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RESEARCH ARTICLE

Rational design of anti-inflammatory lipid nanoparticles for mRNA delivery

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Abstract

Lipid nanoparticles (LNPs) play a crucial role in delivering messenger RNA (mRNA) therapeutics for clinical applications, including COVID-19 mRNA vaccines. While mRNA can be chemically modified to become immune-silent and increase protein expression, LNPs can still trigger innate immune responses and cause inflammation-related adverse effects. Inflammation can in turn suppress mRNA translation and reduce the therapeutic effect. Dexamethasone (Dex) is a widely used anti-inflammatory corticosteroid medication that is structurally similar to cholesterol, a key component of LNPs. Here, we developed LNP formulations with anti-inflammatory properties by partially substituting cholesterol with Dex as a means to reduce inflammation. We demonstrated that Dex-incorporated LNPs effectively abrogated the induction of tumor necrosis factor alpha (TNF-α) in vitro and significantly reduced its expression in vivo. Reduction of inflammation using this strategy improved in vivo mRNA expression in mice by 1.5-fold. Thus, we envision that our Dex-incorporated LNPs could potentially be used to broadly to reduce the inflammatory responses of LNPs and enhance protein expression of a range of mRNA therapeutics.

KEYWORDS
anti-inflammation, dexamethasone, gene delivery, lipid nanoparticles, mRNA

1 | INTRODUCTION

Ionizable lipid nanoparticles (LNPs) are the most clinically advanced non-viral delivery platform for RNA therapeutics, as illustrated by the clinical success of Onpattro and the Pfizer/BioNTech and Moderna COVID-19 mRNA vaccines.[1,2] They are typically composed of

Abbreviations: C, cholesterol; D (or Dex), dexamethasone; LNP, lipid nanoparticles; MW, molecular weight.

Hanwen Zhang and Xuexiang Han contributed equally to this work.

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ionizable lipids, cholesterol, polyethylene glycol (PEG)-conjugated lipids, and phospholipids.[3–5] Ionizable lipids can electrostatically interact with RNA molecules and facilitate their intracellular delivery, phospholipids and cholesterol improve the overall membrane stability of LNPs, and PEG-conjugated lipids reduce protein adsorption and prolong the circulation time of LNPs in vivo.[6–8] LNPs can protect and deliver mRNA therapeutics to target cells and tissues by overcoming biological barriers.[9,10] However, the LNP/mRNA complex can interact with the innate immune system and trigger immune responses.[7,11] While mRNAs can be modified to be immune-silent,[12,13] the LNPs themselves have been shown to induce strong inflammatory responses in immune cells.[14–16] LNPs can activate the immune system by interacting with pattern recognition receptors (PRRs) on antigen presenting cells (APCs), such as toll-like receptors (TLRs).[7,15,17,18] Previous studies have shown that the interaction of LNPs with PPRs will subsequently trigger the release of pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α), suggesting the general onset of an innate immune response.[14,15,19] The inflammatory responses can then reduce the translation efficiency of mRNA and provoke immune-related adverse effects.[20,21] Therefore, premedication with anti-inflammatory drugs and antihistamines is needed for LNP-based mRNA therapeutics in the clinic.[22] Hence, there is an urgent need to develop LNP formulations that can suppress unwanted innate immune responses to not only improve the safety of LNPs but also enhance the potency of mRNA therapeutics. Such anti-inflammatory LNPs can potentially reduce or avoid premedication with anti-inflammatory drugs and increase the tolerance and therapeutic efficacy of mRNA therapeutics for protein replacement and gene editing therapies.

Corticosteroids possess anti-inflammatory effects,[23,24] and previous studies showed that co-delivering genes and broad-spectrum

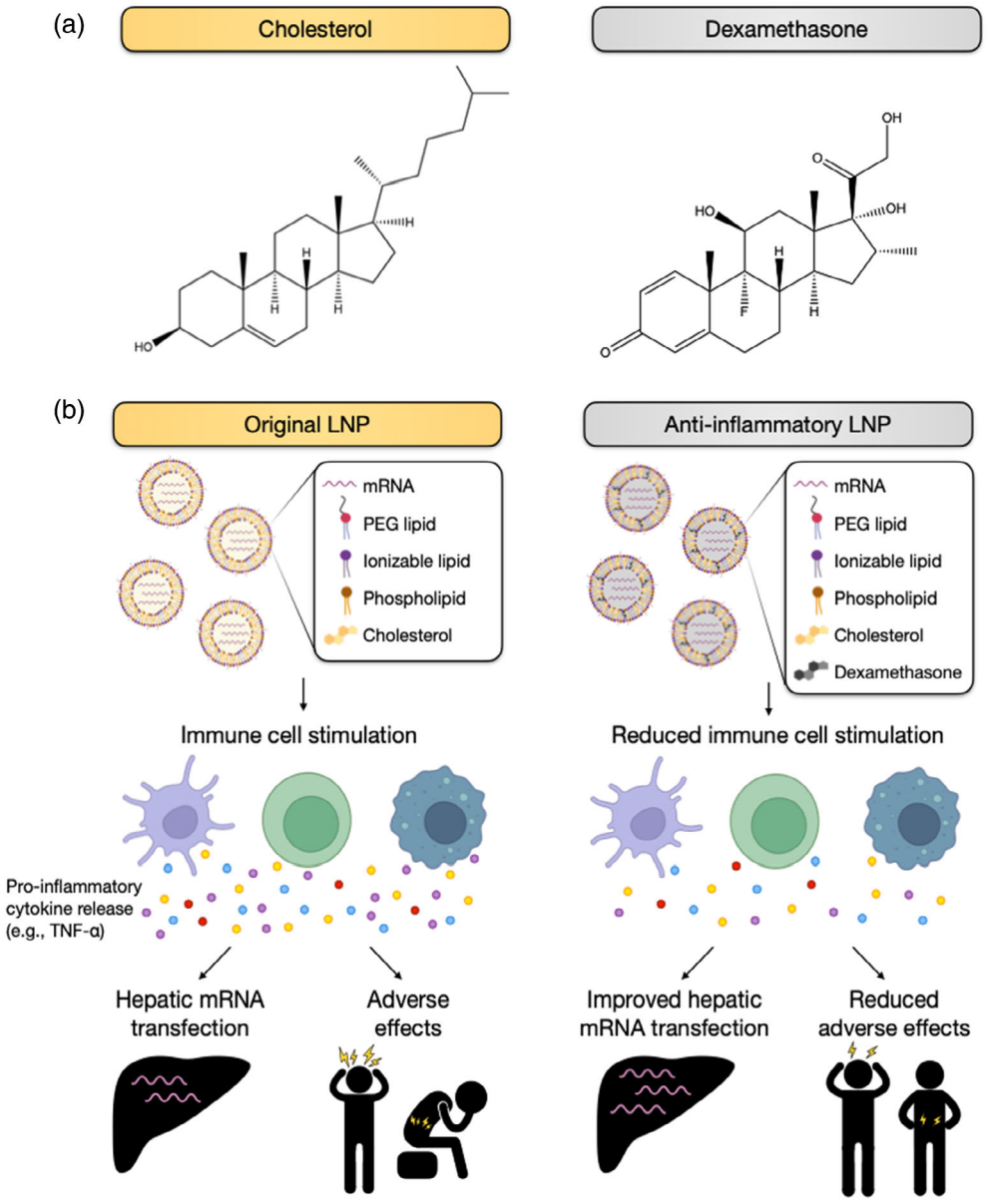

FIGURE 1 Chemical structures of cholesterol and dexamethasone and schematic illustration of anti-inflammatory LNPs to reduce adverse effects and improve mRNA transfection. (a) Chemical structures of cholesterol (left, MW: 386.65 g/mol) and dexamethasone (right, MW: 392.47 g/mol). (b) Anti-inflammatory LNPs suppress the local inflammation caused by LNPs in immune cells leading to reduced adverse effects and enhanced hepatic mRNA transfection. LNPs are proposed to stimulate immune cells such as macrophages. Dex can reduce the release of pro-inflammatory cytokines (e.g., TNF-α), and thus improve hepatic transfection and minimize the adverse effects of LNPs

anti-inflammatory steroids suppress inflammation via inhibiting the transcription of proinflammatory genes.[14–16,25,26] Dexamethasone (Dex) is a commonly used anti-inflammatory corticosteroid. Lipidated Dex was shown to reduce pro-inflammatory cytokines, suppress LNP-triggered immune activation, improve the tolerability of LNPs, and increase the expression of transgene.[14–16,25,27] In addition, Davies et al. recently demonstrated that DLin-MC3-DMA (MC3) LNPs co-delivering RNA therapeutics and anti-inflammatory steroids (rofleponide and budesonide) could suppress the inflammatory response and increase protein expression by 1.2–1.9 fold compared to the original formulation.[16] Dex also shares structural similarities with cholesterol, one of the LNP components responsible for stabilizing LNP structure (Figure 1a).

Inspired by Patel et al.'s previous study on LNPs with cholesterol analog substitutes,[28] we proposed an anti-inflammatory LNP formulation that co-delivers Dex and mRNA. The MC3 formulation that was approved by the FDA for siRNA delivery is explored in this study for further optimization, as previous research has shown that MC3 degrades slowly and is prone to trigger immune responses.[16,29–31] By incorporating Dex directly into the LNP structure, the drug can be delivered to the same cells where LNPs can cause inflammatory responses and is therefore expected to suppress local inflammation caused by LNPs (Figure 1b). From a translational point of view, the inclusion of an original form of Dex into LNPs could potentially face less regulatory hurdles and scale-up challenges than a Dex prodrug, leading to the potential for broader applications of the new LNP formulation. We demonstrate that Dex-incorporated LNPs effectively reduced the production of pro-inflammatory cytokines both in vitro and in vivo and increased hepatic mRNA expression by 1.5-fold.

2 | MATERIALS AND METHODS

2.1 | Materials

DLin-MC3-DMA (MC3) was purchased from MedChemExpress. Dexamethasone was obtained from Sigma-Aldrich (Saint Louis, MO). Other lipids were purchased from Avanti Polar Lipids (Alabaster, AL).

2.2 | Production of the luciferase mRNA

Codon optimized firefly luciferase was cloned into an mRNA production plasmid (optimized 3′ and 5′ UTR and containing a 101 polyA tail), in vitro transcribed in the presence in the presence of N1-methylpseudouridine modified nucleoside (N1mψ), co-transcriptionally capped using the CleanCap™ technology (TriLink) and cellulose purified to remove dsRNA. Purified mRNA was ethanol precipitated, washed, resuspended in nuclease-free water, and subjected to quality control (electrophoresis, dot blot, and transfection into human dendritic cells). mRNA was stored at −80°C until use.[32]

2.3 | Lipid nanoparticle (LNP) formulation and characterization

An ethanol phase containing all lipids and an aqueous phase containing mRNA were mixed using a microfluidic device to synthesize LNPs.[3,6,33] The ethanol phase was composed of the ionizable lipid (MC3), 1,2-distearyol-*sn*-glycero-3-phosphoethanolamine (DSPC), 1,2-dimyristoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[methoxy(polyethyleneglycol)-2000] (C14PEG-2000), cholesterol, and dexamethasone. MC3, DSPC, and C14PEG-2000 were combined at a molar ratio of 50%, 10%, and 1.5%, respectively. The molar ratios of cholesterol and dexamethasone vary by formulation and have a total molar ratio of 38.5%. The aqueous phase contains luciferase mRNA dissolved in 10 mM citrate buffer. The ethanol and aqueous phases were mixed at a flow rate of 1.8 and 0.6 ml/min (3:1), respectively, using Pump33DS syringe pumps (Harvard Apparatus, Holliston, MA). LNPs were placed in 1X PBS for dialysis in a microdialysis cassette (20,000 MWCO, Thermo Fisher Scientific, Waltham, MA) for 2 h and then filtered through a 0.22 μm filter. Zetasizer Nano (Malvern Instruments, Malvern, U.K.) was used to measure the polydispersity index (PDI) and Z-average diameters. mRNA concentration and encapsulation efficiency in each LNP formulation were measured using a modified Quant-iT RiboGreen (ThermoFisher) assay.[34]

2.4 | Cell culture

Human hepatoma cell line HepG2 cells and murine macrophage cell line RAW264.7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA). They were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and incubated at 37°C in a humidified atmosphere with 5% CO_2.

2.5 | In vitro transfection and cytotoxicity

HepG2 cells were seeded in a 96 well plate at a density of $1x10^4$ cells/well and were allowed to grow for 24 h. LNPs with different cholesterol:dexamethasone (C:D) ratios (10:0, 9:1, 7:3, 5:5, 3:7, 0:10) were used to treat cells at a dose of 50 ng mRNA/well for 24 h. Afterwards, luciferase expression and cell viability were tested using a Luciferase Assay Kit (E4550, Promega) and a CellTiter-Glo® Luminescent Cell Viability Assay Kit (G7572, Promega), respectively.

RAW264.7 cells were seeded in a 12-well plate at a density of 2×10^5 cells/well and were allowed to grow for 24 h. LNPs were used to treat cells at a dose of 500 ng mRNA/well for 24 h. The supernatant was collected for TNF-α analysis.

2.6 | Animal experiments

All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania

and were performed following the Guidelines for Care and Use of Laboratory animals at the University of Pennsylvania. Nine 8- to 12-week-old C57BL/6 female mice (Jackson Laboratory, Bar Harbor, ME, ~20 g) were divided randomly into three groups ($n = 3$) and were intravenously injected with either PBS, the original MC3 LNP (C10D0), or the Dex-incorporated LNP (C9D1). For each mouse in the LNP-treated groups, 4 μg of luciferase mRNA was injected. 20 h later, the blood was collected from each mouse through retro-orbital bleeding and the serum was prepared for TNF-α analysis. Bioluminescence imaging was performed with an IVIS Spectrum Imaging system (Caliper Life Sciences, Hopkinton, MA) 20 h after the injection. D-luciferin (PerkinElmer, Waltham, MA) at a dose of 150 mg/kg was injected into mice by intraperitoneal (IP) injection, followed by anesthetization and imaging. The amount of total photon flux was measured using an IVIS imaging system.

2.7 | Cytokine level measurements

The concentration of TNF-α in RAW264.7 cultures and mouse serum were measured using a commercially available ELISA assay kit (Invitrogen).

2.8 | Statistical analysis

Results were analyzed by an unpaired Student's *t*-test using Prism 5 software package (Graphpad, Inc., San Diego, CA), and were expressed as mean values ± standard deviation (SD) or fold-increase. Statistical significance was indicated by a *p*-value of equal to or less than .05.

3 | RESULTS

3.1 | Characterization of LNPs

LNPs were formulated by mixing an aqueous phase containing mRNA and an organic phase containing MC3, 1,2-distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPC), PEG conjugated lipid (C14PEG-2000), cholesterol, and dexamethasone in a microfluidic device (Figure 2). The microfluidic device was designed to enable the formation of LNPs with a uniform size.[33,35] To avoid immune activation by the mRNA, purified 1-methylpseudouridine-containing mRNA was used throughout this study.[36,37]

MC3 LNPs in the absence of Dex (C10D0) and Dex-incorporated LNPs (C9D1) were prepared. The naming convention follows the relative cholesterol:dexamethasone (C:D) ratio. The original formulation C10D0 indicates that the relative C:D molar ratio is 10:0, and the C:D molar percentage in the C10D0 LNP is 38.5%:0%. The C9D1 LNP has a C:D molar ratio of 9:1, and the C:D molar percentage is 34.65%:3.8% (Table 1). Both LNPs had encapsulation efficiencies of >90% and were within the neutral range of ±10mV.[38] Moreover, hydrodynamic sizes as well as polydispersity were similar for both LNPs (Figure 3).

3.2 | In vitro transfection, cytotoxicity, and anti-inflammatory potential of C9D1 LNP

LNPs encapsulating mRNA encoding luciferase—in the presence or absence of Dex—were used to treat HepG2 cells to assess transfection efficiency and cytotoxicity. C9D1 LNP did not show a reduction in transfection efficiency in comparison to C10D0 LNP (Figure 4a). Moreover, C9D1 LNP did not show increased cytotoxicity (Figure 4b). We then further explored the possibility of incorporating more Dex into LNPs (Table 2). Although LNPs could still be formulated, the transfection efficiency dropped significantly as the proportion of Dex increased (Figure 5).

To verify whether the incorporation of Dex can suppress the immune response triggered by the LNPs themselves, the anti-inflammatory effect of C9D1 LNP on murine macrophages (RAW246.7) was evaluated (Figure 4c). After the cells were stimulated with LNPs for 24 h, the concentration of TNF-α in the supernatant was measured by an enzyme linked immunosorbent assay (ELISA). While C10D0 LNP treatment significantly stimulated the production of TNF-α by approximately 2.6 fold in RAW246.7 cells, C9D1 LNP treatment only marginally increased TNF-α levels by 1.2 fold.

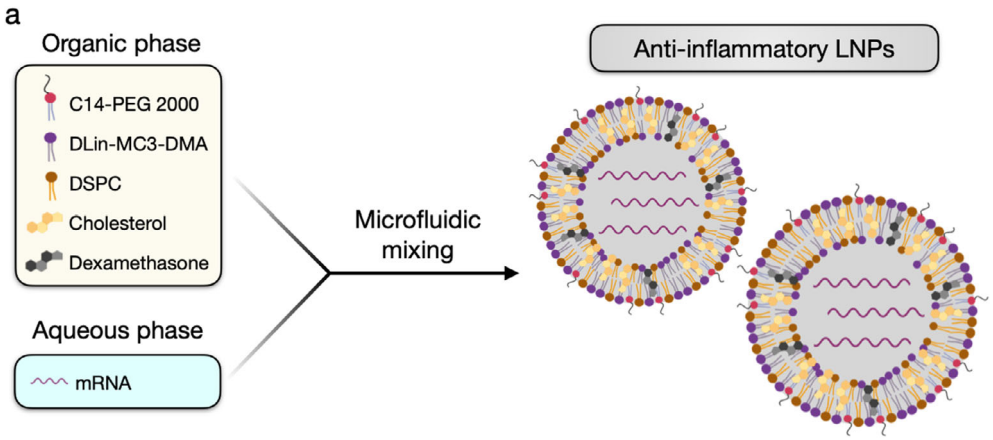

FIGURE 2 Formulation of Dex-incorporated LNPs via microfluidic mixing. mRNA is dissolved in the aqueous phase while PEG conjugated lipid (C14PEG-2000), MC3, DSPC, cholesterol, and dexamethasone are dissolved in the organic phase. The two solutions are rapidly mixed in a microfluidic device to form mRNA-LNPs

TABLE 1 Characterization of LNPs

LNPs	C:D ratio	C:D molar percentage in LNP (%)	Encapsulation efficiency (%)	Z-diameter (nm)	PDI	Zeta-potential (mV)
C10D0	10:0	38.50:0	92.52	71.37 ± 1.74	0.150 ± 0.017	−5.27 ± 0.63
C9D1	9:1	34.65:3.85	93.46	76.84 ± 1.25	0.124 ± 0.046	−0.12 ± 0.10

Note: C:D ratio represents the weight ratio between cholesterol and dexamethasone. Data are presented as mean ± SD ($n = 3$).

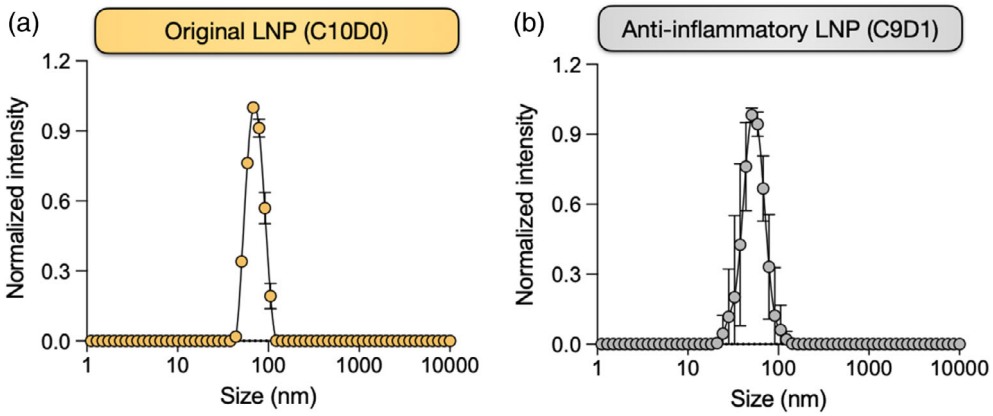

FIGURE 3 Hydrodynamic size of LNPs measured by Dynamic Light Scattering (DLS) (a) Intensity-based size distribution of C10D0 LNP. (b) Intensity-based size distribution of C9D1 LNP. Three representative technical replicates result for each LNP are shown

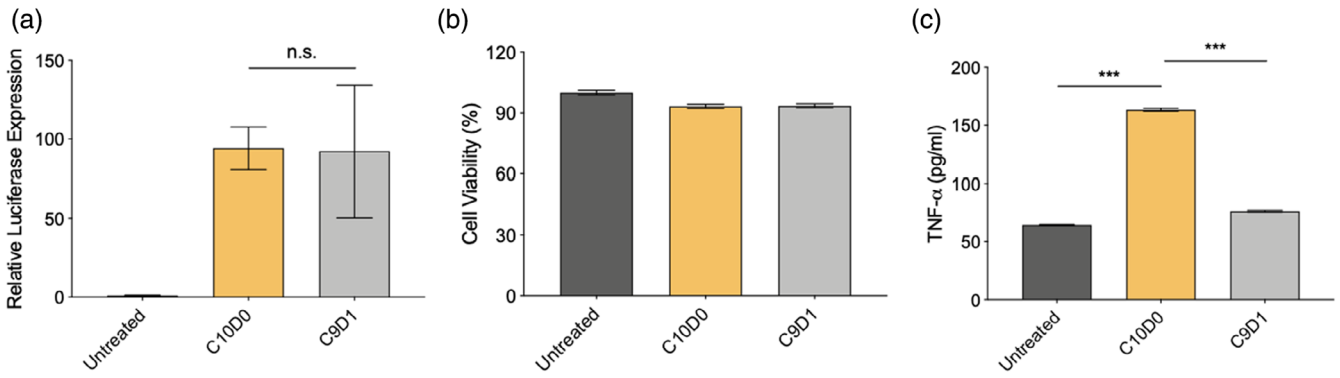

FIGURE 4 In vitro luciferase expression and cell viability in HepG2 cells, and TNF-α levels in RAW246.7 cells, following treatment with mRNA-LNPs incorporating Dex. (a) In vitro luciferase mRNA transfection in HepG2 cells 24 h after treatment. (b) Cell viability of HepG2 cells 24 h after treatment. (c) TNF-α production in RAW246.7 cells 24 h after treatment. Data are presented as mean ± SD ($n = 3$). n.s., non-significant, ***$p < .001$

TABLE 2 Encapsulation efficiency, diameters, polydispersity index, and surface charge for LNPs with increased Dex substitution

LNPs	C:D ratio	C:D molar percentage in LNP (%)	Encapsulation efficiency (%)	Z-diameter (nm)	PDI	Zeta-potential (mV)
C10D0	10:0	38.50:0	92.52	71.37 ± 1.74	0.150 ± 0.017	−5.27 ± 0.63
C7D3	7:3	26.95:11.55	95.59	60.92 ± 2.89	0.033 ± 0.024	−4.07 ± 0.29
C5D5	5:5	19.25:19.25	93.52	81.13 ± 1.79	0.130 ± 0.037	−5.12 ± 0.52
C3D7	3:7	11.55:26.95	95.70	68.10 ± 1.82	0.139 ± 0.039	−4.00 ± 0.77
C0D10	0:10	0:38.5	69.21	76.69 ± 0.786	0.040 ± 0.018	−0.052 ± 0.132

Note: C:D ratio represents the weight ratio between cholesterol and dexamethasone. Data are presented as mean ± SD ($n = 3$).

3.3 | In vivo C9D1 LNP mRNA delivery, transfection, and anti-inflammatory effects

C57BL/6 mice were used to investigate the inflammatory response and mRNA delivery of LNPs. LNPs containing 4 μg of mRNA encoding for luciferase were intravenously (*i.v.*) injected into each mouse. For the C9D1 LNP treatment group, the dose of Dex was 0.62 μg per mouse. Serum from untreated, C10D0 LNP, and C9D1 LNP groups was harvested for TNF-α quantification by ELISA. The results indicate that the original LNPs themselves induce an inflammatory response,

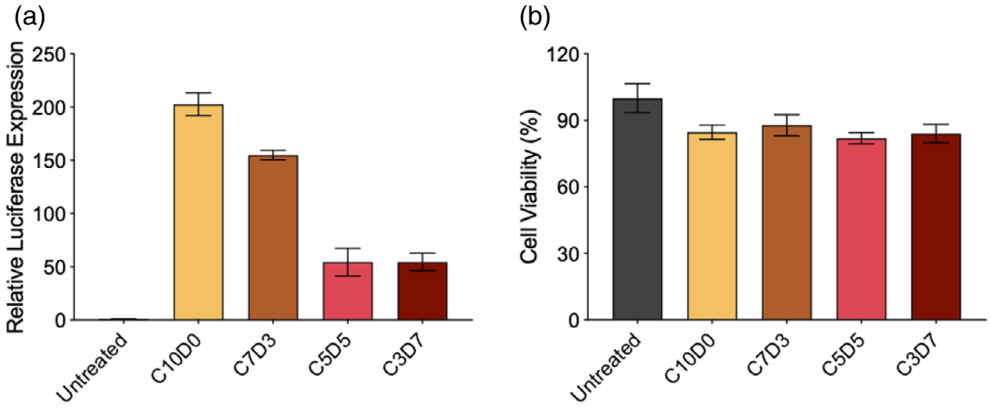

FIGURE 5 In vitro luciferase expression and cell viability in HepG2 cells following treatment with LNPs formulated at different C:D ratios. (a) In vitro luciferase mRNA transfection in HepG2 cells 24 h post-treatment. (b) Cell viability of HepG2 cells 24 h post-treatment. Data are presented as mean ± SD ($n = 3$)

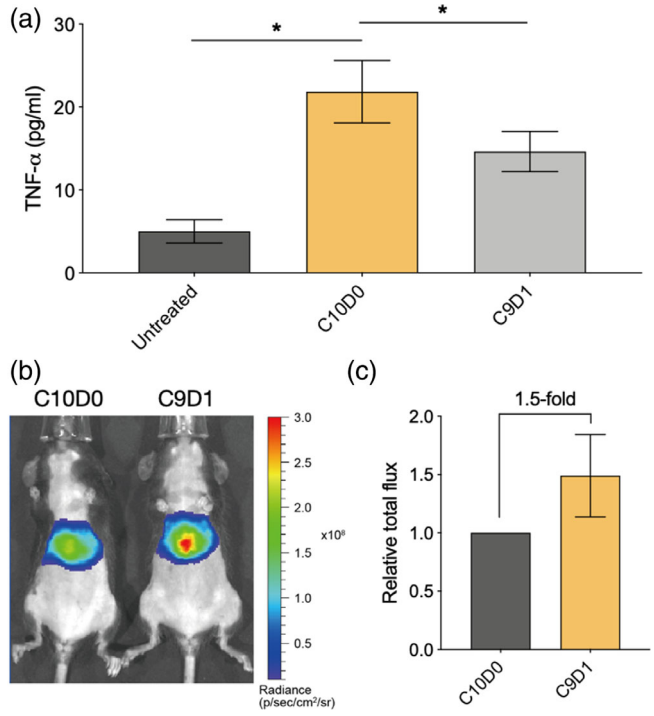

FIGURE 6 In vivo TNF-α levels and mRNA delivery following *i.v.* treatment with C10D0 and C9D1 LNPs. (a) Serum TNF-α levels following treatment with C10D0 or C9D1 LNPs in mice. Serum was collected 20 h after treatment. Data are presented as mean ± SD ($n = 3$). *$p < .05$. (b) In vivo luciferase expression. For each mouse, 4 μg of LNP-formulated luciferase mRNA was *i.v.* injected. Data are presented as mean ± SD ($n = 3$)

as C10D0 LNP-treated mice showed a significantly higher TNF-α level than the untreated control group. Interestingly, the serum TNF-α concentration of C9D1 LNP-treated mice was significantly reduced compared to C10D0 LNP-treated mice (Figure 6a). These results suggest that C9D1 LNP can successfully reduce the inflammatory response triggered by LNPs in vivo.

Next, the in vivo transfection of C9D1 LNP encapsulating mRNA encoding for luciferase was investigated. MC3 LNP is a clinically validated non-viral vector for liver transfection.[39,40] As expected, strong luciferase expression in the liver was observed for both C9D1 LNP- and C10D0 LNP-treated mice (Figure 6b). Interestingly, quantification of the luminescence signal showed a 1.5-fold increase in C9D1 LNP-treated mice compared to C10D0 LNP-treated mice (Figure 6c). These results suggest that the transfection efficiency of C9D1 LNP is significantly improved by 1.5 fold.

4 | DISCUSSION

In the present work, we first used DLS to characterize changes in size and PDI upon substitution with Dex. Beyond using DLS, we sought to evaluate the encapsulation efficiency. Both the original C10D0 LNP and the anti-inflammatory C9D1 LNP showed similar size and PDI and high encapsulation efficiencies (>90%), indiciated that C9D1 LNP efficiently encapsulates mRNA and can potentially have extensive applications in delivering nucleic acids. These results suggest that the replacement of 10% cholesterol with Dex had minimal effect on the size and polydispersity of LNPs, and that Dex-incorporated LNPs with high mRNA encapsulation efficiency were developed for subsequent studies.

Next, we explored C10D0 LNP and C9D1 LNP in vitro. We evaluated the possibility of substituting more cholesterol with Dex, and we found out that increased substitution resulted in reduced encapsulation efficiency, and compromised transfection efficiencies. These results suggest that the replacement of a fraction of cholesterol with Dex is key to maintaining high LNP transfection efficiency. In addition, C9D1 LNP showed minimal immunogenicity and no significant improvements in transfection efficiency, while the pro-inflammatory cytokine TNF- α levels in C9D1 LNP treated cells returned to the untreated level. These results indicate that C9D1 LNP can suppress the immune response in vitro triggered by LNPs themselves. Previous studies used a higher dose of free Dex or Dex prodrug to suppress the inflammatory response induced by LNPs.[15,16,25,27] However, the adverse effects of this regimen to suppress systemic inflammation include abdominal discomfort, skin rash, swelling, and hot flush.[41] In the present work, we showed that an anti-inflammatory LNP that suppresses local immune responses with a low dose of Dex could potentially be a promising alternative to suppressing systemic immune responses with a high dose of corticosteroids.

Finally, we considered the in vivo environment may be more comprehensive in evaluating the anti-inflammatory effect and transfection

efficiency of C9D1 LNP. We showed that transfection efficiency of C9D1 LNP increased by 1.5 fold compared to the original C10D0 LNP accompanied by a significant reduction in TNF- α levels in vivo. This result is in line with previous reports that suggest that suppression of the immune response triggered by LNPs can increase gene expression.[14,15,25] Since transgene expression can be suppressed in the presence of inflammatory cytokines such as TNF-α,[14,15] C9D1 LNP can enhance mRNA transfection by inhibiting the production of inflammatory cytokines (Figures 4c and 6a). Together, C9D1 LNP is a promising formulation that can simultaneously reduce inflammation and enhance protein expression of mRNA-LNP therapeutics.

5 | CONCLUSION

Dex-incorporated LNPs (C9D1) were successfully prepared and demonstrated potent anti-inflammatory effects. C9D1 LNPs were found to suppress the pro-inflammatory cytokine TNF-α to a near-basal level in vitro, and significantly down-regulated TNF-α levels in vivo compared to the original C10D0 LNP. Due to the reduced inflammatory response, the overall mRNA transfection was improved by 1.5-fold in C9D1 LNP-treated mice. Therefore, Dex substitution within LNPs could be a potentially promising strategy to reduce inflammation-related adverse effects of LNPs while enhancing protein expression of mRNA therapeutics.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Hanwen Zhang and Xuexiang Han performed experiments and drafted the manuscript. Hanwen Zhang, Xuexiang Han, Mohamad-Gabriel Alameh, Sarah J. Shepherd, Marshall S. Padilla, Lulu Xue, Kamila Butowska, Drew Weissman, and Michael J. Mitchell discussed the manuscript content. The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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