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

Authors: M. Jubair Hussain[a], Alexander Wilkinson[a], Vincent W Bramwell[a], Dennis Christensen[b], and Yvonne Perrie[a]

[a]School of Life and Health Sciences, Aston University, Birmingham, UK.

[b]Statens Serum Institute, Copenhagen, Denmark.

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

Corresponding author:

Professor Yvonne Perrie
School of Life and Health Sciences,
Aston University,
Aston Triangle,
Birmingham,
B4 7ET.
Tel: +44 121 204 3991
Email address: y.perrie@aston.ac.uk

Date of Manuscript: October 2013

Abstract

Objectives Cationic liposomes of dimethyldioctadecylammonium bromide (DDA) combined with trehalose 6,6-dibehenate (TDB) elicit strong cell mediated and antibody immune responses; DDA facilitates antigen adsorption and presentation, whilst TDB potentiates the immune response. To further investigate the role of DDA, DDA was replaced with the neutral lipid of distearoyl-sn-glycero-3-phosphocholine (DSPC) over a series of concentrations and these systems investigated as adjuvants for the delivery of Ag85B–ESAT-6-Rv2660c, a multistage tuberculosis vaccine.

Methods Liposomal were prepared at a 5:1 DDA-TDB weight ratio and DDA content incrementally replaced with DSPC. The physicochemical characteristics were assessed (vesicle size, zeta potential and antigen loading) and the ability of these systems to act as adjuvants was considered.

Key findings As DDA was replaced with DSPC within the liposomal formulation, the cationic nature of the vesicles decreases as does electrostatically binding of the anionic H56 antigen; however, only when DDA was completed replaced with DSPC did vesicle size increase significantly. Th1 type cell-mediated immune responses reduced. This reduction in responses was attributed to the replacement of DDA with DSPC rather than the reduction in DDA dose concentration within the formulation.

Conclusion These results suggest Th1 responses can be controlled by tailoring the DDA/DSPC ratio within the liposomal adjuvant system.

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

the similarity in alkyl chain lengths and phase transition temperature to that of DDA, factors shown to impact on liposome stability in vivo [10]. Therefore a range of formulations where DSPC systematically replaced DDA within the liposomal system were investigated in combination with the subunit TB vaccine candidate, H56 [11].

Materials and Methods

Materials

Dimethyldioctadecylammonium (DDA), trehalose 6,6-dibehenate (TDB) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were purchased from Avanti Polar Lipids (Alabaster, Alabama, USA). The purity of all the compounds used was > 99%, determined by HPLC. The fusion protein Ag85B-ESAT-6-Rv2660c (H56 antigen) obtained from the Statens Serum Institut (SSI, Copenhagen, Denmark). Tris (Ultra Pure) was purchased from ICN Biomedicals (Aurora, OH). Phosphate buffered saline (PBS) tablets were purchased from Sigma-Aldrich Co. Ltd. (Dorset, UK). Methanol and chloroform (extra pure) were purchased from Fisher (UK). Ultima Gold scintillation fluid and [^{3}H] thymidine were obtained from Perkin Elmer (Waltham, MA). Double distilled water was used in preparation of all solutions.

Liposome preparation: lipid hydration

Liposome formulations were prepared by the previously established method of lipid hydration [12]. Briefly, lipids were dissolved in a chloroform:methanol mixture (9:1 v/v), with DDA and TDB set to concentrations of 1.25 mg and 0.25 mg TDB per mL respectively, representing a 5:1 DDA-TDB weight ratio. The level of DDA within the formulation was incrementally replaced with increasing levels of DSPC, with levels of TDB remaining fixed. Lipid mixtures were added to a round bottomed flask and upon solvent extraction via rotary evaporation and N_2 flushing, a dry film was produced. The lipid film was hydrated in Tris-buffer (10 mM, pH 7.4) for 20 min at 10 °C above the main gel-to-liquid phase transition of DDA at ~47 °C [7, 13] or DSPC at ~55 °C [14] to completely hydrate the film and form liposomes.

Characterisation of liposomes for particle size and zeta potential

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 dd$H_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

vaccine (0.05 mL/dose) three times, with two week intervals between each immunisation. At scheduled time points, blood samples were taken and stored at -20 °C for future analysis.

Upon experiment termination, mice were culled and the spleens were collected. Spleen cell suspensions were produced upon light grinding through a fine wire mesh into 10 ml RPMI 1640 cell culture medium (W/O Glutamine) supplemented with 10% (v/v) FBS and 1% (v/v) PSG (BioSera, East Sussex, UK). Cell suspensions were centrifuged at 1000 RPM for 10 min at 15 °C and upon supernatant removal, the pellet was resuspended in 10 mL RPMI, before repeated centrifugation prior to pellet resuspension in 5 mL RPMI. Single cell suspensions were used to evaluate splenocyte proliferation and antigen specific cytokine responses. For assessment of splenocyte proliferation, H56 was added to sterile 96 well cell culture plates (Greiner Bio-One Ltd, Gloucestershire, UK) with a positive control of concanavalin A (2 µg/mL). 100 µL of spleen cell suspensions were added and incubated at 37 °C, 5% CO_2, and upon 72 hours incubation, 40 µL of [^{3}H] thymidine at 0.5 (µCi) in supplemented RPMI was added per well and incubated for 24 hours. Well contents were harvested onto quartz filter mats (Skatron/Molecular Devices, Berkshire, UK) using a cell harvester (Titertek Instruments, Alabama, USA) and transferred to 20 mL scintillation vials (Sarstedt, Leciester, UK) containing 5 mL scintillation cocktail (Ultima Gold, PerkinElmer, Cambridgeshire, UK). Incorporation of [^{3}H] thymidine in cultured cells was measured with a scintillation counter according to standard operating procedures.

Evaluation of H56 specific antibody isotypes

Serum samples were assessed for levels of IgG1 and IgG2b antibodies by the enzyme-linked immunosorbent assay (ELISA). The ELISA plates (96 well, flat bottomed, high binding, Greiner Bio-One Ltd, Gloucestershire, UK) were firstly coated with 3 µg/mL H56 antigen prior to overnight incubation at 4 °C. All plates were washed three times with PBST wash buffer (40 g NaCI, 1 g KCI, 1 g KH_2PO_4, 7.2 g Na_2HPO_4, ($2H_20$) per 5 litres of ddH_20, incorporating ~0.4 mL of Tween 20) using a using a plate washer (Microplate washer, MTX Lab Systems, INC., Virginia, USA) with subsequent blotting to remove unbound antigen. Plates were blocked by coating each well with 100 µL of Marvel in PBS (dried skimmed milk powder, 4% W/V, Premier Foods, Hertfordshire, UK) and incubated for one hour at 37 °C before washing three times with PBST buffer. 140 µL of serum sample was serially diluted in PBS (70 µL

sequentially), added to the washed ELISA plates and incubated for one hour at 37 °C. Known positive serum and pooled naïve mice sera were used as positive and negative controls respectively. Plates were washed five times with PBST buffer before the addition of 60 µL/well of horseradish peroxidise (HRP) conjugated anti-mouse isotype specific immunoglobulins of IgG1 and IgG2b (AbD serotec, Oxfordshire, UK), to identify anti-H56 antibodies. Plates were washed a further five times with PBST buffer before adding 60 µL/well substrate solution (colouring agent: 6x 10 mg tablets of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS; Sigma, Dorset, UK) in citrate buffer (0.92g Citric Acid + 1.956g NA_2 HPO_4 per 100 ml) incorporating 10 µL of hydrogen peroxide (30% H_2O_2/100 ml) and incubation for 30 min at 37 °C. Absorbance was read at 405 nm using a microplate reader (Bio-Rad Laboratories, model 680, Hertfordshire, UK).

Quantification of cytokines via the sandwich ELISA

Isolation of splenocyte cell suspensions and plating onto 96 well cell culture plates was conducted as summarised in section 2.6.1. The cells were subsequently incubated for 48 hours at 37 °C, prior to supernatant removal and storage at -70 °C for future analysis. Quantification of the cytokines, IL-2, IL-5, IL-10 and IFN-γ within cell culture supernatants took place using each specific DuoSet ELISA development kit (R&D Systems, Oxfordshire, UK). The plates were firstly coated with 100 µL capture antibody per well and incubated at room temperature overnight. The plates were then washed three times with PBST buffer before blocking and incubation at room temperature for one hour before washing a further three times. 100 µL/well of samples/standards was then added to each well and incubated for two hours at room temperature. The plates were washed three times before adding 100 µL of cytokine specific detection antibody per well and incubating for two hours at room temperature, prior to washing three times and adding 100 µL of streptavidin-horseradish peroxidise (HRP) per well (diluted 1/200). The plates were then covered to avoid exposure to direct light and incubated at room temperature for 20 min. After three washes, 100 µL of substrate solution was added per well (1:1 mixture of colour reagent A and B: stabilised hydrogen peroxide and stabilised tetramethylbenzidine (TMB) respectively) and the plates were covered and incubated at room temperature for 20 min. The experimental reaction was stopped by adding 50 µL stop solution (2N H_2SO_4) per well and the optical density was then determined using a microplate reader at 450 nm (Bio-Rad Laboratories, model 680, Hertfordshire, UK).

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

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

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

Conclusion

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

Acknowledgements

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

References

[1] Cox JC and Coulter AR. Adjuvants- a classification and review of their modes of action. Vaccine 1997; 15: 248-256.

[2] Henriksen-Lacey M et al. Liposomes based on dimethyldioctadecylammonium promote a depot effect and enhance immunogenicity of soluble antigen. J. Controlled Release 2010; 142: 180-186.

[3] Korsholm KS et al. T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection. Immunology 2009; 129: 75-86.

[4] Henriksen-Lacey M et al. Comparison of the depot effect and immunogenicity of liposomes based on DDA, DC-Chol and DOTAP: Prolonged liposome retention mediates stronger Th1 responses. Mol. Pharmaceutics 2011; 8: 153-161.

[5] Holten-Andersen L et al. Combination of the Cationic Surfactant Dimethyl Dioctadecyl Ammonium Bromide and Synthetic Mycobacterial Cord Factor as an Efficient Adjuvant for Tuberculosis Subunit Vaccines. Infect. Immun. 2004; 72: 1608-1617.

[6] Davidsen J et al. Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from M. tuberculosis (trehalose 6,6'-dibehenate)-a novel adjuvant inducing both strong CMI and antibody responses. Biochim. Biophys. Acta. 2005; 1718: 22-31.

[7] Werninghaus K et al. Adjuvanticity of a synthetic cord factor analogue for subunit Mycobacterium tuberculosis vaccination requires FcRgamma-Syk-Card9-dependent innate immune activation. J. Exp. Med. 2009; 206: 89-97.

[8] Henriksen-Lacey M et al. Liposomal cationic charge and antigen adsorption are important properties for the efficient deposition of antigen at the injection site and immunogenicity of the vaccine J Controlled Release 2010; 145: 102-108.

[9] Henriksen-Lacey M et al. The vesicle size of DDA:TDB liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production. J Control Release 2011; 154: 131-137.

[10] Senior J, Gregoriadis G. Stability of small unilamellar liposomes in serum and clearance from the circulation: the effect of the phospholipid and cholesterol components. Life Sciences. 1982; 30: 2123-2136.

[11] Aagaard C et al. A multistage tuberculosis vaccine that confers efficient protection before and after exposure. Nat. Med. 2011; 17: 189-194

[12] Bangham AD et al. Diffusion of univalent ions across the lamellae of swollen phospholipids. J. Mol. Biol. 1965; 13: 238-252.

[13] Christensen D et al. Trehalose preserves DDA/TDB liposomes and their adjuvant effect during freeze-drying. Biochim. Biophys. Acta. 2007; 1768: 2120-2129.

[14] Ali S et al. A differential scanning calorimetry study of phosphocholines mixed with paclitaxel and its bromoacylated taxanes. Biophys. J. 2000; 78: 246-256.

[15] Agger EM et al. Cationic liposomes formulated with synthetic mycobacterial cordfactor (CAF01): a versatile adjuvant for vaccines with different immunological requirements PloS One 2008; 3: 1-10.

[16] Christensen D et al. CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations. Int. J. Pharm. 2010; 390: 19-24.

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

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

	DDA/DSPC/TDB		
Factor	100/150/50 µg	100/0/50 µg	Significance
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	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
IgG2b (serial end point dilution, log_{10})			
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

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.

Figures.

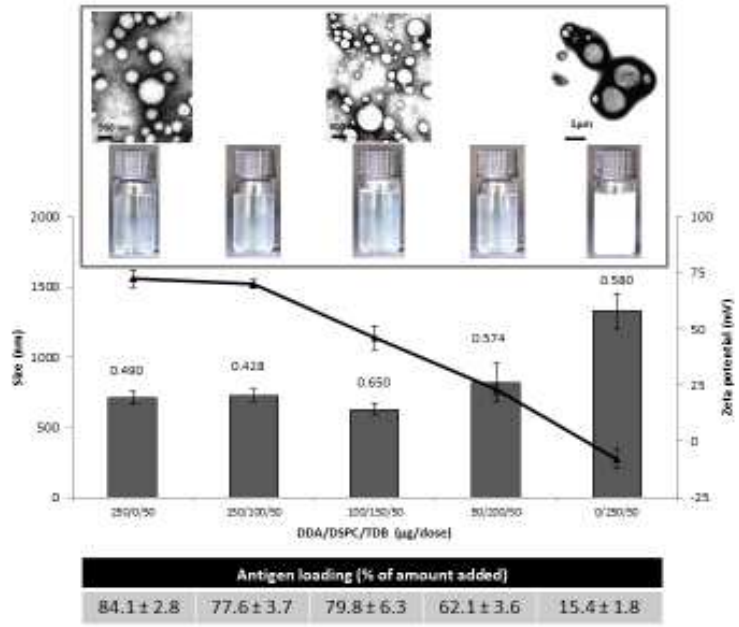

Figure 1

Figure 1. Physico-chemical characteristics of liposomes prepared with varying ratios of DDA and DSPC combined with TDB. A) TEM and visual images of liposomes prepared. B) Vesicle size, polydispersity and zeta potential together with C) H56 antigen loading. Vesicles were prepared via lipid hydration in Tris buffer (10 mM, pH 7.4), with systems surface adsorbed with 0.1 mg/ml H56 antigen and measured in 1 mM Tris buffer. Results represent the mean average ± standard deviation (n=3).

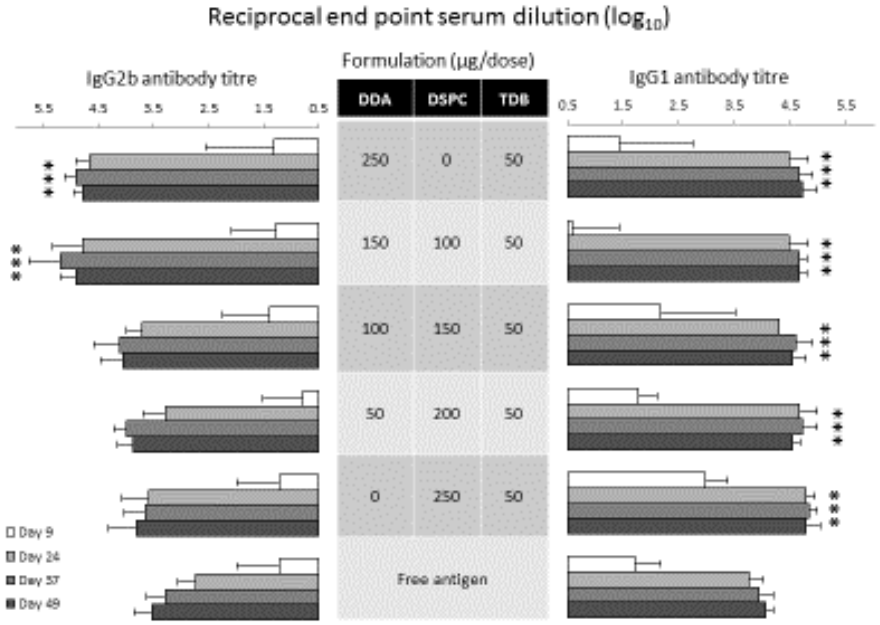

Figure 2

Figure 2. H56 specific antibody titres generated by DDA-TDB and its cationic replacement with DSPC for IgG1 and IgG2b. Values represent µg/dose, with sera collected before the first immunisation and on days 9, 24, 37 and 49 thereafter, and analysised for anti-H56 antibodies by ELISA. Results signify the reciprocal end point dilution (log10) compared with untreated control sera (n=5 ± SD). Significance is illustrated as $p<0.05$ increase compared to H56 vaccination group.

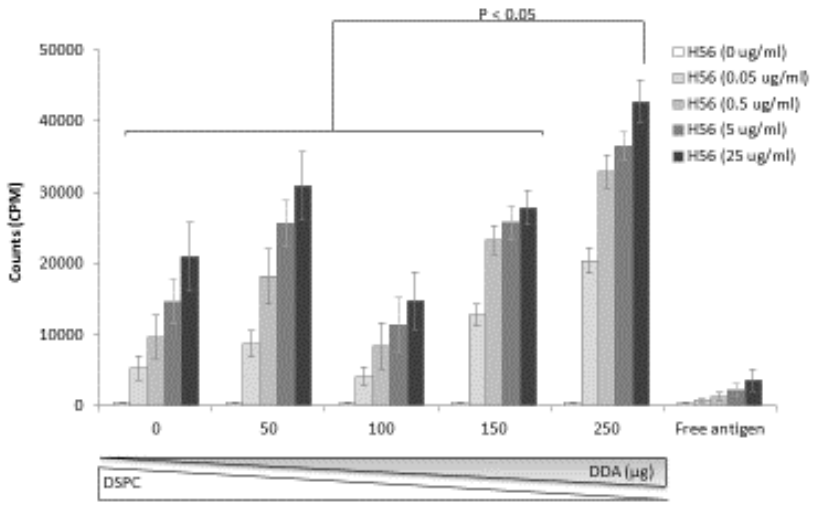

Figure 3

Figure 3. Spleen cell proliferation in response to stimulation/re-stimulation with H56 antigen upon replacement of cationic content within DDA-TDB. Formulation values represent µg/dose, with DDA-TDB and DSPC-TDB set to a 5:1 weight ratio (DDA/DSPC/TDB at 250/0/50 and 0/250/50 µg/dose respectively). H56 antigen specific splenocyte proliferation was indicated by the level of [^{3}H]Thymidine incorporation into cultured splenocytes at antigen concentrations of 0-25 µg/ml. ConA was used as a positive control at 2µg/mL with all counts in the region of 100,000 CPM. The results displayed denote the mean average for each group with associated standard error at n=5. Significance is illustrated between the liposomal vaccination groups, and comparisons shown against one another are upon re-stimulation with H56 vaccine antigen at 25 µg/mL.

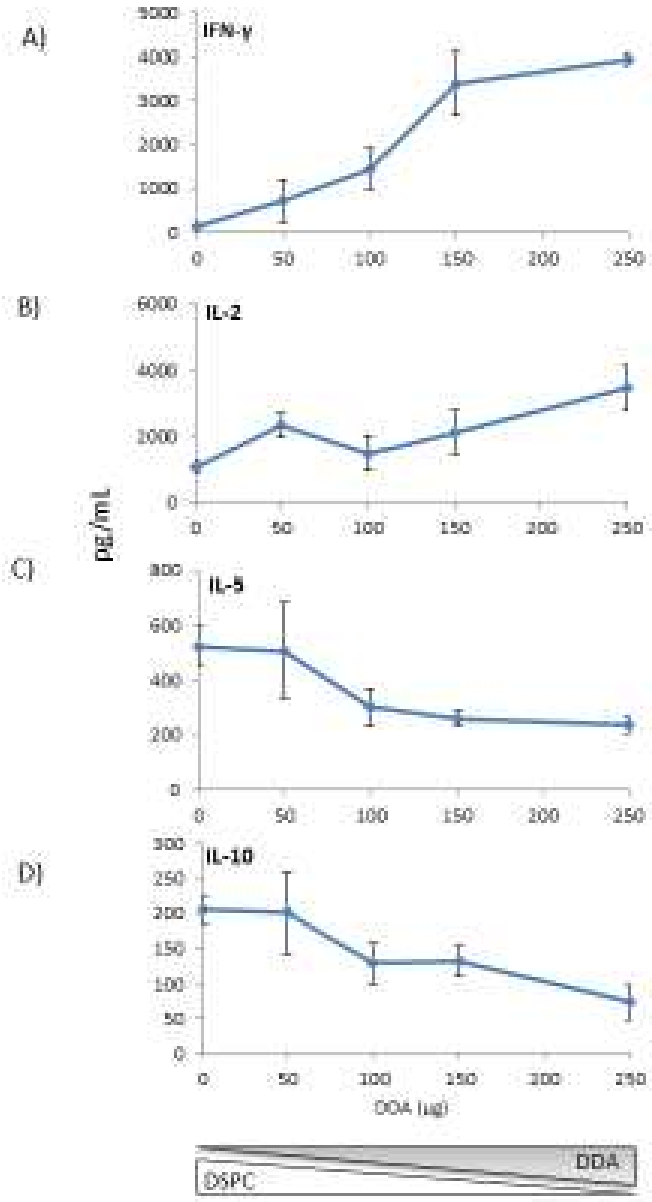

Figure 4

Figure 4. IFN-γ, IL-2, -5, and -10 cytokine production from splenocytes (A–D) derived from mice immunised with H56 combined with DDA/DSPC/TDB liposomes. Mice received 3 injections with 2-week intervals; splenocytes were obtained 3 weeks post the final immunisation. Splenocytes were restimulated for 48 h in the presence of H56 (5 µg/ml). Cytokines were measured from splenocyte using sandwich ELISAs.