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Lipid conjugation of TLR7 agonist Resiquimod ensures co-delivery with the liposomal Cationic Adjuvant Formulation 01 (CAF01) but does not enhance immunopotentiation compared to non-conjugated Resiquimod+CAF01

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**Abstract**

Pattern recognition receptors, including the Toll-like receptors (TLRs), are important in the induction and activation of two critical arms of the host defence to pathogens and microorganisms; the rapid innate immune response (as characterised by the production of Th1 promoting cytokines and type 1 interferons) and the adaptive immune response. Through this activation, ligands and agonists of TLRs can enhance immunotherapeutic efficacy. Resiquimod is a small (water-soluble) agonist of the endosome-located Toll-like receptors 7 and 8 (TLR7/8). However due to its molecular attributes it rapidly distributes throughout the body after injection. To circumvent this, these TLR agonists can be incorporated within delivery systems, such as liposomes, to promote the co-delivery of both antigen and agonists to antigen presenting cells. In this present study, resiquimod has been chemically conjugated to a lipid to form a lipid-TLR7/8 agonist conjugate which can be incorporated within immunogenic cationic liposomes composed of dimethyldioctadecylammonium bromide (DDA) and the immunostimulatory glycolipid trehalose 6,6’– dibehenate (DDA:TDB). This DDA:TDB-TLR7/8 formulation offers similar vesicle characteristics to DDA:TDB (size and charge) and offers high retention of both resiquimod and the electrostatically adsorbed TB subunit antigen Ag85B-ESAT6-Rv2660c (H56). Following immunisation through the intramuscular (i.m.) route, these cationic liposomes form a vaccine depot at the injection site. However, immunisation studies have shown that this biodistribution does not translates into notably increased antibody nor Th1 responses at the spleen and draining popliteal lymph node. This work demonstrates that the conjugation of TLR7/8 agonists to cationic liposomes can promote co-delivery but the immune responses stimulated do not merit the added complexity considerations of the formulation.

**Key Words:** Cationic liposomes, TLR agonist, Resiquimod, vaccine adjuvant, tuberculosis, biodistribution.
Introduction

Cationic liposomes composed of dimethyldioctadecylammonium bromide (DDA) and the immunostimulatory glycolipid trehalose 6,6′ – dibehenate (otherwise known as DDA:TDB or CAF01) have been shown to be a potent adjuvant and produce a Th1-biased immune response, when in combination with a range of sub-unit vaccines including chlamydia, influenza, HIV and tuberculosis vaccine (e.g. [1-5]). The adjuvanticity of DDA:TDB is in part ascribed to surface charge; the cationic nature of these liposomes allow them to adsorb anionic antigens and thereby mediate co-delivery of antigen and adjuvant to antigen presenting cells (APCs). DDA:TDB liposomes have also been shown to promote the formation of an antigen/adjuvant ‘depot’ at the injection site, followed by a sustained release to the draining lymph nodes [5, 6]. By following the fate of radio-labelled liposomes and antigen, biodistribution studies have demonstrated that the cationic charge (resulting from the quaternary ammonium present in the structure of the surfactant DDA), the high antigen adsorption, and the membrane rigidity of the DDA:TDB liposomes at body temperature (Tm ~ 42°C) promote the formation of this depot [5, 6]. By this means, APCs are recruited to the site of injection where they engulf the liposomal-antigen system. Subsequently, these immune cells become activated and move to the draining lymph nodes where they present the antigen to T cells and activating them [6]. The presence of TDB within the formulation also plays an important role promoting enhanced activation of APCs through interaction with the C-Lectin type receptor (CLR) Mincle [7, 8].

To further potentiate liposomal adjuvants, immunostimulatory agonists of Toll-like receptors can be included within the formulation. For example, CD8+ immunopotentiators, such as polyinosinic:polycytidylic acid (polyI:C) (TLR3) and unmethylated CpG oligodeoxynucleotides (CpG ODN) (TLR9) have be formulated within the liposome vaccine adjuvants in order to enhance their ability to promote immune responses [9-11]. Small molecule agonists, such as the TLR7/8 agonist resiquimod, can also be considered. However, their efficacy to act as a vaccine adjuvant can be variable; due to their molecular attributes, upon injection they are rapidly distributed throughout the body. This limits the ability of small molecule agonists to promote local activation of dendritic cells and hence activation of the immune response [12]. Therefore formulating these agonists to remain at the injection site may be important for optimal adjuvant activity, through either topical or dermal application [13-15], as well as incorporation within liposomes [16, 17]. Resiquimod is a synthetic imidazquinoline compound with potent activity as an anticancer and antiviral agent as well as a vaccine adjuvant [12, 18, 19]. Several studies evaluating the incorporation of resiquimod as a vaccine adjuvant have been carried out with a wide range of antigens and animal species. In general, TLR7/8 agonists increase the production of Th1 cytokines (such as IFN-γ, IL-2 and TNF-α)
whereas the production of Th2 cytokines (such as IL-5) is inhibited [20-22]. Unfortunately, studies have shown limited effectiveness of TLR7/8 agonists when compared to other TLR agonists [23, 24]. This might be due to their small size (e.g. resiquimod ~ 500 Da) and therefore, their fast distribution from the application site which results in reduced co-delivery and decreased adjuvant effect [25]. Another disadvantage is the systemic side effects observed after administration of small size TLR7/8 agonists [26]. In order to prolong the retention time of these TLR7/8 agonists at the site of application and reduce these side effects, different approaches through formulation design and delivery have been evaluated. This includes encapsulation in liposomes [27], topical application of antigen and resiquimod [28-30], conjugation of TLR7/8 agonists to antigens [31], polymers [32] or polysaccharides [33], modification of the molecular structure of the agonist itself [16, 34], and combination of TLR7/8 agonists with other adjuvants [35]. However, despite these studies, vaccine responses have been variable and the adjuvant efficacy of these systems has yet to be fully exploited.

Therefore the aim of this study was to consider if the adjuvant action of the cationic DDA:TDB liposomal adjuvants could be further potentiated by conjugating a TLR7/8 agonist to the liposome construct. To achieve this, we chemically synthesised a lipid-TLR7/8 agonist constructed from the phospholipid 1,2-distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and resiquimod. This conjugate can be incorporated into the CAF01 liposome system such that the TLR agonist can be displayed on the liposome surface alongside the H56 tuberculosis antigen (Ag85B-ESAT-6-Rv2660c) to potentially enhance vaccine adjuvant activity.

**Methods**

**Materials**

Dimethyldioctadecylammonium bromide (DDA), 1,2 - distearoyl-sn-glycero-3-phosphoethanolamine (DSPE) and the immunostimatory glycolipid trehalose 6,6′-dibehenate (TDB) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA). The Toll-Like Receptor (TLR) agonist, resiquimod was purchased from Sigma Aldrich (St. Louis, MO). Hydrogen peroxide, succinic anhydride, triphenylphosphine, sephadex™ G-75, sodium hydroxide, sodium thiosulphate, magnesium sulphate, hydrogen peroxide, sodium chloride, concanavalinA (ConA), diisopropyl azodicarboxylate (DIAD), heparin, sodium hydroxide, crystal sky blue (Pontamine blue), phospho-buffered saline (PBS) tablets, 2,2’-azino-bis (3-ethylenbenzthiazoline-6-sulfonic acid (ABTS), citric acid, protease inhibition mixture, sodium azide, HEPES buffer and Triton-X 100 were purchased from Sigma Aldrich (St. Louis,
Foetal Bovine serum (FBS) and RPMI 1640 cell culture medium (without L-glutamine) were from Biosera, UK. Penicillin-streptomycin-glutamine (PSG; 100 x liquid) was from Invitrogen. For radiolabelling, tritium-labelled cholesterol (\(^{3}H\)-Chol) was obtained from GE Healthcare (Amersham, UK), IODOGEN\textsuperscript{R} pre-coated iodination tubes from Pierce Biotechnology (Rockford, IL) and \(^{125}\text{I}\) (NaI in NaOH solution), SOLVABLE\textsuperscript{TM} and UltimaGold\textsuperscript{TM} scintillation fluid were purchased from Perkin Elmer (Waltham, MA). Ag85B-ESAT6-Rv2660c (H56 TB subunit vaccine antigen) was provided by Statens Serum Institut, Denmark at a concentration of 1.6 mg/mL. Methanol, ethyl acetate, and chloroform (all HPLC grade) were purchased from Fisher Scientific (Leicestershire, UK). Tris-base, obtained from IDN Biomedical, Inc (Aurora, Ohio) was used to make Tris buffer and adjusted to pH 7.4, unless otherwise stated. Trehalose [D-Trehalose (99 %) anhydrous] was obtained from Acros Organics. Duoset Sandwich ELISA kits and solutions (IFN-\(\gamma\), IL-17, IL-2, IL-6, IL-10, IL-1\(\beta\), IL-18 and IL-33) were obtained from R & D Systems. Deuteriated chloroform and dimethylsulfoxide (DMSO) were both purchased from Cambridge Isotope Laboratories (MA, USA). Silica gel 60 and TLC Silica 60 plates were purchased from Merck (Darmstadt, Germany). All antibody-specific immunoglobulins (IgG, IgG1 and IgG2b) were purchased from AbD SeroTec. Double distilled water was used to make buffers and solutions used.

**Chemical Synthesis**

**Step 1 formation of the succinyl - linker:** DSPE lipid and succinic anhydride (SA) were dissolved in chloroform/methanol (9:1 v/v) at a ratio of 1:5 M/M and the reaction was completed at room temperature overnight (Figure 1). The progress of the succinylation reaction [36] was monitored by TLC and gave a complete conversion. For work up the mixture was ‘quenched’ by the addition of sodium hydroxide (NaOH) at a 1M concentration. The organic phase was dried with magnesium sulphate (MgSO\(_4\)) and the succinyl – DSPE linker was obtained in quantitative yield as solid intermediate.

**Step 2 Conjugation of resiquimod by Mitsunobu reaction:** The succinyl – DSPE linker and resiquimod were added together during this reaction at a 1:1 M/M ratio for complete reaction. A solution of DIAD, diisopropyl-azodicarboxylate in THF (1 M) was added to the mixture under Nitrogen gas atmosphere followed by 1.0 eq. of solid TPP, triphenylphosphine. The reaction was performed at room temperature over 2 hours, under magnetic stirring and controlled by TLC. Upon completion of the reaction as described previously excess water was ‘quenched’ by the addition of sodium hydroxide (NaOH) at a 1M concentration. The crude conjugated adduct was purified by
column chromatography with ethyl acetate using silica gel. The DSPE -resiquimod conjugate was fully analysed by MS, IR and NMR spectroscopy to confirm the product.

Preparation of liposomes

Liposome formulations were prepared by the previously established lipid film hydration method [37]. Stock lipid solutions were dissolved in a chloroform:methanol mixture (9:1 v/v) and DDA and TDB mixed to a final concentration of 1.25 mg and 0.25 mg DDA and TDB per mL respectively, representing a 5:1 DDA/TDB weight ratio (8:1 molar ratio). To investigate the incorporation of resiquimod onto the liposomes formulations, DDA:TDB liposomes were formulated with inclusion of the DSPE-resiquimod conjugate to a final concentration of 0.185 mg per mL. To serve as a control, resiquimod was added to pre-formed DDA:TDB:DSPE liposomes at the same molar ratio. Therefore each dose contained 0.4 μmol lipid (DDA), 0.05 μmol TDB, 0.032 μmol DSPE lipid and 0.032 μmol resiquimod. Lipid mixtures were added to a round bottomed flask and upon solvent extraction via rotary evaporation and N₂ 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 (DDA at ~47 °C). The subunit protein antigen, H56 (Ag85B-ESAT6-Rv2660c) was added at an in vivo dose of 5 μg (0.1 mg/mL formulation).

Characterisation of particle size and zeta potential for liposome-delivery systems

The intensity mean diameter of all liposome formulations were measured using a Malvern Zetasizer Nano-ZS (Malvern Instruments, Worcs., UK) via dynamic light scattering (DLS). Vesicle size and zeta potential were measured in triplicate at 25 °C by diluting liposomes 1 in 10 in Tris buffer (1 mM, pH 7.4).

Radiolabelling of Antigen

The protein antigen, H56 (Ag85B-ESAT6-Rv2660c), was radiolabelled with ¹²⁵I using pre-coated iodination tubes [(or IODOGEN® tubes) Pierce Biotechnology, Rockford, IL). Separation of ¹²⁵I radiolabelled protein from free ¹²⁵I was carried out using a Sephadex G-75 gel column, pre-soaked in ddH₂O and equilibrated with Tris buffer. This method was carried out as described previously [5].

Radiolabelling of TLR agonist
During this investigation it was required to determine the agonist loading and also to track the biodistribution of TLR agonist in vivo. Resiquimod or DSPE-conjugated resiquimod was radiolabelled with $^{125}$I in an IODO-GEN® tube and left for 1 hour with intermittent swirling. Following the radiolabel, these two products can be incorporated within liposome formulations as described. Due to the radiolabelling procedure not being 100% efficient there may still be some free $^{125}$I. This was removed by using sodium thiosulphate (Na$_2$SO$_3$), which will convert free iodine to iodide (I$_2$ $\rightarrow$ 2I$^-$), followed by extended dialysis (using 3 kDa dialysis tubing).

**Antigen and agonist loading in simulated in vivo conditions**

Radiolabelled H56 or resiquimod (antigen and TLR agonist respectively) were added to each liposome formulation at an in vivo concentration of 5 μg and 10 μg per dose (0.1 or 0.2 mg/mL) respectively, and left to adsorb to the liposome for 45 minutes with intermittent swirling. Surface-associated and unadsorbed antigen/agonist was separated from liposomes by diluting the suspension to 1 mL using Tris buffer, followed by centrifugation on an Optima Max-XP Ultracentrifuge (Beckman-Coulter Inc., Fullerton, CA) at a speed of 125,000 x g (45 minutes at 4 °C). The quantity of radiolabelled antigen or TLR agonist ($^{125}$I-H56, and $^{125}$I-Resiquimod or $^{125}$I-Resiquimod-DSPE) 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 and adsorption of protein antigen or TLR agonist was then determined by calculating the % radioactivity in the liposome pellet fraction. Antigen and Agonist retention studies were undertaken in simulated in vivo conditions (50% FCS in Tris buffer, 37 °C) with samples processed as above at periodic intervals.

**Biodistribution studies to investigation the localisation of vaccine components upon intramuscular injection**

Experimentation strictly adhered to the 1986 Scientific Procedures Act (UK). All protocols have been subject to ethical review and were carried out in a designated establishment. Groups of four 6–8 week old female BALB/c mice were housed appropriately and given a standard mouse diet ad libitum. Four to six days prior to each vaccination, mice were injected subcutaneously (s.c.) with 200 μl pontamine blue (Sigma Aldrich, 0.5% w/v in PBS). Pontamine blue is phagocytosed by monocytes [38] and is therefore a suitable marker for aiding location of lymph nodes during dissection. Liposomes composed of DDA in combination with TDB, with either DSPE-Resiquimod conjugate
(DDA:TDB-Res) or DSPE with post-LH addition of resiquimod (DDA:TDB:Res) and the tracer lipid ³H-cholesterol were produced with the addition of trehalose (10% w/v) to the hydrating Tris buffer in order to maintain isotonicity. ¹²⁵I-labelled Resiquimod or DSPE-Resiquimod were incorporated with the liposomes as appropriate. Subsequently, unlabelled H56 antigen was added to the various liposome formulations at an in vivo dose of 5 µg (0.1 mg/mL). Each immunisation dose contained 0.4 µmol lipid (DDA), 0.05 µmol TDB, 0.032 µmol DSPE lipid and 0.032 µmol resiquimod and 5 µg H56. Mice were injected im (50 µL) into the left quadricep. Each immunisation dose contained 100 kBq ¹²⁵I (radiolabelled agonist) and 100 kBq ³H-Chol (radiolabelled liposomes). At time points 1, 4 and 8 days post injection (pi), mice were terminated by cervical dislocation. Tissue from the injected muscle site (SOI), local draining popliteal lymph node (PLN) were removed and processed as described previously [39, 40] to determine the proportion of ³H (liposome) and ¹²⁵I (agonist) in the tissues.

Immunisation studies

All experiments were undertaken in accordance with the Scientific Procedures Act of 1986 (UK). Female C57BL/6 mice, 6-8 weeks old (Charles River, UK) were split into 5 groups of 5 mice. Vaccine formulations were prepared by the lipid film-hydration method with the liposomes adsorbing H56 antigen at a final concentration of 0.1 mg/mL (5 µg dose). These formulations were prepared with the addition of trehalose (10 % w/v) to the hydration buffer in order to maintain isotonicity. All mice were immunised intramuscularly into the left quadricep (50 µL/dose) three times (days 0, 14 and 28) and at scheduled time points, blood samples were taken from the tail and stored at -20 °C for future analysis of antibodies.

Evaluation of H56-antigen specific antibody isotypes

Samples of blood sera were collected on day 46 for the detection of IgG, IgG1 and IgG2b antibodies (AbD Serotec, Oxford, UK). Blood (50 µL) was collected via tail-bleeding using capillary tubes coated with 1% (w/v) heparin (Sigma Aldrich). Blood was diluted 10-fold in PBS and centrifuged at 10,000 x g (room temperature for 5 minutes) to obtain blood sera and frozen at ~ 20 °C for future analysis. Standard ELISA protocol was used to detect antibodies against H56. Plates were coated with H56 antigen (5 µg/mL in PBS) before overnight incubation at 4 °C. The following day, plates were washed and blocked with skimmed milk powder (4 % w/v in PBS) for 1 hour. Serially diluted (100 µL) serum was added to washed plates and incubated at 37 °C for 1 hour. In order to detect anti-H56
antibodies, goat anti-mouse IgG (1:750) and IgG1(1:4000) were added to wells and incubated for 1 hour at 37 °C followed by washing and addition of 2,2’-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) [ABTS] substrate solution in citrate buffer incorporating 5 μl of 30% H₂O₂/50 ml citrate buffer. Absorbance was read at 405 nm (BioRad, Herts., UK) and the results expressed as the mean of 5 mice per group ± SD of the log₁₀ of the reciprocal end-point dilution.

Cytokine analysis from restimulated splenocytes and popliteal lymph nodes

At the final time point (day 49) of the vaccine study, mice were terminated and spleens were collected. Spleen cell suspensions were produced by mashing through a fine wire mesh into 10 mL RPMI 1640 cell culture medium supplemented with 10% FBS and 1% PSG. Cell suspensions were washed twice with complete RPMI (cRPMI) and resuspended to a final concentration of 8 x 10⁶ cell/mL. Cells were plated in 96-well cell culture plates (100 µL/well) restimulated with either cRPMI alone (negative control), H56 antigen diluted in cRPMI to final concentrations of 0.05, 0.5 or 5 µg/mL, or with concanavalin A (positive control) to a final concentration of 2 µg/mL.

Following 72 h incubation at 37 °C, supernatants were removed and pooled according to group and restimulation condition. Duoset® Capture ELISA kits were used according to the manufacturer’s instructions to detect IL-2, IL-5, IL-6, IL-10 and IFN-γ (R&D, Abingdon, UK) in the supernatants. During this protocol, ELISA plates were coated overnight with capture antibody (at room temperature). The following day, plates were washed followed by blocking with 1 % BSA (in PBS). Samples and serially diluted standards were added to washed plates and incubated for 2 hours at room temperature. Cytokines were detected by addition of detection antibody, streptavidin-HRP conjugate (1:200 dilution), TMB substrate solution and stop solution (2N H₂SO₄). The OD at 450 nm was measured (BioRad, Herts., UK) and a sigmoidal standard curve for each cytokine standard was created to determine cytokine concentrations in these supernatant samples obtained from restimulated splenocytes.

Statistical analysis
Data was tested by one-way analysis of variance (ANOVA) followed by the post-hoc Tukey test in order to compare the mean values of different groups. Differences were considered to be statistically significant at p < 0.05 in all studies. All experiments were carried out in triplicate.

**Results**

**Preparation of the lipid-Resiquimod conjugate**

Various chemical approaches conjugating a lipid with a TLR agonist were investigated. Resiquimod, besides its well-known immunogenicity, also has a chemical structure that enables lipid conjugation. DSPE contains a nucleophilic amine and resiquimod contains a tertiary alcohol, making the anhydride of succinic acid an ideal linker agent. Experimentally, succinic anhydride SA was found the best linker molecule. SA formed in quantitative yield the stage 1 succinamide intermediate containing a free carboxylic acid for subsequent reaction (Figure 1). Generally, agents to activate the carboxylic acid are used for esterification, but here a tertiary alcohol had to be coupled and this was performed best with the Mitsunobu Reaction. The Mitsunobu reaction is a unique dehydration-condensation reaction between alcohols and various other nucleophiles [41-43]. The tertiary alcohol of resiquimod is essential for TLR7/8 agonist properties and chemically this is providing a reactive substrate for SN1 nucleophilic substitutions reactions. Under Mitsunobu conditions with DIAD as activating agent, the carboxylic acid acted as nucleophile and was coupled in presence of triphenyl phosphine into the conjugated DSPE – resiquimod. Thus, the chemically lipid bound TLR agonist resiquimod was obtained in only 2 chemical steps in high yields as a solid material and only one chromatographic purification was required. The covalently bound TLR agonist resiquimod, which is an approved pharmaceutical ingredient, is a key feature in this approach. In the experimental section the fully optimised reaction is reported and the ease of monitoring the reaction by TLC was found ideal in a pharmaceutical laboratory setting. Products and by-products of the reaction, such as triphenyl phosphine oxide [41, 44] indicate the progress of the reaction and the stage 1 intermediate can be in situ converted into the fully conjugated final product, which was then purified by column chromatography to give an analytical pure material.
Figure 1. Conjugation of DSPE to Resiquimod. Step 1 includes the linker formation with SA and in step 2 the succinylated 1,2-distearoyl-sn-glycero-phosphoethanolamine (DSPE) is conjugated to resiquimod in a Mitsunobu reaction.

Formulation of cationic liposomes displaying a lipidated TLR7/8 agonist

As mentioned, DDA:TDB liposomes have been shown to be effective adjuvants in a range of studies [1, 4, 11, 45-49] and the aim of this work was to further potentiate the efficacy of these liposomes through the presence of resiquimod on the liposome surface. Therefore, to consider the impact of the addition of the lipid-TRL agonist conjugate on the formulation, a range of physico-chemical studies were conducted to compare DDA:TDB liposomes to liposomes prepared from DDA:TDB:DSPE mixed with resiquimod (DDA:TDB:Res) and liposomes prepared with the DSPE-resiquimod conjugate (DDA:TDB-Res). All formulations were prepared alone or with the addition of H56 antigen (Figure 2).

Liposomes without the addition of H56 antigen were in the range of 400 to 600 nm, with PDI values around 0.2 to 0.5 irrespective of the presence of resiquimod (Figure 2A). The addition of H56, which electrostatically binds to the cationic liposomes, results in a general trend of increased vesicle size and PDI across all three formulations (Figure 2A). With regard to the cationic nature of the
liposomes, this remains similar and high for all three liposome formulations, with and without the presence of the H56 antigen (approximately 60-70 mV; Figure 2B).

The cationic nature of these liposomes promotes high antigen loading at the doses used, with approximately 85% antigen loading for all three liposome formulations (Figure 3A). To consider the association of resiquimod with the liposome formulations, the loading of resiquimod was also measured. Simple mixing of resiquimod with cationic liposomes (DDA:TDB:Res) resulted in low agonist association (approximately 15%; Figure 3A), as would be expected given there is little ability of electrostatic interactions between the cationic liposomes and resiquimod. In contrast, over 85% resiquimod was incorporated within the liposome formulation using the lipid-resiquimod conjugate (DDA:TDB-Res; Figure 3A). Antigen and agonist retention to these various liposomes was also tested in a simulated in vivo environment (50% FCS in Tris buffer (10 mM); 37 °C). Following a burst release over the initial 3 hours of the study (10-20%), antigen release stabilised over the rest of the 96-hour period (Figure 3B). This demonstrates that antigen can be retained by the delivery system even under simulated in vivo conditions with the presence of conjugated resiquimod making no significant difference. In terms of TLR7/8 agonist retention, high levels of resiquimod conjugate was retained by the DDA:TDB liposomes (DDA:TDB-Res; Figure 3C). This confirms that both antigen and lipidated resiquimod can be efficiently retained by cationic liposomal adjuvants in simulated in vivo conditions. This may be important as recent studies have suggested that the co-localisation of antigen and immunostimulators is important for optimal vaccine adjuvant activity [8] as simultaneous delivery of these to the same antigen presenting cell is crucial for the downstream vaccine-mediated immune responses.
Figure 2. Characterisation of liposome product. The vesicle size and PDI (A) and zeta potential (B) of the DDA:TDB liposomes with and without the addition of H56 antigen (DDA:TDB and DDA:TDB:H56), and either mixed with resiquimod (DDA:TDB:Res) or with resiquimod conjugated to the liposome (DDA:TDB-Res). All results represent mean ± SD of 3 independent liposome batches.

Figure 3. Antigen and Agonist loading and retention on liposomes; A) shows the Antigen (H56) and Agonist (resiquimod) loading on the three liposome formulations; B) shows the antigen retention with the three liposome formulations over 98 h; C) shows resiquimod retention when conjugated to the liposomes. All results represent mean ± SD of 3 independent liposome batches.

Conjugation of a TLR7/8 agonist to cationic liposomes avoids rapid distribution and promotes a depot of both liposomes and TLR agonist at the injection site.

Previous studies have suggested that the ability to form a depot is important for the function of many adjuvants [5, 6, 51] and through a range of studies, we have demonstrated the depot forming effect of DDA:TDB liposomes [5, 6, 39]. Based on this, we compared the biodistribution of the three liposome formulations to consider the ability of the liposome formulation containing conjugated resiquimod (DDA:TDB-Res) to retain the TLR agonist with the liposomes (Figure 4).
Incorporation of DSPE (either lipid alone or the lipid-TLR7/8 conjugate, both present at the same molar ratio) had no significant effect on liposome retention at the injection site all time points measured, with all formulations studied showing a liposome depot effect (Figure 4A). At day 1 pi, DDA:TDB gave liposome dose retention of ~ 85 % (in-line with previous studies [5, 51]) with approximately 30 % of the dose remaining at day 8. With the addition of DSPE in the bilayer and free resiquimod in the formulation (DDA:TDB:Res), or the liposome formulation with conjugated resiquimod (DDA:TDB-Res,) similar levels of liposomes remained at the injection site (70-75 % remaining at day 1, 40 % after 4 days, and 25 % of the dose remaining at 8 day pi; figure 4A). This is in line with the data presented in Figures 2 and 3, which demonstrates that the presence of the additional lipid (DSPE) with or without resiquimod conjugated made no impact on the measured physico-chemical attributes nor the biodistribution of the vesicles.

When considering resiquimod retention at the depot site, high levels (70 %, 40 % and 24 % of the dose at days 1, 4 and 8 pi respectively; Figure 4B) were retained at the depot site along with the liposomes, when resiquimod was conjugated to the liposomes. In contrast, after intramuscular injection of resiquimod either alone (Res) or mixed with DDA:TDB liposomes (DDA:TDB:Res) only low levels (6% or less) were detected after 24 h (Figure 4B), demonstrating the rapid clearance of resiquimod from the injection site when not conjugated to the liposomes. These results demonstrate the ability of the cationic liposomes with conjugated resiquimod to form a depot at the injection site.

![Diagram](https://via.placeholder.com/150)
When considering the movement to the draining lymph node (the popliteal lymph node; PLN), the cationic liposomes were shown to drain at a similar rate irrespective of the presence of free or conjugated resiquimod (Figure 4C). When tracking the presence of the agonist at the PLN, free resiquimod was shown to quickly drain to the lymph node and then rapidly clear (Figure 4D). In the case of resiquimod conjugated to DDA:TDB liposomes, the movement of resiquimod to the PLN maps closely to that of the liposomes, again demonstrating that the conjugation of resiquimod to the cationic liposomes facilitates the movement of the antigen-adjuvant complex together from the site of injection to the local draining lymph node.

Conjugation of a TLR7/8 agonist to cationic liposomes does not improve antibody responses.

The ability of H56 vaccine antigen either delivered alone or in combination with the 3 different liposome formulations (DDA:TDB, DDA:TDB:Res, DDA:TDB-Res) to induce IgG (total), IgG1 and IgG2b antibody isotypes was investigated using antibody ELISAs (Figure 5). Resiquimod alone was also used as a negative control.

In general, all three liposome formulations induced similar immune responses for IgG, IgG1 and IgG2b these responses with no significant difference between the three formulations. All three formulations did promote significantly higher (P < 0.05) than responses generated in mice immunised with antigen alone. Also resiquimod alone-immunised mice (negative control) did not promote detectable antibody responses for all three antibody isotypes investigated (Figure 5).

Figure 5. H56-antigen specific antibody responses in the blood sera (IgG, IgG1, IgG2b). Blood was collected at day 46 from mice immunised with H56 in combination with either DDA:TDB, DDA:TDB:Res or DDA:TDB-Res. As negative controls, resiquimod and H56 antigen were injected alone. Mice received 3 injections with 2-week intervals. Results represent the mean of 5 mice per experimental group ± SD.
Co-delivery of Mincle and TLR7 agonist by liposomes does not influence Th1 cytokine production in restimulated cells from spleen and lymph nodes

Both Mincle and TLR7 activation have been demonstrated to promote a vaccine-mediated skewing of the immune profile to a more Th1 directed response [16, 58]. Therefore, we investigated if dual activation of both receptors by the formulated DDA:TDB-Res liposomes would further boost Th1 responses (Figure 6). Mice were vaccinated with H56 antigen in combination with DDA:TDB-Res, DDA:TDB mixed with Res (DDA:TDB:Res) or DDA:TDB liposomes and the supernatants of restimulated splenocytes and popliteal lymph nodes (PLN) were assayed for the presence of cytokines IFN-γ, IL17, IL-2, IL-6, IL-5 and IL-10. In line with previous reports highlighting the strong Th1-mediating effects of DDA:TDB liposomes [4, 47, 48], high levels of IFN-γ and low levels of IL-5 and IL-10 were noted with cells from both the spleen and local lymph node (Figure 6A and B respectively). The results in figure 6 show that all three liposome formulations enhanced cellular immunity but the benefit of including the resiquimod is not clear. Across the cytokines tested, whilst there are some improvements in IFN-γ production this is already high for the cationic liposomes. This data, combined with the antibody data, suggests that whilst conjugation of resiquimod to the liposomes successfully co-delivers the liposomes with the resiquimod and the antigen, there is no notable improvement in immune response profiles.
Figure 6. Cytokine production (IFN-γ, IL-17, IL-2, IL-5, IL-6 and IL-10) from cultured restimulated spleen (A) and popliteal lymph node (B) cells derived from mice immunised with H56 in combination with either DDA:TDB, DDA:TDB:Res, DDA:TDB:Res. As negative controls, resiquimod and H56 antigen were injected alone. Mice received 3 injections with 2-week intervals and cells were obtained 3 weeks post the final immunisation. Cells were restimulated for 72 hrs in the presence of H56 (at 5 μg/mL). Cytokines were detected from spleen (A) and popliteal lymph node (B) cell supernatants and measured using sandwich ELISAs. Results represent the mean of 5 mice per group ± SD.

Discussion

In this study we have demonstrated that the TLR7 agonist resiquimod can be redesigned to include a DSPE lipid tail, which allows for insertion into cationic liposomes stabilised by TDB. The lipidated resiquimod was stable and remains bound to the DDA:TDB liposomes. In contrast, simple mixing of resiquimod with cationic liposomes resulted in low agonist loading, as expected given there is little ability of electrostatic interactions between the cationic liposomes and resiquimod in its native state. Thus it is reasonable to expect that incorporation of lipidated resiquimod to the DDA:TDB liposomes allowed for co-delivery of Mincle and TLR7 agonists to the same antigen presenting cell.

Many studies have demonstrated the ability of TLR7 agonists to boost vaccine-induced immune responses [58,59]. Further boosting or redirection of immune responses by co-adjuvantage of TLR7 agonists with other adjuvants has also been described. E.g. alum precipitated TLR7 agonists (alum-TLR7) were shown to boost antibody titers to a glycoconjugate vaccine (CRM197-MenC), an acellular pertussis vaccine and a protein-based vaccine against Staphylococcus aureus in mice. Furthermore, this inclusion of a TLR7 agonist with Alum re-directed the alum-induced Th2 flavoured response towards a more Th1-biased immune profile [60-62]. Using anionic liposomes, Fox et al.
demonstrated that co-delivery of the TLR4 agonist GLA and the TLR7 agonist imiquimod increased Th1 responses [27]. Cationic liposomes containing TDB (CAF01) already induce a strong Th1 profile associated with high levels of CD4+ T cells producing IFN-γ [58]. Interestingly, we found that formulating the TLR7 agonist resiquimod into the liposomes made no notable impact on the IFN-γ, IL-2 and TNF-α responses upon reactivation of splenocytes. Therefore, there is no clear evidence to support a distinctive increase in immune responses and why the more complex resiquimod conjugated formulation should be adopted over the simple cationic liposome formulation. However, it is possible that there could be a greater benefit seen in humans or larger animals.

Resiquimod as a TLR7 and 8 agonist has been reported to induce Th1 cytokine responses through a variety of mechanisms. It activates the DCs by binding the TLR7 which are largely located in the endosomal compartments on DCs giving rise to the production of type I IFN [54, 55], upregulation of MHCII and the co-stimulatory receptors CD80 and CD86 [63]. TLR8 is mainly expressed on macrophages and monocytes which generate TNF-α and IL-12 cytokines and thus, contribute to the increased Th1 response [53, 54]. However, despite resiquimods dual ligation of both TLRs, in mice it only exerts its effect by binding to TLR7 as TLR8 is non-functional in mice [56].

As we have demonstrated here, lipidation of the TLR7 agonist resiquimod was necessary for the compound to stably associate with cationic liposomes. However, the lipidation may also in itself have an effect on activation of the immune response. Other lipidated TLR7/8 agonists have been described, e.g. the imidazoquinoline 3M-052 bearing a fatty acyl C18 lipid moiety [16]. Notably, it was found that this compound formulated in dioleoylphosphatidylcholine liposomes resulted in a boost of local immunity at the site of injection and in draining lymph nodes rather than more systemic effects, which may have the advantage that it minimizes the cytokine storm-like effects that is one concern for the small-molecule TLR7/8 agonists [16, 32].

An important consideration for experimental vaccines is translation of the immune profile from small animal species to humans. Whilst no vaccines including TLR7/8 ligands are yet licensed, studies in monkeys have shown that including a TLR7/8 agonist allows for boosting of anti-HIV Envelope IgG responses compared to when alum was used alone [64, 65]. Furthermore, the capability of co-adjuvantation with TLR7 agonists to redirect the alum-induced T cell responses towards IFN-γ producing Th1 cells was also demonstrated in monkeys [67]. It was recently found that a TLR7/8 agonist can boost antibody titers to pneumococcal conjugate vaccine at birth [66]. Immunity is impaired at the extremes of age. Particularly, neonates and elderly have poor responses to several TLR agonists and vaccine responses are therefore impaired in these populations. Dual targeting of pathogen recognition receptors is one promising strategy to boost the low vaccine-
induced immune responses observed in neonates and elderly [66, 67]. The potential to overcome the impaired immunity of specific age or immunocompromised risk groups is therefore also an important application for future studies of the adjuvant combination developed in the present study.

Conclusions

During these studies we have been able to design and synthesise a novel conjugate between lipid and TLR7/8 agonist, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine and resiquimod respectively. This novel lipid-resiquimod conjugate can be effectively incorporated into cationic liposomes. These liposomes form a depot at the injection site allowing the liposomes, antigen and TLR7/8 agonist to be co-located and co-presented. However, this did not notably enhance antibody nor cytokine responses, with strong Th1 cytokine responses seen with the cationic liposomes irrespective of the presence of the conjugated resiquimod. This suggests that despite co-delivery, the presence of the TLR7/8 on the cationic liposome formulation was not able to further potentiate the strong responses generated by this cationic adjuvant.

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Highlights

1. Resiquimod is a small water-soluble agonist of Toll-like receptors 7 and 8.
2. A lipid conjugate of Resiquimod was synthesised using a two-step process.
3. The lipid-resiquimod conjugate was incorporated into a liposomal adjuvant.
4. Co-delivery of antigen, TLR and liposomal adjuvant was achieved.
5. This co-delivery did not immune responses.