

Effects of Cationic Adjuvant Formulation particle type, fluidity and immunomodulators on delivery and immunogenicity of saRNA

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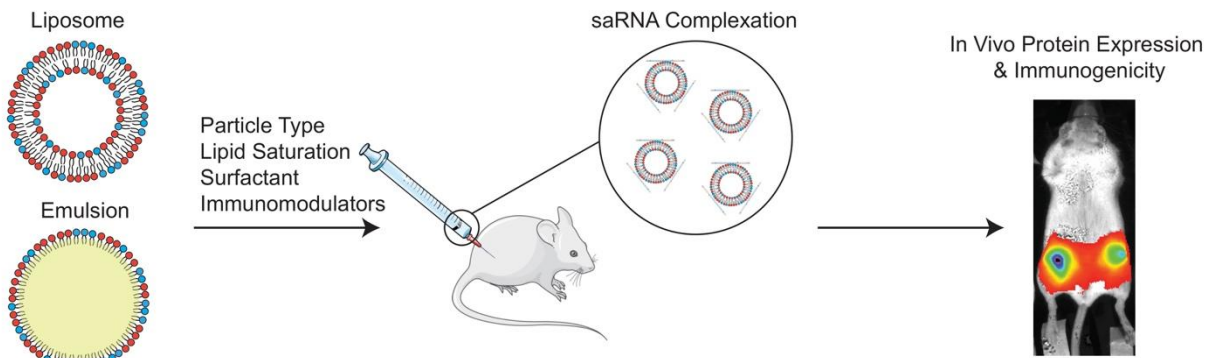
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Abstract

Self-amplifying RNA (saRNA) is well suited as a vaccine platform against chlamydia, as it is relatively affordable and scalable, has been shown to induce immunity against multivalent antigens, and can result in protein expression for up to 60 days. Cationic adjuvant formulations (CAFs) have been previously investigated as an adjuvant for protein subunit vaccines; here we optimize the CAFs for delivery of saRNA *in vivo* and observe the immunogenicity profile in the context of both cellular and humoral immunity against the major outer membrane protein (MOMP) of *Chlamydia trachomatis*. We tested both liposomal and emulsion based CAFs with solid and fluid phase lipids, with or without the TLR agonists R848 and 3M-052, for *in vitro* transfection efficiency and cytotoxicity. We then optimized the RNA/delivery system ratio for *in vivo* delivery using saRNA coding for firefly luciferase (fLuc) as a reporter protein *in vivo*. We observed that while the fluid phase liposome formulations showed the highest *in vitro* transfection efficiency, the fluid and solid phase liposomes had equivalent luciferase expression *in vivo*. Incorporation of R848 or 3M-052 into the formulation was not observed to affect the delivery efficiency of saRNA either *in vitro* or *in vivo*. MOMP-encoding saRNA complexed with CAFs resulted in both MOMP-specific cellular and humoral immunity, and while there was a slight enhancement of IFN- γ^+ T-cell responses when R848 was incorporated into the formulation, the self-adjuvanting effects of RNA appeared to dominate the immune response. These studies establish that CAFs are efficient delivery vehicles for saRNA both for *in vitro* transfections and *in vivo* immunogenicity and generate cellular and humoral responses that are proportionate to protein expression.

Graphical Abstract



Keywords

saRNA, liposomes, emulsion, TLR agonist, chlamydia, vaccine, MOMP

1. Introduction

Despite effective treatment options, the global annual incidence of chlamydia continues to exceed 130 million cases, thus strongly motivating the development of a vaccine [1]. While pre-clinical studies in mice, guinea pigs and non-human primates have demonstrated protection from challenge, only one vaccine candidate has so far moved on to clinical testing [2]. Recent evidence suggests that an effective vaccine may require the induction of memory T- [3] and/or B cells [4], provide protection against multiple serovars, and be scalable and affordable for inclusive distribution to low and middle income countries (LMIC) [5]. Messenger RNA (mRNA) vaccines are especially well suited for this vaccine indication as they are relatively cheap to produce at scale, can induce responses to multivalent antigens [6], and when delivered as alphavirus-derived self-amplifying mRNA (saRNA) express antigens for up to 60 days from a single injection [7], which provides a potential window for establishment of memory T-cells [8].

Cationic adjuvant formulations (CAFs), based on cationic liposomes or emulsions, have previously been paired with protein and peptide immunogens and been shown to induce robust T-cell and antibody responses [9-16]. The first generation CAF-adjuvant CAF01, consisting of cationic surfactant dimethyldioctadecyl ammonium surfactant (DDA) and glycolipid based immunomodulatory trehalose dibehenate (TDB), has been shown in humans to induce T-cell immunity that is detectable for up to 150 weeks after immunization [17] and with no adverse reactions after repeated administration in pre-clinical animal models [18] and humans [17]. It has furthermore shown to induce protective immunity against chlamydia in combination with several different chlamydia vaccine candidates, including Hirep1 and CTH93 in minipigs and MOMP and CTH522 in mice [19, 20]. The combination of CTH522 and CAF01 has recently been tested in a Phase I clinical trial (NCT02787109; publication in preparation). Based on these promising attributes, we hypothesized that CAFs may be well suited as a delivery vehicle for a saRNA based chlamydia vaccine, which requires a delivery vehicle to promote cellular uptake and expression.

While other liposome constructs have previously been used for delivery of mRNA [7, 21, 22], it has not yet been established whether the CAF adjuvants can act as delivery systems for mRNA-based antigens and whether the adjuvant effect observed for protein based antigens can also be found for mRNA. RNA is known to possess adjuvanting properties by activation of innate sensing mechanisms, through toll-like receptors (TLRs) such as TLR3, TLR7 and TLR8, and cytosolic pattern recognition receptors such as PKR, OAS, MDAS, and RIG-1 [23, 24]. However, suppression of innate recognition of RNA by nucleotide modification has been shown to inhibit Type I interferon (IFN) production by preventing activation of TLR7, TLR8 and other innate immune sensors, and subsequently enhance translation [25, 26]. Thus, it may be favorable to pair the antigen and adjuvant effects by employing a delivery vehicle that is both immunostimulatory and provides efficient delivery of RNA based antigens.

Here, we explore the CAFs as a delivery vehicle for saRNA encoding the major outer membrane protein (MOMP) of *Chlamydia trachomatis*. We utilized both CAF01 [17, 18, 27-30], and next-generation CAFs comprising liposomes and emulsions based on solid or fluid phase surfactants/oils, and with or without incorporation of the Mincle receptor agonists TDB or MMG, the TLR7/8 receptor agonists R848 or 3M-052 and/or the TLR3 receptor agonist Poly I:C. First, we used saRNA coding for reporter protein firefly luciferase (fLuc), to establish the transfection efficiency and cytotoxicity profile *in vitro*. We then performed an *in vivo* dose titration study in mice of the same saRNA in combination with the different CAFs to establish the optimal RNA/CAF ratio, and subsequently compare the delivery efficiency. Based on these studies we selected the most optimal combinations for *in vivo* testing using saRNA coding for chlamydia MOMP to evaluate vaccine induced immunogenicity including Ag-specific antibody and T-cell responses.

2. Materials & Methods

2.1 saRNA Synthesis & Purification

Self-amplifying RNA derived from the Venezuelan Equine Encephalitis Virus (VEEV) (Fig. 1) encoding either firefly luciferase (fLuc) or the MOMP of *Chlamydia trachomatis* was prepared using *in*

vitro transcription. pDNA was transformed into *E. coli* and cultured in 50 mL of LB with 100 µg/mL carbenicillin (Sigma Aldrich, UK) and isolated using a Plasmid Plus MaxiPrep kit (QIAGEN, UK). pDNA concentration and purity was measured on a NanoDrop One (ThermoFisher, UK) and subsequently linearized using MluI for 2h at 37 °C and heat inactivated at 80 °C for 20 min. Capped *in vitro* RNA transcripts for cell transfections were synthesized using 1 µg of linearized DNA template in a mMessage mMachine™ reaction (Promega, UK) and purified using a MEGAclear™ column (Promega, UK) according to the manufacturer's protocol. Uncapped *in vitro* RNA transcripts were synthesized using 1 µg of linearized DNA template in a MEGAScript™ reaction (Promega, UK) according to the manufacturer's protocol. Transcripts were then purified by overnight LiCl precipitation at -20 °C, pelleted by centrifugation at 14,000 RPM for 20 min, washed 1X with 70% EtOH, centrifuged at 14,000 RPM for 5 min, and the resuspended in UltraPure H₂O. Purified transcripts were then capped for use in animal experiments using the ScriptCap™ m⁷G Capping System (CellScript, Madison, WI, USA) and ScriptCap™ 2'-O-Methyltransferase Kit (CellScript, Madison, WI, USA) simultaneously according to the manufacturer's protocol. Capped transcripts were then purified by LiCl precipitation, resuspended in UltraPure H₂O and stored at -80 °C.

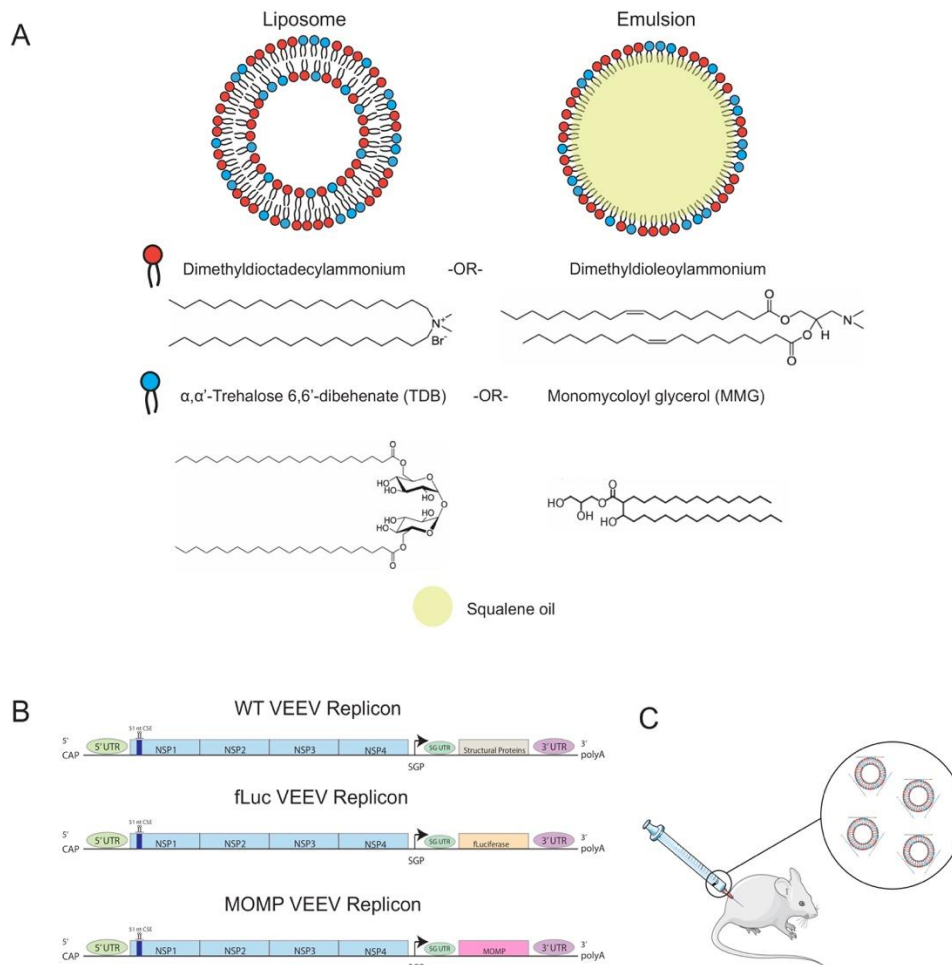


Fig. 1. Schematic of cationic adjuvant formulation (CAF) vaccines. A) Components of liposome and emulsion CAFs, B) structure of VEEV RNA replicons used for luciferase and immunogenicity studies, C) schematic of intramuscular injection of CAF-complexed RNA replicon formulations.

2.2 Production of Cationic Adjuvant Formulations

Squalene, squalane, Tween 60 + 80, Span 60 + 80, R848 and Poly I:C were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyldioctadecylammonium (DDA) bromide, Dimethyldioleoylammonium (DODA) chloride, α,α' -trehalose 6,6'-dibehenate (TDB) and monomycoloyl glycerol-1 (MMG-1) were purchased from Clausson Kaas (Farum, DK), and DODAC was obtained from Northern Lipids (Burnaby, BC, CA). 3M-052 was a gift from 3M (Maplewood, MN). All other chemicals were used at analytical grade and purchased from commercial suppliers.

CAF01a and CAF01b was prepared by the lipid film hydration method as previously described [31]. Weighed amounts of DDA (CAF01a) or DODA (CAF01b) and TDB were dissolved in 99% (v/v) EtOH and mixed in a glass vial at a molar ratio of 89:11. The lipid mixture was dried under a gentle N₂ stream for 2 h followed by air-drying overnight to remove trace amounts of EtOH. The lipid film was rehydrated in Tris-buffer by HSM by using a Heidolph Silent Crusher equipped with a 6F shearing tool (Heidolph Instruments GmbH, Schwabach, DE) at 60°C and 26,000 rpm for 15 min. The final lipid concentration in the resulting dispersion was 2.5/0.5 mg/ml DDA/TDB. CAF08a was prepared by admixing 0.1 mg/ml R848 into the final CAF01 formulation. CAF08b was prepared by admixing 0.04 mg/ml (final concentration) 3M-052 (a gift from 3M Drug Delivery Systems) dissolved in EtOH to the lipid mixture before drying the lipid film.

CAF09 was prepared by the film method for CAF01. Weighed amounts of DDA and MMG were dissolved in 99% (v/v) EtOH and mixed in a glass vial at a molar ratio of 82:18. The lipid mixture was dried under a gentle N₂ stream for 2 h followed by air-drying overnight to remove trace amounts of EtOH. The lipid film was rehydrated in 10 mM Tris-buffer pH 7.4 by HSM for 15 min as described above. Poly(I:C) was slowly added to a concentration of 0.5 mg/ml. The final concentration of CAF09 was 2.5/0.5/0.5 mg/ml DDA/MMG-1/Poly I:C.

CAF19a and CAF19b emulsions were prepared as previously described [13]. CAF19a: weighed amounts of MMG-1 and DDA and CAF19b: weighed amounts of Span 80, MMG-1 and DODAC, respectively, were dissolved in 99% (v/v) EtOH and mixed in glass vials. The mixtures were dried under a gentle N₂ stream for 2 h followed by air-drying overnight to remove trace amounts of EtOH. Span 60 was weighed into the CAF19a dry surfactant mixtures, as it is poorly soluble in EtOH. The oil phase consisting of Squalane (CAF19a) or Squalene (CAF19b) was weighed into the vials and heated at 60°C for 10 min with intermittent mixing to melt the surfactants and the oil phase. The water phase consisted of Tween 60 (CAF19a) or 80 (CAF19b) dissolved in tris(hydroxymethyl)aminomethane (Tris)-buffer (10 mM, pH 7.4), and it was added to the oil phase after melting. A pre-emulsion was prepared by high shear mixing (HSM) by using a Heidolph Silent Crusher equipped with a 6F shearing tool (Heidolph

Instruments GmbH, Schwabach, DE) at 60°C and 26,000 rpm for 5 min, which was subsequently microfluidized by using a LV1 Low Volume Homogenizer (Microfluidics, Westwood, MA, USA) with six passes at 20,000 psi. The final emulsions were sterile-filtered through a 0.22 µm filter (Sartorius Stedim Biotech GmbH, Goettingen, DE) prior to vaccination. CAF26a/b was prepared by admixing 0.04 mg/ml (final concentration) 3M-052 dissolved in EtOH to the lipid mixture before drying the lipid film.

Table 1. Cationic Adjuvant Formulation characteristics.

CAF	Particle Type	Lipid Saturation	Surfactant(s)	Oil	Immunomodulators
01a	Liposome	Saturated	Dimethyldioctadecylammonium	-	TDB
01b	Liposome	Unsaturated	Dimethyldioleoylammonium	-	TDB
08a	Liposome	Saturated	Dimethyldioctadecylammonium	-	TDB/R848
08b	Liposome	Saturated	Dimethyldioctadecylammonium	-	TDB/3M-052
09	Liposome	Saturated	Dimethyldioctadecylammonium	-	MMG/Poly I:C
19a	Emulsion	Saturated	Dimethyldioctadecylammonium/ Span60/Tween60	Squalane	MMG
19b	Emulsion	Unsaturated	Dimethyldioleoylammonium/ Span80/Tween80	Squalene	MMG
26a	Emulsion	Saturated	Dimethyldioctadecylammonium	Squalane	MMG/3M-052
26b	Emulsion	Unsaturated	Dimethyldioleoylammonium	Squalene	MMG/3M-052

2.3 In Vitro Transfections

Transfections were performed in HEK 293T.17 (ATCC, USA) cells that were maintained in culture in complete Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, ThermoFisher, UK) containing 10% fetal bovine serum (FBS), 5 mg/mL L-glutamine and 5 mg/mL penicillin streptomycin (ThermoFisher, UK). HEK cells were plated at a density of 50,000 cells per well 48 hours prior to transfection. The ratio of CAF to RNA, as indicated in each figure legend, was either 0.05, 0.25, 0.5 or

2.5 μ L of CAF per 100 ng of RNA. The RNA complexes were added to each well in a total volume of 100 μ L in transfection medium, containing DMEM and 5 mg/mL L-glutamine. Cells were allowed to transfect for 4 hours, and then the media was replaced with 100 μ L of complete DMEM until the appropriate timepoint.

2.4 Luciferase Assay

After 24 hours from the initial onset of transfection, 50 μ L of media was removed from each well and 50 μ L of ONE-Glo™ D-luciferin substrate (Promega, UK) was added and mixed well. Then, the total 100 μ L was transferred to a white 96-well plate and analyzed on a FLUOstar Omega plate reader (BMG LABTECH, UK) with a gain of 4000. The average of three control wells was subtracted from each value to account for any auto-luminescence from the cells.

2.5 MTS Assay

For cell viability, 20 μ L of CellTiter-Blue reagent (Promega, UK) was added to each well and allowed to incubate for 2 hours. The plate was then analyzed for absorbance on a FLUOstar Omega plate reader (BMG LABTECH, UK) and normalized to the media control.

2.6 In Vitro Imaging Studies

All animals were handled in accordance with the UK Home Office Animals Scientific Procedures Act 1986 in accordance with an internal ethics board and a UK government approved project and personal license. Food and water were supplied *ad libitum*. Female BALB/c mice (Charles River, UK), 6-8 weeks of age were placed into groups (n=5) and housed in a fully acclimatized room. Mice were immunized intramuscularly (IM) in both hind legs with 5 μ g of fLuc saRNA formulated with 2, 10 or 50 μ L of CAF in PBS, to a total injection volume of 50 μ L. Linear polyethyleneimine (PEI) with a molecular weight of 40,000 Da (PEI MAX™, Polysciences, Germany) was used as a positive delivery control and formulated at a ratio of 5:1 PEI to RNA. After 7 days, the mice were injected intraperitoneally (IP) with 100 μ L of XenoLight RediJect D-Luciferin Substrate (Perkin Elmer, UK) and allowed to rest for 10 minutes. Mice were then anesthetized using isoflurane and imaged on an In Vivo Imaging System (IVIS) FX Pro (Kodak Co., Rochester, NY, USA) equipped with Molecular Imaging Software Version 5.0 (Carestream Health,

USA) for 10 minutes. Signal from each injection site was quantified using Molecular Imaging software and expressed as relative light units (RLU).

2.7 MOMP Immunogenicity Study

Mice were immunized intramuscularly (IM) in one hind leg with 5 µg of MOMP saRNA formulated with 2 µL of CAF in PBS, to a total injection volume of 50 µL, and boosted after 3 and 6 weeks. PEI was used as a delivery/positive control as described above. Tail bleeds were collected prior to each vaccination and three weeks after the final boost. Blood was collected and centrifuged at 10,000 RPM for 5 min. The serum was harvested and stored at -20 °C.

2.8 MOMP-Specific ELISA

A semi-quantitative immunoglobulin ELISA protocol was performed as previously described [32]. Briefly, 0.5 µg/mL of rMOMP coated ELISA plates were blocked with 1% BSA/0.05% Tween-20 in PBS. After washing, diluted samples were added to the plates and incubated for 1 hr, washed, and a 1:4,000 dilution of anti-mouse IgG-HRP (Southern Biotech, UK) was used. Standards were prepared by coating ELISA plate wells with anti-mouse Kappa (1:1,000) and Lambda (1:1,000) light chain (Serotec, UK), blocking with PBS/1%BSA/0.05% Tween-20, washing and adding purified IgG (Southern Biotech, UK) starting at 1000 ng/mL and titrating down with a 5-fold dilution series. Samples and standards were developed using TMB (3,3',5,5'-Tetramethylbenzidine) and the reaction was stopped after 5 min with Stop solution (Insight Biotechnologies, UK). Absorbance was read on a spectrophotometer (VersaMax, Molecular Devices) with SoftMax Pro GxP v5 software.

2.9 MOMP-Specific IFN-γ ELISpot

Splenic lymphocytes were harvested as previously described [33]. IFN-γ ELISpots (Mabtech, UK) were performed according to the manufacturer's protocol using two separate MOMP peptide pools consisting of 15-mers overlapping by 11 amino acids.

2.11 Statistical Analysis

Graphs and statistics were prepared in GraphPad Prism, version 7.0. Statistical differences were analyzed using either a two-tailed t test or an ordinary one-way ANOVA with multiple comparisons, with $\alpha=0.05$ used to indicate significance.

3. Results

3.1 In Vitro Transfection Efficiency and Cytotoxicity of CAF-complexed saRNA

Because CAF formulations have not been previously employed to deliver RNA, we first sought to characterize the particles (Fig. 2) and evaluate the *in vitro* transfection efficiency and cytotoxicity of CAF-complexed saRNA (Fig. 3). All CAFs were found to have particle sizes between 100 and 250 nm (Fig. 2A), and the liposomal formulations had a positive surface charge while the emulsion formulations had a neutral surface charge (Fig. 2B). We then evaluated the transfection efficiency in HEK293T cells, using doses of 0.25, 0.5 and 2.5 μ L of each of the CAF adjuvants (Fig. 3, Supplementary Fig. 1). As expected the unformulated saRNA had very little luciferase signal ($\sim$ 100 RLU), thus necessitating the use of a delivery vehicle to enhance cellular uptake. We first compared liposomes (CAF01) and emulsions (CAF19) with saturated (a) and unsaturated (b) surfactants (Fig. 3A). We observed that the liposomes and emulsions with saturated surfactants (CAF01a, CAF19a) had equivalent luciferase expression and did not exhibit dose dependency. Interestingly, when an unsaturated surfactant was used in the liposomal formulation (CAF01b) the luciferase expression was enhanced up to two orders of magnitude and exhibited dose dependency, whereas the use of an emulsion based on the same unsaturated surfactant (CAF19b) completely inhibited saRNA delivery/luciferase expression. We then compared how the incorporation of different immunomodulators (R848, 3M-052, Poly I:C) into liposomes with a saturated surfactant affected the *in vitro* luciferase expression (Fig. 3B). We observed that incorporating R848 (CAF08a) had no effect on luciferase expression, whereas 3M-052 (CAF08b) caused an inverse dose dependent increase in luciferase expression. Incorporation of Poly(I:C) (CAF09) inhibited luciferase expression, possibly due to alterations in the availability of cations in the formulation or induction of Type I IFN that inhibits protein expression. We then sought to observe how incorporation of the immunomodulator 3M-052 into saturated and unsaturated emulsion formulations affected luciferase

expression (Fig. 3C). There was no change in luciferase expression upon incorporation of 3M-052 into the emulsion formulation with saturated surfactant (CAF26a), however; there was no luciferase expression with either of the emulsions with unsaturated surfactants (CAF19b, 26b), suggesting that membrane rigidity is an important factor for cytosolic delivery of saRNA. Based on these observations, we investigated whether the CAFs cause cytotoxicity as an explanation for the low transfection efficiency of CAF 19b and 26b. All CAFs were observed to cause minor amounts of cytotoxicity (10-50% toxicity) in HEK cells (Fig. 3D), compared to un-transfected cells. Surprisingly, saRNA alone induced some cytotoxicity (~12%). There appeared to be no dose dependence of the cytotoxicity induced by CAFs over the tested range (0.25 to 2.5 μ L, Supplementary Fig. 1). Interestingly, the toxicity profiles of CAF 19b and 26b were no different to that of the other CAFs, and thus the lack of transfection efficiency must be due to other mechanisms, such as instability of the fluid phase emulsions in a liquid transfection environment. Based on the promising *in vitro* results, we aimed to establish whether selected CAFs were able to efficiently deliver saRNA *in vivo*.

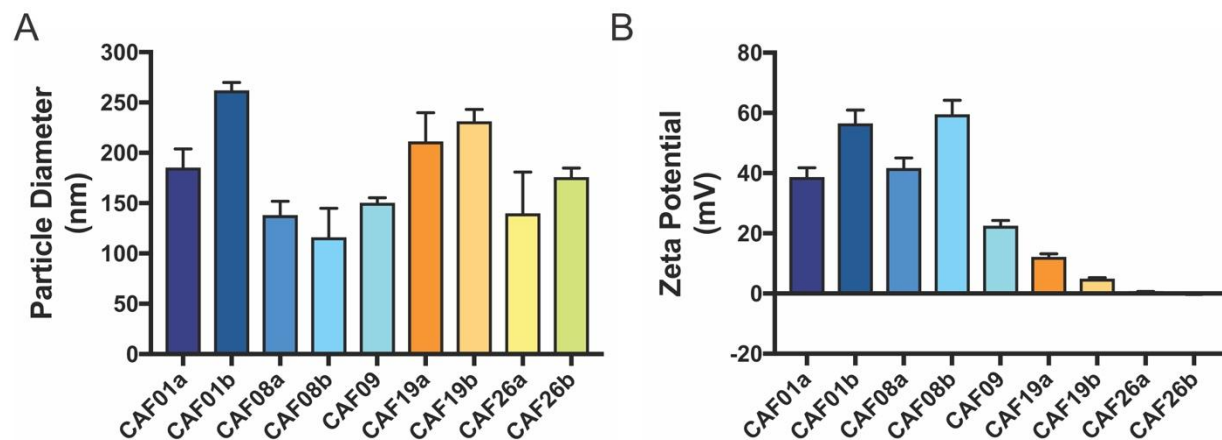


Fig. 2. Particle characterization of CAFs as determined by dynamic light scattering. A) Particle size and B) surface charge measurements for each formulation. Bar represents the mean $\pm$ standard deviation for n=3 measurements.

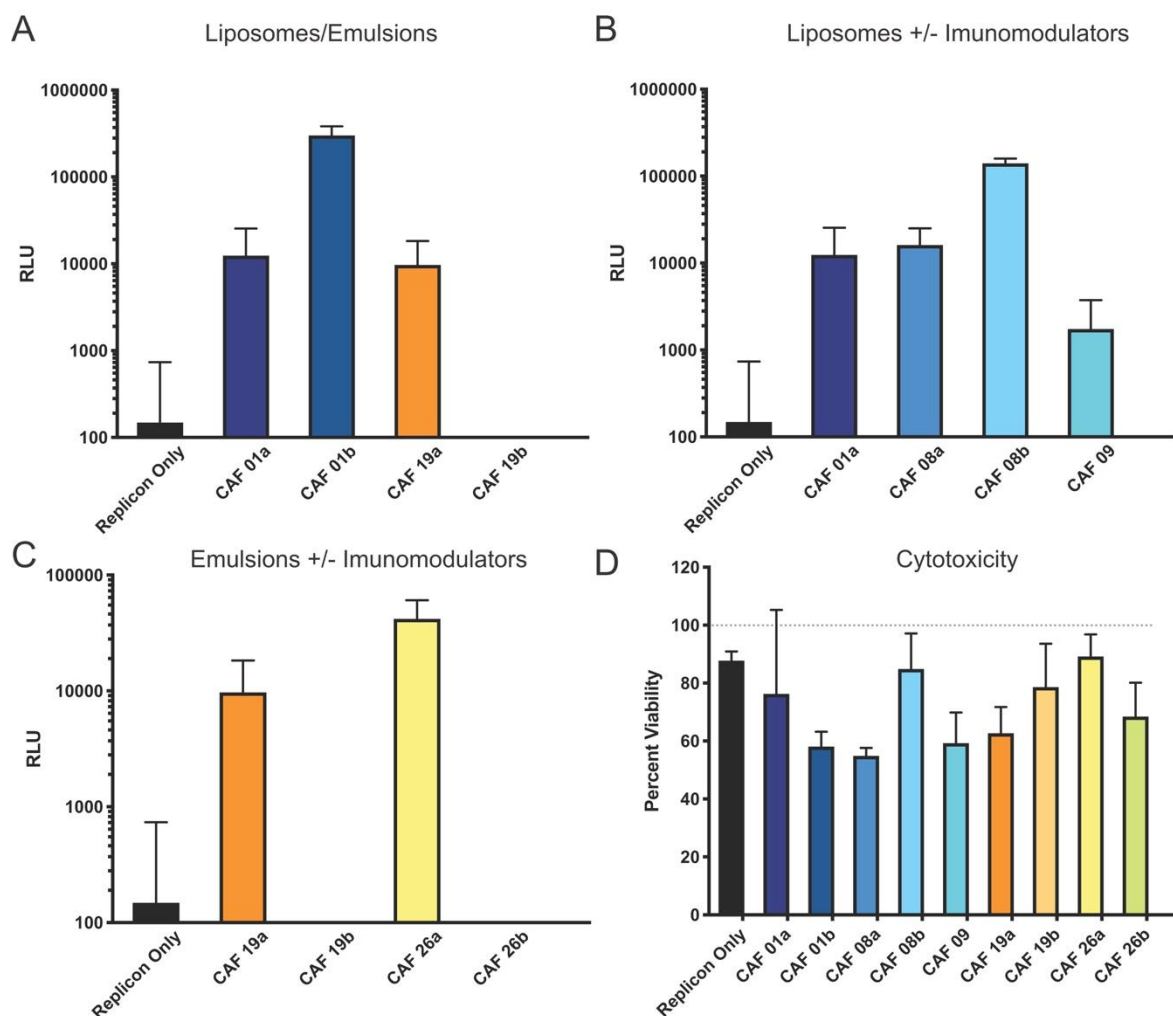


Fig. 3. In vitro transfection and cytotoxicity of CAF complexed fLuc RNA replicons in HEK 293T cells. A-C) Luciferase expression of 0.1 μ g RNA alone or complexed with varying doses of CAF after 24 hours represented as mean relative light units (RLU) $\pm$ standard deviation for A) liposome or emulsion CAFs with saturated/unsaturated surfactants, B) liposome CAFs with saturated surfactant and immunomodulators, C) emulsion CAFs with immunomodulators. D) Cytotoxicity of CAF complexed RNA after 24 hours, normalized to no treatment control. Values expressed as percent viability $\pm$ standard deviation.

3.2 In Vivo Dose Titration of CAF-complexed saRNA

The CAF adjuvants were observed to mediate efficient cellular uptake *in vitro*, thus warranting *in vivo* testing as an saRNA delivery vehicle; we therefore opted to do an *in vivo* dose titration. We chose to use CAF01a and CAF01b at doses of 2, 10 and 50 μ L with 5 μ g of saRNA encoding fLuc (Fig. 4), as these CAFs had been observed to have either no dose dependence (CAF01a) or increasing transfection

efficiency with decreasing dose (CAF01b). Mice were injected and imaged after 7 days (Fig. 4A) due to previous data suggesting that the peak luciferase expression from VEEV saRNA occurs at that timepoint [34]. We used PEI as a positive delivery control as it has previously been used for saRNA formulations *in vivo* [35]. We observed that PEI, and the 2 μ L dose of CAF01a and CAF01b had equivalent luciferase expression after 7 days (10,000-30,000 RLU) (Fig. 4B,C). No luciferase signal was observed when using higher doses of the CAFs, except for one leg of one mouse in the CAF01b 50 μ L dose group. We postulate that the lack of signal was due to aggregation in the formulation liposomes upon addition of the saRNA, which resulted in visible particulates and likely limited cellular uptake. These observations confirmed that 2 μ L was the appropriate dose of CAF to use for immunogenicity studies.

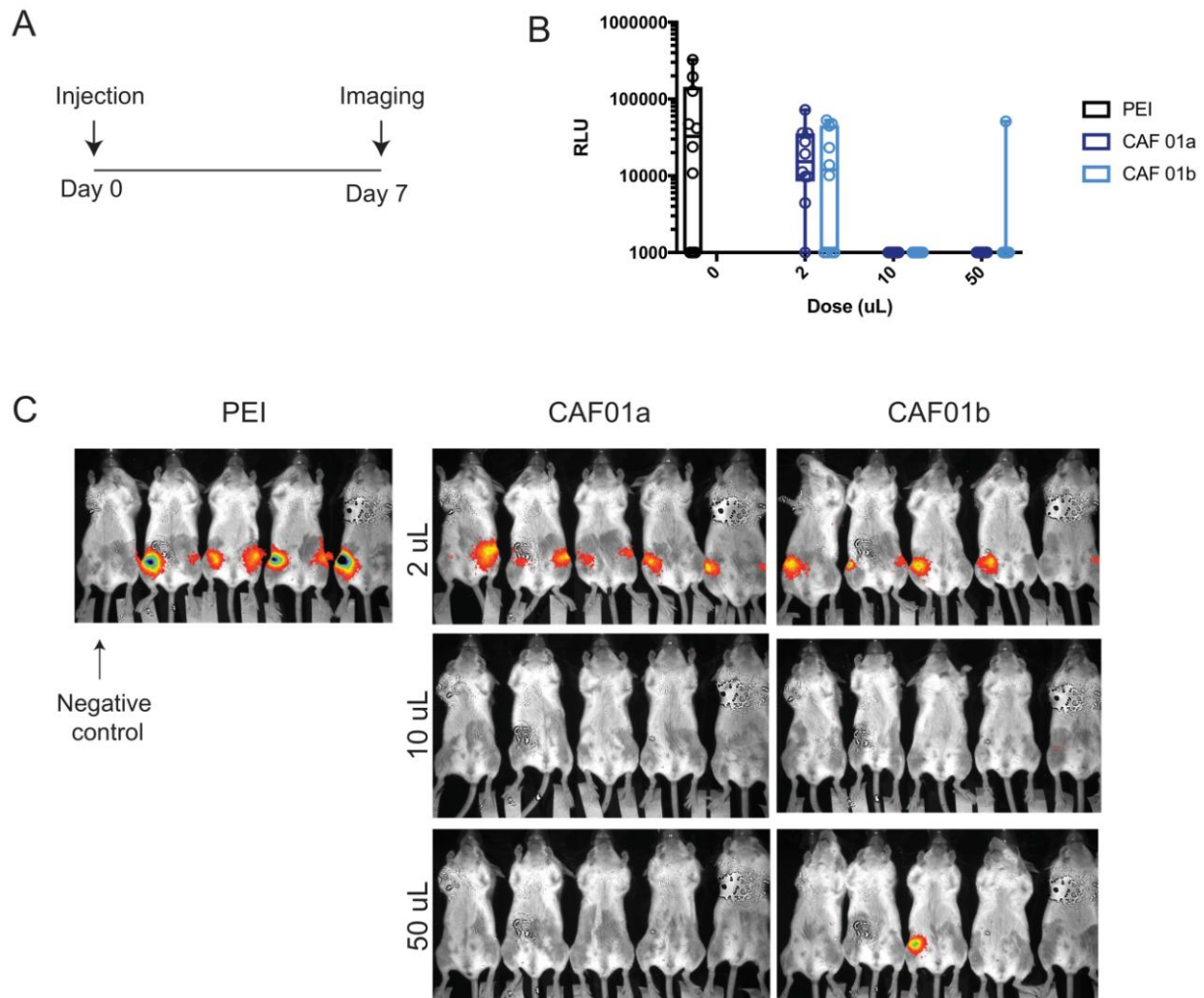


Fig. 4. In vivo titration of CAF01a and CAF01b formulations with 5 µg fLuc RNA replicon. A) Injection and imaging schedule, B) quantification of luciferase expression represented as box and whisker plots with mean, minimum and maximum, C) images of in vivo luciferase expression.

3.3 In Vivo Delivery Efficiency of CAF adjuvants

Based on the results of the *in vivo* dose titration (Fig. 4), we then sought to determine which CAFs had the highest delivery efficiency of saRNA (Fig. 5). In order to minimize the number of groups of animals used, we chose to test five CAFs that allowed us to answer whether components of the formulations, such as the particle type, surfactant saturation and immunomodulator (Table 1), affected the delivery of saRNA. Thus, we compared CAF01a, CAF01b, CAF08b, CAF19a and CAF19b with luciferase expression as the primary outcome. Comparison of CAF01a and CAF01b indicates the role of the fluidity phase of the lipid whereas CAF01a versus CAF08b indicates the role of co-stimulation of TLR7/8 receptor. Comparing CAF01a with CAF19a indicates the role of using a liposomal- versus emulsion-based formulation, and finally CAF19a versus CAF19b indicates the role of the fluidity of the lipids in the emulsions. We used an identical dosing and imaging schedule to the dose titration study, with imaging 7 days after injection (Fig. 5A). We observed that CAF01a, CAF01b, CAF08b and CAF19b had equivalent saRNA delivery *in vivo*, resulting in luciferase expression of ~100,000 RLU (Fig. 5B,C). CAF19a was found to have poor delivery of saRNA, resulting in an average luciferase expression of ~1,000 RLU, however, these differences were not statistically significant after adjusting for multiple comparisons. While there is inherent variation *in vivo*, CAF01a, CAF08b and CAF19b had high luciferase expression and relatively less variation, indicating that these were the most efficient delivery vehicles for saRNA. Due to efficient delivery of saRNA *in vivo* and the possibility of adjuvant effects in the context of a vaccine, CAF01a and CAF08b were chosen to move forward in immunogenicity studies.

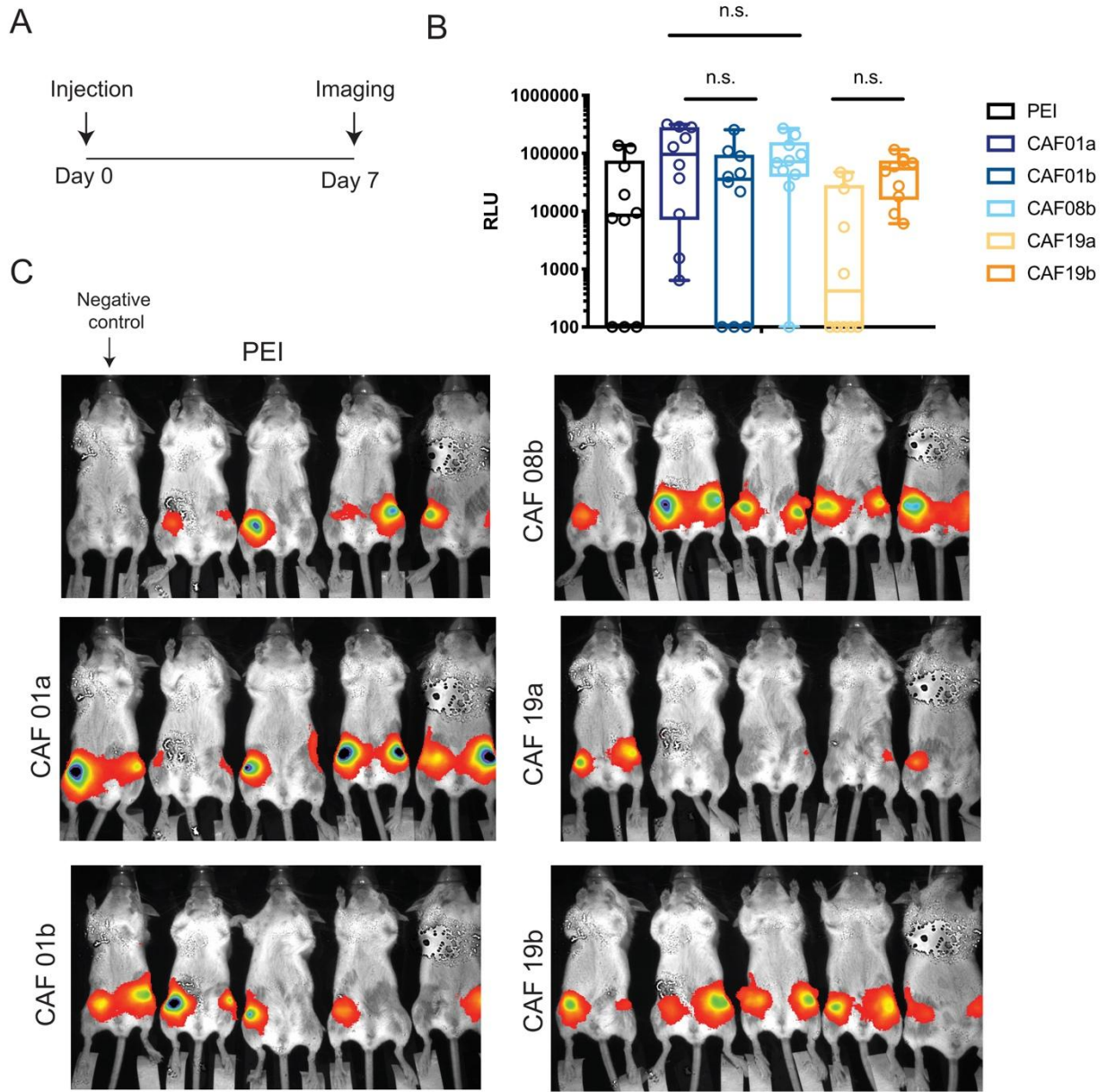


Fig. 5. In vivo evaluation of luciferase expression from formulations of CAF 01a, CAF01b, CAF08b, CAF19a, and CAF19b complexed with 5 μ g fLuc RNA replicon. A) Injection and imaging schedule, B) quantification of luciferase expression represented as box and whisker plots with mean, minimum and maximum, C) images of in vivo luciferase expression.

3.4 Induction of MOMP-Specific IgG Antibodies by CAF-complexed saRNA

Because enhanced protein expression may not be equivalent to immunogenicity we sought to determine the immunogenicity of the CAFs using MOMP as a model immunogen, given the previous observations using these formulations and recombinant MOMP [36]. The immunogenicity of MOMP

saRNA was assessed in BALB/c mice immunized with an intramuscular prime/dual boost regimen. Because CAF01a, CAF01b, CAF08b and CAF19b were found to have equivalent saRNA delivery efficiency *in vivo* (Fig. 5), the saRNA was formulated with either PEI (positive control), 2 μ L CAF01a or 2 μ L CAF08b, in order to determine whether the inclusion of immunomodulators in the CAFs affect the immunogenicity of the vaccine. Female mice were immunized at three-week intervals (Fig. 6A), and serum was collected prior to each regimen and three weeks after the final immunization to assess the MOMP-specific IgG titers. After three weeks, all three formulations had equivalent antigen-specific IgG titers ($\sim$ 40 ng/mL). After the boost regimen, the MOMP saRNA formulated with CAF08b was observed to have the highest antigen-specific IgG titers at both 6 and 9 weeks (80 and 100 ng/mL, respectively) but were not statistically significantly higher than the PEI or CAF01a formulated saRNA (40 and 60 ng/mL after 6 and 9 weeks, respectively). These results indicate that while CAF01a and CAF08b deliver saRNA equivalently, there is negligible effects on humoral immunogenicity due to the incorporation of 3M-052 into the formulation. Furthermore, the boost immunizations yielded only minor increases in antibody titers beyond those induced with the single prime vaccination, which could likely benefit from an elongated interval.

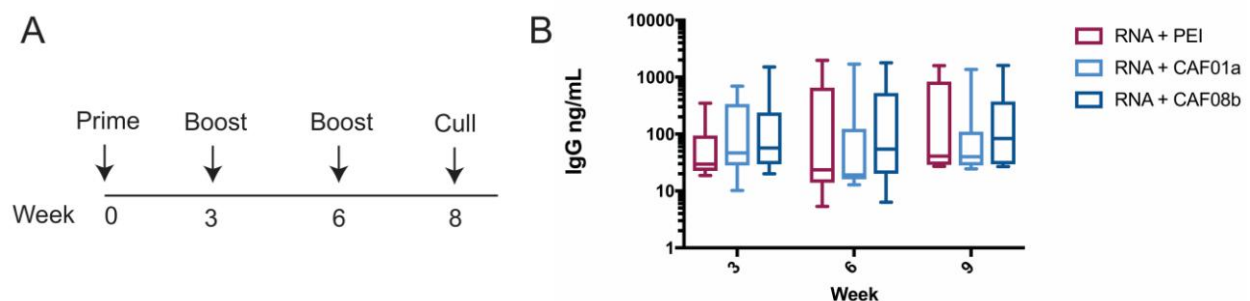


Fig. 6. Immunization schedule and antibody titers after immunization with MOMP saRNA. A) Immunization schedule, B) MOM antigen-specific IgG antibody titers following immunization with prime and two boosts of RNA complexed with PEI, CAF 01a or CAF 08b. Boxes represent maximum, mean and minimum antibody titer as determined by ELISA for $n=10$ at each timepoint.

3.5 IFN- γ Production by MOMP-reactive Splenocytes

T-cell responses were assessed three weeks after the final immunization. All formulations, including PEI-, CAF01a- and CAF08b-complexed saRNA resulted in MOMP-specific IFN- γ^+ T-cell responses as assessed by ELISpot (Fig. 7). The T-cells were consistently more reactive against MOMP peptide pool 2, with 50 SFU/ 10^6 splenocytes reactive against MOMP peptide pool 1 and 80-125 SFU/ 10^6 splenocytes reactive against MOMP peptide pool 2. The T-cell responses to peptide pool 2 were higher for saRNA + PEI and saRNA + CAF08b (125 SFU/ 10^6 splenocytes) than saRNA + CAF01a (80 SFU/ 10^6 splenocytes), however, these differences were not significant. Thus, we conclude that the addition of 3M-052 to the formulation does not significantly affect the cellular (Fig. 7) or humoral (Fig. 6) responses to MOMP saRNA.

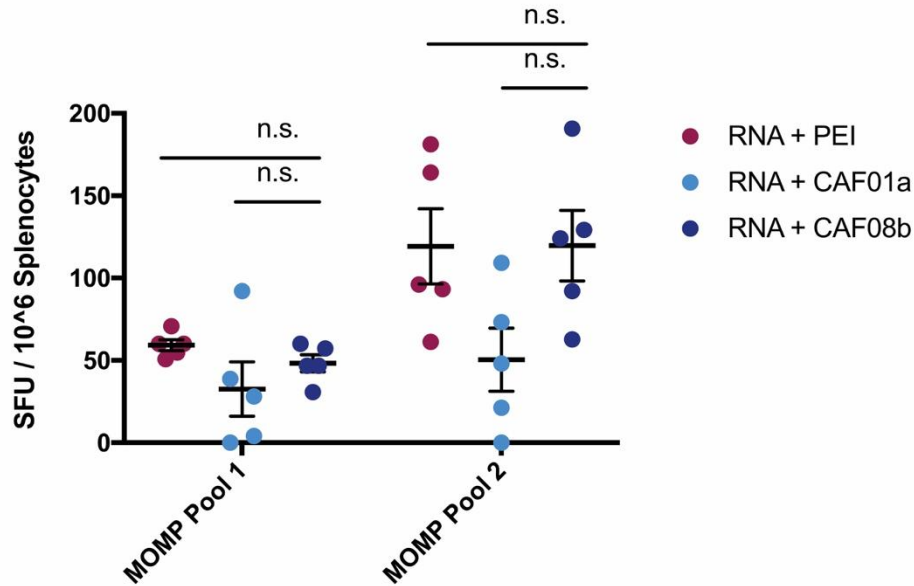


Fig. 7. T-cell ELISpot responses to MOMP peptide pools three weeks after final boost of regimen. Values represent mean number of spots with background subtracted $\pm$ standard deviation for $n=5$. n.s. = not significant.

4. Discussion

These studies demonstrate that CAFs are efficient delivery vehicles for saRNA, both in the context of *in vitro* transfections and *in vivo* immunogenicity. First, we observed the transfection efficiency and cytotoxicity of liposomal and emulsion CAFs with varying surfactant saturation and

immunomodulators. We optimized the formulation of CAFs for maximal *in vivo* protein expression induced by saRNA and show that incorporating immunostimulatory molecules into the CAFs do not inhibit the delivery capacity of these formulations. Finally, we showed that saRNA encoding the MOMP antigen induced MOMP-specific IgG responses as well as IFN- γ ⁺ T-cells, but immunogenicity was unaffected by the incorporation of TLR-agonist 3M-052 into the formulation.

When complexed to saRNA, each of the liposomal CAFs tested were shown to enhance delivery over RNA alone *in vitro*. CAF01a and CAF01b, which are both liposomal formulations with either solid or fluid phase surfactant, exhibited variable dose dependency of transfection efficiency- CAF01a did not exhibit dose dependency whereas CAF01b did. Interestingly, when water soluble R848 was admixed to CAF01a (CAF08a), there was similarly no dose dependency, whereas incorporation of lipid anchored TLR7/8 agonist 3M-052 into the CAF01a to form CAF08b did result in an inverse dose dependent increase in transfection. This implies that both the surfactant saturation state and incorporation of immunomodulators into the formulation can affect the manner in which saRNA adsorbs to the liposomal particle surface. However, the solid phase emulsions (CAF19a and CAF26a) did not exhibit dose dependency whereas the fluid phase emulsions (CAF 19b and CAF 26b) had absolutely no signal. We postulate that this is due to the instability of the fluid phase emulsions in the liquid phase transfections, which are performed in L-glutamine-supplemented DMEM. Interestingly, the transfection efficiency of all the solid phase formulations, including liposomes and emulsions (CAF01a, CAF08a, CAF08b, CAF19a, CAF26a), was approximately equivalent (~10,000 RLU). While we observed some *in vitro* toxicity, this has been previously observed with cationic transfection delivery vehicles [37-39]. The observed toxicity may be synonymous with the adjuvant effects that have been previously observed for the CAFs, which are known to stimulate an immune response through both TLR-dependent (TLR2, 3, 4, 7) and TLR-independent pathways (IL-1, IL-18, MyD88) [40]. However, activation of the different receptors is dependent on the composition of the formulation. Alternatively, the incorporated adjuvants may have better access to the cells *in vitro* than *in vivo* which would lead to more toxicity. Despite

observed cytotoxicity, this does not explain the lack of transfection efficiency of CAF19b and CAF26b, which warrants future investigation.

We observed that lower doses of CAFs resulted in higher luciferase expression *in vivo*, which was equivalent to the protein expression induced by PEI-complexed saRNA (Fig. 4). There was no signal in the 10 and 50 μ L doses, except for one leg of one mouse; we hypothesize that this was due to a small amount of saRNA delivery to the muscle which was amplified and visible due to the self-replicating nature of the RNA. We observed visible aggregation when the saRNA was mixed with the higher doses of CAFs, likely due to the strong interaction between the cationic DDA and the anionic RNA, which cause the particles to fuse. We observed that the solid and fluid phase liposomes (CAF01a, CAF01b) exhibited similar saRNA delivery *in vivo*. Furthermore, incorporating 3M-052 into the formulation (CAF08b) does not interfere with the delivery of saRNA, but may potentially enhance the saRNA expression (Fig. 5). Contrary to the observed *in vitro* results, the fluid-phase emulsion (CAF19b) had higher fLuc expression *in vivo* than the solid phase emulsion (CAF19a), indicating that the fluid phase emulsion is more stable after IM injection. Ultimately, there was not a significant difference between the liposomal and emulsion delivery *in vivo*. Although saRNA has previously been formulated in both liposomes and emulsions [7, 41], to our knowledge this is the first study to systematically compare the two formulation approaches.

We observed that CAF01a and CAF08b had similar fLuc saRNA delivery *in vivo*, but then sought to investigate whether they had similar immunogenicity profiles. Do CAFs act as an adjuvant for saRNA in the same way as for protein antigens? We observed that MOMP-encoding saRNA complexed with CAF01a, CAF08b and even PEI induced similar MOMP-specific IgG responses after 3, 6 and 9 weeks (Fig. 6). Similarly, there were no differences in the IFN- γ ⁺ T-cell responses between the different formulations when stimulated with MOMP peptide pool 1. Re-stimulation with peptide pool 2 resulted in a ~2 fold increase in IFN- γ ⁺ T-cells in response to MOMP with the PEI and CAF08b formulations compared to CAF01a, although these differences were not significant (Fig. 6). Thus, we conclude that the adjuvant effects of CAF08b are similar to those of both PEI [42] and CAF01a, but the immune stimulus

by these formulation is dominated by the inherent RNA innate sensing. 3M-052 is a TLR7/8 agonist that is known to induce both humoral and cellular responses, especially Th1-skewed responses [43]. However, because RNA is known to also stimulate TLR7/8, we hypothesize that any adjuvant effects from these formulations is largely dominated by innate activation triggered by the saRNA itself.

The majority of the antibody response to MOMP was induced by the first immunization, and we observe little benefit from the boosting immunizations. This likely reflects the sustained protein expression associated with saRNA, where immune response would not have been sufficiently contracted after the first immunization to benefit from boosting immunizations [7]. Despite the sustained protein expression associated with saRNA, we did not observe enhanced antibody responses with the repeated boosting immunization schedule in these experiments. We anticipate that wider spacing between immunizations would lead to enhanced MOMP-specific IgG responses. While we observed only minor differences in IFN- γ ⁺ T-cell responses, we were not able to test the temporal profile of these responses over time and can only glean the state of cellular responses at the end of the study. We hypothesize that the CAFs may exhibit more of an adjuvant effect when combined with RNA that is designed to induce less innate sensing, such as non-replicating mRNA with modified nucleotides. However, there is no rationale for using modified nucleotide saRNA because while this may reduce innate sensing of the initial copies of RNA, the subsequent copies would not contain modified nucleotides and thus might actively trigger TLRs. Furthermore, saRNA constructs are relatively long (~9,500 nt), and long RNA transcripts with modified nucleotides have been previously shown to stall transcription [44]. It may be beneficial to further optimize the formulations, as we opted to use the lowest dose of CAFs (2 μ L) because we observed the most efficient saRNA delivery, whereas previous studies with CAFs have utilized a higher dose (50 μ L) [20, 45], and thus devising a formulation method wherein the higher CAF content does not induce particle aggregation may allow for similar saRNA expression of antigen but enhanced formulation-enabled adjuvant effects.

5. Conclusions

Here, we show that CAFs are an efficient delivery vehicle for saRNA both for *in vitro* transfections and *in vivo* immunogenicity. We show that fluid phase liposomes had the highest transfection efficiency *in vitro*, whereas solid phase liposomes and emulsions all had equivalent luciferase expression. Incorporating immunomodulators into the CAFs did not affect saRNA delivery *in vitro* or *in vivo*. We observed that lowest tested dose of CAFs had the highest luciferase expression *in vivo*, and that the fluid phase, solid phase and 3M-052 CAF liposomes had equivalent fLuc saRNA expression *in vivo*, whereas the solid phase emulsions were less efficient as a delivery vehicle. Finally, CAF01a and CAF08b induced both humoral and cellular immune responses that were equivalent to PEI when paired with MOMP-encoding saRNA.

Acknowledgements

We gratefully acknowledge Dormeur Investment Services Ltd for providing funds to purchase equipment used in these studies. We furthermore acknowledge 3M Drug Delivery Systems for providing 3M-052 as a gift.

Funding

AKB is supported by a Whitaker Post-Doctoral Fellowship. Funding was provided by the EPSRC Future Vaccines Manufacturing Research Hub at Imperial College and the FP7 European Commission ADITEC project (Grant No. 280873).

References

- [1] L. Newman, J. Rowley, S. Vander Hoorn, N.S. Wijesooriya, M. Unemo, N. Low, G. Stevens, S. Gottlieb, J. Kiarie, M. Temmerman, Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting, PLoS ONE, 10 (2015) e0143304.
- [2] R.G. Rank, J.A. Whittum-Hudson, Protective immunity to chlamydial genital infection: Evidence from animal studies, Journal of Infectious Diseases, 201 (2010) S168-S177.

- [3] G. Stary, A. Olive, A.F. Radovic-Moreno, D. Gondek, D. Alvarez, P.A. Basto, M. Perro, V.D. Vrbanc, A.M. Tager, J. Shi, J.A. Yethon, O.C. Farokhzad, R. Langer, M.N. Starnbach, U.H. von Andrian, A mucosal vaccine against *Chlamydia trachomatis* generates two waves of protective memory T cells, *Science*, 348 (2015).
- [4] A.W. Olsen, E.K. Lorenzen, I. Rosenkrands, F. Follmann, P. Andersen, Protective Effect of Vaccine Promoted Neutralizing Antibodies against the Intracellular Pathogen *Chlamydia trachomatis*, *Frontiers in Immunology*, 8 (2017) 1652.
- [5] T.B. Poston, S.L. Gottlieb, T. Darville, Status of vaccine research and development of vaccines for *Chlamydia trachomatis* infection, *Vaccine*, (2017).
- [6] K. Bahl, J.J. Senn, O. Yuzhakov, A. Bulychev, L.A. Brito, K.J. Hassett, M.E. Laska, M. Smith, Ö. Almarsson, J. Thompson, A. Ribeiro, M. Watson, T. Zaks, G. Ciaramella, Preclinical and Clinical Demonstration of Immunogenicity by mRNA Vaccines against H10N8 and H7N9 Influenza Viruses, *Mol. Ther.*, 25 (2017) 1316-1327.
- [7] A.J. Geall, A. Verma, G.R. Otten, C.A. Shaw, A. Hekele, K. Banerjee, Y. Cu, C.W. Beard, L.A. Brito, T. Krucker, D.T. O'Hagan, M. Singh, P.W. Mason, N.M. Valiante, P.R. Dormitzer, S.W. Barnett, R. Rappuoli, J.B. Ulmer, C.W. Mandl, Nonviral delivery of self-amplifying RNA vaccines, *Proceedings of the National Academy of Sciences*, 109 (2012) 14604.
- [8] M.F. Bachmann, R.R. Beerli, P. Agnellini, P. Wolint, K. Schwarz, A. Oxenius, Long-lived memory CD8⁺ T cells are programmed by prolonged antigen exposure and low levels of cellular activation, *European Journal of Immunology*, 36 (2006) 842-854.
- [9] G.K. Pedersen, P. Andersen, D. Christensen, Immunocorrelates of CAF family adjuvants, *Seminars in Immunology*, 39 (2018) 4-13.
- [10] S.T. Schmidt, G.K. Pedersen, M.A. Neustrup, K.S. Korsholm, T. Rades, P. Andersen, C. Foged, D. Christensen, Induction of Cytotoxic T-Lymphocyte Responses Upon Subcutaneous Administration of a Subunit Vaccine Adjuvanted With an Emulsion Containing the Toll-Like Receptor 3 Ligand Poly(I:C), *Frontiers in Immunology*, 9 (2018).
- [11] K.S. Korsholm, J. Hansen, K. Karlsen, J. Filskov, M. Mikkelsen, T. Lindenstrøm, S.T. Schmidt, P. Andersen, D. Christensen, Induction of CD8⁺ T-cell responses against subunit antigens by the novel cationic liposomal CAF09 adjuvant, *Vaccine*, 32 (2014) 3927-3935.
- [12] A. Wilkinson, E. Lattmann, C.B. Roces, G.K. Pedersen, D. Christensen, Y. Perrie, Lipid conjugation of TLR7 agonist Resiquimod ensures co-delivery with the liposomal Cationic Adjuvant Formulation 01 (CAF01) but does not enhance immunopotentiality compared to non-conjugated Resiquimod+CAF01, *Journal of Controlled Release*, 291 (2018) 1-10.
- [13] S.T. Schmidt, M.A. Neustrup, S. Harloff-Helleberg, K.S. Korsholm, T. Rades, P. Andersen, D. Christensen, C. Foged, Systematic Investigation of the Role of Surfactant Composition and Choice of oil: Design of a Nanoemulsion-Based Adjuvant Inducing Concomitant Humoral and CD4⁺ T-Cell Responses, *Pharmaceutical Research*, 34 (2017) 1716-1727.
- [14] S.T. Schmidt, S. Khadke, K.S. Korsholm, Y. Perrie, T. Rades, P. Andersen, C. Foged, D. Christensen, The administration route is decisive for the ability of the vaccine adjuvant CAF09 to induce antigen-specific CD8⁺ T-cell responses: The immunological consequences of the biodistribution profile, *Journal of Controlled Release*, 239 (2016) 107-117.
- [15] K. Karlsen, K.S. Korsholm, R. Mortensen, S.M. Ghiasi, P. Andersen, C. Foged, D. Christensen, A stable nanoparticulate DDA/MMG formulation acts synergistically with CpG ODN 1826 to enhance the CD4⁺ T-cell response, *Nanomedicine*, 9 (2014) 2625-2638.
- [16] D. Christensen, M. Henriksen-Lacey, A.T. Kamath, T. Lindenstrøm, K.S. Korsholm, J.P. Christensen, A.-F. Rochat, P.-H. Lambert, P. Andersen, C.-A. Siegrist, Y. Perrie, E.M. Agger, A cationic vaccine adjuvant based on a saturated quaternary ammonium lipid have different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity compared to its unsaturated analog, *Journal of Controlled Release*, 160 (2012) 468-476.
- [17] J.T. van Dissel, S.A. Joosten, S.T. Hoff, D. Soonawala, C. Prins, D.A. Hokey, D.M. O'Dee, A. Graves, B. Thierry-Carstensen, L.V. Andreassen, M. Ruhwald, A.W. de Visser, E.M. Agger, T.H.M.

- Ottenhoff, I. Kromann, P. Andersen, A novel liposomal adjuvant system, CAF01, promotes long-lived Mycobacterium tuberculosis-specific T-cell responses in human, *Vaccine*, 32 (2014) 7098-7107.
- [18] A. Fomsgaard, I. Karlsson, G. Gram, C. Schou, S. Tang, P. Bang, I. Kromann, P. Andersen, L.V. Andreasen, Development and preclinical safety evaluation of a new therapeutic HIV-1 vaccine based on 18 T-cell minimal epitope peptides applying a novel cationic adjuvant CAF01, *Vaccine*, 29 (2011) 7067-7074.
- [19] S. Bøje, A.W. Olsen, K. Erneholm, J.S. Agerholm, G. Jungersen, P. Andersen, F. Follmann, A multi-subunit Chlamydia vaccine inducing neutralizing antibodies and strong IFN- γ + CMI responses protects against a genital infection in minipigs, *Immunology and Cell Biology*, 94 (2015) 185-195.
- [20] A.W. Olsen, F. Follmann, K. Erneholm, I. Rosenkrands, P. Andersen, Protection Against Chlamydia trachomatis Infection and Upper Genital Tract Pathological Changes by Vaccine-Promoted Neutralizing Antibodies Directed to the VD4 of the Major Outer Membrane Protein, *The Journal of Infectious Diseases*, 212 (2015) 978-989.
- [21] K.J. Kauffman, J.R. Dorkin, J.H. Yang, M.W. Heartlein, F. DeRosa, F.F. Mir, O.S. Fenton, D.G. Anderson, Optimization of Lipid Nanoparticle Formulations for mRNA Delivery in Vivo with Fractional Factorial and Definitive Screening Designs, *Nano Letters*, 15 (2015) 7300-7306.
- [22] N. Pardi, S. Tuyishime, H. Muramatsu, K. Kariko, B.L. Mui, Y.K. Tam, T.D. Madden, M.J. Hope, D. Weissman, Expression kinetics of nucleoside-modified mRNA delivered in lipid nanoparticles to mice by various routes, *Journal of Controlled Release*, (2015).
- [23] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, *Nature Immunology*, 11 (2010) 373.
- [24] S. Jensen, A.R. Thomsen, Sensing of RNA Viruses: a Review of Innate Immune Receptors Involved in Recognizing RNA Virus Invasion, *Journal of Virology*, 86 (2012) 2900.
- [25] K. Karikó, M. Buckstein, H. Ni, D. Weissman, Suppression of RNA Recognition by Toll-like Receptors: The Impact of Nucleoside Modification and the Evolutionary Origin of RNA, *Immunity*, 23 (2005) 165-175.
- [26] K. Karikó, H. Muramatsu, F.A. Welsh, J. Ludwig, H. Kato, S. Akira, D. Weissman, Incorporation of Pseudouridine Into mRNA Yields Superior Nonimmunogenic Vector With Increased Translational Capacity and Biological Stability, *Mol. Ther.*, 16 (2008) 1833-1840.
- [27] P. Nordly, F. Rose, D. Christensen, H.M. Nielsen, P. Andersen, E.M. Agger, C. Foged, Immunity by formulation design: Induction of high CD8+ T-cell responses by poly(I:C) incorporated into the CAF01 adjuvant via a double emulsion method, *Journal of Controlled Release*, 150 (2011) 307-317.
- [28] J.M. Leal, M. Mosquini, L.P. Covre, N.P. Staggmiller, R.R. Rodrigues, D. Christensen, H.L.D.M. Guedes, B. Rossi-Bergmann, D.C.D.E.O. Gomes, Intranasal vaccination with killed *Leishmania amazonensis* promastigotes antigen (LaAg) associated with CAF01 adjuvant induces partial protection in BALB/c mice challenged with *Leishmania (infantum) chagasi*, *Parasitology*, 142 (2015) 1640-1646.
- [29] T. Lindenstrøm, E.M. Agger, K.S. Korsholm, P.A. Darrah, C. Aagaard, R.A. Seder, I. Rosenkrands, P. Andersen, Tuberculosis Subunit Vaccination Provides Long-Term Protective Immunity Characterized by Multifunctional CD4 Memory T Cells, *The Journal of Immunology*, 182 (2009) 8047.
- [30] D. Christensen, C. Foged, I. Rosenkrands, C.V. Lundberg, P. Andersen, E.M. Agger, H.M. Nielsen, CAF01 liposomes as a mucosal vaccine adjuvant: In vitro and in vivo investigations, *International Journal of Pharmaceutics*, 390 (2010) 19-24.
- [31] J. Davidsen, I. Rosenkrands, D. Christensen, A. Vangala, D. Kirby, Y. Perrie, E.M. Agger, P. Andersen, Characterization of cationic liposomes based on dimethyldioctadecylammonium and synthetic cord factor from *M. tuberculosis* (trehalose 6,6'-dibehenate)—A novel adjuvant inducing both strong CMI and antibody responses, *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1718 (2005) 22-31.
- [32] A. Badamchi-Zadeh, P.F. McKay, M.J. Holland, W. Paes, A. Brzozowski, C. Lacey, F. Follmann, J.S. Tregoning, R.J. Shattock, Intramuscular Immunisation with Chlamydial Proteins Induces Chlamydia trachomatis Specific Ocular Antibodies, *PLoS ONE*, 10 (2015) e0141209.

- [33] P.F. McKay, A.V. Cope, J.F.S. Mann, S. Joseph, M. Esteban, R. Tatoud, D. Carter, S.G. Reed, J. Weber, R.J. Shattock, Glucopyranosyl Lipid A Adjuvant Significantly Enhances HIV Specific T and B Cell Responses Elicited by a DNA-MVA-Protein Vaccine Regimen, *PLoS ONE*, 9 (2014) e84707.
- [34] A.B. Vogel, L. Lambert, E. Kinnear, D. Busse, S. Erbar, K.C. Reuter, L. Wicke, M. Perkovic, T. Beissert, H. Haas, S.T. Reece, U. Sahin, J.S. Tregoning, Self-Amplifying RNA Vaccines Give Equivalent Protection against Influenza to mRNA Vaccines but at Much Lower Doses, *Mol. Ther.*, 26 (2018) 446-455.
- [35] T. Démoulins, T. Ebsen, K. Schulze, P.C. Englezou, M. Pelliccia, C.A. Guzmán, N. Ruggli, K.C. McCullough, Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure, *Journal of Controlled Release*, 266 (2017) 256-271.
- [36] S. Pal, D.F. Tifrea, F. Follmann, P. Andersen, L.M. de la Maza, The cationic liposomal adjuvants CAF01 and CAF09 formulated with the major outer membrane protein elicit robust protection in mice against a *Chlamydia muridarum* respiratory challenge, *Vaccine*, 35 (2017) 1705-1711.
- [37] M. Thomas, A.M. Klibanov, Enhancing polyethylenimine's delivery of plasmid DNA into mammalian cells, *Proceedings of the National Academy of Sciences*, 99 (2002) 14640.
- [38] F. Nouri, H. Sadeghpour, R. Heidari, A. Dehshahri, Preparation, characterization, and transfection efficiency of low molecular weight polyethylenimine-based nanoparticles for delivery of the plasmid encoding CD200 gene, *International Journal of Nanomedicine*, 12 (2017) 5557-5569.
- [39] Z.Y. He, X. Zheng, X.H. Wu, X.R. Song, G. He, W.F. Wu, S. Yu, S.J. Mao, Y.Q. Wei, Development of glycyrhethinic acid-modified stealth cationic liposomes for gene delivery, *International Journal of Pharmaceutics*, 397 (2010) 147-154.
- [40] E.M. Agger, I. Rosenkrands, J. Hansen, K. Brahimi, B.S. Vandahl, C. Aagaard, K. Werninghaus, C. Kirschning, R. Lang, D. Christensen, M. Theisen, F. Follmann, P. Andersen, Cationic Liposomes Formulated with Synthetic Mycobacterial Cordfactor (CAF01): A Versatile Adjuvant for Vaccines with Different Immunological Requirements, *PLOS ONE*, 3 (2008) e3116.
- [41] L.A. Brito, M. Chan, C.A. Shaw, A. Hekele, T. Carsillo, M. Schaefer, J. Archer, A. Seubert, G.R. Otten, C.W. Beard, A.K. Dey, A. Lilja, N.M. Valiante, P.W. Mason, C.W. Mandl, S.W. Barnett, P.R. Dormitzer, J.B. Ulmer, M. Singh, D.T. O'Hagan, A.J. Geall, A Cationic Nanoemulsion for the Delivery of Next-generation RNA Vaccines, *Mol. Ther.*, 22 (2014) 2118-2129.
- [42] N.C. Sheppard, S.A. Brinckmann, K.H. Gartlan, M. Puthia, C. Svanborg, G. Krashias, S.C. Eisenbarth, R.A. Flavell, Q.J. Sattentau, F. Wegmann, Polyethyleneimine is a potent systemic adjuvant for glycoprotein antigens, *International Immunology*, 26 (2014) 531-538.
- [43] J.P. Vasilakos, M.A. Tomai, The use of Toll-like receptor 7/8 agonists as vaccine adjuvants, *Expert Review of Vaccines*, 12 (2013) 809-819.
- [44] J. Chelliserrykattil, A.D. Ellington, Evolution of a T7 RNA polymerase variant that transcribes 2'-O-methyl RNA, *Nature Biotechnology*, 22 (2004) 1155.
- [45] A. Huber, R.S. Kallerup, K.S. Korsholm, H. Franzyk, B. Lepenies, D. Christensen, C. Foged, R. Lang, Trehalose diester glycolipids are superior to the monoesters in binding to Mincle, activation of macrophages in vitro and adjuvant activity in vivo, *Innate Immunity*, 22 (2016) 405-418.