



The role of helper lipids in optimising nanoparticle formulations of self-amplifying RNA

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ABSTRACT

Lipid nanoparticle (LNP) formulation plays a vital role in RNA vaccine delivery. However, further optimisation of self-amplifying RNA (saRNA) vaccine formulation could help enhance seroconversion rates in humans and improve storage stability. Altering either the ionisable or helper lipid can alter the characteristics and performance of formulated saRNA through the interplay of the phospholipid's packing parameter and the geometrical shape within the LNP membrane. In this study, we compared the impact of three helper lipids (DSPC, DOPC, or DOPE) used with two different ionisable lipids (MC3 and C12–200) on stability, transfection efficiency and the inflammation and immunogenicity of saRNA. While helper lipid identity altered saRNA expression across four cell lines *in vitro*, this was not predictive of an *ex vivo* or *in vivo* response. The helper lipid used influenced LNP storage where DSPC provided the best stability profile over four weeks at 2–8 °C. Importantly, helper lipid impact on LNP storage stability was the best predictor of expression in human skin explants, where C12–200 in combination with DSPC provided the most durable expression. C12–200 LNPs also improved protein expression (firefly luciferase) and humoral responses to a SARS-CoV-2 spike saRNA vaccine compared to MC3 LNPs, where the effect of helper lipids was less apparent. Nevertheless, the performance of C12–200 in combination with DSPC appears optimal for saRNA when balancing preferred storage stability requirements against *in vivo* and *ex vivo* potency. These data suggest that helper lipid influences the stability and functionality of ionisable lipid nanoparticle-formulated saRNA.

1. Introduction

Lipid nanoparticle (LNP) formulations of messenger RNA (mRNA) enabled a rapid and efficient vaccination program in developed countries during the COVID-19 pandemic. Yet, the relatively limited supply and stringent storage requirements of these vaccines limited globally equitable distribution [1]. Self-amplifying RNA (saRNA) vaccines, which can achieve sustained antigen expression through intracellular RNA replication, offer an opportunity to increase global access to RNA vaccines by lowering dosage requirements and associated costs [2,3]. While saRNA LNPs have demonstrated equal protection at an approximately 60-fold lower dose than non-replicating mRNA in mice [3], clinical results suggest that substantial engineering may be required to successfully translate this technology into humans [4]. Tuning the chemical composition and formulation ratio of LNP lipids is one promising approach to improve the delivery efficiency and storage stability of

saRNA [5].

Lipid nanoparticles facilitate the intracellular uptake and endosomal escape of RNA [6] but also augment the innate immune response to translated antigens through as-yet poorly defined mechanisms [7–9]. Typically, LNP formulations are composed of an ionisable lipid, a helper lipid, cholesterol, and a PEGylated lipid. Most studies have focused on the role of ionisable lipids in RNA packaging and endosomal escape [10–13]. Still, helper phospholipids are also known to contribute to both the stability and delivery efficiency of RNA-LNP formulations [14]. Helper lipids such as DSPC, DOPC, and DOPE have varying alkyl chain saturations, chain lengths, and polar head group substitutions that result in different conformational structures in the LNP membrane that can be quantified as a molecular packing parameter, P [15–17]. For example, DSPC and DOPC impart cylindrical geometric structure, while DOPE imparts cone-shaped geometry [14,18,19].

Membrane structure must be finely tuned to maintain LNP storage

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stability while enabling efficient delivery [20–22]. Broadly speaking, cylindrical lipid formulations based on DSPC and DOPC are reportedly more stable and less potent [20,21], while conical DOPE formulations with fusogenic properties transfect more potently at the expense of storage stability [22–24]. It is well known that the lipid chemical identities and formulation ratios impact mRNA LNP storage stability and transfection biodistribution [25,26], but it is unclear if these lessons translate to saRNA, which is $\sim 3\times$ larger than mRNA and contains substantially more secondary structure. Furthermore, it is unknown how ionisable/helper lipid combinations affect LNP saRNA expression and immunogenicity, although direct (membrane destabilisation) and indirect (altered biodistribution) mechanisms have been proposed for LNP mRNA [27].

In this study, we investigated the combinatorial impact of helper lipids (DSPC, DOPC, or DOPE) and ionisable lipids (MC3 or C12–200) (Fig. 1) on the expression, immunogenicity, and storage stability of saRNA LNP vaccines. MC3 and C12–200 have been selected as ionisable lipids since they are potent delivery vehicles for RNA molecules. MC3 is present in the Onpattro formulation [28], which has been granted FDA approval, and C12–200 has been previously optimised for *in vivo* delivery [24]. Previous clinical studies of saRNA vaccines using one of the typical ionisable lipids present in mRNA vaccines against COVID-19 failed to elicit 100% seroconversion [29] or did not induce the same levels of neutralising antibody [30]. Thus, it is of interest to investigate novel lipid combinations in the context of saRNA.

Overall, LNPs with DOPC and DOPE were effective *in vitro*, and DSPC formulations proved optimal for delivery *ex vivo* and *in vivo*. Additionally, our *ex vivo* human skin explant model demonstrates potential as a screening tool since it exhibited similar RNA expression trends to those observed *in vivo*. DSPC formulations exhibited the best stability profile that correlated to their sustained expression *in vivo*, indicating that

storage stability may be a strong predictor of LNP robustness *in vivo*. Collectively, these results demonstrate the importance of optimising saRNA LNP formulations while holistically considering their different aspects: expression, stability, and immunogenicity. An optimal RNA LNP formulation should combine all these aspects in a balanced manner.

2. Material and methods

2.1. *in vitro* transcription of self-amplifying mRNA

Self-amplifying mRNA (saRNA) constructs encoded either firefly luciferase (fLuc) or the novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spike glycoprotein (nCoV spike). Initially, the plasmid DNA template was linearised using *MluI* (New England BioLabs, UK) for 3 h at 37 °C, and linearisation was confirmed by agarose gel electrophoresis. Next, RNA transcripts were synthesised according to the manufacturer's instructions using the mMessage mMachine kit (Invitrogen, Thermo fisher scientific, UK) for *in vitro* purposes and using the MEGAScript T7 kit (Invitrogen, Thermo fisher scientific, UK) for *in vivo* purposes. Transcripts were purified by lithium chloride (LiCl) precipitation. Samples were kept in a LiCl solution overnight at -20°C and then centrifuged at 14000 rpm for 20 min at 4°C . Pellets were washed with 70% ethanol, centrifuged again for 5 min, the ethanol was then removed, and pellets were dried for 5 min at RT. The final product was resuspended in ultrapure H_2O . For RNA transcripts synthesised using the MEGAScript T7 kit, RNA capping was then performed using a Script-Cap™ cap 1 capping system (CELLSCRIPT™, US) according to the manufacturer's instructions. Transcripts produced with the mMessage mMachine kit were synthesised in a co-capping reaction and did not require a capping step. Post-capped samples were purified via another round of LiCl precipitation. The RNA quantification was done using a

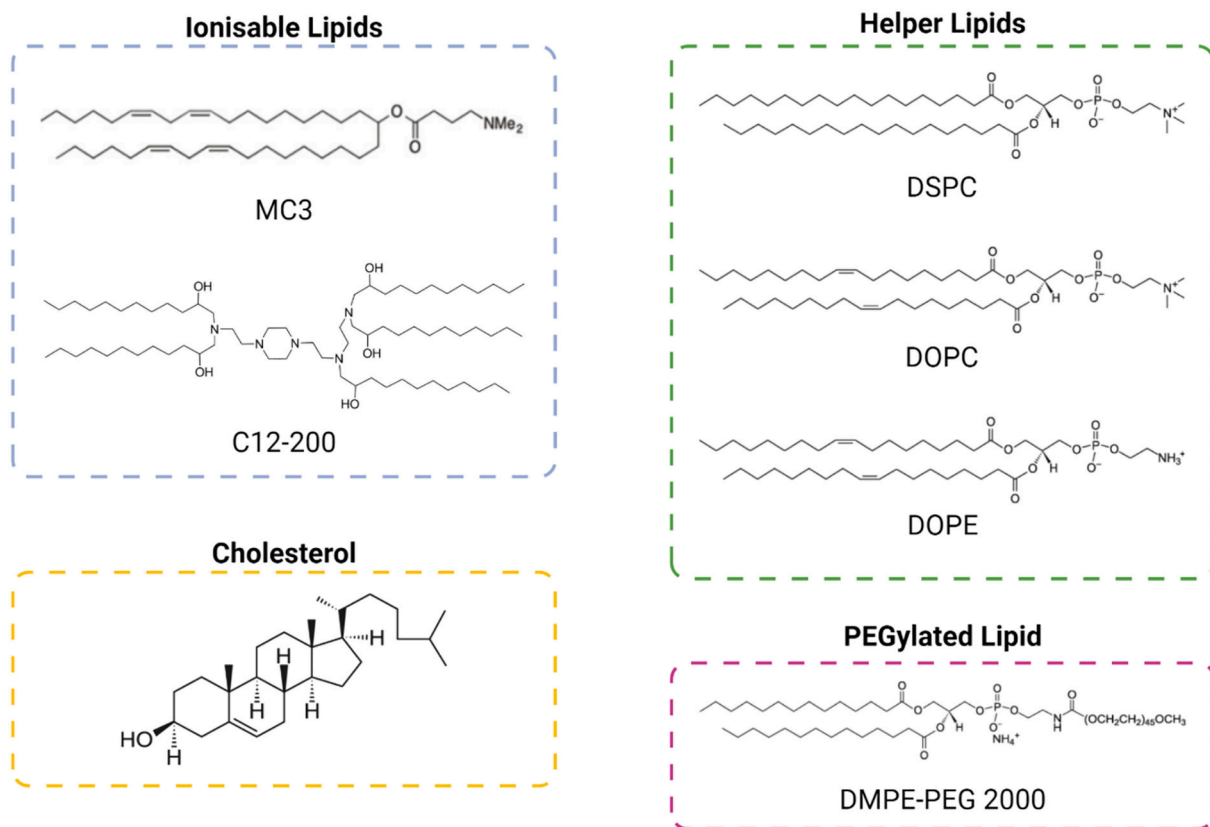


Fig. 1. Library of lipids and their chemical structures. The LNP formulations used in this study are composed of one ionisable lipid (MC3 or C12–200), one helper lipid (DSPC, DOPC, or DOPE), cholesterol, and a PEGylated lipid. LNPs were formulated with different saRNA constructs using a microfluidics device. Created with BioRender.com.

NanoDrop one (Thermo fisher scientific, UK), and RNA quality was evaluated by RNA gel electrophoresis

2.2. LNP formulation

Stock solutions of the following lipids were prepared in absolute ethanol: DLin-MC3-DMA (Biorbyt, UK), C12–200 (Corden Pharma, Switzerland), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) (Avanti® Polar Lipids, US), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) (Avanti® Polar Lipids, US), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) (Avanti® Polar Lipids, US), cholesterol (plant-derived) (Avanti® Polar Lipids, US) and 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DMPE-PEG2000) (Avanti® Polar Lipids, US).

MC3 LNPs were prepared with MC3, a helper lipid (either DSPC, DOPC, or DOPE), cholesterol, and DMPE-PEG2000 at an RNA/lipid (w/w) ratio of 1:50 and a molar ratio of 50:10:38.5:1.5. C12–200 LNPs were prepared with C12–200, a helper lipid (either DSPC, DOPC, or DOPE), cholesterol, and DMPE-PEG2000 at an RNA/lipid (w/w) ratio of 1:55 and a molar ratio of 35:16:46.5:2.5.

saRNA solutions were prepared in 50 mM sodium acetate (Sigma®, US) and 100 mM sodium chloride buffer (Sigma®, US) at pH = 5.5. LNPs were formulated on an Ignite microfluidics device (Precision NanoSystems Incorporated, Vancouver, BC, Canada) using a flow rate ratio of RNA:lipid of 3:1 and a total flow rate of 8 mL/min. The formulated material was then concentrated in Dulbecco's phosphate-buffered saline (DPBS) 1× (Gibco, UK) in approximately 5 times the volume of the total formulation. This diluted sample was spun down using VivaSpin 6 filter tubes with a molecular weight cut-off (MWCO) of 10 kDa (Sartorius, Germany) at 4000 rpm and 18 °C until the desired concentration was achieved.

2.3. RNA encapsulation efficiency by RiboGreen Assay

The quantification of saRNA encapsulated inside lipid nanoparticles was analysed using a Quant-iT RiboGreen assay (ThermoFisher, UK) according to Precision NanoSystems' protocol. Briefly, samples were diluted to a concentration of 6 µg/mL and added 1:1 in either 1× TE Buffer or Triton X-100 (2% in 1× TE buffer), using a black 96-well Costar® plate (ThermoFisher, UK). Samples were incubated at 37 °C for 10 min to allow for the lysis of LNPs and then kept for 5 min at room temperature. RiboGreen reagent was prepared at a 1:100 dilution in 1× TE buffer, and 100 µL was added per well. An RNA standard curve was also prepared as recommended by the protocol. Fluorescence intensity was analysed on a microplate reader (BMG LABTECH, UK) at an excitation of 480 nm and emission of 520 nm. The total saRNA and saRNA concentration on the outside (non-encapsulated) was calculated based on the standard curve. The concentration of encapsulated saRNA was calculated by subtraction (total saRNA minus non-encapsulated saRNA). The percentage of encapsulation efficiency (EE) was calculated as:

$$EE (\%) = 100 \times (\text{RNA encapsulated} / \text{RNA added to assay})$$

2.4. Physicochemical characterisation

Physicochemical analysis was conducted for the evaluation of Z-average diameter, polydispersity index, and zeta potential. Samples were equilibrated at RT and prepared a 1:100 dilution in DPBS 1× (Gibco, UK) at a total volume of 850 µL. Characterisation was performed using a Zetasizer Nano ZS (Malvern instruments, UK) with DTS1070 cuvettes and the following parameters: Material refractive index of 1.529, absorbance of 0.010, dispersant viscosity of 0.8820 cP, refractive index of 1.330, and dielectric constant of 79, as reported previously [31]

2.5. Cell line and culture conditions

The cell lines used in this study were HEK 293 T/17 (ATCC, US), HeLa (ATCC, US), THP-1 (ATCC, US), and immortalised human skeletal muscle cells (hSkMC) (PromoCell, UK). All cells were cultured at 37 °C under 5% CO₂ at conditions similar to those previously described [31].

HEK 293 T/17 and HeLa cells were cultured in DMEM (Gibco, Thermo Fisher, UK) supplemented with 10% foetal bovine serum (FBS) (v/v), 1% L-glutamine (v/v), and 1% penicillin/streptomycin (v/v) (Thermo Fisher, UK). Cells were washed with DPBS 1× (Gibco, UK) and detached with trypsin (TrypLE Express 1×) (Gibco, UK).

THP-1 cells are grown in suspension and cultured in RPMI-1640 Medium (Sigma, UK) supplemented with 10% FBS (v/v), 1% L-glutamine (v/v), and 1% penicillin/streptomycin (v/v) (Thermo Fisher, UK). For passage, cell culture media was collected and centrifuged at 1750 rpm for 5 min. The cell pellet was re-suspended in fresh medium for seeding in new culture flasks (Corning, US).

hSkMCs were cultured with Skeletal Muscle Cell Growth Medium (PromoCell, Germany) supplemented with SupplementMix (PromoCell, Germany). Cells were washed with DPBS 1× (Gibco, UK) and detached with trypsin. Trypsin was neutralised with a 10% FBS in DPBS 1× solution. Cells were centrifuged, and the pellet was re-suspended in a fresh medium for passaging into new culture flasks (Corning, US).

2.6. Cell transfection assay

On the day before the experiment, cells were seeded in 96-well plates at the following concentrations: HEK 293 T/17 and HeLa cells at 50×10^3 cells/well, THP-1 cells at 80×10^3 cells/well, and immortalised hSkMC at 100×10^3 cells/well. For evaluation of transfection efficiency, a dose of 100 ng of saRNA-LNPs was added to each well and transfection was allowed to occur for 24 h. Next, 50 µL of medium was removed from each well and replaced with 50 µL of Bright-Glo™ luciferin substrate (Promega, UK). After 5 min, the whole 100 µL was transferred to white plates (Falcon®, US), and luminescence intensity was analysed using a FLUOstar Omega plate reader (BMG LABTECH, UK).

2.7. Cellular uptake assay

To analyse saRNA LNP uptake in different cell types, saRNA was labelled with Cy5 by incorporating Cy5 UTP (ApexBio, US) at a ratio of 1:5 with regular UTP during the *in vitro* transcription (IVT) reaction. IVT was performed similarly as described above but using the HiScribe™ T7 High Yield kit (New England Biolabs Inc.®, US), according to the manufacturer's instructions. The labelled saRNA was encapsulated into LNPs in the same manner as described in the LNP formulation method above.

Different cell lines were first seeded at different concentrations: HEK 293 T/17 and HeLa at 7.5×10^5 cells/well, hSkMC at 10×10^5 cells/well, and THP-1 at 8×10^5 cells/well. A dose of 1 µg of saRNA LNPs was added to cells and allowed to transfect for 4 h. The cell medium was then discarded, and cells were collected by trypsinisation. Upon centrifugation, cells were re-suspended in their appropriate medium, and half of the Live/Dead control volume was heat killed at 70 °C for 5 min. Samples were then stained with Zombie Violet™ dye (BioLegend®, US) at a dilution of 1:500 for 15 min at room temperature, followed by washes in FACS buffer (PBS with 5% FBS), and a final round of fixation in 2% Formaldehyde (Thermo Fisher Scientific, UK) in PBS, for 15 min at 4 °C. Cells were then washed twice in FACS buffer and analysed using a BD LSRFortessa™ (BD Biosciences, US). Results were analysed using the FlowJo™ software version 10.8.1 (BD, US).

2.8. MSD assay (human cells)

Cryopreserved human peripheral blood mononuclear cells (PBMCs) obtained from a healthy donor were thawed and seeded at 2×10^6 cells/

mL in a 24-well plate using RPMI media (Sigma, UK), supplemented with 10% foetal bovine serum (Gibco, UK), 1% penicillin-streptomycin (Thermo Fisher, UK), and 1% L-glutamine (Sigma, UK). THP-1 cells (ATTC, US) were seeded at 1×10^6 cells/mL using RPMI media supplemented as described above. Cells were transfected with LNPs containing 1 μ g of saRNA for 24 h and 48 h. Cell media was then collected and spun down for 5 min at 1750 rpm, and the supernatant was collected for the MSD assay.

The MSD assay was performed according to the manufacturer's instructions. Briefly, prior to running the assay, plates were coated with a multiplex solution (mix of the linker and biotinylated antibody), washed 3 times with PBS-T solution (0.05% Tween-20 in PBS 1 $\times$), and stored at 2–8 °C. On day of running, 25 μ L of diluent was added to each well, followed by another 25 μ L of either sample or standards. Standard solutions were prepared in a 1:4 serial dilution. Plates were incubated for 1 h at RT using a shaker (Jencons Scientific Ltd., UK). Plates were washed 3 times with PBS-T, 50 μ L of antibody solution was added to each well, and plates were incubated shaking for 1 h at RT again. Finally, plates were washed 3 times with PBS-T, 150 μ L of MSD Gold Read buffer was added to each well, and plates were read on the MESO QuickPlex SQ 120 (MSD, US). The panel of cytokines analysed was: ENA-78, GM-CSF, IFN- β , IL-1 α , IL-1 β , IP-10, MCP-1, MDC, MIP-1 α , MIP-1 β , Eotaxin-2, GRO- α , and MCP-3.

2.9. MSD assay (mice sera)

The MSD assay was performed similarly as described above and according to the manufacturer's instructions. The panel of cytokines analysed was: GM-CSF, IL-1 β , IL-6, KC, IP-10, MCP-1, MIP-1 α , MIP-1 β , IFN- α , and IFN- β .

2.10. Human skin explants culture and imaging

Fresh human skin tissue was obtained from elective mastectomy and abdominoplasty surgeries performed at Charing Cross Hospital, Imperial NHS Trust, London, UK. Patients were instructed to sign an informed consent form, and procedures followed the Local Research Ethics Committee protocols. Processing of the skin tissue began by removing the fat layer, and a circular biopsy of 10 mm diameter was punched from the epidermis and dermis (Acuderm Inc., US). Explants were placed in 12-well plates with 2 mL of complete DMEM (Gibco, Thermo Fisher UK), and cell culture media was replaced daily.

To analyse expression in the skin tissue, explants were transfected with 1 μ g of fLuc encapsulated within different LNP formulations. Micro-fine insulin needles (BD Biosciences, UK) containing 1 μ g of saRNA-LNP in 50 μ L were injected intradermally in a controlled manner. On the day of imaging, media was replaced with 950 μ L of DMEM and 50 μ L of IVISbrite D-luciferin Bioluminescent Substrate in RediJect Solution (Revvity, UK) and explants were allowed to incubate for 10 min. Image acquisition was performed using the Ami HT Optical Imaging System (Spectral Instruments Imaging, US) using a suitable exposure, and image analysis was done using the Aura Image Analysis Software version 4.0.8 (Spectral Instruments Imaging, US).

2.11. *in vivo* studies

6–8-week-old female BALB/c specific-pathogen-free (SPF) mice were purchased from Charles River Laboratories (Bayswater, U.K.) and maintained according to institutional and Home Office guidelines. All experiments were performed in the SPF room in the animal facility on a 12-h light/dark cycle at 20–24 °C with 55% $\pm$ 10% humidity at Imperial College London, St Mary's Hospital Campus. All work was approved by the Animal Welfare and Ethical Review board at Imperial College London, and studies were in accordance with the Animal Research: Reporting of *In vivo* Experiments (ARRIVE) guidelines.

2.12. *in vivo* saRNA expression study with firefly luciferase (fLuc)

BALB/c mice ($n = 5$) were injected i.m. with 5 μ g of fLuc saRNA encapsulated into LNPs. For imaging, animals received a 100 μ L dose of D-Luciferin at 30 mg/mL (Perkin Elmer, UK) via the intraperitoneal route and were imaged after 10 min. The acquisition was performed using the Ami-HT (Spectral Imaging Instruments, US), and image analysis was done with the Aura Image Analysis Software version 4.0.8 (Spectral Imaging Instruments, US).

2.13. *in vivo* immunogenicity studies

BALB/c mice ($n = 5$) were injected i.m. with 1 μ g of SARS-CoV-2 spike protein saRNA from the original Wuhan variant [32] encapsulated into LNPs and received a boost dose vaccination after 21 days. Blood was collected 24 h, 2 and 5 weeks after the first dose. Spleens were harvested at the end of the study for an IFN- γ ELISpot assay.

2.14. Antigen-specific IgG ELISA

MaxiSorp high binding ELISA plates (Nunc) were coated with 1 μ g/mL SARS-CoV-2 spike HexaPro in 100 μ L per well. Standard columns were coated with goat anti-mouse Kappa (Southern Biotech) and Lambda light chains (Southern Biotech) at 1:1000 dilution each. Upon overnight incubation at 4 °C, plates were washed with PBS-Tween 20 0.05% (v/v) and blocked with 1% BSA (w/v) in PBS-Tween 20 0.05% (v/v) for 1 h at 37 °C. After another round of washing, serum samples were diluted and added to the wells at appropriate concentrations, while standards (mouse immunoglobulins) were diluted to a top concentration of 1000 ng/mL and then further diluted in a 5-fold dilution series to generate the standard curve. Plates were incubated for 1 h at 37 °C and washed again. The secondary antibody, an anti-mouse IgG-HRP (Southern Biotech), was added at 1:2000 dilution at a volume of 100 μ L per well, and plates were incubated for 1 h at 37 °C. Results were developed using SureBlue TMB (3,3', 5,5'-tetramethylbenzidine) substrate at 50 μ L/well, and the stop solution (Insight Biotechnologies) was added after 5 min using 50 μ L/well. Absorbance was recorded using a spectrophotometer (VersaMax, Molecular Devices) at 450 nm.

2.15. Pseudotype virus neutralisation assay

HEK-ACE2 cells were seeded at 1×10^4 cells/well in a 96-well plate. Serum samples were heated at 56 °C for 30 min and stored at 4 °C overnight. Cell culture medium was prepared by mixing DMEM with 10% FBS, 1% L-glutamine, and 1% penicillin-streptomycin (complete DMEM). Serum samples were diluted 1:10 in medium, and a further 6 serial dilutions at a ratio of 1:5 were prepared in the same medium (in a total of 50 μ L). Pseudovirus was diluted at 100 TCID₅₀ to a 50 μ L/well volume. The mixture of sera and virus was incubated for 1 h at 37 °C. After incubation, the medium was removed from the cells, and the serum-virus mixture was added. Cells were incubated with the mixture for 24 h at 37 °C. As the SARS-CoV-2 pseudovirus was engineered to express luciferase, the infection percentage was quantified via a luciferase assay, in which 100 μ L of Bright-Glo™ luciferin substrate (Promega, UK) was added to each well. After 5 min, the whole 100 μ L was transferred to white plates (Falcon®, US), and luminescence intensity was analysed using a FLUOstar Omega plate reader (BMG LABTECH, UK).

Data analysis was performed by subtracting the average value of uninfected wells to account for background. The percentage of infection was calculated by considering the virus-infected positive control as 100%, and the percentage of neutralisation was calculated by subtraction (100% - % of infection). IC₅₀ neutralisation titres were calculated using a nonlinear regression model (sigmoidal 4-parameter logistic curve) of log(%neutralisation), which were then interpolated from the curve at $y = 50$, and values were transformed by $x = 10^x$. Calculations were performed in Graph Pad Prism V9 (GraphPad Software, San Diego, CA).

2.16. Interferon (IFN)- γ ELISpot assay

On the final day of the *in vivo* study, mice were culled, and spleens were harvested, processed, and filtered using a 70 μ m cell strainer. The harvested splenocytes were added into SepMate™-15 tubes (STEM-CELL™ Technologies, UK) containing Lymphoprep™ medium (STEM-CELL™ Technologies, UK), then centrifuged at 800 rcf for 30 min at 25 °C. Isolated cells were collected and washed twice with complete DMEM. Cells were plated at 25×10^4 cells/mL, and 1 μ g/mL SARS-CoV-2 peptide pools PepMix™ SARS-CoV-2 (spike glycoprotein) (JPT, Germany) were added to stimulate the cells, with 5 μ g/mL of Concavalin A (Sigma, UK) used as a positive control for stimulation in separate wells. The plates were incubated at 37 °C, 5% CO₂ overnight. Then, according to the manufacturer's instructions, IFN- γ T cell responses were evaluated using an ELISpotPLUS Mouse IFN- γ kit (Mabtech, UK). Once plates were dry, results were quantified using the AID ELISpot reader ELR03 and AID ELISpot READER software (AutoimmunDiagnostika GmbH).

2.17. Statistics

All statistical analyses were performed in Graph Pad Prism V9 (GraphPad Software, San Diego, CA). A statistically significant difference was defined as a *P*-value <0.05 by one-way analysis of variance (ANOVA).

3. Results & discussion

3.1. LNPs with different helper lipids have similar physicochemical characteristics

LNPs were prepared with C12–200, a commonly used ionisable lipid in preclinical development, [24,33] or MC3, the ionisable lipid present in the FDA-approved LNP formulation Onpatro® [28]. The molar ratio between compounds and lipid/RNA ratio of the formulations were based on previously reported studies [10,24,34–37], as well as *in vitro* and *in vivo* studies from our group (Table S1, Fig. S1–S4).

Initial studies assessed the physicochemical characteristics of saRNA-

LNPs generated with the different helper lipids (Fig. 1). All LNPs had diameters below 100 nm, most around 70–80 nm (Fig. 2A), and their polydispersity index was below 0.2 (Fig. 2B). LNP zeta potential was slightly negative, close to neutral (Fig. 2C), as expected due to the presence of PEG on the particle surface [38,39]. RNA encapsulation efficiency, as quantified by the Ribogreen assay, was above 80% for all formulations (Fig. 2D). There were no significant differences in bulk physicochemical properties between any of the formulations.

3.2. LNPs with DSPC exhibit favourable physicochemical and functional stability

The six different LNP formulations were stored at 4 °C for 4 weeks to assess initial stability, and their size and transfection efficiency were longitudinally assessed. Helper lipid identity significantly impacted the loss of LNP transfection potency over time. LNPs formulated with DSPC maintained transfection efficiency with no significant change for 4 weeks (Fig. 3E,F). Although LNPs formulated with DOPC started with the highest levels of transfection efficiency, these decreased by 100-fold and 10-fold by 4 weeks for MC3 and C12–200 formulations, respectively (Fig. 3E,F). In agreement with past reports for mRNA [14,40], LNPs with DOPE had the poorest stability profile, with a significant drop in their transfection efficiency of approximately 1000-fold (Fig. 3E,F) over 4 weeks. A long-term storage stability study using Tris buffer with sucrose as cryoprotectant also demonstrated that DSPC formulations, especially C12–200 DSPC, are the most stable at 4 °C (Fig. S5). DSPC is a phospholipid known for assuming a cylindrical shape, which confers high stability to these LNP formulations [18,19,41]. LNPs formulated with DSPC were the most stable for saRNA, and this is the same phospholipid used in the mRNA-LNP vaccine formulations against COVID-19 [42].

Virtually all LNPs showed some increase in size or polydispersity index during storage (Fig. 3A–D). Changes in PDI reflect the formation of a sub-population of aggregates while the main population remained stable (Fig. S6). Overall, changes in physicochemical characteristics did not appear to affect the expression of DSPC-formulated saRNA, having only affected the expression of DOPC and DOPE formulations. This result suggests that using DLS as an indicator of the formulation stability

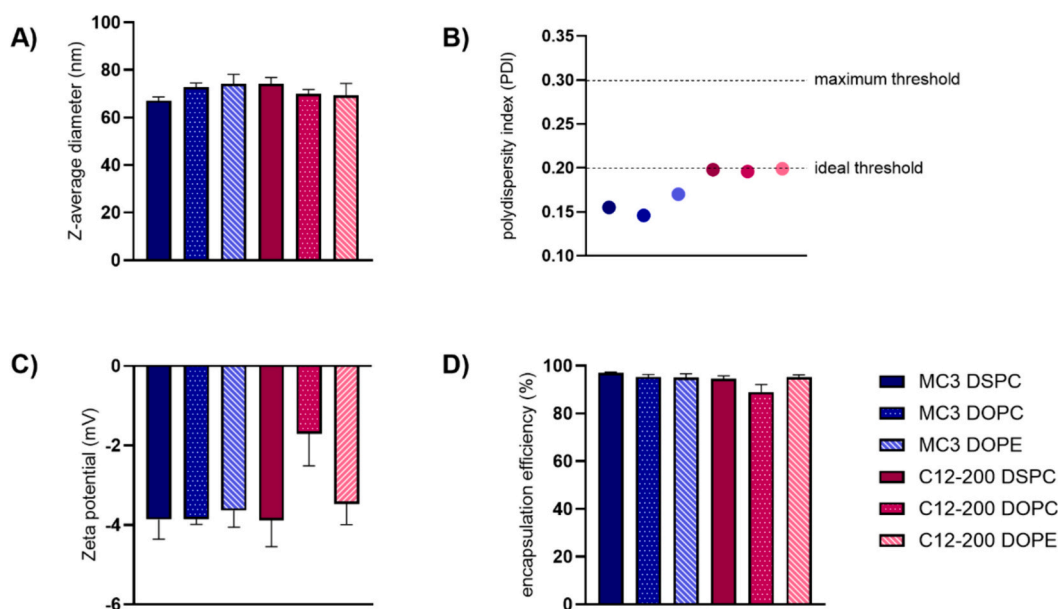


Fig. 2. Physicochemical characterisation and RNA encapsulation efficiency of LNPs with different helper lipids. (A) The hydrodynamic diameter and (B) polydispersity index, or PDI of MC3 and C12–200 LNPs, (C) zeta potential, and (D) RNA encapsulation efficiency measured by Ribogreen assay. Bars represent mean $\pm$ SEM ($n = 3$ independently formulated particle batches). Statistical significance was calculated by One-way ANOVA with Tukey's multi-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

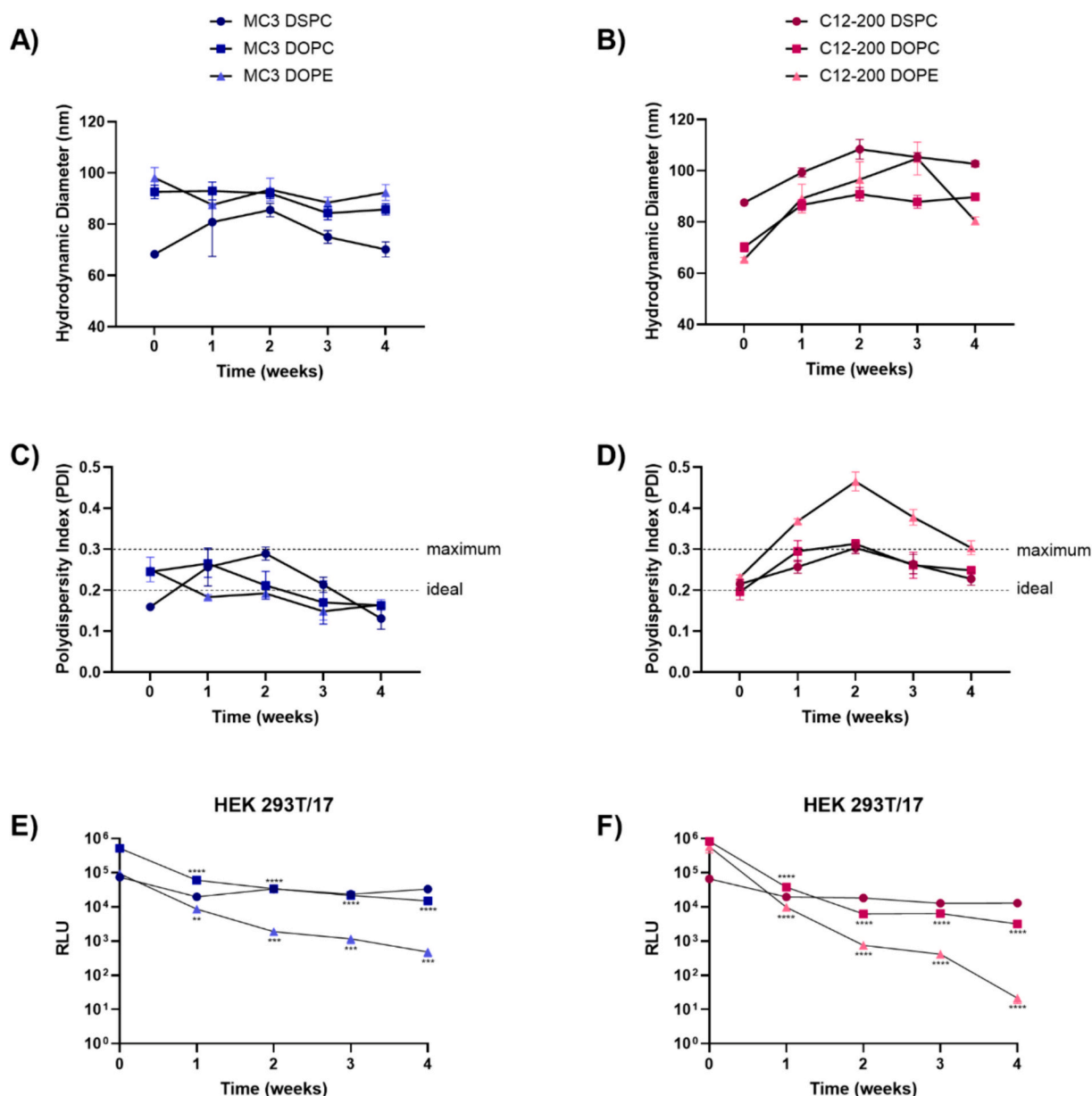


Fig. 3. Storage stability of LNP formulations containing different helper lipids. LNPs were stored at 4 °C for 4 weeks. Physicochemical characteristics and transfection efficiency were assessed weekly. Graphs represent MC3 LNPs' stability in (A,C) physicochemical characteristics and (E) transfection efficiency & C12-200 LNPs' stability in (B,D) physicochemical characteristics and (F) transfection efficiency. PDI: polydispersity index. RLU: relative light units. Bars indicate mean $\pm$ SD, $n = 3$. Statistical significance was calculated by Two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

is insufficient to explain changes in expression, and other methods are needed to address this, such as Raman-based chemical composition analysis. Additionally, differences in lipid purity can influence storage stability, a limitation of this study that must be considered.

3.3. LNP formulations with DSPC are less effective than DOPC or DOPE for *in vitro* transfection

To compare the impact of different lipid combinations on saRNA delivery, we compared luciferase expression levels. LNPs encapsulating saRNA encoding for firefly luciferase (fLuc) were used to transfect four different cell lines: HEK 293 T/17 (human embryonic kidney cells), HeLa (human epithelial cells), hSkMC (human skeletal muscle cells [43]), and THP-1 cells (a monocytic cell line). HEK cells were selected due to their high transfectability [44,45], while the latter three cell lines

were chosen due to the presence of these cell lineages at potential sites of vaccination, either intramuscular or transcutaneous.

For MC3 LNPs, formulation with DOPC resulted in the significantly highest levels of saRNA transfection in all four cell lines (Fig. 4 A-D). In the case of C12-200 LNPs, formulation with either DOPC or DOPE provided similar levels of transfection, while the DSPC formulation delivered the lowest levels of transfection *in vitro* (Fig. 4 A-D). For both ionisable lipids, formulations with DOPC exhibited the highest transfection efficiency in general. The higher transfection levels for DOPC and DOPE over DSPC align with previous studies using the same helper lipids for both DNA and mRNA formulations [20,46], which indicates that helper lipid effects are independent of the nucleic acid being encapsulated. However, this might be different for smaller macromolecules, such as siRNA, which perform better *in vitro* with DSPC [46].

Overall, the transfection potency of LNPs was lower in HeLa and

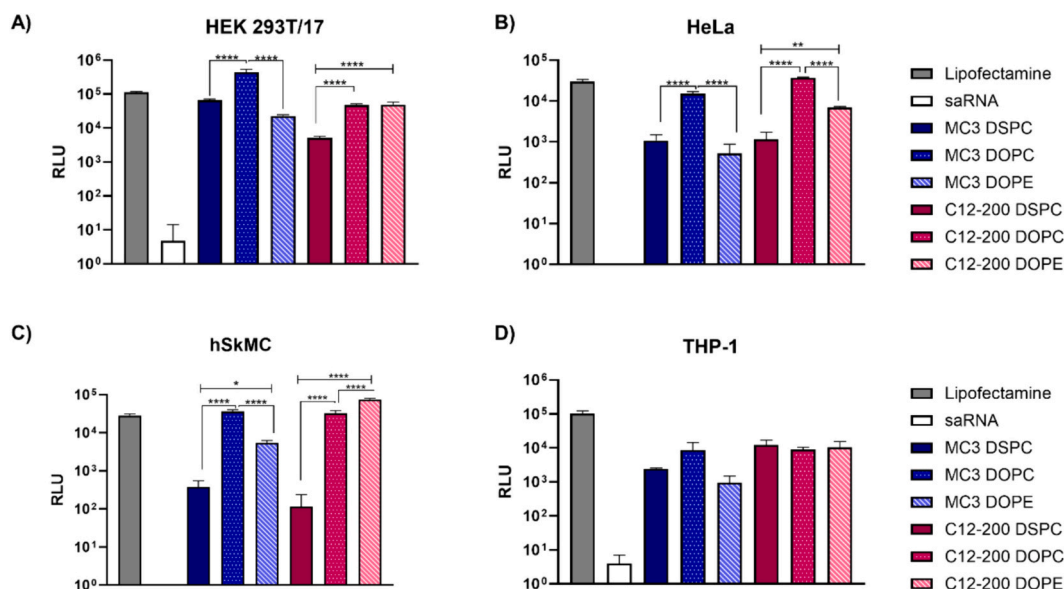


Fig. 4. saRNA expression levels *in vitro* using different cell lines. Cells were transfected with MC3 and C12–200 LNPs encapsulating firefly luciferase (fLuc), at a dose of 100 ng of saRNA for 24 h, and protein expression was analysed via a luciferase assay. (A) Transfection of HEK 293 T/17 cells, (B) Transfection of HeLa cells, (C) Transfection of hSkMC, and (D) Transfection of THP-1 cells. RLU: relative light units, hSkMC: human skeletal muscle cell. Bars represent mean $\pm$ SD ($n = 3$). Statistical significance was calculated by One-way ANOVA with Tukey's multi-comparison test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

THP-1 cells, with the latter exhibiting the lowest transfection levels. Both cells have tighter regulatory mechanisms on RNA translation due to their innate sensing capacity [47,48] in comparison to HEK cells. THP-1 cells also grow in suspension compared to the other cell lines, which can affect the dynamics of cellular uptake and delivery.

3.4. Cellular expression levels are overall correlated to LNP uptake *in vitro*

The varying levels of transfection potency (Fig. 4) prompted the question as to what might be causing these differences in transfection. Cellular transfection levels can be directly linked to the cellular uptake

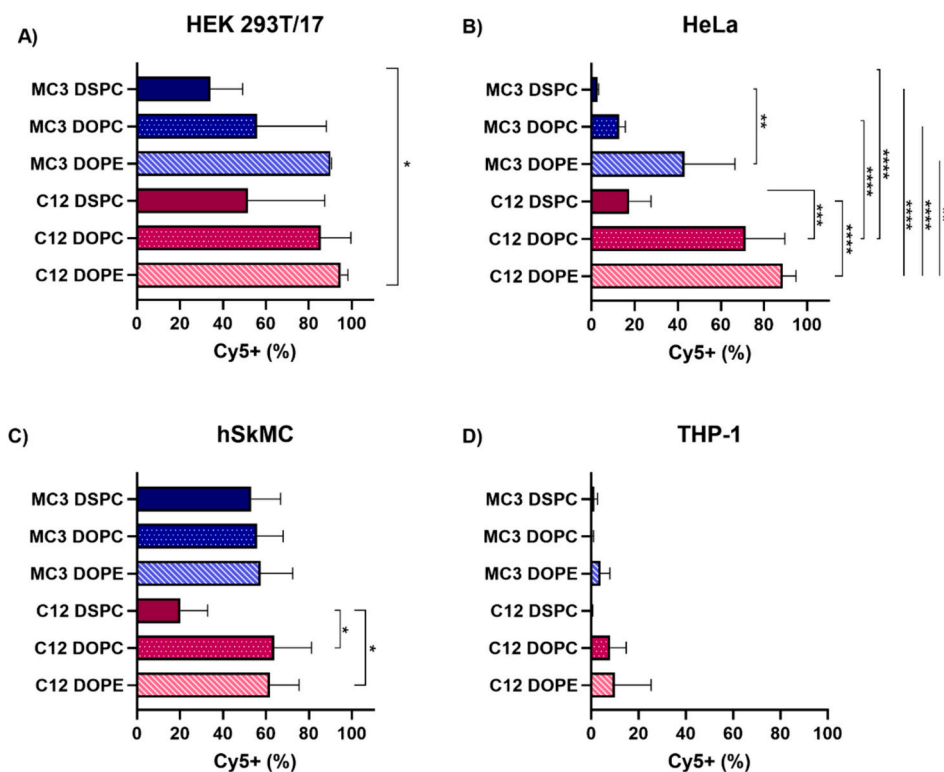


Fig. 5. Cellular uptake of LNPs with different helper lipids in various cell lines. The saRNA encapsulated by the LNPs was labelled with Cy5 and cellular uptake levels were analysed 4 h post transfection by flow cytometry. (A) Uptake in HEK 293 T/17 cells, (B) Uptake in HeLa cells, (C) Uptake in hSkMC, and (D) Uptake in THP-1 cells. hSkMC: human skeletal muscle cells. Bars indicated mean $\pm$ SD ($n = 3$). Statistical significance was calculated by Two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

of LNPs. To determine uptake, saRNA was labelled with Cy5 for direct detection by flow cytometry. Overall, LNPs with DOPE exhibited the best cellular uptake (Fig. 5A–D), followed by those with DOPC. LNPs with DSPC had significantly lower cellular uptake, which correlates with their lower expression in cells (Fig. 4 & 5). Additionally, cellular uptake was lower in THP-1 (all LNPs) and HeLa cells (C12–200 + DSPC and MC3 LNPs) than HEK and muscle cells (Fig. 5A–D), also reflecting expression levels.

3.5. Helper lipids influence in vitro cytokine induction

Following cellular uptake, saRNA expression is influenced by cell intrinsic immunity and the production of inflammatory cytokines produced by antigen-presenting cells. Indeed, an exacerbated type I interferon response can downregulate RNA expression levels [49]. The cytokine profile elicited upon stimulating cells with saRNA-LNPs was evaluated in THP-1 and human PBMCs.

In THP-1 cells, MC3 DOPE induced significantly more cytokines than other formulations, followed by C12–200 DOPE (Fig. 6A). The increased cytokine induction observed for DOPE formulations in THP-1 cells could be explained by their higher LNP uptake. This is in accordance with the fusogenic properties of DOPE as a helper lipid [22,50,51], facilitating cargo delivery and resulting in higher expression and cytokine levels, when compared to other formulations at the same dose.

We also used human PBMCs, known to be responsive to saRNA [52], as a sensitive model to detect the inflammatory nature of the different LNPs. In PBMCs, C12–200 formulations induced more cytokines than MC3, with C12–200 DOPE and MC3 DSPC being the most inflammatory formulations within their respective ionisable lipid groups (Fig. 6B). These LNP responses likely reflect the diversity of PBMCs, where monocytes represent a small fraction of the total population.

3.6. DSPC promotes saRNA expression in human skin ex vivo

We have previously demonstrated that human skin explant cultures can be used to evaluate the translational potential of saRNA delivery formulations; however, no one has reported transfection using classical LNPs in this model [53–55]. Human skin explants (1 cm²) obtained from healthy donors were transfected with 1 µg of LNP encapsulating fLuc saRNA. The time course of expression was followed until there was no significant signal detected. Contrary to our *in vitro* observations, DSPC mediated the highest levels of LNP transfection *ex vivo*, followed by

DOPE and lastly DOPC (Fig. 7). This trend was similar for C12–200 and MC3 LNPs, with C12–200 outperforming MC3 in each respective helper lipid formulation, and where MC3 DOPC LNPs exhibited virtually no transfection. These results highlight a lack of correlation between experiments performed using *in vitro* cell lines and *ex vivo* tissue models, and identify the potential importance of maintaining the spatial organisation of primary cells and associated extracellular matrix structures in LNP assessment. Here, the better performance of DSPC formulations may reflect a higher resilience to a more stringent tissue environment.

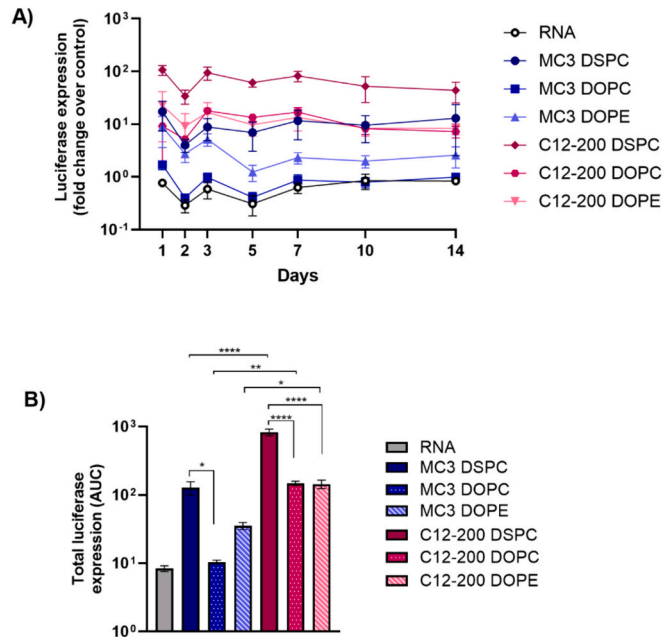


Fig. 7. saRNA expression levels *ex vivo* using human skin explants. Explants of 1 cm² were transfected with MC3 and C12–200 LNPs encapsulating firefly luciferase (fLuc), at a dose of 1 µg of saRNA. (A) Quantification of transfection levels of LNPs with different helper lipids compared to the PBS control (*n* = 3) and (B) area under the curve calculation for each experimental group over 14 days. Statistical significance was calculated by One-way ANOVA with Tukey's multi-comparison test (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

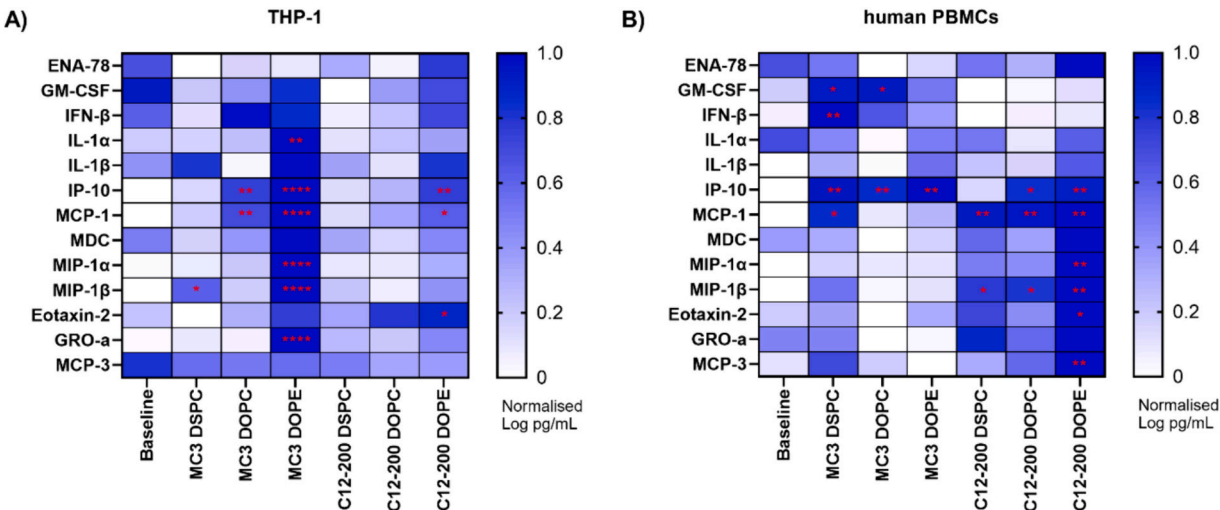


Fig. 6. Cytokine responses to vaccine formulations in THP-1 cells and human PBMCs. The supernatant was collected 48 h after cell transfection with LNPs, and samples were analysed by MSD. Significant differences are plotted compared to the baseline. (A) Cytokine panel of THP-1 cells per formulation (*n* = 2, Two-way ANOVA). (B) Cytokine panel of Human PBMCs per formulation (*n* = 2, Two-way ANOVA). Human PBMCs were obtained from a single healthy donor. Statistical significance was determined in comparison to baseline levels (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001).

3.7. Helper lipids influence in vivo saRNA expression in mice

Based on the saRNA expression results in human skin explants, we inoculated mice ($n = 5$ per group) intramuscularly with the same LNP formulations containing $5 \mu\text{g}$ of fLuc saRNA and analysed the expression time course. Luciferase expression was detectable for up to 20 days in the muscle tissue surrounding the administration site (Fig. 8). As with the skin explants, C12–200 LNPs led to significantly greater expression than MC3 LNPs. The most stable level of expression over time was seen

using C12–200 with DSPC (Fig. 8 A). C12–200 DOPE LNPs achieved an initial higher level of expression than DSPC LNPs but had a more rapid decay over time, while LNPs with DOPC performed poorly compared to the others (Fig. 8). The longer duration of expression in mice compared to skin explants mostly likely reflects reduction in skin explant viability over time, but there were also differences in dose (1 vs $5 \mu\text{g}$), administration site (skin vs muscle) and species (human vs mice), which may influence the outcome. Additionally, the difference in expression between *in vitro* and *ex vivo/in vivo* models could be influenced by the fact

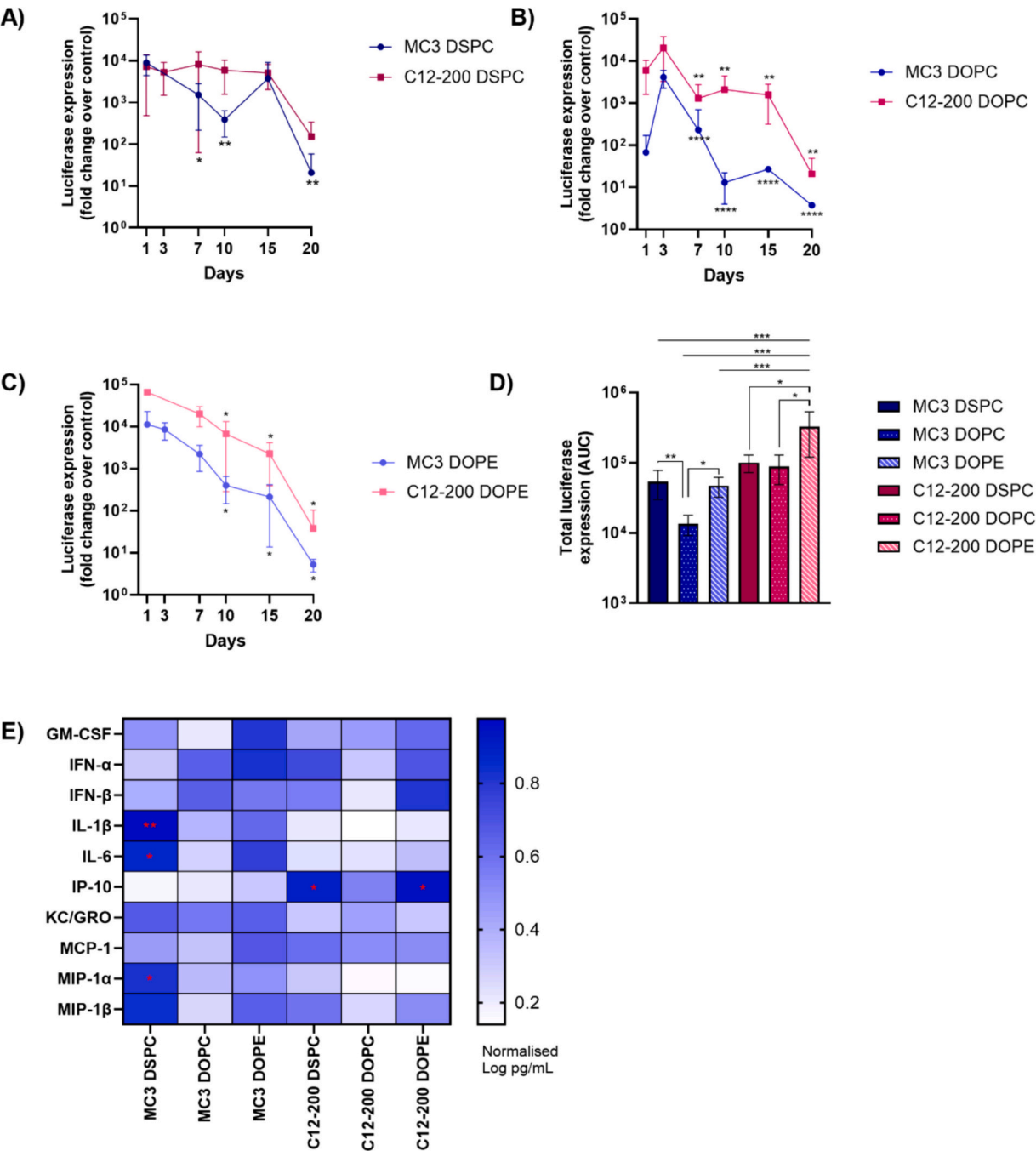


Fig. 8. saRNA expression levels *in vivo* using a murine model. BALB/c mice ($n = 5$ per group) were transfected with MC3 and C12–200 LNPs encapsulating firefly luciferase (fLuc), at a dose of $5 \mu\text{g}$ of saRNA. Quantification of bioluminescence at the administration site over 20 days and normalised against the PBS control group was done for (A) DSPC, (B) DOPC, and (C) DOPE formulations. The area under the curve (D) was calculated from graphs A–C and is represented in bar graphs with mean $\pm$ SD. Statistical significance was calculated by One-way ANOVA with Tukey’s multi-comparison test ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$). (E) Cytokine panel of animals vaccinated with saRNA LNPs at a $5 \mu\text{g}$ dose. Normalisation was performed between formulations, with the highest concentration presented in the strongest blue colour and the lowest concentration in white. Statistical significance was calculated by Two-way ANOVA ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$).

that different IVT kits were employed for saRNA synthesis, which is a caveat of this study.

To determine any relationship between the *in vivo* inflammatory profile of the different LNPs and luciferase expression, cytokine analysis was performed on murine blood samples collected 24 h after LNP administration. All formulations induced significant differences in cytokine expression in comparison to the PBS control group, and this was seen for IL-6, IP-10, KC/GRO, MCP-1, and MIP-1 β (Fig. S7). MC3 LNPs induced higher IL-6 levels while C12–200 LNPs induced significantly higher IP-10 levels, indicating a distinct profile for the different ionisable lipids (Fig. 8E). In fact, higher IP-10 levels were overall correlated with increased bioluminescence (Fig. S8), while high levels of acute IL-6 and MIP-1 β were significantly negatively correlated with luciferase expression at days 10 and 20, respectively (Fig. S8). Helper lipid incorporation also influenced the induced cytokine response. Generally, DOPC was less inflammatory for both MC3 and C12–200 LNPs. However, each helper-ionisable lipid combination provided a unique signature, suggesting such combinations and their associated lipid ratios could be tailored to deliver specific responses.

3.8. Ionisable lipid has a dominant impact over helper lipid in the induction of adaptive immune responses

We then investigated whether these formulations differed with respect to *in vivo* adaptive immunogenicity. BALB/c mice ($n = 5$ per group) were injected with either 1, 0.1, or 0.01 μg of saRNA encoding for the SARS-CoV-2 spike protein with the 6 different LNP formulations and a control group receiving a LNP saRNA vaccine used in a saRNA vaccine clinical trial [29]. Animals were injected with prime and boost vaccines 21 days apart. Blood samples were collected 2 weeks and 5 weeks after the prime vaccination.

C12–200 LNPs induced 10-fold higher levels of binding antibodies

than MC3 LNPs and 10- and 5-fold greater than LNPs used in previous clinical studies (Fig. 9A & C) at weeks 2 and 5 post-immunisation, respectively. For MC3 LNPs, the combination of MC3 with DOPE was significantly higher after the boost, compared to DSPC and DOPC at a 1 μg dose, but these differences were lost at a 0.1 μg dose (Fig. 9A). Any apparent effect of helper lipid on binding antibody titre was minimal for C12–200 LNPs. Despite differences in binding titres, levels of neutralising antibodies were similar between MC3 and C12–200 formulations, with no appreciable impact derived from the choice of helper lipid (Fig. 9B & D).

To determine cellular responses, spleens were harvested and processed to analyse T cell responses via an IFN- γ ELISpot assay. Overall, MC3 and C12–200 LNP formulations exhibited similar levels of IFN- γ producing cells (Fig. 10). MC3 in combination with DSPC had the highest levels of IFN- γ producing cells, and this trend was also observed for C12–200 (Fig. 10). Unexpectedly, C12–200 combined with DOPC exhibited the same levels of IFN- γ producing cells across all three doses administered to mice (1, 0.1, and 0.01 μg) (Fig. 9B). This finding warrants further investigation and validation of C12–200 DOPC ability to induce IFN- γ producing splenocytes at different doses, as it could be a potential formulation for the development of T cell vaccines.

To understand whether differences in cytokine induction might reflect differences in the magnitude of humoral or cellular responses, cytokine induction was assessed 24 h post intramuscular administration of 1 μg of LNP-formulated saRNA. Similar to the fluc imaging study, high levels of IL-6, IP-10, KC/GRO and MCP-1 were detected (Fig. S9), and C12–200 LNPs induced higher levels of cytokines compared to MC3 LNPs (Fig. 11A), reflecting the stronger humoral response with C12–200 LNPs. This reflects previous studies identifying ionisable lipids as the main component responsible for the efficiency of an LNP system *in vivo* [56,57]. C12–200 DSPC formulations induced higher cytokine levels than DOPE (Fig. 11A), while DOPC induced the highest levels of

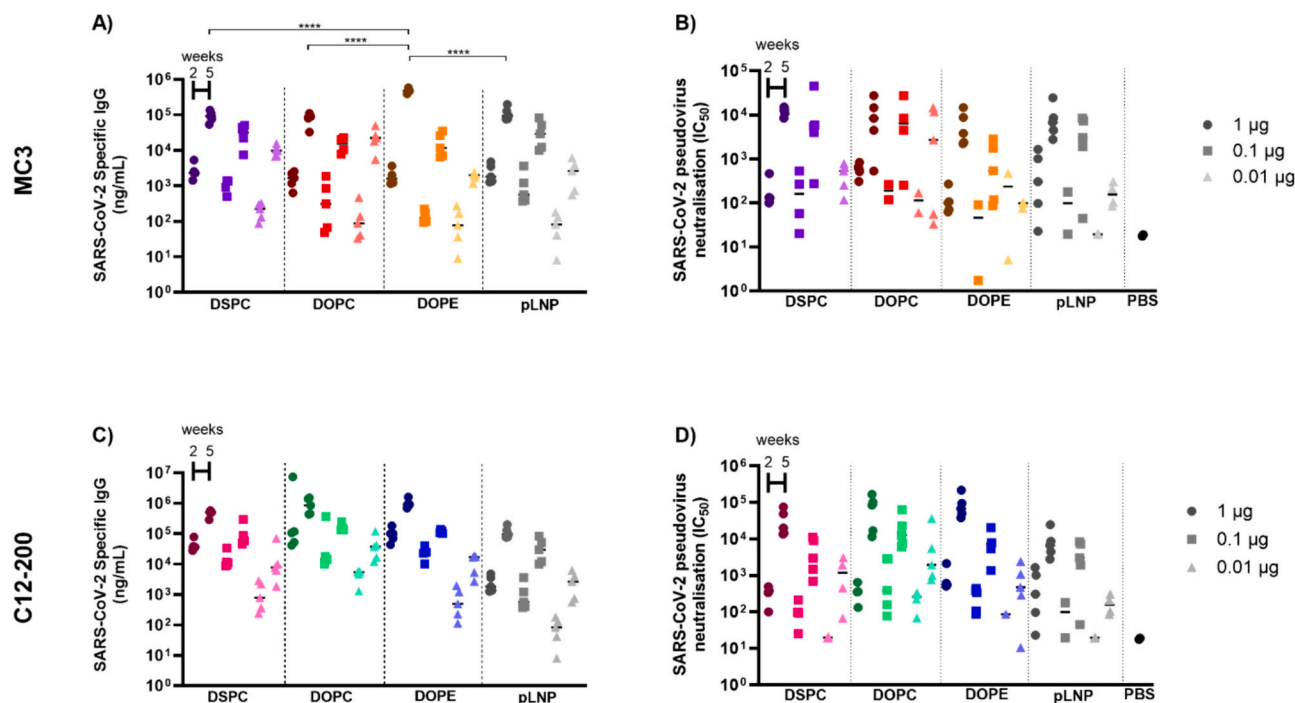


Fig. 9. Antibody quantification and neutralisation against SARS-CoV-2 spike protein in mice vaccinated with different LNP formulations. (A,C) SARS-CoV-2 specific IgG levels of mice vaccinated with LNPs containing different helper lipids and encapsulating 1, 0.1, or 0.01 μg of saRNA, $n = 5$ animals per group. Statistical significance was calculated by One-way ANOVA with Tukey's multi-comparison test (**** $p < 0.0001$). (B,D) SARS-CoV-2 neutralisation titres in sera from mice vaccinated with LNPs containing different helper lipids at three different doses (1, 0.1, 0.01 μg of saRNA), $n = 5$ animals per group. Data for MC3 DSPC at 0.01 μg was too low for IC50 calculations at week 2, and only data for week 5 is presented in the graph. Statistical significance was calculated by Two-way ANOVA. pLNP: proprietary/benchmark LNP.

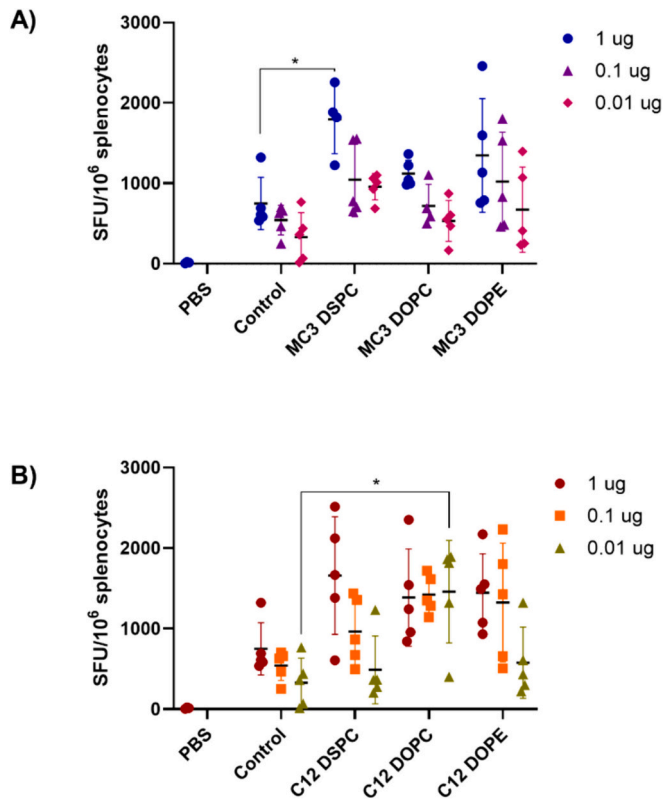


Fig. 10. Cellular responses to SARS-CoV-2 vaccine formulations containing different helper lipids. Quantification of IFN- γ producing splenocytes in mice vaccinated with (A) MC3 LNPs or (B) C12-200 LNPs. SFU: spot forming units. Control is a commercial LNP vaccine formulation against SARS-CoV-2. Symbols indicate mean $\pm$ SD, $n = 5$ animals per group. Statistical significance was calculated by Two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

cytokine change, and in particular, MCP-1 induction was associated with a stronger induction of cellular responses (Fig. 11B), offering a possible explanation for the observed cellular responses even at a 0.01 μ g dose.

4. Conclusion

In this study, we sought to investigate the effect of helper lipids on the expression, immunogenicity and initial stability of saRNA-LNP formulations. Two different ionisable lipids (MC3 & C12-200) were combined with one of three helper lipids (DSPC, DOPC, and DOPE). The LNPs had similar physicochemical characteristics but resulted in different levels of saRNA expression (*in vitro*, *ex vivo*, and *in vivo*). For both MC3 and C12-200, DOPC and DOPE formulations were more potent *in vitro*, with higher cellular uptake and RNA expression levels, while DSPC formulations provided optimal storage stability, *i.e.* they presented stable levels of cellular transfection with moderate changes in physicochemical characteristics. The results presented here are an early indication that storage stability might be an important parameter for the development of novel saRNA vaccines, however, more comprehensive studies are required to establish any correlation.

DSPC formulations exhibited high and stable levels of expression in human skin explants, where C12-200 was more potent than MC3. In this respect, LNP storage stability was a better predictor of expression in human skin than *in vitro* transfection of human cell lines. In addition, skin explants better represent the more stringent tissue environment presented by the spatial organisation of primary cells and associated extracellular matrix structures. C12-200 was also more potent than MC3 with respect to *in vivo* luciferase expression in mice. However, C12-200

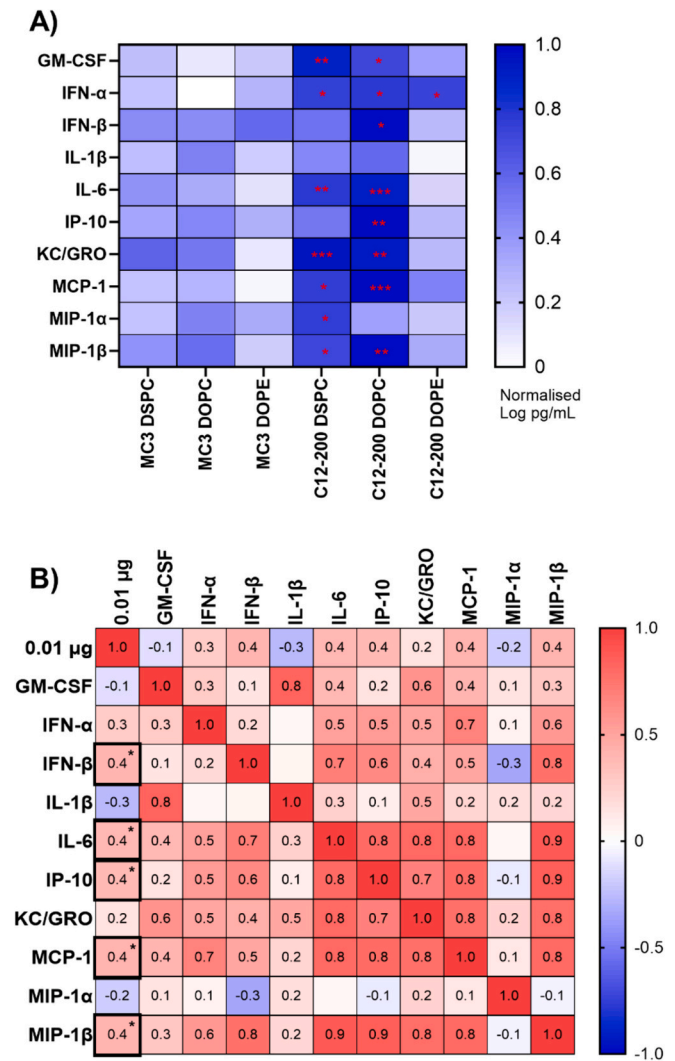


Fig. 11. Cytokine responses to different helper lipids formulations containing SARS-CoV-2 saRNA. Serum samples were collected 24 h after vaccination and (A) samples were analysed by MSD. Significant differences are plotted when formulations are significantly different to their counterparts. (B) Correlation analysis between serum cytokines and IFN- γ producing cells at 0.01 μ g vaccine dose. $n = 5$ animals per group. Statistical significance was calculated by Two-way ANOVA (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

combined with DOPE induced higher expression than DSPC.

While each LNP induced a distinct cytokine profile, it is not yet clear how individual cytokines influence the overall efficacy or safety of RNA vaccines; further work is required to dissect this. It is of note that, the higher induction of IP-10 by C12-200 LNPs was positively correlated with increased luciferase expression. C12-200 also promoted stronger antibody responses than MC3 formulations to saRNA encoding the SARS-CoV-2 spike glycoprotein, where the effect of helper lipids had little apparent impact. The more potent activity of C12-200 reflected a more robust induction of cytokines, in particular, MCP-1 and IFN- α levels (Fig. S10). Induced cellular responses were equivalent for C12-200 and MC3 formulations irrespective of helper lipid at a 1 μ g dose. However, C12-200 in combination with DOPC provided the most robust cellular responses at lower doses (0.1, 0.01 μ g), reflecting stronger induction of MCP-1 (Fig. 11B & S11).

The data presented here indicates that helper lipids can influence both the stability and functionality of LNP-formulated saRNA. As seen previously for mRNA, *in vitro* studies failed to predict performance in both the human skin model and in mice. However, increasing evidence

has raised significant questions over the predictability of the mouse model for human translation [55,58–60]. In this respect, the human skin model may provide an important complementary assay. On the whole, formulating saRNA with C12–200 in combination with DSPC enables an optimised balance of storage stability against *in vivo* and *ex vivo* potency. This formulation proved more potent than that of previous clinical trials [29] and has been selected for future clinical evaluation of saRNA vaccine constructs.

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CRediT authorship contribution statement

Beatriz D. Barbieri: Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **David J. Peeler:** Writing – review & editing, Visualization, Conceptualization. **Karnyart Samnuan:** Investigation. **Suzanne Day:** Investigation. **Kai Hu:** Investigation. **Hadijatou J. Sallah:** Investigation. **John S. Tregoning:** Writing – review & editing, Resources. **Paul F. McKay:** Supervision, Resources, Methodology, Investigation, Conceptualization. **Robin J. Shattock:** Writing – review & editing, Supervision, Resources, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare no conflict of interest.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jconrel.2024.08.016>.

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