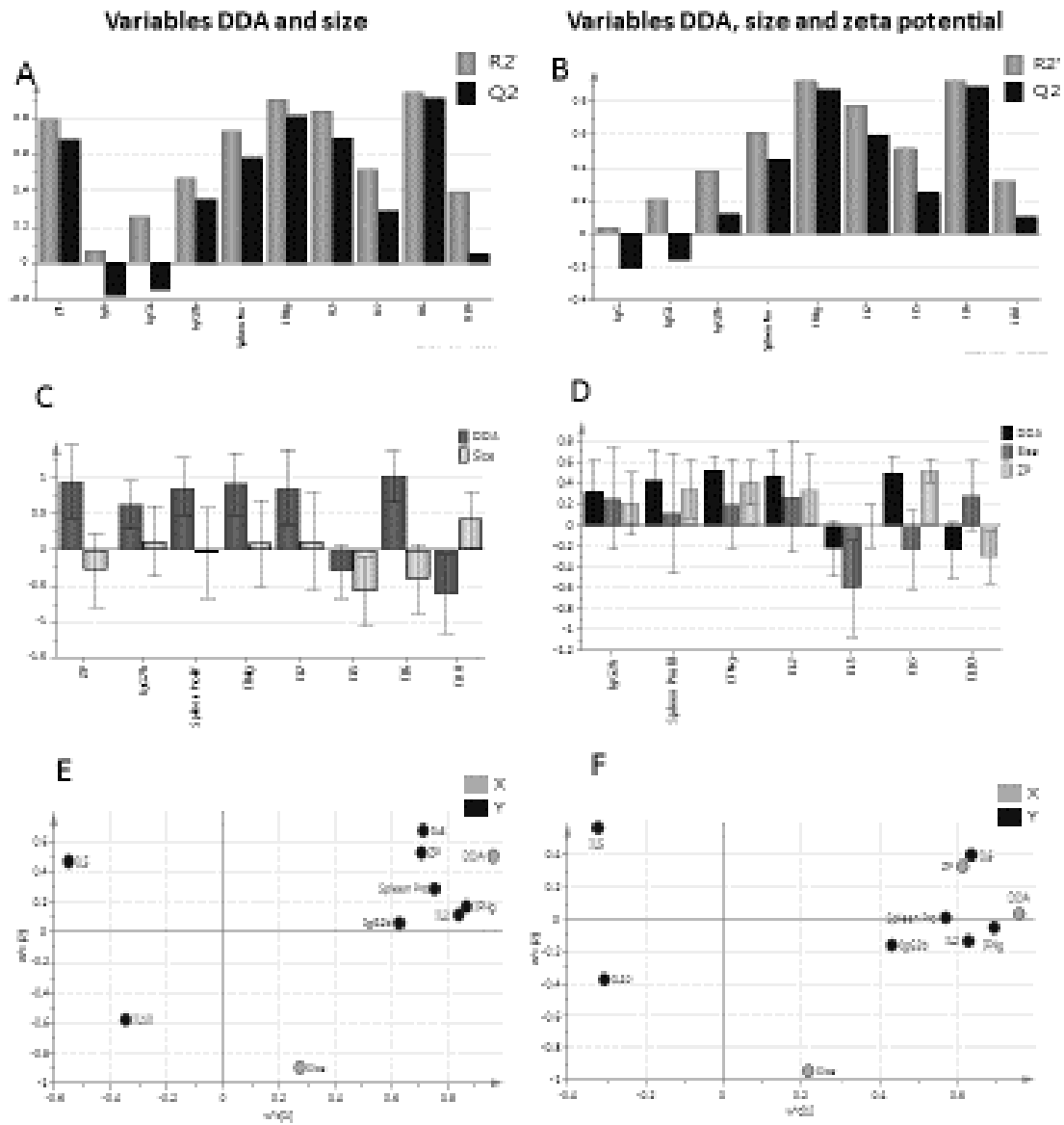
693 **Figure 3**

694 **Figure 3.** Spleen cell cytokine production in response to re-stimulation with H56  
 695 antigen at 0, 0.5 and 5  $\mu\text{g/mL}$ , quantified for A: IFN- $\gamma$ , B: IL-2, C: IL-5, D: IL-10 and E:  
 696 IL-6. Results represent mean average cytokine production of five spleens per  
 697 vaccination group +/- standard error.

698

699





700

701 **Figure 4.** X/Y overview plot indicating the cumulated R<sup>2</sup> and Q<sup>2</sup> values for each  
 702 response for A) DDA and size and B) DDA, size and zeta potential. Well modelled  
 703 responses show a R<sup>2</sup> and Q<sup>2</sup> value above 0.5 IgG and IgG1 responses show poor  
 704 model fit (negative Q<sup>2</sup>), that indicates noise and no correlation between the X and the  
 705 Y variables for those responses (statistical insignificance). PLS analysis results with  
 706 Coefficient overview, displaying the coefficients for all responses to interpret how the  
 707 X-variables affect the Y-variables for C) DDA and size and D) DDA, size and zeta  
 708 potential. Loading scatter plot, where the relation between X and Y- variables are  
 709 displayed for E) DDA and size and F) DDA, size and zeta potential.

710

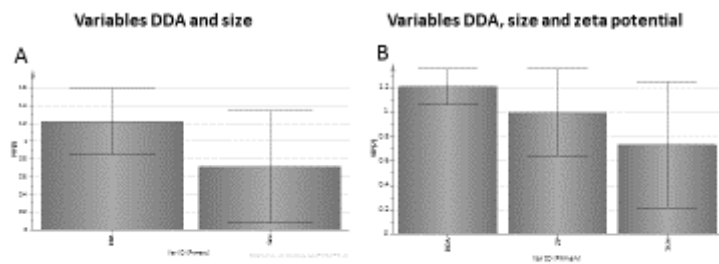


Figure 5

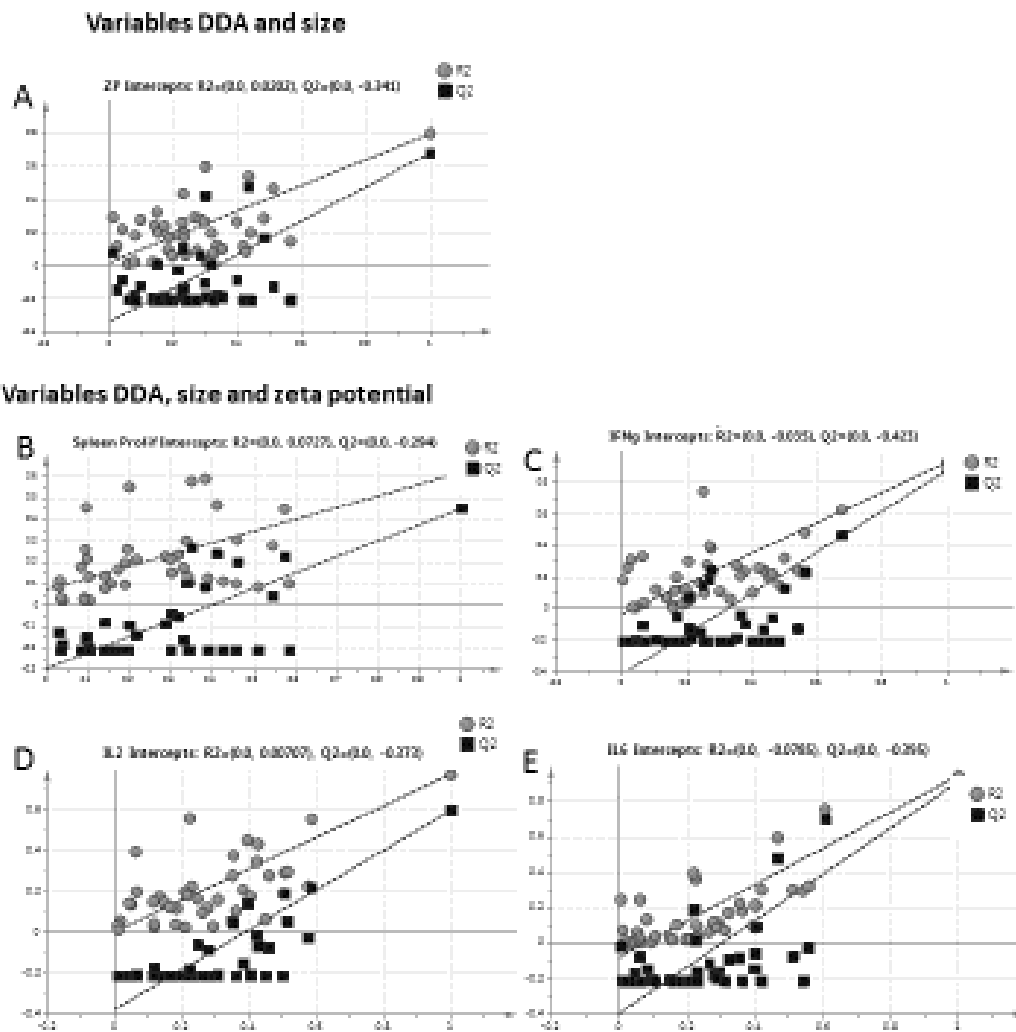
711

712 **Figure 5.** VIP plot (variable importance for projection) summarizing the importance of  
 713 the variables liposome size and zeta potential. The VIP plot is sorted from high to low  
 714 and indicates the value of the variable zeta potential as the most important X-variable  
 715 in the PLS model for A) DDA and size and B) DDA, size and zeta potential.

716

717

718



719

720 **Figure 6.** Permutations plot for A: zeta potential, B: spleen proliferation, C: IFN-  $\gamma$  , D:

721 IL-2, E: IL-6. Model validity was assessed for 40 permutations. The correlation

722 between permuted Y-vector to the original X-vector is depicted by the horizontal

723 correlation axis. The criteria for model validity have been selected as the intercept of

724 the  $Q^2$  regression line at or below zero.

725