Vaccine 37 (2019) 1503–1515

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

A lipidic delivery system of a triple vaccine adjuvant enhances mucosal immunity following nasal administration in mice

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ARTICLE INFO

Article history:
Received 4 September 2018
Received in revised form 19 December 2018
Accepted 20 January 2019
Available online 7 February 2019

Keywords:
Liposomes
Vaccine
Adjuvant
Self-assembled systems
Nanoparticles
Intranasal delivery

ABSTRACT

We previously developed an highly efficacious combination adjuvant comprised of innate defense regulator (IDR)-1002 peptide, poly(I:C) and polyphosphazene (TriAdj). Here we aimed to design and test the in vivo efficacy of a mucoadhesive nasal formulation of this adjuvant. To determine the physical properties of the formulation, the effect of addition of each individual component was characterised by gel electrophoresis and fluorescence quenching using rhodamine-poly(I:C). Cationic liposomes comprised of didodecyl dimethylammonium bromide (DDAB), dioleoyl phosphatidylethanolamine (DOPE) (50:50 or 75:25 mol:mol) and DDAB, L-α-phosphatidylcholine (egg PC) and DOPE (40:50:10 mol:mol:mol) were prepared by the thin-film extrusion method. The liposomes and TriAdj were combined by simple mixing. The formed complex (L-TriAdj) was characterized by dynamic light scattering, zeta potential, and mucin interactions. We found that IDR-1002 peptide, polyphosphazene and poly(I:C) self-assembled in solution forming an anionic complex. Exposure of RAW267.4 mouse macrophage cells to TriAdj alone vs. L-TriAdj indicated that DDAB/DOPE (50:50) and DDAB/EPC/cholesterol (40:50:10) complexation reduced TriAdj toxicity. Next, TriAdj-containing cationic liposomes were prepared at several molar ratios to determine optimal size, stability and desired positive charge. Transmission electron microscopy showed rearrangement of lipid structures on binding of liposomes to TriAdj and to mucin. Stable particles (<200 nm over 24 h) showed mucin binding of DDAB/DOPE + TriAdj was greater than DDAB/EPC/DOPE + TriAdj. To verify in vivo efficacy, mice were administered the DDAB/DOPE + TriAdj complex intranasally with ovalbumin as the antigen, and the immunogenic response was measured by ELISA (serum IgG1, IgG2a, IgA) and ELISpot assays (splenocyte IL-5, IFN-γ). Mice administered adjuvant showed a significantly greater immune response with L-TriAdj than TriAdj alone, with a dose-response proportionate to the triple adjuvant content, and an overall balanced Th1/Th2 immune response representing both systemic and mucosal immunity.

1. Introduction

Adjuvants are crucial components of vaccines that improve immunogenicity, direct the response to facilitate long-term protection, enhance the efficacy of vaccines in newborns, elderly or immunocompromised persons, and reduce the amount of antigen or the number of doses required to elicit effective immunity. Recently, a combination adjuvant platform has been developed comprised of three components (a triple adjuvant – "TriAdj"), namely (1) poly(I:C) (polyinosinic-polycytidylic acid, a TLR agonist); (2) host defense peptide IDR-1002 [1–4]; and (3) polydi(p-oxyphenylpropionate) phosphazene, also known as PCEP ("polyphosphazene"), a cationic polymer adjuvant. This triple adjuvant forms a stable complex and has been demonstrated to be highly effective in a wide range of animal and human vaccine candidates [5]. The ratio of components that produces an immune response following intramuscular or subcutaneous administration has been optimized through extensive *in vivo* testing, demonstrating that TriAdj administered with various vaccine antigens induces effective long-term humoral and cellular immunity. This adjuvant offers an excellent opportunity for use in present and future generations of vaccines against multiple infectious agents. However, the

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https://doi.org/10.1016/j.vaccine.2019.01.058
0264-410X/

efficacy by the nasal and other mucosal routes to maximize mucosal immunity still requires enhancement. The purpose of the present study was to devise and evaluate a mucoadhesive lipidic carrier for this triple adjuvant combination in order to improve its efficacy as nasal vaccine adjuvant.

The combination adjuvant TriAdj proved highly effective in a number of species and for a number of diseases in prior work. For example, an earlier onset of immunity, higher humoral and cell-mediated immune responses were found in mice and pigs after immunization with Bordetella pertussis antigen pertussis toxoid (PTd), filamentous hemagglutinin (FHA) and pertactin (prn) [6,7]. The adjuvant also proved highly effective with an RSV vaccine consisting of the RSV F protein formulated with the combination adjuvant and tested in mice, cotton rats and lambs [5,8,9]. This includes intranasal administration of the vaccine. Furthermore, both experimental vaccines for pertussis and RSV were highly effective in the presence of maternal antibodies [6,10]. The adjuvant also enhanced both humoral and cell-mediated immunity to the chlamydia outer membrane protein in mice, koalas and sheep [11–13]. Other examples include antigens from influenza, parainfluenza, bovine virus diarrhea virus, to name a few. These were highlighted in a review by Garg et al. [14].

It is now widely recognized that especially for respiratory diseases, the induction of both local and systemic immunity can substantially improve the level of protection [15–17]. The advantage of intranasal administration lies in the ability to induce both local and systemic immunity, in addition to its ease of administration. Indeed, vaccines are increasingly being administered mucosally, both in humans and in animals. An intranasally administered vaccine can, for example, be delivered in a carrier that is adherent to the nasal mucous and may penetrate to the mucosa itself. The carrier may provide a depot effect or have its own immunostimulatory effects as an adjuvant. Various positively-charged mucoadhesive particulate carriers are currently under investigation to generate mucosal immunity with nasally administered vaccines such as those used for hepatitis B or HIV [18–23]. The mucoadhesive lipidic carrier described in the present study was hypothesized to enhance the adjuvant effect due to the inclusion of cationic lipid. This nasal formulation was thus a positively charged lipid nanocarrier comprised of cationic lipids and phospholipids at a defined ratio relative to TriAdj. The particle size and charge of the lipidic carrier described here were by design ideal for attracting dendritic cells in the mucosal tissues [24–26]. Secondary objectives of this study were to determine if the composition of the carrier and the dose of the triple adjuvant, TriAdj, would influence its efficacy. The tolerability in macrophages was assessed and its ability to activate an immune response in mice following nasal administration with ovalbumin antigen was determined.

2. Materials and methods

2.1. Materials and chemicals

Poly(I:C) double-stranded RNA adjuvant was purchased from Sigma-Aldrich (Canada). Polydi(p-oxyphenylpropionate) phosphazene, also known as PCEP (“polyphosphazene”), sodium salt (average molecular weight $\sim 1800 \times 10^3$) was synthesized and purified to 100% by Idaho National Laboratory (INL, Idaho Falls, ID USA). The polyphosphazene tested endotoxin free. IDR-1002 cationic peptide adjuvant was synthesized by CPC Scientific, Sunnyvale, CA) (MW 1652 Da). The sequence of IDR-1002 is: Val-Gl n-Arg-Trp-Leu-Ile-Val-Trp-Arg-Ile-Arg-Lyz-NH_2 and provided in powder form as the trifluoroacetate salt at 94.1% purity and used as provided. Rhodamine labeled poly(I:C) was purchased from InvivoGen (San Diego, USA), agarose was purchased from Invitrogen, gel loading dye 6x from New England Biolabs Inc. (Ipswich, MA, USA), and sterile syringe filters 0.2 μm were from Millipore. Dimethyldioctadecylammonium bromide (DDAB) and 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (Egg PC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Lipids 1,2-dioleoyl-*sn*-glycerol-3-phosphoethanolamine (DOPE), egg L-a-phosphatidylcholine (EPC) were purchased from Avanti Polar Lipids (Alabaster, USA) and cholesterol was from J.T Baker. Cell line RAW 264.7 was obtained from American Type Culture Collection (ATCC®TIB-71™), MTS (tetrazolium compound [3-(4,5-dimethyl thiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt; MTS) cell proliferation assay kit was from Promega (USA). Tissue culture medium Dulbecco’s modified Eagle’s medium (DMEM high glucose, GE Health Care, Canada) and 1% penicillin-streptomycin were from Gibco, Canada. General chemicals Tris base, ethidium bromide, ascorbic acid, potassium phosphate monobasic, hydrochloric acid, boric acid and dextrose were purchased from Sigma-Aldrich Canada. Porcine gastric mucin (Type II) and ovalbumin (Ova) from chicken egg white were purchased from Sigma Aldrich Canada.

2.2. Preparation of TriAdj

TriAdj was prepared mixing 150 μg of Poly(I:C), 300 μg of IDR-1002 peptide and 150 μg polyphosphazene in 1:2:1 (w/w/w) ratio in a volume of 1 mL [5]. The diluent was sterile-filtered (0.2 μm) dextrose (5% (w/v) (D5W) and the preparation was carried out on ice and stored at 4 °C for use within 3 days [8]. The formation of a nondissociable complex was confirmed by agarose gel electrophoresis and fluorescence quenching that occurs upon interaction of the components (See Supplementary Data).

2.3. Preparation of liposomes

Pre-formed liposomes were used for preparing a lipidic complex with TriAdj, in order to readily control the proportions of lipid components as well as the homogeneity of the mixture of lipids while in the aqueous environment required for TriAdj. The liposomes were prepared by the thin-film extrusion method. Lipids at the appropriate molar ratios such as DDAB/DOPE 75:25, DDAB/DOPE 50/50, DDAB/Egg PC/DOPE 40:50:10, or Egg PC/cholesterol (90/10) were dissolved in chloroform. The preparation was dried under a stream of filtered air to form a thin film in a glass tube; the thin film was dried under vacuum in a lyophilizer 6 h to remove the organic solvent. The dried lipid films were rehydrated using D5W. After hydration of the lipid films, the lipid suspensions were subjected to freeze-thaw 10 times resulting in formation of multilamellar vesicles (MLVs). The resulting preparation was extruded 6 times at 55–60 °C through polycarbonate filters (0.1 μm Whatman, Sigma-Aldrich, St. Louis, USA) with an extruder apparatus (Lipex Extruder), forming liposomes of approximately 100 nm. The mean diameter of the liposomes was determined by dynamic light scattering and zeta potential was measured in the D5W diluent, both at 23 °C (Malvern, Nano ZS). Liposomal lipid concentration was quantified by a phosphorous assay described below.

2.4. Phosphorous assay

A modified version of the Fiske and Subbarow phosphorus assay was used to quantify phospholipids as well as TriAdj [27]. Phosphorus standard (0.65 mM solution, SigmaAldrich Canada) was aliquoted in triplicate into six separate standards tubes: 0 μmoles (0 μl) blank, 0.0325 μmoles (50 μl), 0.065 μmoles (100 μl), 0.114 μmoles (175 μl), 0.163 μmoles (250 μl), and 0.228 μmoles

(350 μl). Separately, samples representing approximately 0.1 μmoles phosphorus were placed into the bottoms of each sample tube in triplicate. To all tubes 450 μl of 8.9 N H_2SO_4 (Mallinckrodt) were added followed by heating in an aluminum block in a chemical fume hood at 200–215 °C for 25 min, then allowed to cool 5 min before adding 150 μl H_2O_2 (Fisher). All tubes were heated for an additional 30 min to a colorless state, then cooled to ambient temperature. Then 3.9 mL deionized water followed by 500 μl of ammonium molybdate(VI) tetrahydrate solution (2.5% w/v) were added to all tubes with vortexing. Next, 500 μl ascorbic acid solution (10% w/v) were added to all tubes, which were vortex-mixed thoroughly. Tubes were covered with a glass marble to prevent evaporation and to avoid pressure buildup during heating at 100 °C for 7 min. After cooling the tubes to ambient temperature, a spectrophotometer was used to measure absorbance at 820 nm. Phosphorus in the samples was quantified by comparison to the standard curve prepared by linear regression analysis of the absorbance values (λ = 820 nm) of the standard samples (linear range of 0–0.23 μmoles/mL ($r^2 > 0.99$).

2.5. Preparation of TriAdj formulations for nasal administration

2.5.1. Preparation of L-TriAdj

The phosphorus (P) concentration was determined as described above. The molar ratio of P from the liposomes to P from TriAdj was set as 0.5:1, 1:1, 2:1 and 3:1 to span a range of molar charge ratios (negative to positive), in order to determine empirically the composition necessary to achieve a cationic supramolecular assembly, i.e. positively charged lipidic nanoparticles. The goal was to establish component ratios that would facilitate favorable polyvalent polymer interactions between the cationic liposomes and the anionic TriAdj resulting in condensation into discrete complexes [28] rather than gross aggregation. The total P content was determined for the various liposome preparations and for TriAdj. This information was used to devise molar ratios required to approximate the desired charge ratios of lipidic complex of liposomes plus TriAdj (L-TriAdj). The molar ratio of P from the liposomes to P from TriAdj was set as 0.5:1, 1:1, 2:1 and 3:1 (ratios 1, 2, 3, 4). Liposomes and TriAdj were separately diluted in D5W and subsequently, consistent volume ratios of the two components were mixed to achieve different P molar ratios. The combination of lipids and TriAdj to form L-TriAdj was performed by vortex mixing cationic liposomes with TriAdj for 2 min followed by a 30 min incubation at ambient temperature.

2.5.2. Preparation of $CaCl_2$ microparticle vaccine for in vivo studies

As a point of comparison, the triple adjuvant was prepared as microparticles as previously described by Garlapati et al. without further physical characterization [3,6,29]). Polydi(p-oxyphenylpropionate) phosphazene, also known as PCEP (“polyphosphazene”) was obtained by custom synthesis at Idaho National Laboratory. Polyinosinic-polycytidylic acid [poly(I:C)] [30] (99% purity) was purchased from Sigma Aldrich Canada. IDR-1002 (VQRWLIVWRIRK) [29] was obtained from Genscript. Microparticles were prepared by a coacervation method, with poly(I:C) first mixed with IDR-1002 peptide at 37 °C for 30 min, and the PCEP and Ova antigen separately combined. The poly(I:C)-peptide mixture was then combined with the polyphosphazene and antigen mixture, followed by dropwise addition of 6.2%NaCl at a ratio of 1.95 mL of NaCl to 1 mL of 0.2% PCEP. The weight ratio of poly(I:C), IDR-1002 peptide and PECP was 10:20:10 μg. After 20 min at RT, 8% $CaCl_2$ solution was added to achieve a 1:200 dilution followed by 10 min incubation at RT on a rocker. To collect the microparticles, the suspension was centrifuged at 1390*g* for 10 min, washed with ddH_2O and resuspended in phosphate-buffered saline. The pooled supernatants from these final steps have been used to estimate Ova antigen lost during formation of the microparticles. After filtering through a 0.2 μm low protein binding syringe filters, typical encapsulation efficiency is approximately 70%.

2.6. Particle size and zeta potential analysis

The average particle size (nm) and polydispersity index (PDI) of liposomes and TriAdj and L-TriAdj were determined by dynamic light scattering. Surface charge was estimated by zeta potential measurements (Malvern, Nano ZS) in D5W at 23 °C. Samples were measured in triplicate. Particle size and features were observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) at the Western College of Veterinary Medicine Imaging Centre (See Supplementary Data). SEM was performed on dried samples mounted on copper 200 mesh grids and sputter-coated with 5 nm chromium where indicated to avoid electrostatic charge dissipation. Imaging was performed using a Hitachi SU8010 electron microscope with a voltage of 10–80 kV. TEM was performed on dried samples mounted on copper 200 mesh grids and stained with 1% phosphotungstic acid and observed at 80 kV power using a Hitachi HT7700 electron microscope.

2.7. Mucin interaction assessment

Mucin in deionized water (5 mg/mL) was mixed with L-TriAdj or liposomes and incubated for 30 min prior to particle sizing and zeta potential analysis, performed at 23 °C (Malvern, Nano ZS). Samples were measured in triplicate. Multimodal analysis with number weighting was used for the particle sizing. Interactions were also observed by scanning electron microscopy (SEM) and transmission electron microscopy (TEM) as described above.

2.8. Cytotoxicity assay

Cytotoxicity of TriAdj vs. L-TriAdj was assessed by MTS assay in a mouse macrophage cell line, RAW 267.4 (macrophage; Abelson murine leukemia virus transformed, ATCC – TIB71) [31,32]. Cells were cultivated in DMEM (Dulbecco’s modified Eagle’s medium) high glucose [10% FBS, 1% antibiotics (1% penicillin-streptomycin)], at 37 °C and 5% CO_2 and plated at 5000 cells/well 24 hrs before treatment. Cells were treated with TriAdj or L-TriAdj comprised of DDAB/DOPE (50/50) or DDAB/EPC/DOPE (40/50/10) as the lipid component, containing the equivalent of 0.5 μg poly(I:C), 1 μg IDR-1002 peptide and 0.5 μg polyphosphazene/well in a volume of 100 μl/well and incubated at 37 °C. CellTiter 96® Aqueous One Solution Reagent (Promega) was used per manufacturer’s instructions and absorbance read (λ = 490 nm). Significant differences were determined by one-way ANOVA with Tukey’s post-hoc test (n = 4, $p < 0.05$).

2.9. Intranasal vaccination in mice

Two *in vivo* studies were conducted with intranasal administration of an Ova vaccine in mice. Animal studies were conducted upon peer-reviewed protocol approval of the University of Saskatchewan Animal Research Ethics review board. First, two different lipid compositions of L-TriAdj as well as 2 different doses of TriAdj with a constant weight ratio of polyphosphazene:peptide:poly(I:C), i.e. 1:2:1 or 5:10:5 (μg:μg:μg). Female Balb/c mice, 5–6 weeks old, were randomly divided into 7 groups (n = 8/group). All groups except PBS control and Ova control received 1 μg Ova antigen mixed with the adjuvant (20 μl in one nostril). Groups: A: PBS control; B: Ova control (1 μg) (antigen only, no adjuvant); Groups C-G received Ova antigen along with the indicated adju-

vant: C: TriAdj (5:10:5); D: L-TriAdj as DDAB/DOPE 50/50 (mol/-mol) (TriAdj 1:2:1); E: L-TriAdj as DDAB/DOPE 50/50 (TriAdj 5:10:5); F: L-TriAdj as DDAB/EPC/DOPE 40/50/10 (TriAdj 1:2:1); G: L TriAdj as DDAB/EPC/DOPE 40/50/10 (TriAdj 5:10:5).

Second, a comparison of L-TriAdj coformulated with the Ova antigen versus a calcium microparticle formulation of TriAdj [29] was performed in a similar way as described above with 20 μl vaccine administered intranasally. Controls received Ova alone (1 μg or 10 μg/dose) The remaining groups received triple adjuvant as the 5:10:5 ratio of poly(I:C): IDR-1002 peptide: polyphosphazene, where the formulation was varied as TriAdj microparticles (MP), L-TriAdj DDAB/DOPE (50/50 mol/mol) or "soluble" TriAdj, and the dose of Ova was 1 or 10 μg/dose for each formulation.

In both studies, the mice were vaccinated at day 0 and boosted on day 28. Serum was collected on days 0, 14, 28, 42, 56, and 70 for individual plasma IgG1 and IgG2a ELISAs. IgA levels were measured in the second study at day 70. Mice were euthanized and spleens were collected on day 70. Each spleen was used for lymphocyte activation assays by the ELISpot method.

2.10. ELISA assays

ELISAs were performed on the sera as previously described [10]. Plates were coated with Ova and incubated at 4 °C overnight with sera diluted 1:4. To detect IgG1 and IgG2a, biotin-labeled goat anti-mouse IgG1 or IgG2a was added (IgG1: Invitrogen Cat # A10519,

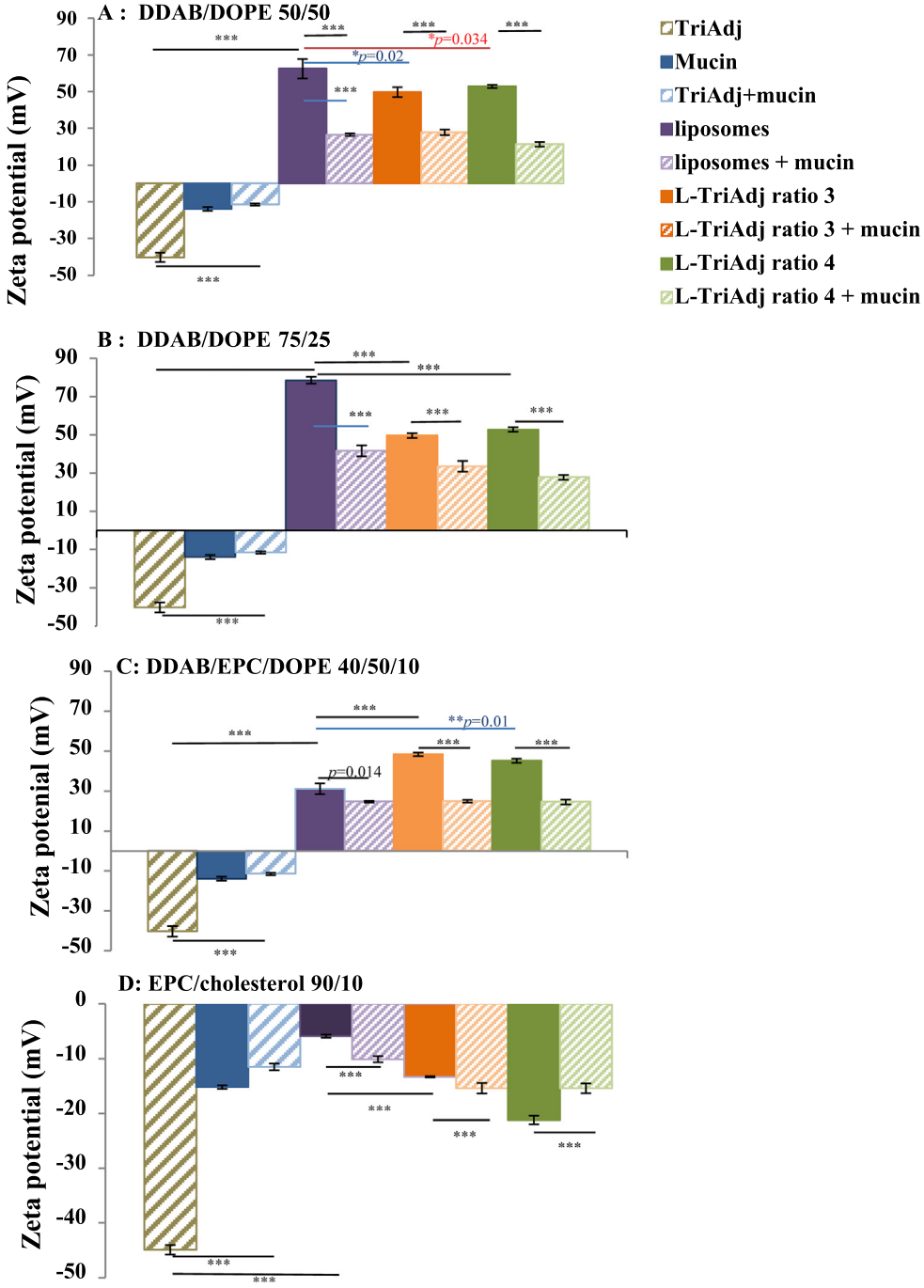

Fig. 1. Zeta potential of cationic liposomes ± TriAdj changes upon interaction with mucin. Data represent mean ± SD (n = 3). Ratio 3 and 4 refer to the molar phosphorus ratio of liposomes:TriAdj, of 2:1 (mol/mol) and 3:1, respectively. EPC; (Egg PC) 1,2-dioleoyl-*sn*-glycero-3-phosphocholine.

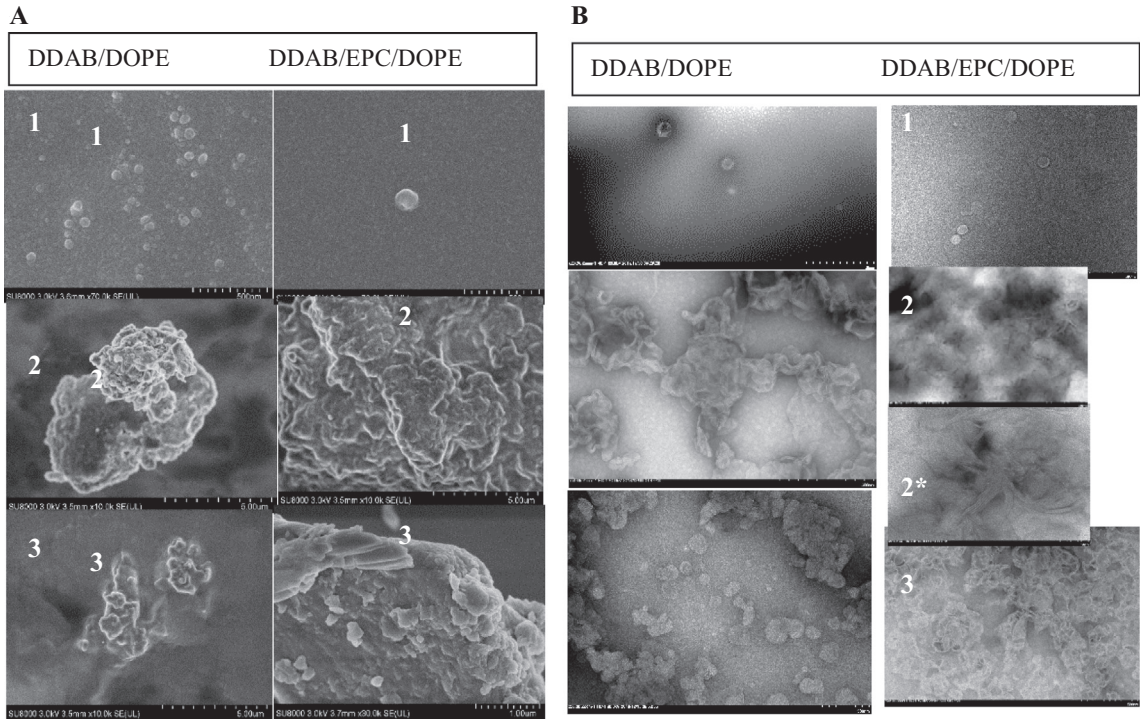

Fig. 2. Representative SEM (A) and TEM (B) images of liposomes, L-TriAdj and L-TriAdj with mucin.

IgG2a: Invitrogen Cat # M32315). To detect IgA, the starting dilution of the serum was 1 in 100 and the antibody used was goat anti-mouse IgA Biotin Conjugate (Invitrogen Cat # M31115). Streptavidin-alkaline phosphatase (AP) was added next as 100 μl of a 1:5000 dilution (Jackson ImmunoResearch Laboratories Inc., 016–050–084). A colorimetric reaction was developed by adding 100 μl of 1 mg/mL of p-nitrophenyl phosphate (Sigma-Aldrich, N3254) as the AP substrate. Plates were read with a Biorad iMark Microplate Reader. Data are expressed as titres, which represent the dilution factor required to generate an absorbance reading two standard deviations above the mean of the negative control.

2.11. Elispot assays

Spleens were harvested from the mice at day 70 post-vaccination, placing them in 10 mL Minimal Essential Medium (MEM, Gibco) on ice. The splenocytes were sieved through a 40 μm strainer (BD Falcon) and the cells pelleted at 1000 rpm for 10 min at 4 °C. The cell pellet was resuspended in 5 mL Gey solution and incubated at room temperature for 10 min. To this suspension 9 mL MEM was subsequently added followed by twice centrifugation as described above. The final pellet was resuspended in 5 mL AIM V media (Gibco) and the cells counted with trypan blue staining. ELISpot assays were performed as described previously [3,10,33]. Briefly, ELISpot plates (Millipore, Billerica, MA, USA) were coated overnight with IL5 or IFN-γ at 2 μg/mL (BD Biosciences cat # 551,216 and 554393). Spleen samples were then added in triplicate at a concentration of 1×10^7 cells/mL and incubated overnight. Splenocytes were stimulated with two different concentrations of Ova: 5 μg/mL and 10 μg/mL. Spots representing IFN- γ or IL-5 secreting cells were developed with biotinylated IFN-γ- or IL-5-specific goat anti-mouse IgG (BD Biosciences, 554410, 554397), followed by AP-conjugated streptavidin and BCIP/NBT (Sigma-Aldrich, B5655) as the substrate. Spots were counted with an AID ELISpot Reader (Autoimmun Diagnostika GmbH, Germany).

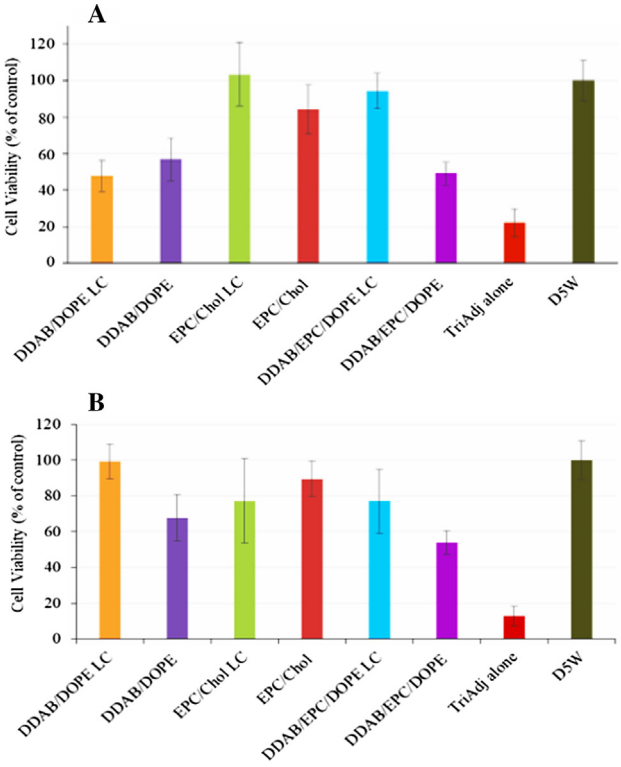

Fig. 3. MTS cytotoxicity assay in RAW264.7 cells after 24 h exposure in 96-well plates. The TriAdj concentration was constant at 0.5:1:1 μg/well (Fig. 2A) and 0.25:0.5:0.25 μg/well (Fig. 2B). *TriAdj alone was significantly more toxic ($p < 0.01$) compared to liposomes comprised of DDAB/DOPE (50:50 mol:mol); EPC/Chol (90:10); or DDAB/EPC/DOPE (40:50:10) or as lipid complexes (LC) with TriAdj.

2.12. Statistical analyses

Data are represented as mean ± standard deviation unless otherwise indicated. GraphPad Prism 5.0 was used for ANOVA

analysis with Tukey's post hoc test with significance set to $p < 0.05$. For the ELISA results, the data were homoscedastic based on analysis of variance of the residuals but not normally distributed. Therefore, comparison between treatment groups was performed on rank order-transformed data with the Kruskall-Wallis test (Statistics, STATEXT) and post-hoc Tukey test. Quartile analysis on rank order data was also used to illustrate comparative maximal response values from the ELISA data. Where indicated on the graphs, symbols indicate: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

3. Results

3.1. Particle size and zeta potential analysis of L-TriAdj

The mean diameter of all the liposome formulations was <200 nm and for those containing DDAB, the zeta potential was highly positive. P ratios of 0.5:1 and 1:1 (liposomes:TriAdj) consistently resulted in gross visible aggregation and were not used further, likely representing samples with a net neutral surface charge. For L-TriAdj containing DDAB/DOPE (75/25 mol/mol) at a 3:1 P ratio, aggregation was also observed and this composition was also eliminated from further consideration. As L-TriAdj, DDAB/DOPE (50/50 mol/mol) produced particles that were smaller and more homogeneous than DDAB/DOPE (75/25). (Supplementary Data, Tables S1 and S2), therefore, DDAB/DOPE (50/50) L-TriAdj was used for the in vivo studies. The zeta potential values of DDAB/DOPE (50/50) and DDAB/DOPE (75/25) liposomes were 62.5 and 78.6, respectively. For L-TriAdj the corresponding zeta potential values were reduced (by the addition of negatively charged Tri-Adj) to 49.7 and 56.4, which were stable over 24 h. A similar zeta potential was measured for DDAB/DOPE (50/50 or 75/25) prepared as L-TriAdj at 2:1 or 3:1 phosphorus molar ratio (ratio 3 and 4, respectively), over 24 h, in the range of 45–55 mV. (See Supplementary Data Fig. S3) The TriAdj composition at weight ratios of 5:10:5, 6:25:12.5:6.25 or 12.5:25:12.5 (μg:μg:μg) of poly(I:C):IDR-1002-peptide:polyphosphazene did not significantly influence the particle size or zeta potential of L-TriAdj using these lipid formulations (data not shown). Both the size analysis and zeta potential of L-TriAdj over 24 hr was found to be consistently stable. Whole vaccine, comprised of L-TriAdj with Ova, was stable for 24 h (Supplementary Data).

3.2. Structure and mucin interaction studies

To assess the potential for mucoadhesion, the zeta potentials of liposomes, TriAdj and L-TriAdj was measured before and after the addition of mucin (5 mg/mL). Zeta potential is a measurement of the electrical potential difference between the particle surface and the bulk liquid phase. Here, a change in zeta potential was used as a surrogate measure of mucin binding because the zeta potential value would be expected to change if mucin adsorbed or bound to the particle surface. It does not reflect the affinity or the specificity of binding, which would require more elaborate testing. Fig. 1 shows that cationic liposomes alone including DDAB/DOPE 50/50 (Fig. 1A), DDAB/DOPE 75/25 (Fig. 1B) and DDAB/EPC/DOPE 40/50/10 (Fig. 1C) showed initial zeta potential values of 62.5, 78.6 and 31 mV, respectively, and these decreased significantly upon addition of TriAdj (to form L-TriAdj) consistent with complex formation. These results were similar for at 2:1 or 3:1 phosphorus molar ratio (liposomes: TriAdj), indicated in the figure as ratio 3 and 4, respectively). TriAdj alone had a modest negative potential (−5 mV). When mucin was added to L-TriAdj, the zeta potential further decreased, consistent with an interaction. As a control, EPC/Chol 90/10 (Fig. 1D) showed a slight change in the zeta potential of liposomes (−33 mV) when mixed with TriAdj and mucin, perhaps due to nonspecific interactions. Phosphatidylcholine (PC) is a glycerophospholipid with a choline headgroup, possessing a zwitterionic character but a net formal charge of 0.

Fig. 2 indicates the ultrastructure of the liposomes, L-TriAdj and the association of L-TriAdj with mucin by scanning electron microscopy (SEM, Fig. 2A) and by transmission electron microscopy (TEM, Fig. 2B). Both DDAB/DOPE and DDAB/EPC/DOPE formulations were visualized. Samples had to be air-dried before microscopy which caused some degree of aggregation. Additional images of mucin alone, TriAdj alone and liposomes or TriAdj mixed with mucin are available in the Supplementary Data. The liposomes were seen to be on the order of 100–200 nm in agreement with the results of dynamic light scattering analysis of the lipo-

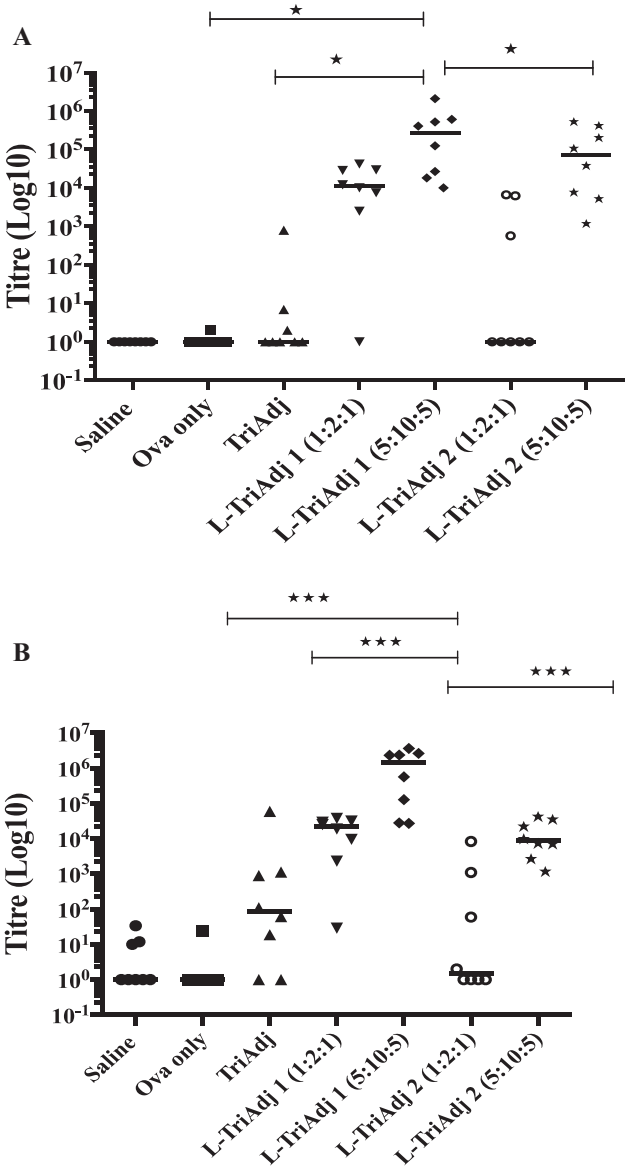

Fig. 4. ELISA results of IgG2a (A) and IgG1 (B) response in mice after nasal vaccine administration of TriAdj with ovalbumin (Ova) as the antigen and either 1:2:1 or 5:10:5 (μg:μg:μg) TriAdj per dose. L-TriAdj was formulated with DDAB/DOPE (50:50 mol/mol) or DDAB/EPC/DOPE (40/50/10 mol/mol). ●Saline control ■ Antigen only ▲TriAdj ▼L-TriAdj (1:2:1 wt ratio) DDAB/DOPE ◆ L-TriAdj DDAB/DOPE (TriAdj 5:10:5) ⬢ L-TriAdj (DDAB/EPC/DOPE (TriAdj 1:2:1) * L-TriAdj (DDAB/EPC/DOPE (TriAdj 5:10:5).

somes in suspension. L-TriAdj is seen on chromium-coated samples to be irregularly globular by SEM and variably sized. The appearance is similar when mucin was present, with larger globular structures noted. TEM analysis of liposomes alone showed vesicles with 1–3 lamellae, with a mean diameter of about 100 nm. L-TriAdj images clearly show deformation and reformation of lipid structures on the TriAdj particles, with the surface appearing to be covered with deformed liposomes or multilamellar nonvesicular regions. Unincorporated liposomes were quite scarce. Liposomes alone also interacted with the mucin globules but did not deform/reform, unlike with L-TriAdj, rather covering the mucin surface (Supplementary Data). Interaction of L-TriAdj with mucin was seen by TEM as strand-like areas and larger globules.

3.3. Cytotoxicity assay

An MTS cytotoxicity assay was performed using RAW264.7 mouse macrophage cells after 24 h exposure to the lipid adjuvant (Fig. 3), whereby TriAdj content was kept constant at either 0.5 μg:1 μg:0.5 μg/well (Fig. 2A) and 0.25 μg: 0.5 μg: 0.25 μg/well (Fig. 2B). TriAdj alone was significantly more toxic ($p < 0.01$) compared to TriAdj complexed with liposomes (L-TriAdj).

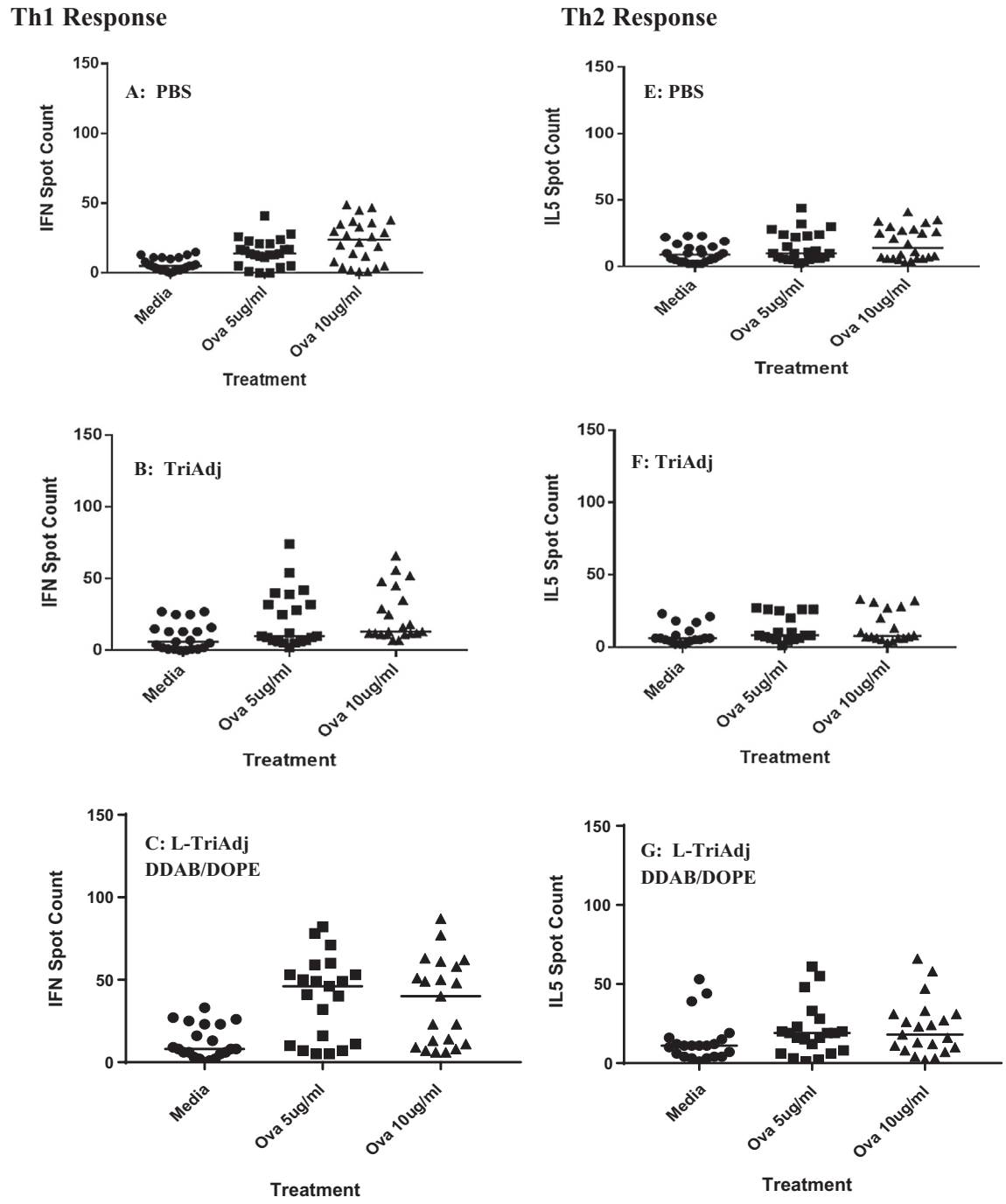

Fig. 5. ELISpot results from spleen lymphocytes harvested from the vaccinated mice which received TriAdj (5:10:5 wt ratio) or L-TriAdj, showing Ova antigen-stimulated secretion of IFN-γ (left side of the figure) or IL5 (right side), respectively. Data represent response from triplicate samples from individual mice and the horizontal bar represents the median value (n = 8).

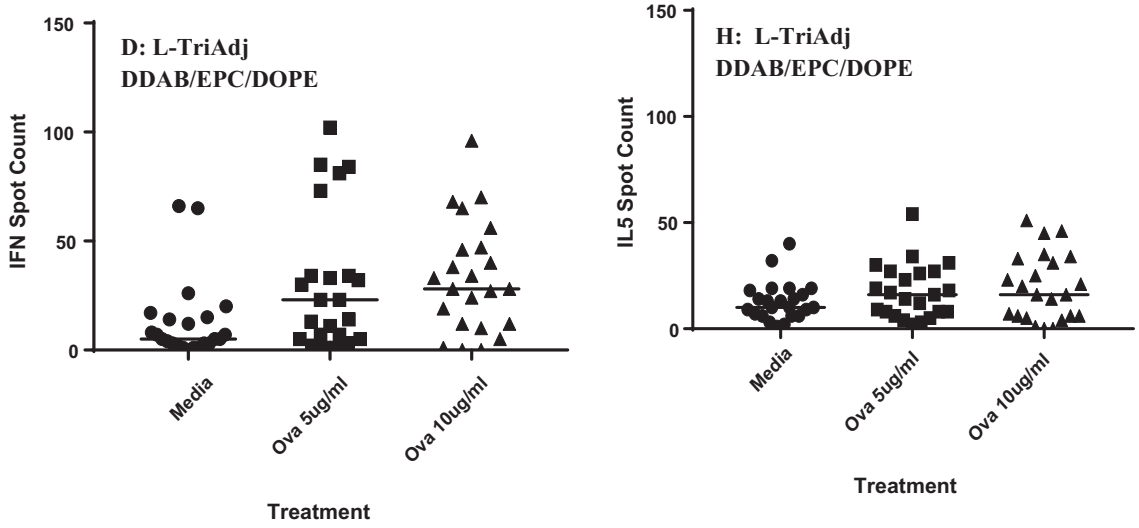

Fig. 5 (*continued*)

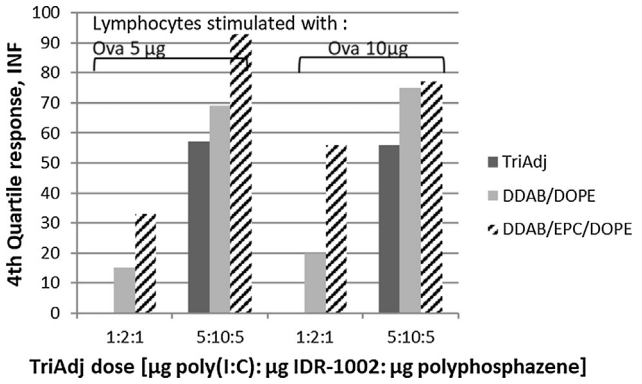

Fig. 6. Effect of TriAdj dose on the immune response to the adjuvanted ovalbumin vaccine in mice. Data represent 4th quartile of IFN-γ response from each treatment group (n = 8/group).

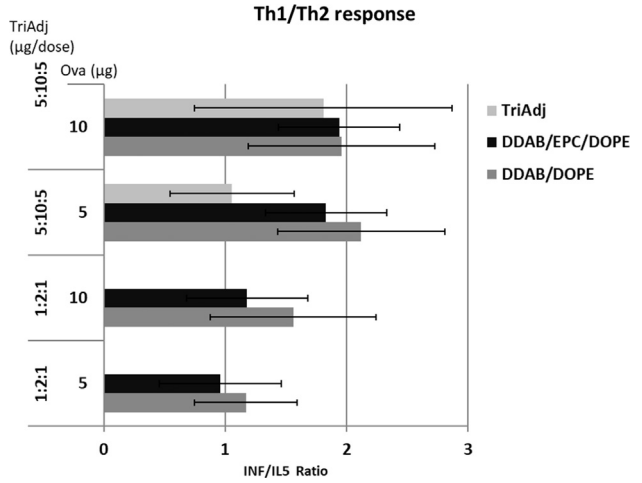

Fig. 7. Th1/Th2 response ratio for the triple adjuvant: The ratio of ELISpot values for IFN and IL5 for each mouse vaccinated with TriAdj or L-TriAdj + Ova antigen are expressed as mean ± SD (n = 7). TriAdj dose of 1:2:1 or 5:10:5 µg and lipid composition are as in Fig. 4. The spleen lymphocytes from the vaccinated mice were exposed in triplicate to 5 or 10 µg ovalbumin ex vivo and secretion of IL5 and IFN were measured. The ratio of these values reflects the balance of cellular (Th1) vs humoral (Th2) type response. *Significantly different from L-TriAdj DDAB/DOPE with 5:10:5 µg TriAdj and stimulated with 5 µg Ova ($p = 0.05$).

3.4. In vivo studies

The results obtained from the first in vivo study in mice are illustrated in Fig. 4 and showed a significantly greater immune response following intranasal administration of the lipid-based adjuvant complexed with the lower dose of ovalbumin antigen (Ova), compared to the non-lipidic TriAdj complex. At a higher dose of Ova both groups performed equally well. To assess humoral (Th2 type) vs. cellular (Th1 type) immune responses to vaccination, serum levels of IgG1 (typical of Th2 responses) and IgG2a (Th1) were measured at 0, 6 and 10 weeks by ELISA (Fig. 4A and B). L-TriAdj comprised of DDAB/DOPE with TriAdj at 5:10:5 wt ratio of poly (I:C): IDR-1002 peptide: polyphosphazene generated significantly higher IgG1 levels compared to TriAdj alone ($p < 0.01$), but this was not the case for DDAB/EPC/DOPE-TriAdj. Rank-order transformation of the IgG1 titre values revealed that groups receiving L-TriAdj based on DDAB/DOPE at both doses of TriAdj (1:2:1 and 5:10:5), or DDAB/EPC/DOPE formulated with TriAdj at a 5:10:5 wt ratio, produced statistically significantly higher ($p < 0.01$) IgG1 titres than the groups receiving non-lipidic TriAdj at a 5:10:5 wt ratio. Comparison of the rank order data further showed a significant difference in IgG1 responses between mice receiving L-TriAdj at a 1:2:1 vs. 5:10:5 wt ratio of TriAdj ($p < 0.05$). Furthermore, the median IgG2a responses of mice in groups receiving the lipid formulations were significantly higher than those receiving TriAdj alone as the adjuvant (Fig. 4B). There were significant differences between the rank-order transformed IgG2a values from groups receiving doses of TriAdj at a 1:2:1 vs. 5:10:5 ratio for both DDAB/DOPE and DDAB/EPC/DOPE-based L-TriAdj ($p < 0.01$). However, there was no statistically significant difference in IgG2a response when comparing the two lipid-based adjuvants at the 5:10:5 ratio at week 10.

Lymphocytes were isolated from the spleens of vaccinated mice and their response to the Ova antigen was assessed *ex vivo* by measurement of secreted IFN-γ and IL-5 (ELISPOT assay). Fig. 5 demonstrates the cellular Th1 response (IFN-γ; left side) and the humoral Th2 response (IL-5; right side). A balanced Th1/Th2 response is considered desirable for a vaccine while a Th1 type response is essential for vaccines intended for viral infections. Secretion of IL-5 from lymphocytes obtained from the vaccinated mice was not significantly different between the various treatment groups (Fig. 5E-5H). However, ELISPOT results for secretion of IFN-γ from Ova-stimulated splenocytes (Fig. 5A-5D) showed a greater proportion of strong responders in the groups vaccinated with L-TriAdj at

the 5:10:5 wt ratio compared to TriAdj alone as the adjuvant. This dose-response to the triple adjuvant content within L-TriAdj, illustrated in Fig. 6, showed that lymphocytes from vaccinated mice stimulated with a recall dose of 5 or 10 μg Ova had a higher level of IFN release for those groups that received L-TriAdj at 5:10:5 wt ratio of the adjuvant. Fig. 7 demonstrates an analysis of the polarization of the T cell response relative to lipid composition, adjuvant dose and Ova antigen dose, indicating that with both TriAdj and L-TriAdj, a desirable balanced response was obtained (N. B. a value < 1 would imply a relatively greater Th1 type response. while a value > 1 would imply a stronger Th2 response).

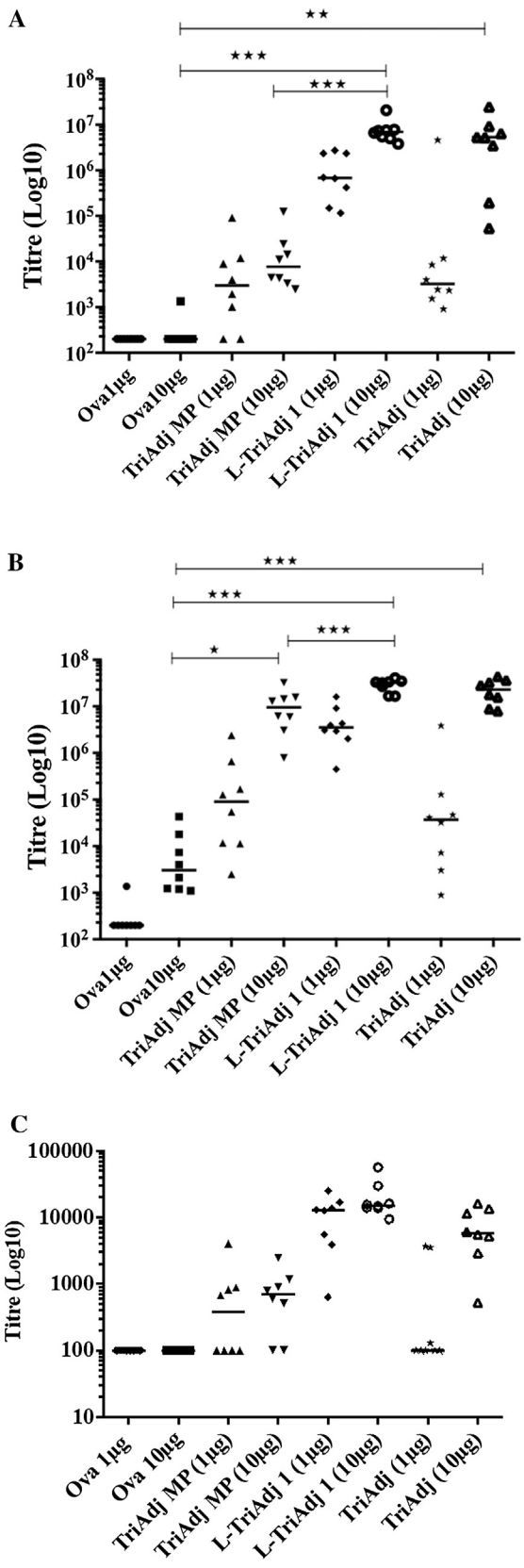

Fig. 8. ELISA results of IgG2a (A), IgG1 (B) and IgA (C) response in mice after nasal vaccine administration ovalbumin (Ova) at 1 μg or 10 μg Ova/dose and TriAdj formulated as L-TriAdj or TriAdj MP.

A second in vivo study in mice compared the adjuvant ability of TriAdj formulated as calcium microparticles (MP) [29] vs. L-TriAdj or TriAdj alone (Fig. 8). These studies assessed plasma IgG2a levels (Fig. 8A), IgG1 levels (Fig. 8B) and IgA (Fig. 8C), as measured by ELISA assay, at 0, 6, and 10 weeks in mice receiving intranasal Ova vaccines (1 or 10 μg/dose with a booster dose administered intranasally at week 4) adjuvanted with TriAdj, TriAdj MP, or L-TriAdj. PBS and Ova without adjuvant served as controls. At 6 weeks, for MP and lipidic formulations of TriAdj, the IgG1 titres were similar for mice vaccinated with 1 vs. 10 μg Ova, and a similar trend could be observed for IgG2a titres. In contrast, soluble TriAdj required 10 μg Ova to enable the generation of IgG1 and IgG2a titres comparable to those achieved with 1 μg Ova with L-TriAdj as the adjuvant. At 6 weeks, TriAdj MP with 1 μg Ova generated lower IgG2a titres when compared to L-TriAdj with 1 μg Ova, whereas the IgG1 titres were similar at the same antigen dose (1 or 10 μg Ova). At the high dose of antigen (10 μg Ova), there was no significant difference in IgG1 titres between groups receiving the vaccine adjuvanted with TriAdj, TriAdj MP or L-TriAdj, however, for IgG2a, TriAdj MP induced a lower titre than the other two adjuvant groups at 10 μg Ova/dose. Furthermore, L-TriAdj outperformed the other adjuvants at an Ova dose of 1 μg in terms of IgG2a response, demonstrating its potential for an antigen dose-sparing effect. ELISA results for IgA showed a stronger response to the lipidic formulation than the other test vaccines with Ova at 1 or 10 μg/dose (Fig. 8C), clearly demonstrating that mucosal immunity was achieved. This is particularly evident at the 1 μg dose of Ova, where the proportion of strong IgA responders is greater in the group receiving the lipidic formulation of TriAdj compared to soluble or microparticle preparations.

ELISpot results from the second in vivo study (Fig. 9) illustrate the IFN- γ (Fig. 9A–F, left-side) and IL-5 responses (Fig. 9G–L, right side) from lymphocytes obtained from the spleens of the vaccinated mice were assessed by ELISPOT assay following *ex vivo* stimulation with Ova antigen at 5 or 10 μg/ml. Thus, this data not only compared the effect of adjuvant formulation and antigen dose, but also the range of responses to antigenic recall at two doses. Assessing the response of lymphocytes from vaccinated mice revealed that within each formulation group and antigen dose, the median response of the lymphocytes to the Ova recall was similar at both 5 vs. 10 μg/mL Ova, based on the IFN-γ and IL-5 ELISPOT results and for both the L-TriAdj and MP groups. However, a greater response was noted in IL-5 and IFN- γ values when 10 μg Ova antigen was included in the vaccine compared to 1 μg Ova. Similar IL-5 and IFN-γ values were measured from groups receiving L-TriAdj with just 1 μg Ova antigen compared to TriAdj MP with 10 μg of Ova in the vaccine. Fig. 10 illustrates the median IgG2a titres at 6 weeks. Consistent with the first in vivo study, in those animals receiving antigen with L- TriAdj, the IgG2a antibody titres and INF-γ secretion from lymphocytes of vaccinated mice indicate a strong cell-mediated response for both the lipidic and MP formulations.

4. Discussion and conclusions

The combination of lipid nanocarrier with TriAdj undergoes a super-molecular self-assembly process which results in lipidic

nanoparticles of ideal diameter and charge. The composition facilitates adherence to mucin and may permit its penetration which would be promoted both by the cationic liposomes and the peptide IDR-1002. The lipid composition was comprised of cationic lipid (DDAB), for immunostimulation and mucin association, as well as "helper lipid" (DOPE) to aid endosomal escape. Modulation of both liposomal surface charge density and, theoretically, liposomal membrane fluidity was achieved by inclusion of egg phosphatidylcholine (EPC). The assembly process of cationic liposomes and TriAdj was reproducible and generated stable, condensed L-TriAdj particles with adjuvant activity in excess of that achieved by the TriAdj alone. The ratio of components that produced stable lipidic complexes of the TriAdj adjuvant was assessed in detail (Supplementary Data). Thus, the balance of charged polyelectrolyte components incorporated into the lipidic adjuvant promoted self-assembly and condensation, and an overall cationic charge inhibited gross aggregation and facilitated mucin interaction as indicated by its effects on the measured zeta potentials. The

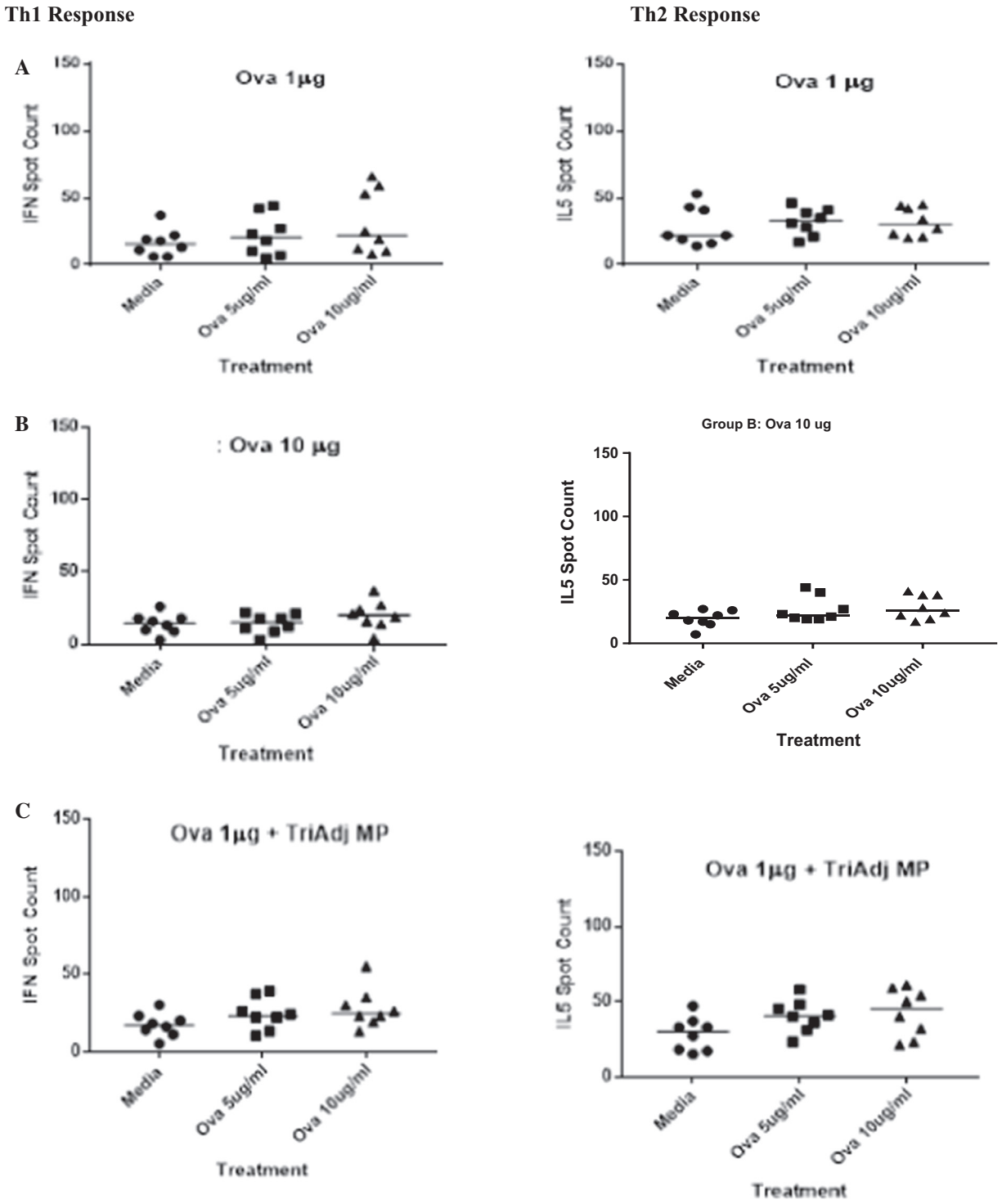

Fig. 9. Data represent *ex vivo* Ova antigen-stimulated splenocyte secretion of IFN-γ and IL5 by ELISPOT assay. Data represent triplicate samples from individual mice and the horizontal bar represents the median value (n = 8).

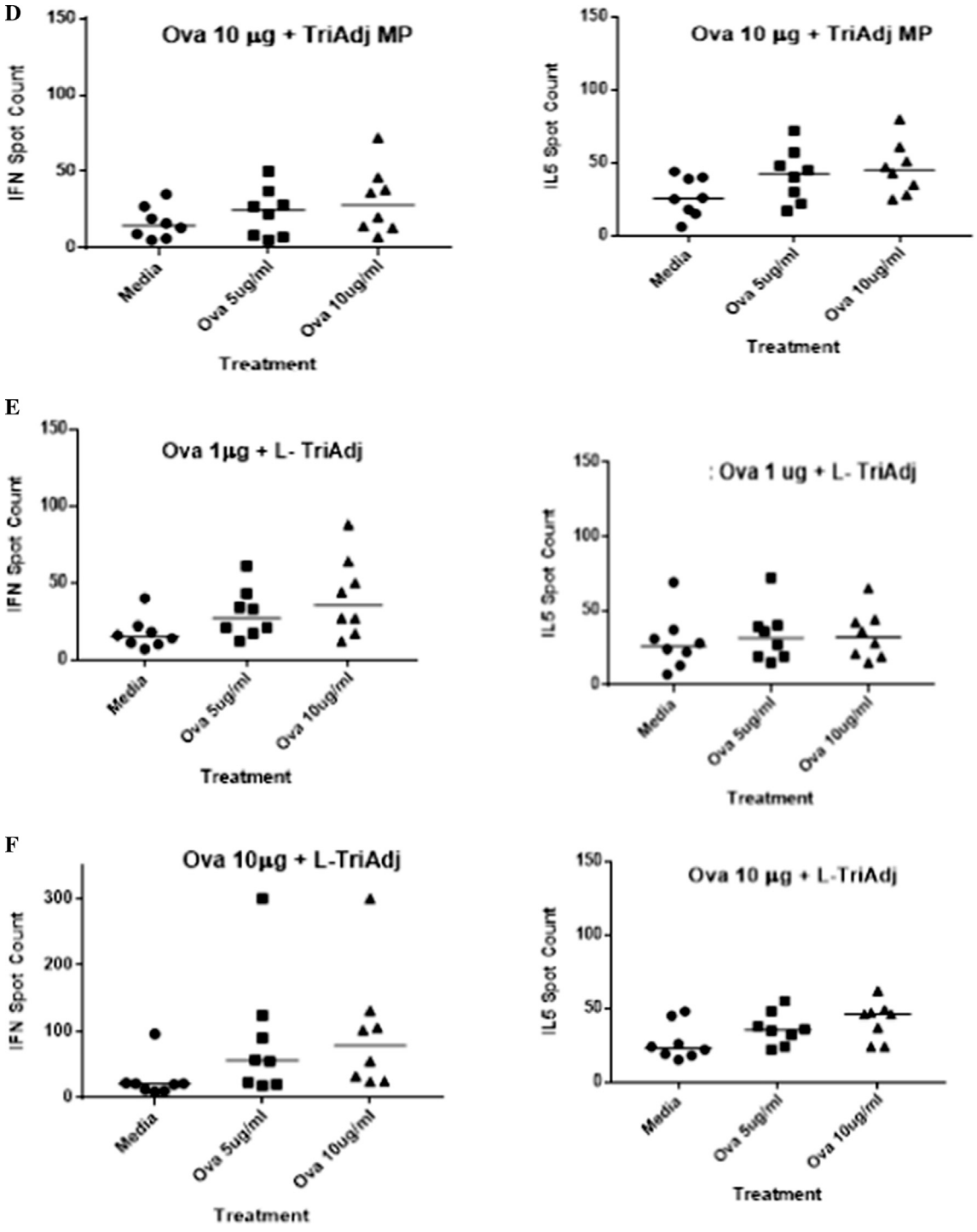

Fig. 9 (*continued*)

condensation of components also generated relatively small particles (<200 nm) that would be of a diameter amenable to cellular uptake. Analysis of whole-vaccine (antigen + adjuvant) size and 24 h stability indicated a submicron particle size range also (Supplementary Data). Ideally, the antigen and adjuvant would be taken up by the same APC, so binding of the antigen to the lipidic adjuvant would be advantageous. The effect of the antigen physicochemical features on lipidic adjuvant binding as well as the impact of binding on the structure of the antigen may need to be further explored with the relevant therapeutic vaccine antigen [34]. This in turn might be affected by lipid parameters such as degree of saturation, membrane fluidity and packing parameters [35]. For example, phosphatidylcholine, which is a neutral diacyl phospholipid with one unsaturation (16:1/18:0) would be expected to be in lamellar phase in the liposomes used to prepare L-TriAdj. However, the lipid phase organization after binding and its subsequent effect on adjuvant processing within the endosome, which may impact release of the various components, is not known. Although the EPC-containing liposomes (DDAB/EPC/DOPE 40/50/10 M ratio) would have a lower cationic surface charge density, the lipid content was normalized to the same total molar content of cationic lipid as DDAB/DOPE (50/50) for combining into

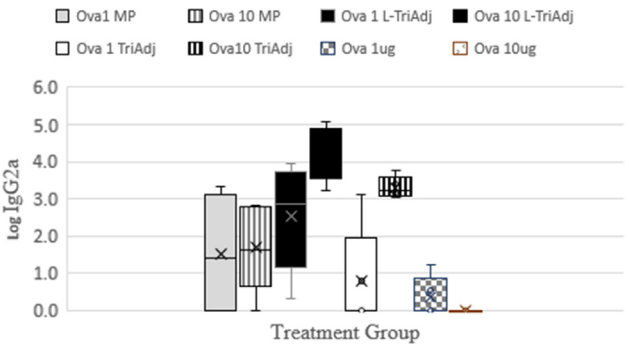

Fig. 10. IgG2a activity at 6 weeks post-vaccination is greater in mice receiving Ova+ L-TriAdj vaccine, based on IgG2a plasma levels. Data represent log values (n = 8); X represents median value.

lipidic particles with TriAdj. It can only be speculated that the difference in the *in vivo* adjuvant effect with the Ova vaccine might be related either to differential APC uptake or endosomal processing, which bears further study. Other research groups have noted the importance of lipid composition on the degree of immunostimulation as well as the tissue transit of cationic lipid-based adjuvants [36–40]. It is also important to note that the *in vitro* cytotoxicity studies showed that formulation of TriAdj with lipids reduced the cytotoxicity of TriAdj towards RAW267.4 mouse macrophages *in vitro*, reducing concerns over the potential for toxicity. Future *in vivo* studies will include examining the histopathology of the nasal cavity following nasal administration of L-TriAdj adjuvanted vaccines.

It should be noted that the other components of TriAdj, namely poly (I:C) and polyphosphazene, were varied in tandem with the peptide at previously optimized ratios and also contributed to the adjuvant activity. Mixed adjuvants provide a distinct advantage by activating different aspects of the immune response and lowering the antigen dose or number of doses required to generate a response of sufficient strength to protect the host following challenge with the infectious agent. Poly(I:C) is a synthetic version of double-stranded RNA which alerts the immune system by nature of its pathogen-associated molecular pattern (PAMP), activates an innate immune response via Toll-like receptor 3 (TLR3). It not only drives a Th1/cellular response, but also modulates the duration of response, promoting apoptosis of dendritic cells [41], which is important for resolution of immune responses. Polyphosphazene is a synthetic anionic polymer with immunostimulatory properties that also serves as a polyelectrolyte binding agent [3,42]. Another critical component of TriAdj is the cationic innate defense regulatory (IDR) peptide 1002, which has multiple immune modulatory roles including recruitment and selective activation of neutrophils and dendritic cells [3–5,43]. Through the use of rational proportions of cationic and helper lipid which enabled mucoadhesive particle formation, there was enhancement of an established adjuvant by the nasal route of administration resulting in a balanced Th1/Th2 immune response *in vivo*. The trend of influence of the adjuvant dose and the lipid composition on the Th2/Th1 balance bears further study because this may inform future studies with therapeutic vaccines where a specific type of response is required. A particulate formulation also has the potential for a depot effect, residing in the nasal tissues for an extended time for ongoing exposure.

Here we have demonstrated a novel intranasal adjuvant platform that can be used for a wide range of vaccines for the induction of both local and systemic immunity [44]. We have demonstrated an enhanced IgG (Figs. 4 and 8) and IgA (Fig. 8C) response as well as an antigen dose-sparing effect, in that the lipidic formulation enabled a stronger response at the lower doses of antigen compared to non-lipidic preparations of TriAdj. The advantages of the intranasal formulation are to deliver the vaccine via mucosal surfaces, thereby avoiding the use of needles, and inducing mucosal and systemic immunity simultaneously, which is key to enhanced protection against respiratory infections. These studies add to the growing understanding of the relationship between lipid composition or particle features of cationic lipid carriers of vaccine adjuvants and how they direct or influence the immune response [38,39,45,46]. Using a variety of compositional variations to permit formulation optimization as well as the tools for physical characterization to ensure reproducible fabrication, it will be feasible to design carriers that generate specific responses in the context of payload type. The lipidic adjuvant is expected to be useful for a variety for vaccine types and formulations including inactivated, subunit and modified live vaccines. Future studies will focus on therapeutic nasal vaccines with the DDAB/DOPE (50/50) composition of L-TriAdj that generated the most vigorous immune response at the lowest antigen dose.

Conflict of interest

The author declare that there is no conflict of interest

Acknowledgements

The authors acknowledge funding from the President's NSERC Fund, University of Saskatchewan, the College of Pharmacy and Nutrition of the University of Saskatchewan, the Agriculture Development Fund from the Government of Saskatchewan and the Saskatchewan Cattlemen's Association, and to REWH from a Canadian Institutes for Health Research grant FDN-154287). REWH holds a Canada Research Chair in Health and Genomics and is a UBC Killam Professor. In-kind support for the in vivo studies was provided by VIDO-InterVac, Saskatoon, SK.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.01.058.

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