# Scale-Independent Microfluidic Production of Cationic Liposomal Adjuvants and Development of Enhanced Lymphatic Targeting Strategies

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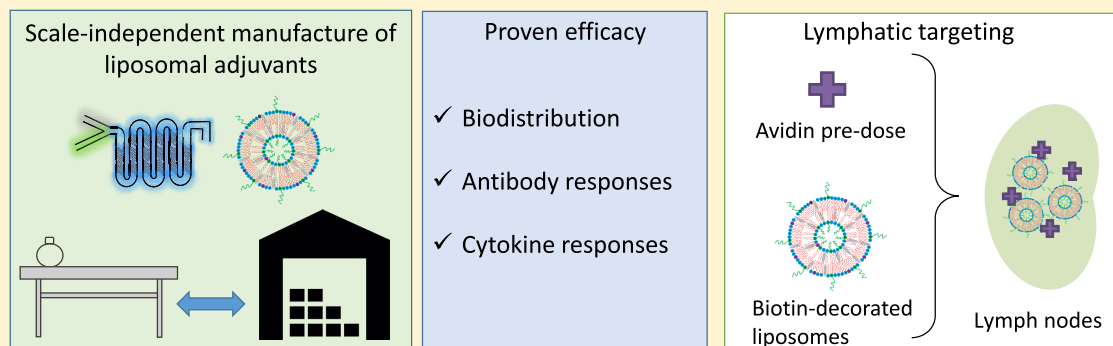
ABSTRACT: Cationic liposomes prepared from dimethyldioctadecylammonium bromide (DDAB) and trehalose 6,6′-dibehenate (TDB) are strong liposomal adjuvants. As with many liposome formulations, within the laboratory DDAB:TDB is commonly prepared by the thin-film method, which is difficult to scale-up and gives high batch-to-batch variability. In contrast, controllable technologies such as microfluidics offer robust, continuous, and scale-independent production. Therefore, within this study, we have developed a microfluidic production method for cationic liposomal adjuvants that is scale-independent and produces liposomal adjuvants with analogous biodistribution and immunogenicity compared to those produced by the small-scale lipid hydration method. Subsequently, we further developed the DDAB:TDB adjuvant system to include a lymphatic targeting strategy using microfluidics. By exploiting a biotin−avidin complexation strategy, we were able to manipulate the pharmacokinetic profile and enhance targeting and retention of DDAB:TDB and antigen within the lymph nodes. Interestingly, redirecting these cationic liposomal adjuvants did not translate into notably improved vaccine efficacy.

KEYWORDS: *microfluidics, manufacture, vaccine adjuvants, cationic liposomes, lymphatic targeting*

1. INTRODUCTION

Liposomes have been extensively studied as vaccine adjuvants. However, current production methods for liposomes are costly, multistep, and generally limited to batch production. Given that the cost of vaccines is a key contributing factor in global accessibility, low-cost scalable production of vaccine adjuvants is required to ensure an affordable supply chain. To address this and bring down the costs of liposomal adjuvants, streamlining their manufacturing process is essential. Recently, the application of microfluidics has been demonstrated for a range of nanoparticles and liposomes (e.g., see refs 1−5). Microfluidics as a manufacturing platform offers a scale-independent alternative to batch production; the nanoparticle product attributes have been shown to be process-controlled in terms of particle size, and high protein loading can be achieved compared to other production methods.[1]

In the manufacture of liposomal adjuvants, control of the physicochemical attributes is vital given that these are often critical quality attributes. Indeed, a range physicochemical attributes have been shown to impact on the immunological

properties of liposomal adjuvants, including particle size,[6−9] charge,[10−12] lipid composition,[11,13,14] fluidity,[14−17] and degree of pegylation.[18−21] Furthermore, several of these physicochemical attributes also dictate the pharmacokinetic properties of both the liposomal adjuvant and the subunit antigen and the recruitment of antigen presenting cells (APCs) to the injection site.[8] Thus, by modifying these attributes, both the pharmacokinetic and immunogenic profile can be manipulated. In particular, the use of cationic lipids has a strong impact on both the adjuvanticity of liposomes and their retention at the site of injection. For example, the use of the cationic lipid *N,N′*-dimethyl-*N,N′*-dioctadecylammonium bromide (DDAB) has been shown to favor the absorption of subunit antigens onto the liposomal surface, promote retention of both the adjuvant and antigen at the injection site, and promote strong cell-mediated

immune responses.[10] To formulate liposomal adjuvants, DDAB is often used in combination with the synthetic immunopotentiator α,α'-trehalose-6,6'-dibehenate (TDB) to improve the stability of the liposomes and enhance the immunogenicity of the formulation.[22] TDB is a synthetic analogue of trehalose 6,6'-dimycolate (TDM), a mycolic acid from the mycobacterial cell wall from *Mycobacterium tuberculosis*, and it is combined with DDAB at a weight ratio of 5:1 (DDAB:TDB).[22] The adjuvanticity of DDAB:TDB is generated via the Syk−Card9−Bcl10−Malt1 pathway. In this way, TDB activates macrophages and dendritic cells (DCs).[23] Moreover, interaction with the Mincle receptor (a C-type lectin receptor expressed in macrophages) which also stimulates MyD88-dependent Th1/Th17 responses may contribute to DDAB:TDB efficacy.[24,25] Through this pathway, DDAB:TDB promotes strong cellular and humoral immune responses based on high IFN-γ and IL-17 secretion, low IL-5 production, and high IgG antibody production.[26,27] The pharmacokinetic profile of DDAB:TDB and numerous variants has also been investigated to consider potential links between biodistribution and vaccine efficacy. By dual radiolabeling of the adjuvant and the antigen, it has been shown that cationic liposomes form a depot at the injection site, followed by a sustained release to the draining lymph nodes.[15,28] However, it has also been shown that direct injection of DDAB:TDB into the lymph node promotes a strong response.[29] Indeed, Mohanan et al. showed the importance of the route of administration for the DDAB:TDB liposomal formulation; while no significant differences were found between subcutaneous, intramuscular, or intradermal vaccination, intralymphatic administration of DDAB:TDB liposomes resulted in significantly higher IgG2a and IFN-γ responses.[29] Thus, the potential to redirect an increased dose of DDAB:TDB to the draining lymphatics and further enhance immune responses is an interesting consideration. To promote retention at the draining lymphatics, a biotin−avidin complex formulation can be adopted. Studies carried out by Phillips et al.[30] demonstrated the ability of this high-affinity complex to improve the accumulation and retention of liposomes into the draining lymph nodes. By this means injection of biotin-coated liposomes, in combination with an adjacent intramuscular injection of avidin, become localized/trapped in the draining lymph nodes because of the formation of avidin−biotin-coated liposome complexes.[30,31] Thus, exploiting the high affinity between biotin and avidin resulted in higher accumulation of liposomes in the lymph nodes (up to 14%) in comparison to biotin-coated liposomes injected without avidin (2% of the injected dose).[30]

As with many liposome formulations, the common method for preparing DDAB:TDB liposomal adjuvants within the laboratory is via the hydration method (LH),[22,32] which results in the formation of large multilamellar vesicles (MLVs) which are heterogeneous in nature. In order to reduce the size and lamellarity of these liposomes, sonication or high-shear mixing can be applied. This produces small unilamellar vesicles (SUVs), and in the case of DDAB:TDB, this can reduce the particle size from approximately 500 to 200 nm and a polydispersity (PDI) between 0.2 and 0.4.[17,32] However, these processes are difficult to scale-up and are generally limited to small-scale laboratory production. Therefore, to address the need for scale-independent manufacture of cationic liposomal adjuvants, we have investigated the use of microfluidics for the production of DDAB:TDB. We have then applied this method to develop a modified biotinylated DDAB:TDB formulation that can

promote liposome and antigen drainage to, and retention within, the lymphatics in order to test the impact this has on vaccine efficacy.

2. MATERIALS AND METHODS

2.1. Materials. The cationic surfactant dimethyldioctadecylammonium (DDAB) bromide, the immunopotentiator trehalose 6,6'-dibehenate (TDB), and 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine-N-[biotinyl(polyethylene glycol)-2000] (DSPE-PEG(2000)-biotin) were purchased from Avanti Polar Lipids Inc. (Alabaster, United States). Avidin (egg white) and cholesterol were purchased from Sigma-Aldrich Company Ltd. (Poole, U.K.). Hybrid 56 (H56) tuberculosis vaccine candidate was gifted by Statens Serum Institut (Copenhagen, Denmark). Tris-base was obtained from IDN Biomedical Inc. (Aurora, OH, United States) and used to make 10 mM Tris buffer and adjusted to pH 7.4 using HCl. The radionucleotides iodine ^{125}I (NaI in NaOH solution) and tritium ^{3}H-cholesterol (tritium-labeled cholesterol in ethanol) and Ultima Gold scintillation fluid were purchased from PerkinElmer (Waltham, MA, United States). Sodium hydroxide (NaOH) and hydrogen peroxide (H_2O_2) were purchased from Sigma-Aldrich Company Ltd. (Poole, U.K.). Bicinchoninic acid protein assay (BCA) kit and Sephadex G-75 superfine were purchased from Fisher Scientific (Leicestershire, U.K.). IODO-GEN precoated iodination tubes from Pierce Biotechnology (Rockford, IL) and scintillation vials from Sardsted Ltd. (Leicester, U.K.) were used. Horseradish peroxidase (HRP) enzyme (HRP-streptavidin), purified rat anti mouse IFN-γ and IL-17, biotin conjugates IFN-γ and IL-17, IL-17 standard, and mouse IL-5 ELISA set were purchased from Becton Dickinson (BD biosciences, New Jersey, United States). Mercaptoethanol, concanavalinA (conA), Tween 20, Bovine serum albumin (BSA), carbonate-bicarbonate buffer tablets, sulfuric acid, IFN-γ standard, skimmed milk powder, heparin, bovine serum albumin, sodium chloride (NaCl), sodium azide (NaN$_3$), Triton X-100, and protease inhibitor cocktail were purchased from Sigma-Aldrich Company Ltd. (Poole, U.K.). Tetramethylbenzidine (TMB) substrate, isotype-specific immunoglobulins (Goat antimouse IgG1 and IgG2c), Penicillin-Streptomycine (10 000 U/mL), L-glutamine 200 mM, sodium pyruvate 100 mM, MEM nonessential amino acids solution (100×), RPMI 1640 media, fetal bovine serum (FBS), HEPES (1 M), and phosphate buffered saline (10×) were purchased from Fisher Scientific - UK Ltd. (Loughborough, U.K.).

2.2. Manufacture of Liposomes. Two techniques for the production of liposomes were applied and compared: LH and microfluidics (MF). For the LH method, liposomes were prepared by a modification of the Bangham method.[33] Briefly, lipid stocks of DDAB and TDB were dissolved in a mixture of chloroform and methanol (9:1 v/v). The required amount of lipid solution was transferred to a round-bottom flask to reach the appropriate final concentration (5 mg/mL DDA and 1 mg/mL TDB). Organic solvent was removed under vacuum with a rotary evaporator for 15 min at 200 rpm (rpm). The lipid film was hydrated with the desired amount of 10 mM Tris buffer (pH 7.4) at 60 °C for 20−30 min.

The preparation of liposomes by microfluidics was conducted on the Nanoassemblr Benchtop system from Precision Nanosystems Inc. Stocks of DDAB and TDB were prepared in 2-propanol (IPA) and mixed to the desired concentration (in general 20 mg/mL of DDAB and 2 mg/mL TDB). Selected speeds (total flow rates (TFRs)) and ratios between the aqueous and organic phase (flow rate ratios (FRRs)) were investigated,

with FRRs of 1:1, 3:1, and 5:1 (solvent to aqueous phase) and TFRs of 5, 10, and 15 mL/min tested. During the process, samples were heated to ensure the lipids stayed dissolved and thus the heating block was set at 60 °C. To prepare biotinylated liposomes, DSPE-PEG(2000)-biotin was added to the DDAB:TDB formulation at a 20 mol % ratio in order to investigate the effect of the biotin−avidin complex in the distribution of these particulate systems within the body. Concentrations ranging primarily between 0.3 and 24 mg/mL total lipid were tested. H56 antigen (5 μg per vaccine dose (50 μL)) was mixed with preformed liposomes after production. Solvent was removed by dialysis (dialysis tubing Mw 12 000−14 000 Da, Sigma-Aldrich, Poole, U.K.) against Tris buffer. For free-antigen removal, a 300 000 Da MWCO membrane was used (Spectra-Por, Spectrum Laboratories, Breda, The Netherlands).

2.3. Determination of the Particle Size, PDI, and Zeta Potential. The size of liposomes was determined by dynamic light scattering (Zetasizer nano ZS, Malvern PANalytical Ltd., Worcestershire, U.K.). Samples manufactured using the LH were measured approximately 1 h after preparation to allow samples to cool down, whereas the samples manufactured using microfluidics were measured directly after purification. For zeta potential measurement, samples were diluted in the same fashion as for the size determination. Three measurements of each sample at 25 °C were taken.

2.4. Quantification of Lipid Recovery. Quantification of the lipid recovery was performed by high-performance liquid chromatography (HPLC, YL Instruments Co. Ltd. Korea) using a Sedex 90LTD ELSD detector (Sedex Sedere, Alfortville, France) as described previously.[34] A Luna 5 μ C18(2) column (Phenomenex, Cheshire, U.K.), pore size of 100 Å, was used. Lipids were dissolved in chloroform:methanol (9:1 v/v), and liposomal samples were injected without preparation. HPLC-ELSD settings were kept constant as follows: 30 μL injection volume in a partial loopfill injection mode, 100 μL loop volume, and 15 μL tubing volume. Column temperature was maintained at 35 °C, whereas the ELSD temperature was set at 52 °C in all the runs. Nitrogen was used as a carrier gas at 3.5 psi inlet pressure. Clarity DataApex version 4.0.3.876 was used for data analysis.

2.5. Quantification of Antigen Loading. Quantification of the antigen loading on the liposomal formulations was performed by reverse phase HPLC (RP-HPLC) using an ultraviolet (UV) detector (Agilent Technologies, Edinburgh, U.K.) as described previously.[35] A Jupiter 5 μ C18(2) column (Phenomenex, Cheshire, U.K.), pore size 300 Å, was used as stationary phase. For the preparation of the standards and samples, antigen alone or liposomes loaded with H56 were diluted in 50% Tris/IPA (1:1 v/v).[36] Mobile phase A contained 90% H_2O, 10% acetonitrile, and 0.1% TFA, whereas mobile phase B contained 70% acetonitrile, 30% H_2O, and 0.1% TFA. The instrument settings were as follows: 50 μL injection volume, flow rate 1 mL/min, UV wavelength 210 nm, and column temperature 60 °C.

2.6. Stability of Liposomes in Simulated in Vivo Conditions. Stability of the antigen-loaded liposomal formulations was assessed in terms of size, PDI, and zeta potential under simulated in vivo conditions. Briefly, liposomes were placed in a water bath at 37 °C with 50% FBS, and aliquots were taken at specific time points in line with the biodistribution time points.

2.7. In Vivo Studies. All in vivo studies were conducted under the regulations of Directive 2010/63/EU. All protocols have been subjected to ethical review and were carried out in a designated establishment. During all studies, mice were weighed weekly and examined to detect any significant change in their health. All mice had a healthy weight, characteristic of the strain and age, with no significant differences between groups.

2.7.1. Biodistribution Studies of Liposomal Adjuvants and Their Associated Antigen. Inbred female BALB/C mice (3 mice per time point) were obtained from the Biological Procedure Unit at the University of Strathclyde, Glasgow. All mice used were 6−10 weeks of age at the start of the experiment. Liposomal formulations were radiolabeled with ³H-Cholesterol by incorporation of the isotope into the lipid bilayer.[37] For isotonicity, 10% w/v trehalose was added to the filtered buffer (0.22 μm filter). Radiolabeled H56 antigen was added to the hydrated formulation at the desired concentration (0.1 mg/mL) for surface loading onto the DDAB:TDB formulation. This formulation was used as a control because its biodistribution vaccine efficacy has been reported previously.[8,10,11,13,18,38] All liposomal formulations contained a final concentration of 250 μg DDAB/50 μg TDB/5 μg H56 per vaccine dose (50 μL). To investigate the effect of the biotin−avidin complex in the distribution of these DDAB:TDB liposomes, DSPE-PEG(2000) biotin was added to the DDAB:TDB formulation at a 20 mol % ratio. When necessary, avidin (200 μg per dose) was injected intramuscularly 2 h prior to main immunization with the biotinylated formulation in the same quadriceps (adjacent injections).[30]

Mice were injected intramuscularly with 50 μL of the radiolabeled formulation in the right quadriceps, and mice were terminated after 6, 24, 48, 96, and 192 h. Specific organs and tissues were isolated: inguinal lymph node (ILN), mesenteric lymph node (MLN), popliteal lymph node (POP), spleen, and the site of injection. These organs and tissues were added to a scintillation vial containing 2 mL of NaOH 10 mM for dissolution. Mice carcasses were dissolved with 60 mL of NaOH 10 mM for mass balance calculation. All vials containing NaOH were incubated in an oven at 60 °C overnight. All scintillation vials containing 2 mL of either carcasses, organs, or tissues were bleached with 200 μL of H_2O_2 and incubated for 2 h in the oven at 60 °C.

2.7.2. Immunization Studies. Five female C57BL/6 mice (6−10 week-old) per group were injected i.m. (50 μL) into the right quadriceps. Mice received 3 immunizations at 2 week intervals (days 0, 14, and 28) and were terminated 3 weeks after the last immunization (on day 49). For the quantification of serum immunoglobulins, 50 μL of blood was collected via tail-bleed on days 7, 21, and 49. Blood was collected in a heparinized Eppendorf (1% w/v heparin) and centrifuged for 10 min at 10 000 g (Mini centrifuge Mikro200, Hettich (Tuttlingen, Germany)) in order to separate blood cells from serum. A standard direct enzyme-linked immunosorbent assay (ELISA) was carried out to detect immunoglobulins IgG1 and IgG2c in serum. Maxisorp flat bottom 96-well plates (Fisher Scientific, Loughborough, U.K.) (high binding and affinity) were coated by passive absorption overnight at 4 °C with 0.5 μg/mL H56 antigen diluted in carbonate buffer 0.05 N pH 9.6 (100 μL/well). Plates were washed with washing buffer (PBS pH 7.2, 10 mM containing 0.2% Tween 20) and blocked for 1.5−2 h at room temperature with 200 μL/well of PBS (pH 7.2, 10 mM) containing 2% BSA to block any nonspecific binding. Samples were added to the plates (serial dilution). Plates were then
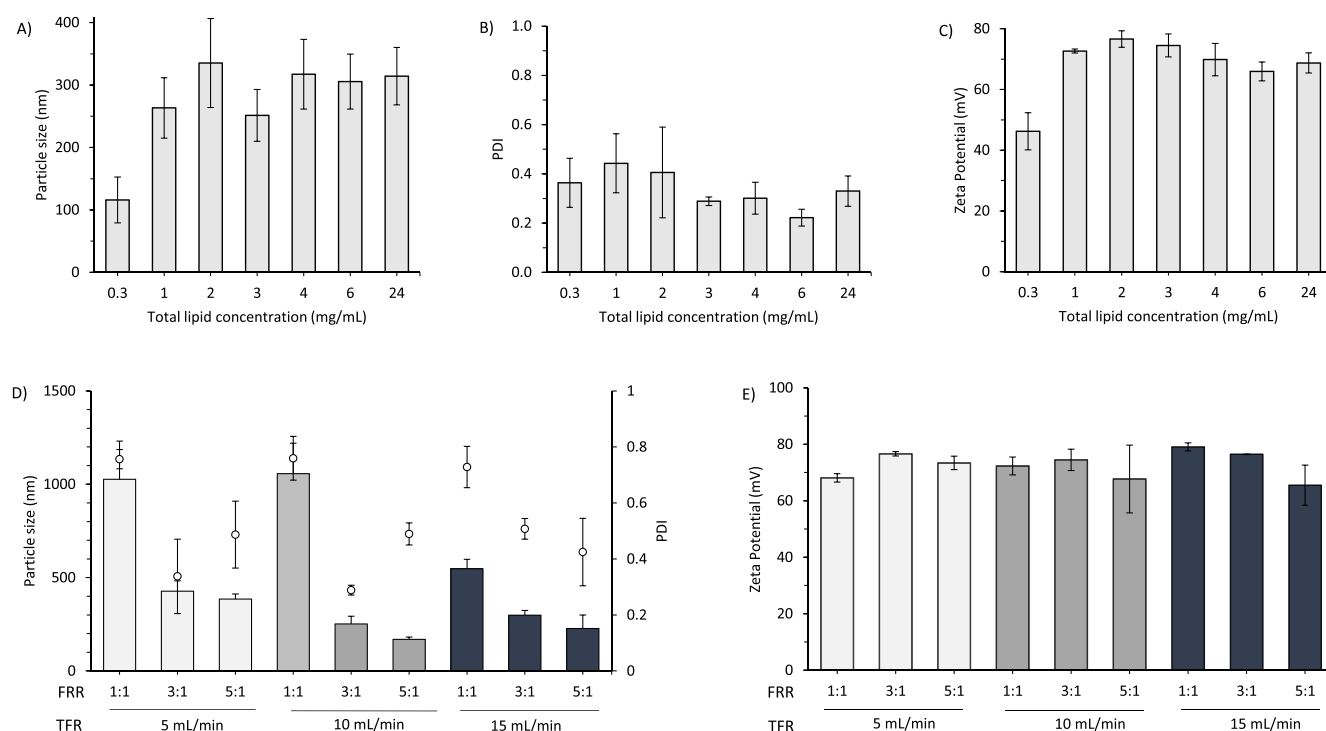
Figure 1. Establishing the operating parameters for DDAB:TDB using microfluidics. The effect of lipid concentration on the physicochemical characteristics of the DDAB:TDB adjuvant formulations manufactured using microfluidics. DDAB:TDB liposomes were prepared at TFR 10 mL/min and FRR 3:1, varying the initial concentration from 0.3 to 24 mg/mL, and (A) particle size, (B) PDI, and (C) zeta potential were measured. The impact of the different parameters adopted during microfluidics formulation of DDAB:TDB liposomes was tested [(D) particle size (bars) and PDI (open circles) and (E) zeta potential of the DDAB:TDB liposomes manufactured by using microfluidics]. Results represent the mean ± SD from at least 3 independent experiments.

incubated at room temperature for 2 h. HRP-conjugated antibody was diluted in 1% BSA in PBS (1:20000 and 1:5000 for IgG1 and IgG2c, respectively) and added in a volume of 100 μL/well to the washed plates. Plates were incubated for an hour, and TMB substrate was added to the plates at 100 μL/well. The reaction was stopped by adding 0.2 M sulfuric acid, and the absorbance at 450 nm was measured (with wavelength correction 570/620 nm). Results were plotted as the Log10 of the end point titer giving an optical density value (OD$_{450}$) of 0.1 or higher.

Spleens and popliteal lymph nodes from each mouse were isolated and processed on day 49 as described before.[35] Cells were counted and diluted in complete RPMI to a final concentration of 2×10^6 cells/mL and plated (100 μL) on a Nunclon 96-well round-bottom (Fisher scientific, Loughborough, U.K.). Cells were stimulated with 100 μL of ConA (5 μg/mL), RPMI media, or H56 antigen (5 μg/mL) and incubated at 37 °C, 5% CO$_2$, and 95% humidity for 72 h. Supernatants were harvested and stored at −20 °C for further processing. The sites of injection (i.e., the right quadriceps) were excised 3 weeks after the last immunization (on day 49), and the method from Sharp et al. for the analysis of cytokines at the site of injection was followed.[39] Quadriceps muscles were removed from the bone and weighed out individually. Individual muscles were homogenized in 2.5 mL of homogenization buffer 500 mM NaCl/50 mM Hepes, pH 7.4 containing 0.1% Triton X-100, 0.02% NaN$_3$, and 1% v/v protease inhibitor cocktail. Homogenates were sonicated twice for 15 s and centrifuged at 3600 rpm for 20 min at 4 °C. The supernatants were removed and stored at −20 °C.

Supernatants from restimulated splenocytes and lymph nodes were analyzed using a sandwich ELISA protocol for the production of cytokines IL-17 and IFN-γ. Plates were coated with the specific capture antibody diluted in carbonate buffer, overnight at 4 °C. Plates were blocked for 1.5−2 h at room temperature with PBS containing 2% milk powder. Samples and standards were diluted in 2% BSA in PBS and incubated at room temperature for 2 h. Then the biotin conjugate was diluted in 1% BSA in PBS (IL-17 1:2000, IFN-γ 1:5000), and 100 μL were added on each well. Plates were incubated for an hour at room temperature followed by incubation with 100 μL/well of HRP-streptavidin in 1% BSA (1:5000). TMB substrate was added to the plates (100 μL/well), and after approximately 15 min the reaction was stopped with 0.2 M sulfuric acid. For the quantification of IL-5 cytokine production, the manufacturer's instructions were followed (ELISA IL-5 kit, BD Biosciences). All experiments were carried out in duplicate, and absorbance was measured at 450 nm with wavelength correction (570/620 nm).

2.8. Statistical Analysis. All experiments were carried out at least in triplicate unless otherwise stated. Means and standard deviations are plotted on the graphs. Statistical analysis of data was calculated by one-way analysis of variance (ANOVA). Where significant differences are indicated, differences between means were determined by Tukey's post hoc test.

3. RESULTS AND DISCUSSION

3.1. Production and Optimization of DDAB:TDB Liposomes Using Microfluidics. The use of microfluidics as a technique for the production of liposomes has already been demonstrated in several studies with process parameters,
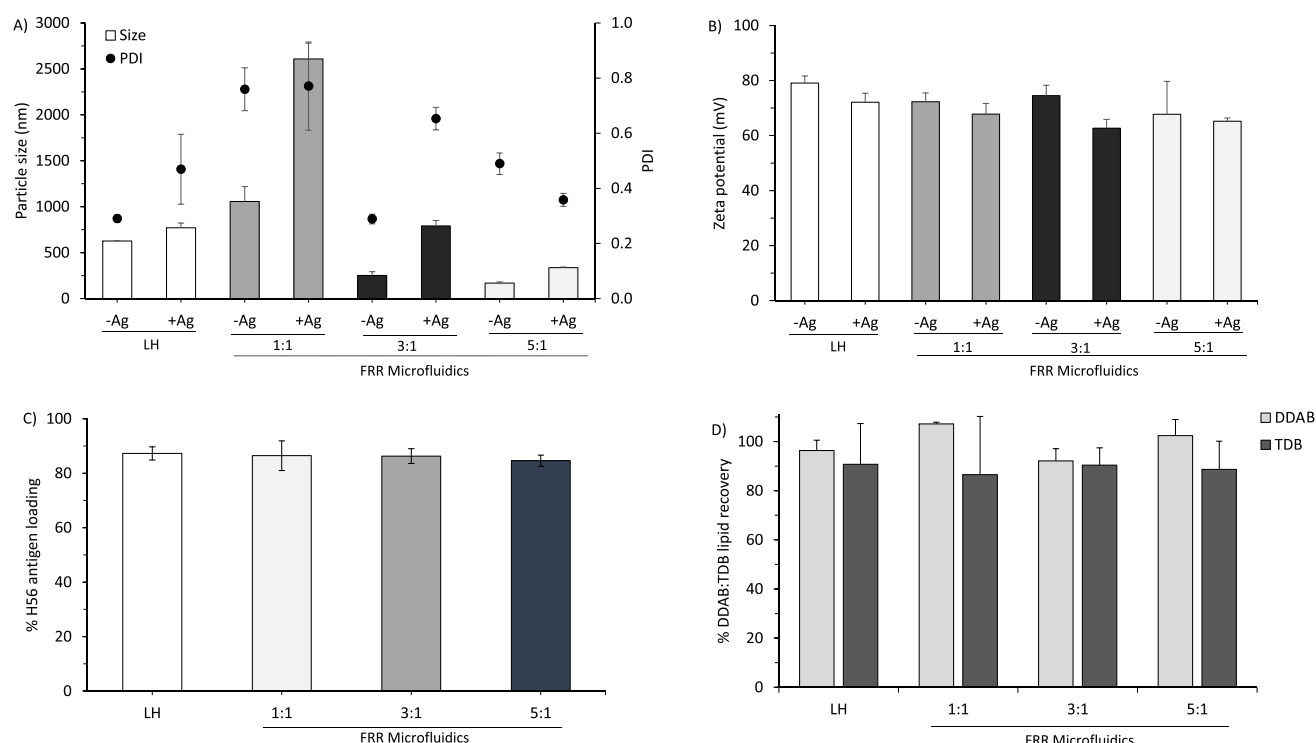
Figure 2. Comparing production methods for liposomal adjuvants. Physicochemical characteristics: (A) particle size and PDI, (B) zeta potential, (C) H56 antigen loading, and (D) lipid recovery of the DDAB:TDB liposomes produced using either the LH method or microfluidics at different flow rate ratios (1:1, 3:1, and 5:1). Results represent the mean ± SD of at least 3 independent batches. Ag = H56 antigen; −Ag represents without H56 antigen; +Ag represents with H56 antigen.

including FRR, TFR, and initial lipid concentration, all being shown to be important considerations.[1−3,35,40] Therefore, initially, these parameters were investigated and optimized for the production of DDAB:TDB.

3.1.1. Lipid Concentration, Flow Rate, and Flow Rate Ratios. Initial studies considered the impact of initial lipid concentration of the DDAB:TDB liposomes in terms of size, PDI, and zeta potential. Previous studies carried out by Davidsen et al. showed that DDAB:TDB immunological responses were optimal when the DDAB:TDB molar ratio was fixed at 8:1 (5:1 weight ratio).[22] Therefore, variation of the initial lipid concentration while keeping the DDAB:TDB molar ratio constant was examined. Initial liposomes concentrations of 0.3, 1, 2, 3, 4, 6, and 24 mg/mL were formulated at constant microfluidic parameters: TFR of 10 mL/min and FRR of 3:1 (Figure 1). When prepared with low lipid concentrations, the particle sizes were low (approximately 120 nm; Figure 1A). However, at higher concentrations (1−24 mg/mL) the particle size was between 250 and 350 nm with no significant differences (Figure 1A). At all concentrations tested, the DDAB:TDB liposomes were more heterogeneous in nature (PDI 0.2 to 0.4; Figure 1B) compared to previously studied liposome formulations (e.g., see ref 1) where PDIs of below 0.2 were achieved. In terms of zeta potential, all formulated liposomes were highly cationic with values between +60 and +75 mV for initial lipid concentrations from 1 to 24 mg/mL (Figure 1C). These results show that the DDAB:TDB adjuvants can be formulated at initial lipid concentration between 1 and 24 mg/mL with no significant difference in size, PDI, or zeta potential. Previous studies carried out by Joshi et al. have also shown that the initial lipid concentration used for the microfluidic

production of liposomes is a critical process parameter; for PC:Chol liposomes it was shown that concentrations above 3 mg/mL gave reproducible physicochemical characteristics of the formed liposomes, and at concentrations below this the particle size was increased.[3] Forbes et al. also showed that increasing initial lipid concentration from 0.3 mg/mL to 2 mg/mL decreased the particle size of neutral liposomes (PC:Chol, DMPC:Chol, DSPC:chol, and DPPC:chol) and at concentrations above 4 mg/mL the particle size plateaued.[1]

After optimization of the initial DDAB:TDB concentration for the production of DDAB:TDB, the effect of the TFR and FRR on particle size, PDI, and zeta potential was evaluated (panels D and E of Figure 1, respectively). At all three flow rates tested (5, 10, and 15 mL/min) it can be seen than increasing the FRR (from 1:1 to 5:1) reduces the liposome size and through the combined selection of FRR and TFR liposome sizes of 1000 to 160 nm could be prepared (Figure 1D) which, were highly cationic in nature (Figure 1E). However, at lower FRRs, the increased particle size was also paired with high PDI (0.7−0.8; Figure 1D). The smallest particle size and PDI combination was achieved at a FRR of 3:1 and a TFR of 10 mL/min (approximately 250 nm; 0.3 PDI; Figure 1D).

The TFR and FRR are important factors to consider when producing liposomes as they impact on the polarity of the organic solvent-aqueous phases when mixing within the micromixer and therefore influence the physicochemical attributes of the produced liposomes.[2,41] The influence of the FRR has been reported previously in several studies, and it is noted that increases in the aqueous phase during the liposome production create a narrow solvent stream which consequently favors the production of small size particles due to reduced
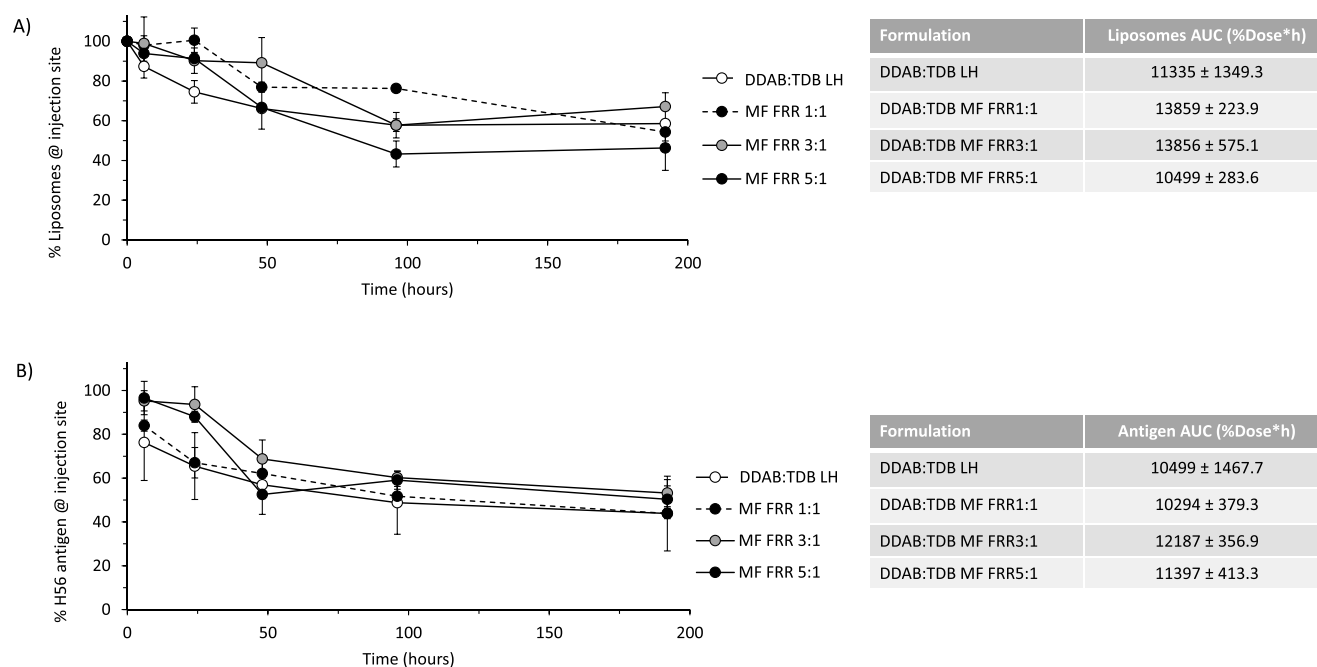
Figure 3. Movement from the injection site of DDAB:TDB liposomal adjuvants to their associated antigen produced by LH and MF. Percentage of (A) liposomes and (B) antigen retained at the site of injection. Dual labeling of liposomes and antigen by incorporating either ^{3}H-lipid or ^{125}I-antigen was used for the detection of the liposomes and antigen, respectively, at different time points. Liposomes were manufactured using either the LH method or microfluidics (FRR 1:1, 3:1, and 5:1). Results represent the mean of 3 mice ± standard deviation.

particle fusion.[42] Indeed, studies by Kastner et al.,[2] Joshi et al.,[3] and Forbes et al.[1] have shown that increasing the FRR from 1:1 to 5:1 reduced the liposomes size with neutral or anionic liposomes. Results from another study from Kastner et al. also demonstrated this flow rate/particle size interaction with cationic liposomes containing 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP).[40] Studies carried out with different microfluidic technology also report the effect of the FRR on particle size.[40,43−45] In addition to higher flow rate ratios producing a smaller solvent stream, the lower organic solvent concentration may also reduce particle fusion and subsequently promote the formation of larger particles.[40,45] Regarding PDI, the high values obtained for FRR 5:1 may result from the high dilution, lower lipid concentrations, lower diffusion rates, and more variable nucleation.[40,46]

Interestingly, with the manufacture of DDAB:TDB, the speed at which the particles are manufactured through the system was shown to impact the liposome size contrary to previous studies.[1,46,47] Generally with microfluidics, the rapid mixing within the microfluidic cartridge results in the organic solvent being diluted very quickly, hindering the formation of large particles.[48] In the case of DDAB:TDB dissolved in 2-propanol (IPA), high flow rates (>10 mL/min) combined with higher (>3:1) flow rate ratios may promote better mixing and nanoprecipitation of DDAB and TDB to form liposomes. These results highlight the importance of considering the liposome composition when designing the microfluidic production process. In the case of DDAB:TDB, the particle size of DDAB:TDB liposomes was controlled by both the FRR and TFR. On the basis of these results, a TFR of 10 mL/min was selected for producing liposomes in further studies. This was due to the ability to produce liposomes in different particle size ranges (combined with the lower PDI) through control of the FRR.

3.1.2. Antigen Loading and Lipid Recovery of DDAB:TDB Liposomes. Following optimization of the microfluidic method, DDAB:TDB liposomes were produced using the LH as well as microfluidics (FRR 1:1, 3:1 and 5:1 at TFR 10 mL/min), and the particle size, zeta potential, antigen loading, and lipid recovery were measured (Figure 2). DDAB:TDB produced by the LH were approximately 600 nm in size, with a PDI of 0.3, and a highly cationic surface charge of +80 mV which promotes high (>90%) antigen loading (Figure 1A−C, respectively) in line with previously reported studies.[13,22,49] Addition of 0.1 mg/mL H56 antigen generally results in a small increase in size and PDI and reduction in zeta potential as would be expected from the addition of an anionic subunit antigen to a cationic liposome formulation (Figure 2). Results from DDAB:TDB produced via microfluidics followed a similar trend, with particle sizes tending to increase irrespective of initial particle size, and all formulations showing a high zeta potential and high (>90%) antigen loading (Figure 2A−C), with no significant difference in antigen loading across the different DDAB:TDB formulations tested (Figure 2C). In terms of lipid recovery, across all 4 formulations lipid recovery of both DDAB and TDB was high, with no loss in the microfluidic process, ensuring the 5:1 weight ratio of DDAB to TDB was maintained after production (Figure 2D).

3.2. Liposome and Antigen Movement from the Injection Site to the Draining Lymphatics. DDAB:TDB liposomes were labeled with ^{3}H-tritium cholesterol, and the H56 antigen was labeled with ^{125}I; the pharmacokinetic profile of the DDAB:TDB liposomes manufactured using different technologies after intramuscular injection was studied. All four liposomal formulations tested contained the same total lipid and antigen concentration (300 μg of total lipid and 5 μg of H56 per 50 μL dose). The percentage of liposomes (Figure 3A) and antigen (Figure 3B) at the site of injection (right quadriceps) at various time points and the area under the curve (AUC) for each
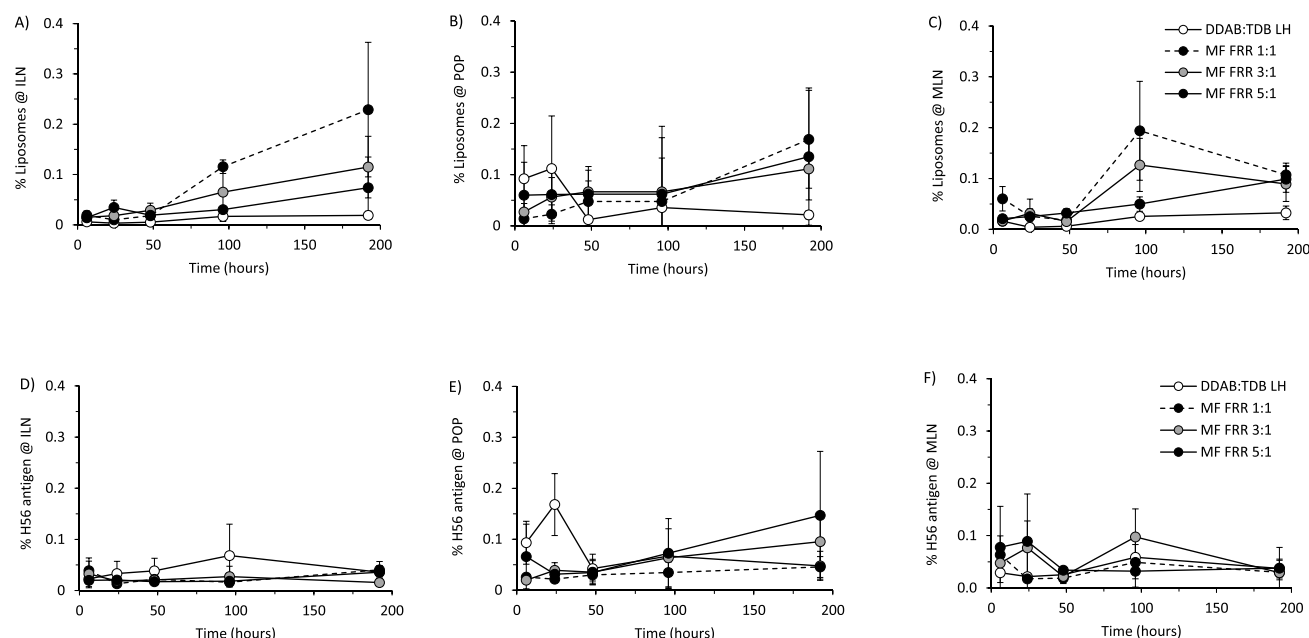
Figure 4. Percentage of liposomes (A−C) and antigen (D−F) detected at the lymph node [inguinal (A and D), popliteal (B and E), and mesenteric (C and F)] after intramuscular injection. Dual labeling of liposomes and antigen by incorporating either ^{3}H-lipid or ^{125}I-antigen was used for the detection of the liposomes and antigen, respectively, at different time points. Liposomes were manufactured using either the LH method (DDAB:TDB LH) or microfluidics (MF) (FRR 1:1, 3:1, and 5:1). Results represent the mean of 3 mice ± standard deviation.

formulation were measured. The method of DDAB:TDB (and the resulting particle size and PDI) does not make a significant difference on the clearance of the liposomes nor the antigen from the injection site, as shown by the similar clearance profiles and AUC, which are not significantly different across the 4 formulations (Figure 3). Drainage from the injection site followed a similar trend for all 4 liposome formulations, with between 90% and 100% of the dose remaining after 24 h, dropping to approximately 50% after 8 days (Figure 3A). These results for DDAB:TDB produced by the LH method (which was used as our control) are in line with previously published data, showing that 1 day post injection, DDAB:TDB liposomes begin to drain steadily from the injection site and on day 8 between 40% and 60% of the initial dose is still detectable there.[8] The depot effect and slow clearance from the injection site of DDAB:TDB may be attributed to the cationic nature of the liposomes, which results in the cationic liposomes aggregating with interstitial proteins and becoming trapped at the injection site irrespective of their difference in size. Indeed, previous work investigating the role of particle size using DDAB:TDB liposomes prepared via LH and subsequent sonication to produce small (<200 nm), medium (500−600 nm), and large (~1500 nm) vesicles showed no significant difference in the drainage of the liposomes or their adsorbed antigen from the site of injection.[8] However, this slow clearance of DDAB:TDB may also be related to their high bilayer rigidity, as substitution of DDAB with dioctadecyldimethylammonium bromide (DODAB) did promote a more rapid clearance from the injection site. The mechanism of action behind the adjuvant effect of DDAB is also attributed to its cationic nature and its ability to associate antigens and promote cellular uptake.[50,51] Work by Korsholm et al.[51] using stimulated immature bone marrow-derived dendritic cells with fluorescently labeled ovalbumin (OVA) showed that adsorption of OVA onto DDAB enhanced the cellular acquisition of the antigen.

Furthermore, inhibition of active cellular processes by OVA stimulation at 4 °C or by the addition of cytochalasin D reduced the cellular uptake, suggesting that active actin-dependent endocytosis is the predominant uptake mechanism.[51]

Besides the site of injection, the detection of liposomes and antigen in the lymph nodes was also investigated as vaccine delivery of antigens to the lymphatics can be important for the protection against most diseases, such as tuberculosis (TB).[52] The popliteal (POP) lymph node is the first draining lymph node where the formulations will move after intramuscular injection in the mouse quadriceps, followed by the inguinal lymph node (ILN). These lymph nodes are representative of the local biodistribution of the formulations, whereas mesenteric lymph nodes (MLN) were isolated as representation of their systemic biodistribution. Correlating with the high doses remaining at the injection site, low levels of DDAB:TDB and H56 were detected in the POP (less than 0.3% of the initial vaccine dose administered) with no significant differences between the four different formulations (Figure 4).

3.3. Delivery and Retention of the Cationic Liposomes to the Draining Lymph Nodes by Exploiting a PEG-Biotin/Avidin Complex. While DDAB:TDB is known to promote strong immune responses,[53−57] studies based on the immunization with fluorescent labeled tuberculosis antigen (Ag85-BESAT-6) and adjuvant (DDAB:TDB) demonstrated the localization of low amounts of vaccine components in the draining lymph nodes after subcutaneous immunization. However, its efficient targeting to the DCs induces potent Th1 and Th17 responses. Moreover, both antigen and adjuvant have to target the DCs at the same time in order to elicit Th1/Th2 responses because previous activation of DCs by free antigen decreases the generated immune responses.[55,58] Therefore, increasing delivery to the lymphatics may further enhance immune responses.[55,58] Indeed, Mohanan et al. demonstrated that intralymphatic administration of DDAB:TDB liposomes
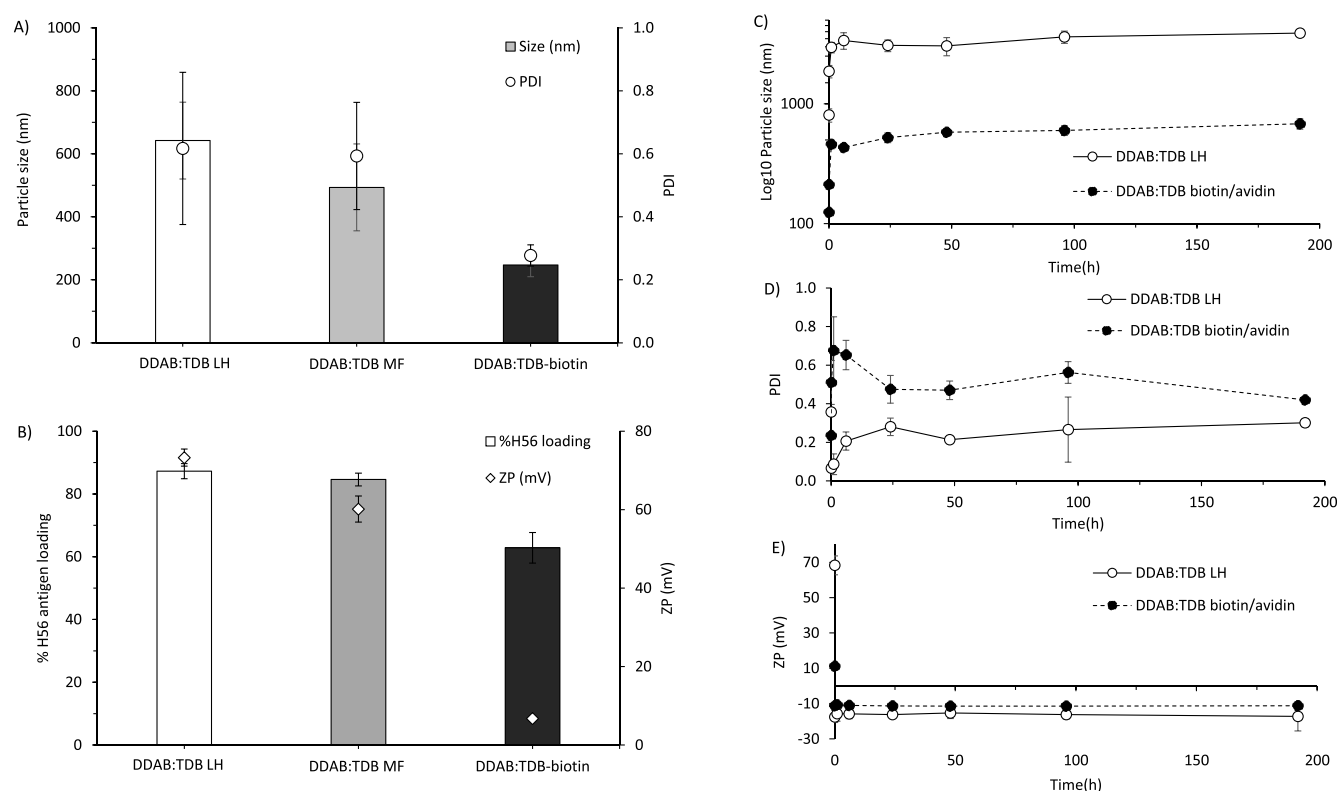
Figure 5. Physicochemical characteristic comparison of the DDAB:TDB formulations using LH and microfluidics and DDAB:TDB liposomes incorporating a DSPE-PEG(2000)-biotin. (A) Particle size (bars) and PDI (dots); (B) H56 antigen loading (bars) and zeta potential (diamonds); stability study of the liposomal formulations under simulated in vivo conditions (50% FCS at 37 °C), (C) particle size, (D) polydispersity and (E) zeta potential. Results represent the mean ± SD from at least 3 independent experiments.

resulted in significantly higher IgG2a and IFN-γ responses compared to other routes of administration.[29] Therefore, to achieve this, we explored the use of biotinylated liposomes in combination with predosing of avidin to promote retention of the vaccine components in the lymphatics.[59−61] Previous work by Medina et al. demonstrated the importance of the injection order to increase the lymph node targeting, showing better results when avidin was injected 2 h prior to injection of biotinylated liposomes.[59] Therefore, this dosing strategy was adopted.

To produce biotinylated DDAB:TDB liposomes, DSPE-PEG(2000)-biotin was incorporated within the formulation. DSPE-PEG(2000)-biotin was added to the DDAB:TDB at a 20 mol % ratio and prepared using microfluidics TFR 10 mL/min and FRR 3:1. The 20 mol % ratio was selected as previous studies have shown that by incorporating 20 mol % of PEG within DDAB:TDB liposomes, the depot effect can be blocked.[18,19] Therefore, it was hypothesized that the presence of DSPE-PEG(2000)-biotin on the DDAB:TDB liposomes would allow them to move from the injection site to the draining lymphatics, where they would then complex with the avidin and be retained. As seen previously, antigen was adsorbed onto the surface of the preformed liposomes. The concentration of lipids and antigen was adjusted to match the desired in vivo dose (300 μg of total lipid and 5 μg of H56, per 50 μL). Figure 5 summarizes the physicochemical characteristics of the different liposome formulations. DDAB:TDB-biotin liposomes were significantly ($p < 0.05$) smaller (~250 nm) in size with an associated lower PDI (0.25−0.30) when compared to the DDAB:TDB LH or DDAB:TDB MF (Figure 5A). This PDI,

while slightly higher than would be required for an intravenous injection, suggests a low level of size heterogeneity which is useful for robust characterization of the formulation. However, as shown in Figure 3, variations in particle size alone do not impact on clearance of the liposomes from the injection site, so a slightly wider size range would not impact on biodistribution. The addition of DSPE-PEG(2000)-biotin resulted in a reduction in zeta potential and antigen loading (+7 mV and ~60% antigen loading, respectively; Figure 5B). The ability of the PEG to mask the cationic charge (as shown in Figure 5B) and thereby circumvent aggregation in the presence of biological media is shown in Figure 5C−E. Formulations were incubated at 37 °C in 50% FCS and characterized in terms of size, PDI, and zeta potential. The highly cationic formulation DDAB:TDB LH aggregates when it is administered and comes in contact with the serum proteins with the particle size increasing from ~800 to ~1800 nm after 3 min, and up to ~3000 nm after 1 h with no further change (Figure 5C). Simultaneously, there is an increase in the PDI (Figure 5D) and a rapid drop in the zeta potential (+70 to −20 mV; Figure 5E) as a result of the cationic liposomes aggregating with the anionic proteins present in FCS. On the other hand, the DDAB:TDB-biotin formulation showed a much lower degree of aggregation, with the vesicle size increasing from 150 to 200 nm in the first 3 min, up to 460 nm after 1 h, and reaching 700 nm after 8 days (Figure 5C). Overall, these pegylated vesicles show less aggregation (Figure 5C,D) and less reduction in zeta potential (from +10 to −10 mV; Figure 5E).

On the basis of these results, the ability of the DDAB:TDB-biotin formulation to avoid aggregation at the injection site was demonstrated. Therefore, to compare the biodistribution of
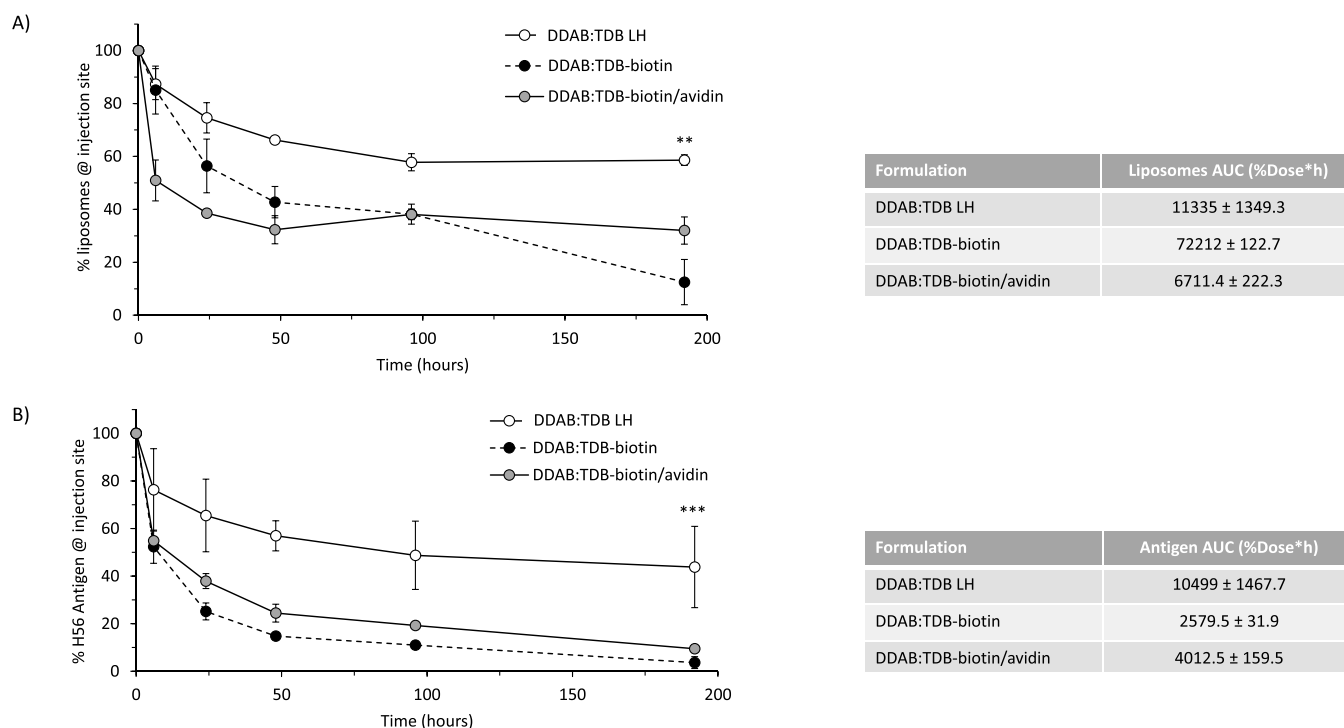
A)

Formulation	Liposomes AUC (%Dose*h)
DDAB:TDB LH	11335 ± 1349.3
DDAB:TDB-biotin	72212 ± 122.7
DDAB:TDB-biotin/avidin	6711.4 ± 222.3

B)

Formulation	Antigen AUC (%Dose*h)
DDAB:TDB LH	10499 ± 1467.7
DDAB:TDB-biotin	2579.5 ± 31.9
DDAB:TDB-biotin/avidin	4012.5 ± 159.5

Figure 6. Movement from the injection site of DDAB:TDB-biotin liposomes and their associated antigen with or without previous administration of avidin. Percentage of (A) liposomes and (B) antigen retained at the site of injection. Dual labeling of liposomes and antigen by incorporating either ^{3}H-lipid or ^{125}I-antigen was used for the detection of the liposomes and antigen, respectively, at different time points. Liposomes were manufactured using either the LH method (DDAB:TDB LH) or microfluidics (DDAB:TDB-biotin). Results represent the mean of 3 mice ± standard deviation. Significant differences are shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
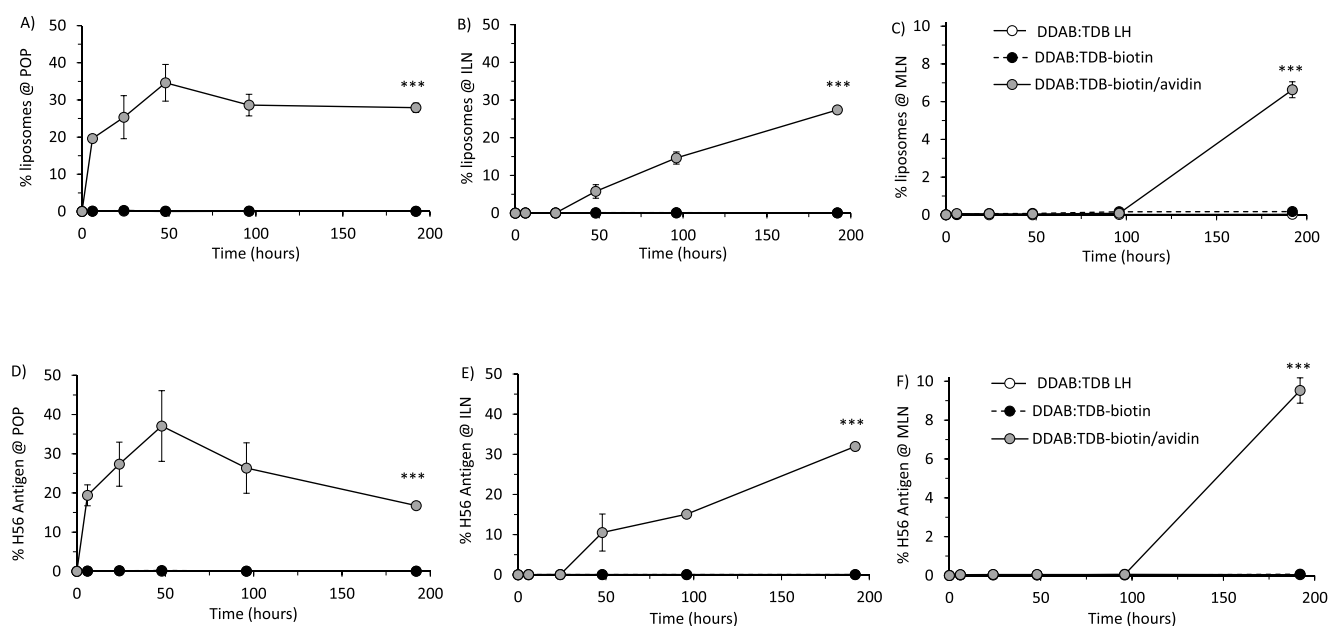

Figure 7. Percentage of biotin-DDAB:TDB liposomes (A−C) and antigen (D−F) detected at the local lymph node [inguinal (A and D), popliteal (B and E), and mesenteric (C and F)] after intramuscular injection with or without previous administration of avidin. Dual labeling of liposomes and antigen by incorporating either ^{3}H-lipid or ^{125}I-antigen was used for the detection of the liposomes and antigen, respectively, at different time points. Liposomes were manufactured using either the LH method (DDAB:TDB LH) or microfluidics (DDAB:TDB-biotin). Results represent the mean of 3 mice ± standard deviation. Significant differences are shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

these formulations in vivo, DDAB:TDB produced by LH was compared to DDAB:TDB-biotin alone, or DDAB:TDB-biotin with mice being predosed with avidin 2 h prior (200 μg/dose; intramuscularly). Figure 6 shows the percentage of liposomes
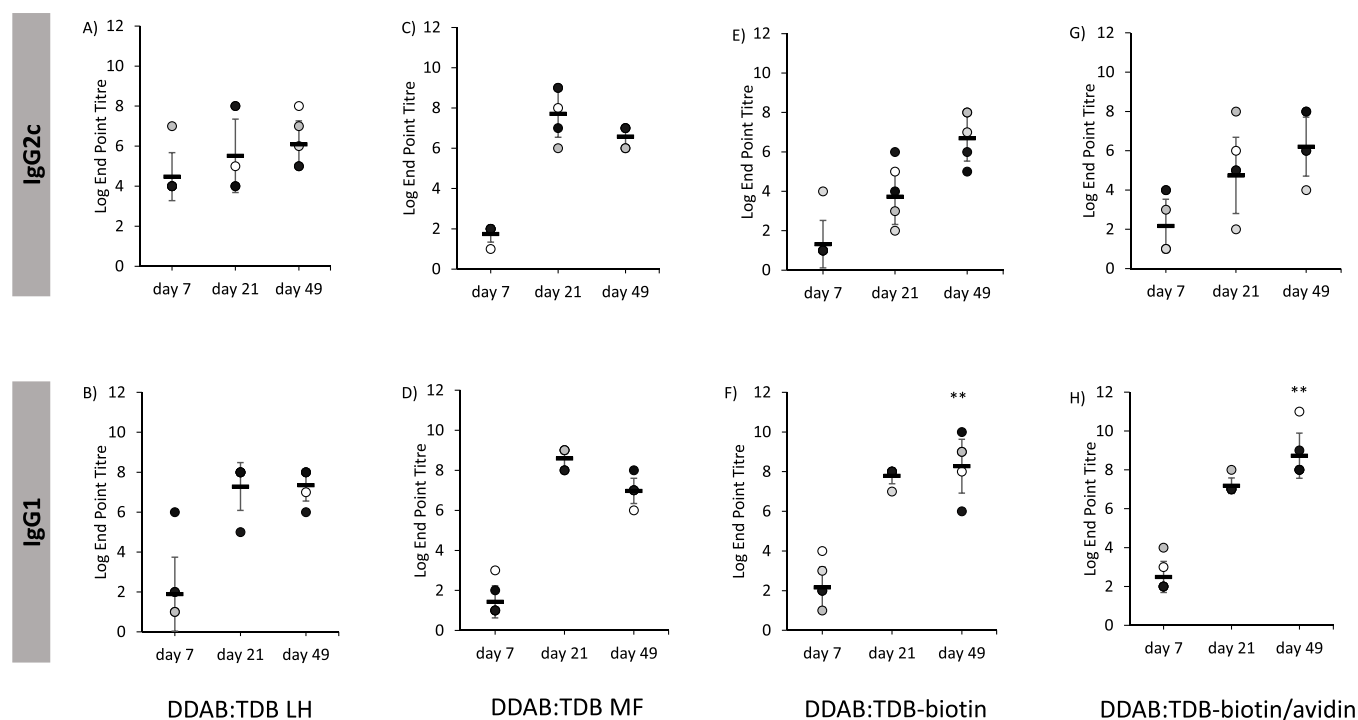
Figure 8. Antigen-specific IgG1 and IgG2c responses after intramuscular immunization using various DDAB:TDB liposomal adjuvants. Five C57BL/6 mice were intramuscularly immunized with H56 combined with different adjuvants, and humoral response was analyzed in blood. H56-specific IgG1 and IgG2c serum response detected by ELISA on sera collected on days 7, 21, and 49 after i.m. immunization. Antibody titers were expressed as the reciprocal of the highest dilution with an OD value ≥0.1 after background subtraction, and responses from each of the 5 mice are individually plotted for each time point. Significant differences at the end of the study are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.
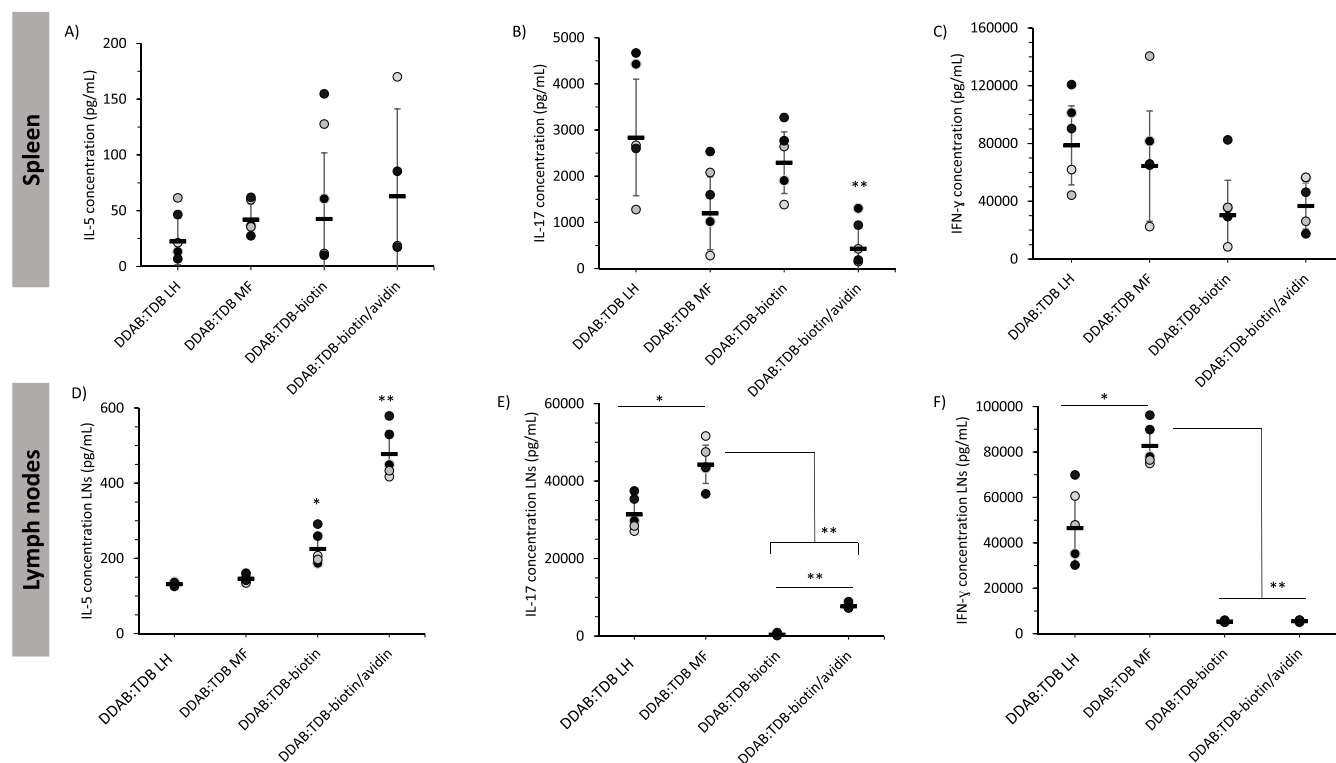

Figure 9. Cytokine production in splenocyte and popliteal lymph node (POP) culture supernatants: IL-5, IL-17, and IFN-γ. C57BL/6 mice were intramuscularly immunized with H56 combined with different adjuvants, and spleens and POPs were collected 3 weeks after the last immunization. Values, expressed as picograms per milliliter, are reported as the mean value ± SD of H56-stimulated of five animals per group. Significant differences are shown as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Figure 10. Cytokine production at the injection site (SOI): (A) IFN-γ, (B) IL-5, and (C) IL-17. C57BL/6 mice were intramuscularly immunized with H56 combined with different adjuvants, and the sites of injections were excised 3 weeks after the last immunization. Values, expressed as picograms per miligram, are reported as the mean value ± SD of H56-stimulated of five animals per group. Significant differences are shown as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.
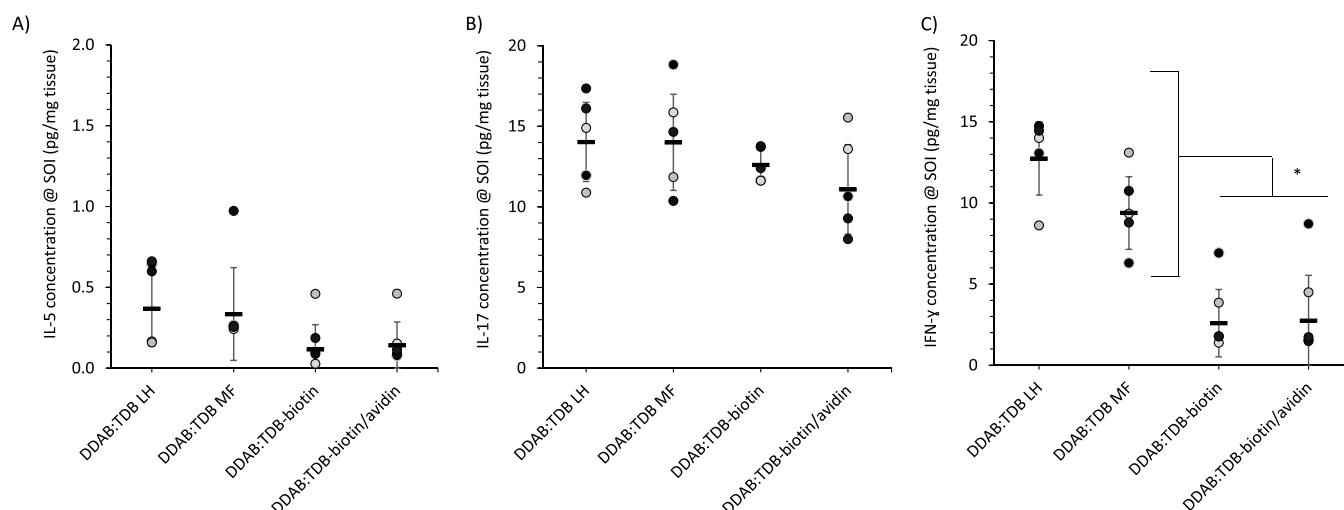
and antigen at the injection site at the chosen time points. The results show the ability of the DDAB:TDB-biotin liposomes to move from the injection site more rapidly than DDAB:TDB liposomes, irrespective of the avidin predosing (Figure 6A). This is confirmed by comparison of the AUC, which is significantly ($p < 0.05$) lower with the DDAB:TDB-biotin (approximately 6000−7000% dose·h) compared to the nonbiotinylated formulation (11 300% dose·h) (Figure 6A). A similar profile can be seen with the antigen, with more rapid clearance from the injection site when delivered with the biotinylated liposomes (irrespective of avidin predosing) (Figure 6B). These results demonstrate that the presence of the DDAB:TDB-biotin liposomes, with their reduced size and charge, can move more rapidly from the injection site. The results also show that the predosing with avidin 2 h prior to injection does not result in aggregation at the injection site.

The ability of pegylation to promote the movement of DDAB:TDB from the injection has been previously shown[18,19] with pegylated DDAB:TDB liposomes showing a more rapid clearance from the injection site to the draining lymphatics. However, with the biotinylated liposomes we also want retention at the lymphatics. From Figure 7, it can be seen that while both biotinylated liposomes may move rapidly from the injection site, only when mice received a predose of avidin were the DDAB:TDB-biotin liposomes retained at the lymph nodes, with up to ~30% of the DDAB:TDB-biotin liposomes being detected at the ILN, ~40% at the POP, and approximately ~8% at the MLN (panels A−C of Figure 7, respectively). This is compared to low (<0.5%) levels of DDAB:TDB-biotin without predosing of avidin, or DDAB:TDB LH. Again a similar trend can be seen with the antigen, with high levels of antigen being recorded at each of the 3 lymph nodes studied only when delivered with DDAB:TDB-biotin in combination with predosing of avidin (Figure 7D−F). The MLNs were analyzed in order to check the systemic distribution of the particles carrying antigen within the body (Figure 7C,F), and the results suggest that reaching the MLNs takes longer compared to the other lymph nodes analyzed in this study. The results also demonstrate that it is possible to drive the movement of the

liposomal adjuvants and antigen to a range of lymph nodes by the formation of the avidin/biotin complex.

3.4. DDAB:TDB Vaccine Efficacy Studies. Given that comparable pharmacokinetic profiles were identified between the biodistribution of DDAB:TDB produced at the different FRRs (Figure 3), DDAB:TDB produced at a flow rate ratio of 5:1 was tested for vaccine efficacy in vivo. This was compared to the traditional DDAB:TDB LH lab-scale formulation and the lymphatic targeting DDAB:TDB-biotin (with and without avidin predosing). Mice (groups of 5) were immunized three times with a 2 week interval between immunizations with DDAB:TDB LH (positive control, traditional method), DDAB:TDB MF (microfluidics), DDAB:TDB-biotin (no avidin predosing), or DDAB:TDB-biotin/avidin (avidin predosing), and antibody titers (Figure 8), cytokine responses (Figure 9), and cytokine production at the injection site (Figure 10) were measured.

3.4.1. Vaccine Efficacy of DDAB:TDB Liposomal Adjuvants Produced by Lab-Scale and Scale-Independent Microfluidic Production. In terms of H56-specific IgG1 (Th2) and IgG2c (Th1) secretion, when comparing between the DDAB:TDB formulation prepared by small lab-scale LH method (Figure 8A,B) and those prepared by scale-independent microfluidic processing (Figure 8C,D), both liposomal adjuvants produced high and comparable antibody responses with no significant difference at each of the three time points measured.

Antigen-specific T-cell responses (IL-5, IL-17, and IFN-γ) were analyzed in the supernatant of restimulated splenocytes from immunized mice using the ELISA assay (Figure 9). IFN-γ and IL-17 cytokines are frequently used as markers for the determination of the vaccine efficacy against TB.[62,63] Furthermore, these three cytokines are characteristics of the immunological DDAB:TDB profile. The DDAB:TDB immunological fingerprint is distinguished by the production of high levels of IFN-γ and IL-17 (Th1/Th17 stimulation) and low levels of IL-5 cytokine (Th2 stimulation), which correlates with the results shown here (Figure 9A−C). These results confirm that the DDAB:TDB liposomal adjuvants can be produced by the rapid and scale-independent microfluidics manufacturing

process. Interestingly within the lymph nodes, while the cationic liposomal adjuvants manufactured using microfluidics showed IL-5 responses (Figure 9D) similar to those prepared by the LH method, DDAB:TDB produced by microfluidics prompted significantly ($p < 0.05$) higher levels of IFN-γ and IL-17 cytokines compared to DDAB:TDB prepared by LH (Figure 9E,F). This may result from subtle differences in size between the two formulations which could influence interactions with APCs and the production of cytokines in the draining lymph nodes.[8]

To investigate this further, the same cytokines were also analyzed at the site of injection. Supernatants from the injection site were analyzed by ELISA, and results were normalized by individual mouse muscle weight. For the cationic liposomal adjuvants produced by either LH or microfluidics, negligible levels of IL-5 and high production of IL-17 and IFN-γ were observed (Figure 10). Both manufacturing techniques resulted in comparable cytokine production, which correlated to the similar biodistribution profile (Figures 3 and 4), as a result of the high retention of the vaccine components (liposomes and antigen) at the injection site due to the cationic nature of the formulations. These results demonstrate the ability to manufacture a highly effective liposomal adjuvant formulation using a scale-independent microfluidic production method.

3.4.2. Redirecting and Redirection of DDAB:TDB in the Draining Lymphatics Using Biotin–Avidin Complexation. Using this microfluidic production method for the cationic liposomal adjuvants, we investigated the biotin–avidin DDAB:TDB system as a vaccine adjuvant. Immunization with the DDAB:TDB-biotin did not result in any notable differences in the antibody profiles irrespective of the predosing with avidin with all four formulations promoting similar immune response profiles (Figure 8). When considering the cytokine profiles (Figure 9), target and retention of the DDAB:TDB formulation to the lymphatics did not improve immune responses with no notable differences in cytokine production from stimulated splenocytes across the four DDAB:TDB formulations (Figure 9A–C). However, when considering cytokine levels induced by cells isolated from the draining lymph nodes, differences in immune response profiles can be seen; biotinylated liposomes combined with avidin predosing resulted in significantly ($p < 0.05$) higher levels of IL-5 compared to the other groups (Figure 9D) and immunization with DDAB:TDB-biotin (with and without avidin predosing) significantly ($p < 0.05$) reduced IFN-γ and IL-17 cytokine levels (Figure 9E,F). Considering cytokine levels at the injection site, IL-5 and IL-17 production was not significantly influenced by biotinylation of DDAB:TDB (with or without avidin predosing) (panels A and B of Figure 10, respectively); however, IFN-γ levels were reduced (Figure 10C), again irrespective of predosing with avidin. These results show that while adopting a biotin–avidin complexing strategy can increase delivery and retention of cationic liposomal adjuvants and their antigen to local lymph nodes, this does not translate into improved immune response and may promote a more Th2 biased response. This may have caused trapping of activated lymphocytes in the nodes and a reduced response. It may also be a result of the PEG-biotin conjugate used within the formulation, which allows the liposomes to move from the injection site by blocking aggregation of the liposomes when they come in contact with biological moieties. Similar results were shown when pegylation of DDAB:TDB was investigated as a mean to block the formation of the depot at the injection site with Kaur et al. demonstrating that surface pegylation of

DDAB:TDB liposomes was able to allow the cationic liposomes and antigen to move from the injection site, but reduced immune responses were noted.[18,19] This suggests that the presence of the PEG moieties incorporated into the liposomal bilayer reduces immune responses despite helping to promoting the accumulation of the vaccine components into the lymph nodes. It has been reported that the incorporation of PEG into particles reduces particle uptake by macrophages (e.g., see refs 64–66), therefore, it is hypothesized that pegylation of the DDAB:TDB results in reduced interactions with the APCs and subsequently reduces its adjuvant activity.

4. CONCLUSIONS

Traditionally, the liposomal adjuvant formulation DDAB:TDB has been manufactured in a batch scale manner using either the LH method or high-shear mixing. Production of DDAB:TDB using these techniques can be an inefficient and time-consuming process, especially if considered for larger-scale industrial manufacture. If large-scale production of a TB vaccine is to be achieved for global immunization, flexible manufacturing methods that are easily and rapidly translated from bench to production must be developed. Our results demonstrate the ability of a microfluidic platform to produce DDAB:TDB liposomal adjuvants with matching physicochemical properties, pharmacokinetic profiles, and immunological activity compared to DDAB:TDB produced by traditional small batch-scale methods. Additionally, the use of microfluidics allows for the control of particle size through modification of process parameters. Therefore, microfluidic-based manufacture can be used to support the rapid translation of particulate-based adjuvants from bench to production. This manufacturing process was also used to prepare biotin-coated DDAB:TDB liposomes. By using this formulation, with or without injection of avidin 2 h in advance, faster clearance from the injection site was achieved, and predosing with avidin promoted retention of the biotinylated DDAB:TDB liposomes at the draining lymph nodes. Interestingly, redirecting the cationic liposomal adjuvants, and their associated antigen, did not improve immune responses and may skew the responses to a more Th2 profile.

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Notes

The authors declare no competing financial interest.

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