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Dexamethasone prodrugs as potent suppressors of the immunostimulatory effects of lipid nanoparticle formulations of nucleic acids

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ABSTRACT

Lipid nanoparticles (LNPs) are playing a leading role in enabling clinical applications of gene therapies based on DNA or RNA polymers. One factor impeding clinical acceptance of LNP therapeutics is that LNP formulations of nucleic acid polymers can be immunostimulatory, necessitating co-administration of potent corticosteroid immunosuppressive agents. Here, we describe the development of hydrophobic prodrugs of a potent corticosteroid, dexamethasone, that can be readily incorporated into LNP systems. We show that the presence of the dexamethasone prodrug LD003 effectively suppresses production of cytokines such as KC-GRO, TNFα, IL-1β and IL-6 following intravenous administration of LNP loaded with immune stimulatory oligodeoxynucleotides containing cytosine-guanine dinucleotide motifs. Remarkably, LD003 dose levels corresponding to 0.5 mg/kg dexamethasone achieve a greater immunosuppressive effect than doses of 20 mg/kg of free dexamethasone. Similar immunosuppressive effects are observed for subcutaneously administered LNP-siRNA. Further, the incorporation of low levels of LD003 in LNP containing unmodified mRNA or plasmid DNA significantly reduced pro-inflammatory cytokine levels following intravenous administration. Our results suggest that incorporation of hydrophobic prodrugs such as LD003 into LNP systems could provide a convenient method for avoiding the immunostimulatory consequences of systemic administration of genetic drug formulations.

1. Introduction

Nucleic acid-based therapeutics such as antisense oligodeoxynucleotides (ASO) or short-interfering RNA (siRNA) for gene silencing, and messenger RNA (mRNA) or plasmid DNA (pDNA) for gene expression can be potent inducers of the innate immune response in vertebrates [1, 2]. Therapeutic use of nucleic acid-based macromolecules often requires sophisticated delivery vehicles, which can further exacerbate this response [3–5]. Lipid nanoparticles (LNP) that contain ionizable amino-lipids are the most clinically advanced delivery system for nucleic acid therapeutics [6]. LNP systems can give rise to “flu-like” symptoms and hypotension from the activation of toll-like receptors and increases in serum cytokine levels even when they contain a payload (e.g. siRNA) that has been engineered to minimize immunostimulatory potential [7–9]. Prophylactic administration of corticosteroids reduces LNP-siRNA mediated immune stimulation [8], but breakthroughs can occur for a variety of nanoparticle formulations [10, 11]. Furthermore, while siRNA can be designed and synthesized *in silico* with modifications that reduce immune stimulation, larger nucleic acids such as mRNA or pDNA are more difficult to modify chemically. Immune responses can also result in the rapid clearance of subsequent administrations of LNP [12–17], greatly limiting the utility of such formulations. Thus, the potential immunostimulatory properties of LNP and other formulations of genetic drugs are a major challenge for clinical advancement of gene therapies in general.

Dexamethasone is a potent synthetic corticosteroid used for the treatment of a number of inflammatory and autoimmune conditions such as Crohn's disease, asthma, ulcerative colitis, rheumatoid arthritis and immune thrombocytopenia. It has also been used for certain hematological malignancies [18, 19], and prophylactically to reduce inflammatory responses to therapeutic treatments such as antibiotics and some chemotherapeutics [20, 21]. It has been shown previously that co-administration of dexamethasone with LNP greatly reduces immune stimulation [8, 22]. Here, we demonstrate that the incorporation of a dexamethasone prodrug directly into LNP containing various types of nucleic acid cargos can greatly reduce the level of pro-inflammatory

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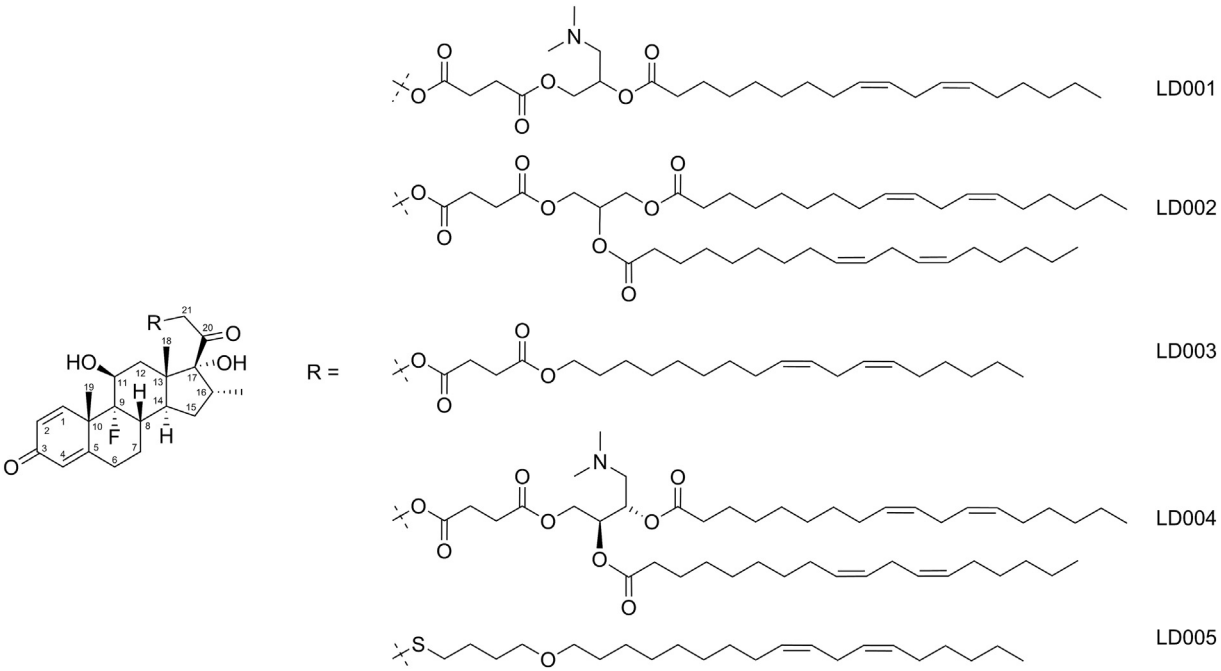

Fig. 1. Structures of lipophilic dexamethasone prodrugs. Dexamethasone prodrugs of varying hydrophobicity were synthesized. LD001-LD004 contain a succinate linker between dexamethasone and either one or two C18 hydrocarbon moieties. LD001 and LD004 also contain a tertiary amine group. LD005 is composed of dexamethasone conjugated with a single C18 hydrocarbon chain via an ether linkage.

Table 1
Prodrug and lipid nanoparticle parameters.

Prodrug	Predicted LogP (or LogD)[a]	Particle diameter (nm)	PdI	% CpG ASO entrapment	% Prodrug entrapment
LD001	5.1/7.6	46	0.08	100%	~ 40
LD002	15.0	46	0.09	100%	> 95
LD003	8.9	49	0.06	99%	> 95
LD004	11.6/14.0	48	0.04	98%	~ 60
LD005	10.4	50	0.06	99%	> 95

[a] LogD predicted at two pH values (pH 4/pH 7.4).

cytokines induced by the LNP. In order to incorporate dexamethasone directly into the particle, lipophilic acyl/alkyl moieties were chemically conjugated to dexamethasone via biodegradable linkers. Various dexamethasone prodrugs were synthesized and tested to better understand the requirements for effective incorporation within the LNP. Finally, we show these prodrugs ameliorate pro-inflammatory cytokine induction using LNP that contain ASO, siRNA, mRNA or plasmid DNA.

2. Material and methods

2.1. Materials

1,2-Distearoyl-sn-glycero-3-phosphocholine (DSPC) was purchased from Avanti Polar Lipids (Alabaster, AL) and cholesterol was purchased from Sigma-Aldrich (St. Louis, MO). Amino-lipids 3-(dimethylamino) propyl(12Z,15Z)-3-[(9Z,12Z)-octadeca-9,12-dien-1-yl]henicosa-12,15-dienoate (DMAP-BLP), (6Z,9Z,28Z,31Z)-Heptatriaconta-6,9,28,31-tetraen-19-yl4-(dimethylamino)butanoate (DLin-MC3-DMA) and 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) were synthesized by BioFine International (Vancouver, BC) [23, 24]. (*R*)-2,3-bis(octadecyloxy)propyl-1-(methoxy polyethylene glycol 2000) carbamate (PEG-DMG) and (*R*)-2,3-bis(stearyloxy)propyl-1-(methoxy poly(ethylene glycol)2000 carbamate (PEG-DSG) were synthesized as previously described [25]. Phosphorothioated unmethylated cytosine-guanine containing oligodeoxynucleotide (CpG) complementary to the initiation codon region of the human/mouse c-myc proto-oncogene (5'-AACGTTGAGGGGCAT-3′) was purchased from TriLink Biotechnologies (San Diego, CA). This sequence has been previously identified as highly immunostimulatory when administered to mice due to the presence of the CpG dinucleotide motif [5, 15, 26, 27]. The sense and antisense sequences of siRNA targeting coagulation factor VII (siFVII) are 5′-GGAucAucucAAGucuuAcT*T-3′ and 5′-GuAAGAcuuGAGAuGAuccT*T-3, respectively with 2'fluoro-modified nucleotides represented in lower case and phosphorothioate linkages by asterisks. siFVII was synthesized by Integrated DNA Technologies (Coralville, IA) [25, 28]. *In vitro* transcription of firefly luciferase mRNA was carried out as described previously [29]. pGL4.51 plasmid DNA (Promega Madison, WI) was prepared using a Qiagen EndoFree Plasmid Giga Kit as per manufacturer's instructions (Germantown, MD).

2.2. Synthesis of lipophilic-dexamethasone prodrugs

Detailed description of the synthesis of dexamethasone prodrugs LD001-LD005 can be found in Supplemental Methods.

2.3. Preparation of lipid nanoparticles

LNP containing various types of nucleic acids (CpG, siRNA, mRNA, pDNA) were prepared by rapid mixing through a T-junction mixer as previously described [30, 31]. Briefly, amino-lipid, DSPC, cholesterol and PEG-DMG were dissolved in ethanol at the mole ratio of 50, 10, 38.5 and 1.5 respectively. Dexamethasone prodrugs were incorporated at 1, 4 or 10 mol% at the expense of amino-lipid. Amino-lipids were chosen based on previous work. For CpG and siRNA, DMAP-BLP [23, 28, 32] was used while DLin-MC3-DMA [24] and DLin-KC2-DMA [33] were used for the delivery or mRNA and pDNA, respectively. Nucleic acids were dissolved in 25 mM acetate buffer at pH 4.0 such that the final mixture would have a defined nucleic acid to lipid weight/μmol ratios (0.056 for CpG and siRNA, 0.028 for mRNA and pDNA). Ethanolic and aqueous mixtures were mixed together at a 1:3 volume and flow rate ratio with final flow rates > 10 mL/min at room temperature. Resulting mixtures containing 25% ethanol were dialyzed against

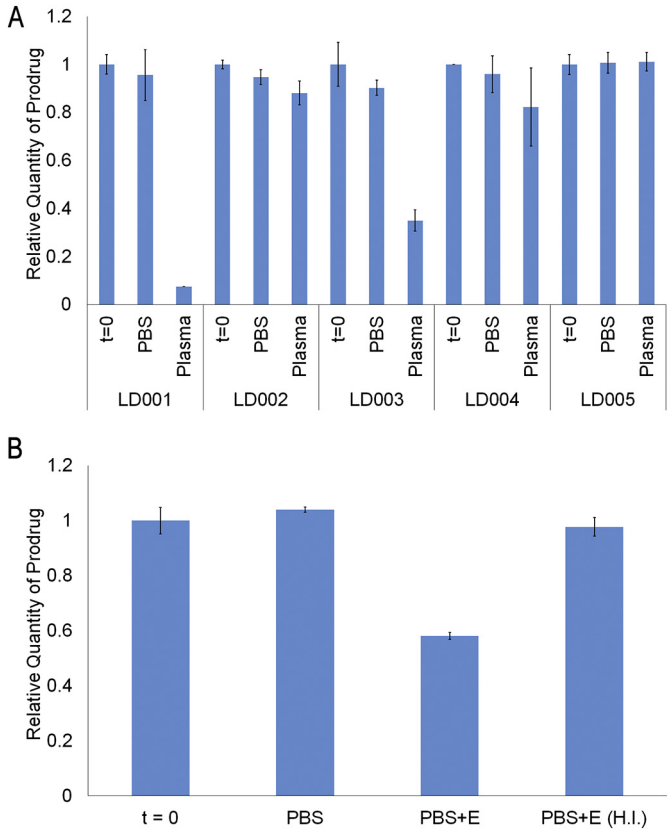

Fig. 2. Dexamethasone prodrugs containing a succinate linker are degradable by esterases. Dexamethasone prodrugs were formulated into LNP containing nucleic acid and incubated with esterases. The amount of intact prodrug was determined by UPLC analysis and the data was normalized to amounts in pre-incubation mixtures ($t = 0$). A) Dexamethasone prodrugs (LD001-LD005) in LNP were incubated in mouse plasma for 1 h at 37 °C. (B) LD003 containing LNP and incubated for 4 h at 37 °C with either 10 U of purified porcine esterase (PBS + E) or heat inactivated porcine esterase (H.I.). Error bars represent ± s.d. of at least three replicates.

phosphate buffered saline (PBS) overnight. Particle size was determined by dynamic light scattering (Malvern Zetasizer NanoZS, Worcestershire, UK). CpG concentration and entrapment efficiency were determined by measuring the absorbance at 260 nm after lipids were extracted using the Bligh and Dyer method [34]. siRNA and mRNA concentrations were determined using the Quant-iT RiboGreen RNA Assay while pDNA concentrations were determined using the Quanit-iT PicoGreen dsDNA Assay according to manufacturer's protocols (ThermoFisher, Waltham, MA). LNP exhibiting < 85% nucleic acid entrapment were not used for further experimentation. Lipid concentrations were determined by measuring the cholesterol content (Cholesterol E Assay, Wako Chemicals, Richmond, VA).

2.4. In vitro degradation of prodrugs

To determine the biodegradability of the LD001–005, 1 mg/mL LNP was incubated in mouse plasma (Cedarlane, Burlington, Ontario) or PBS supplemented with 10 U of purified porcine esterase (Sigma-Aldrich, St. Louis, MO) for up to 4 h at 37 °C. Post incubation, four volumes of chloroform/methanol (2:1) were added and the mixture was vortex mixed. Samples were centrifuged at 13,000*g* for 5 min and the upper phase was discarded. The remaining organic phase was dried down under vacuum and the resulting lipid extract was dissolved in methanol/acetonitrile (1:1). Quantity of parent dexamethasone prodrug was determined by ultra high performance liquid chromatography (UHPLC) on a Waters Acquity H-Class UHPLC System equipped with a BEH C18 column (1.7 µm, 2.1 × 100 mm) and a photodiode array detector. Separation was achieved at a flow rate of 0.5 mL/min, with a mobile phase consisting of a linear methanol-water gradient (85:15 to 100:0) over 5 min at a column temperature of 55 °C. The absorbance at 239 nm was measured and the analyte concentration was determined using calibration curves.

2.5. In vitro LNP tolerability by MTT and hemolysis

In vitro LNP tolerability was determined by performing an MTT cell proliferation assay on cultured HeLa cells. Briefly, 10,000 cells/well were seeded in 96-well plates, grown overnight and treated with various concentrations of LNP (5×10^{-7} to 5 mg/mL, with or without prodrug) for 24 h. Cells were then incubated with 1 mg/mL of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO) in 100 µL of complete media for 2 h followed by an overnight incubation with 100 µL of 20% sodium dodecyl sulfate dissolved in dimethylformamide/water (1:1) at pH 4.7 at 37 °C. Absorbance at 570 nm was measured after the overnight incubation and % viability was determined by normalizing the absorbance of LNP treatment with PBS controls.

The ability of LNP to cause hemolysis was assessed as follows. Briefly, human erythrocytes (RBC) (Innovative Research, Novi, MI) were washed three times with cold isotonic saline (0.9% *w/v* NaCl in water) and resuspended in pH 7.4 PBS to a final concentration of 4% vol/vol. LNP (with or without LD003) were then incubated with the RBC solution for 1 h at 37 °C. RBC solutions incubated with saline or 0.2% vol/vol Triton X-100 were used as controls. Samples were then cooled and centrifuged for 5 min at 1000 ×*g* at 4 °C and the supernatant was removed and the absorbance at 562 nm was determined. % hemolysis was determined by normalizing the absorbance at 562 nm to Triton X-100 treated samples.

2.6. In vivo immune suppression by prodrugs

6–8 weeks old female C57Bl/6 mice (Charles River Laboratories, Wilmington, MA) were injected intravenously with LD-LNP containing CpG (10 mg/kg), pDNA (1 mg/kg) or mRNA (3 mg/kg). Animals were euthanized 2 and/or 4 h post-injection and blood was collected via intracardiac sampling. Plasma was separated from whole blood by centrifugation and analyzed for proinflammatory cytokines using the Mesoscale Proinflammatory Multiplex kit (Rockville, MD) or the custom Mouse Magnetic Luminex Assay (R&D Systems, Minneapolis, MN). For histological demonstration of immune suppression, C57Bl/6 mice were injected subcutaneously in the interscapular space with LNP or LD003-LNP containing siRNA (5 mg/kg) and reinjected with the same formulation 7 days later. Mice were euthanized 10 days after the initial injection and the interscapular tissue at the injection site was collected, sectioned and stained with hematoxylin and eosin (Wax-it, Vancouver BC). Control dexamethasone (dexamethasone-21-phosphate; Sigma-Aldrich, St. Louis, MO) treated mice were injected 30 min prior to the injection of LNP. All procedures were approved by the Animal Care Committee at the University of British Columbia and were performed in accordance with guidelines established by the Canadian Council on Animal Care.

2.7. In vivo gene silencing in the mouse FVII model

6–8 weeks old female C57Bl/6 were injected intravenously with LD-LNP containing siRNA targeting the mouse FVII gene at siRNA doses of 0.03, 0.1 and 0.3 mg/kg. Animals were euthanized 24 h post injection and blood was collected via intracardiac sampling. Serum was separated from the whole blood and FVII quantity was determined using the Biophen VII assay (Aniara, Mason, OH) according to manufacturer's protocol. All procedures were approved by the Animal Care Committee at the University of British Columbia and were performed in accordance

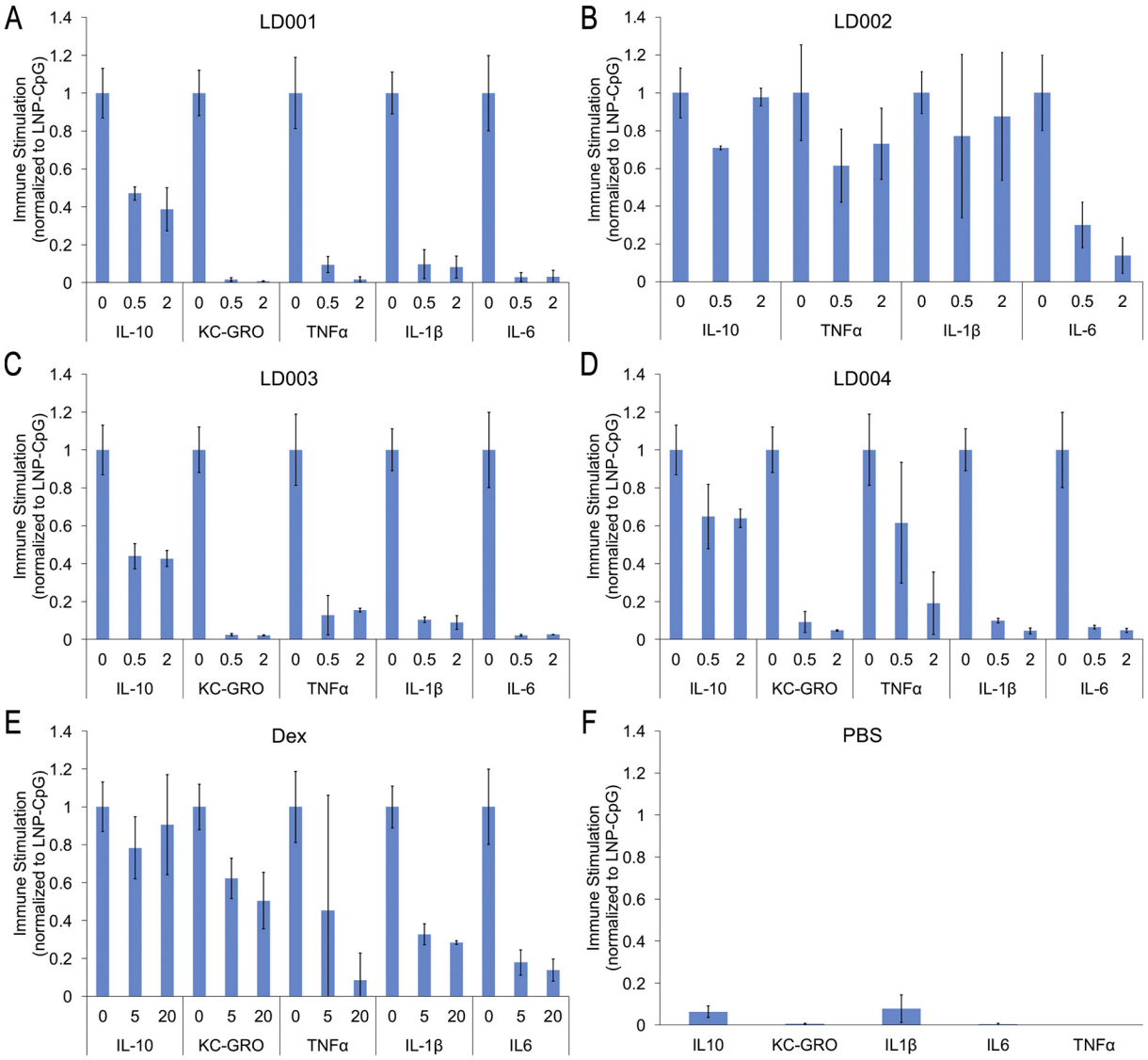

Fig. 3. Incorporation of dexamethasone prodrugs can dramatically suppress pro-inflammatory cytokine production in mice treated with LNP-CpG. Mice were injected with LNP-CpG at doses corresponding to 10 mg/kg CpG oligonucleotide. (A-D) Dexamethasone prodrugs (LD001–004) were formulated into LNP-CpG corresponding to 0.5 and 2 mg/kg dose of dexamethasone. Blood was collected after 2 h and the levels of IL-10, KC-GRO, TNFα, IL-1β, and IL-6 were measured in the plasma. (E) Cytokine levels observed for mice receiving free dexamethasone co-injected with LNP-CpG (10 mg/kg) at 0, 5 and 20 mg/kg dexamethasone dose levels. (F) Cytokine levels observed for mice injected with PBS. Data is normalized to the cytokine levels measured for mice treated with LNP-CpG. Error bars represent ± s.d. using four animals.

with guidelines established by the Canadian Council on Animal Care.

3. Results and discussion

Nucleic acid based therapeutics has potential utility for vaccines, protein expression, gene silencing and gene editing [35–37]. However, nucleic acid polymers can be potent activators of the innate immune response [2], which can potentially be ameliorated by chemical modifications [38, 39]. While such modifications are feasible for smaller oligonucleotides such as siRNA and ASO, they are less straightforward for larger constructs such as pDNA or mRNA. Furthermore, delivery of nucleic acids often requires complex nanoparticle delivery vehicles such as LNP that can further enhance immune stimulation. As a result, co-administration of potent immunosuppressive agents such as dexamethasone, which has been used prophylactically to reduce inflammatory responses to antibiotics and chemotherapeutics [40], is often used in conjunction with LNP formulations of siRNA [8, 22].

Given that LNP systems accumulate in phagocytic cells of the immune system following systemic administration [41, 42], direct association of dexamethasone with LNP offers the possibility of delivering the drug more specifically to immune cells, potentially lowering the dose required and simplifying clinical use. To test this, we synthesized five hydrophobic derivatives of dexamethasone (LD001-005) by attaching hydrocarbon moieties via a biodegradable succinate linker to the hydroxyl group located on carbon 21 of dexamethasone (Fig. 1). The compounds were synthesized with one or two hydrocarbon chains, with some including an ionizable tertiary amine specifically to mimic the amino-lipids that are routinely used to encapsulate nucleic acids in LNP [24, 31]. Compound LD001 contains a single 18-carbon (C18) hydrocarbon moiety and an ionizable amine, whereas LD002 contains two C18 hydrocarbon chains and no ionizable group. LD003 contains a single C18 hydrocarbon moiety, while LD004 contains two C18 hydrocarbon moieties and an ionizable amine. LD005, which closely resembles LD003 as it contains a single C18 chain and no ionizable function, does not have a biodegradable succinate linker or ester bonds, thereby serving as a negative control.

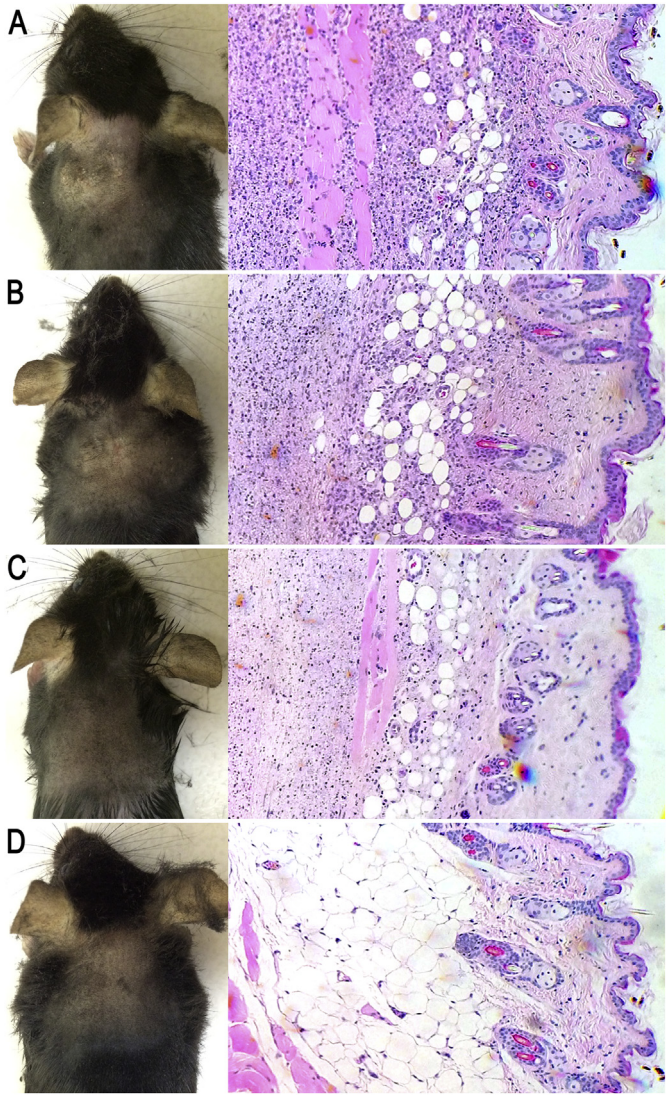

Fig. 4. Incorporation of dexamethasone prodrug LD003 reduces immune cell infiltration at the injection site following subcutaneous administration of LNP-siRNA. (A) C57Bl/6 mice were injected s.c. on day 1 and day 7 with LNP-siRNA (80 nm diameter) at an siRNA dose of 5 mg/kg. (B) 30 min prior to the injection of LNP-siRNA, animals were injected i.p. with 5 mg/kg dexamethasone. (C) The injected LNP-siRNA systems contained 10 mol% of LD003. (D) Control animals injected with PBS. LNP-siRNA was re-administered 7 days after the initial injection to mimic the treatment regimen necessary to maintain gene silencing. Mice were euthanized after a total of 10 days of treatment and the inter-scapular space was collected, sectioned and stained. Representative images from four mice per treatment group are shown.

The physical properties and formulation characteristics of LD001–005 prodrugs are summarized in Table 1. The relative hydrophobicity of the compounds is reflected by their predicted octanol-water partitioning coefficient, logP or logD values (for non-ionizable or ionizable compounds, respectively). The logP (or logD) values ranged from 5.1 for LD001 to 15.0 for LD002. As shown in Table 1, all LNP formulations containing 10 mol% of dexamethasone prodrug have particle diameters of approximately 50 nm with polydispersity indices (PdI) < 0.1. This indicates that incorporation of the prodrugs did not significantly affect LNP size or homogeneity. Essentially complete entrapment (routinely over 95% entrapment) of LD002, LD003 and LD005 was observed, whereas somewhat lower trapping efficiencies were observed for the prodrugs containing the ionizable amines (LD001 and LD004). Therefore, the likelihood of entrapment cannot be predicted by hydrophobicity alone, and the ionizable moiety may affect entrapment. This is not surprising since LNP are formed by the rapid mixing of lipids dissolved in ethanol with a low pH buffer to entrap nucleic acid, where the ionizable tertiary amine is charged. The prodrug containing a charged amine group may be more water soluble, thus reducing its incorporation into the hydrophobic core of the LNP.

Since the biological activity of LNP-associated prodrugs requires the release of the active dexamethasone compound, prodrugs must degrade in esterase-rich environments. To ascertain potential bioavailability, LNP containing the five prodrugs were incubated with mouse plasma (which has high esterase activity [43, 44]) and the amount of intact prodrug was determined at various times. As shown in Fig. 2A, incubation for 1 h resulted in degradation of approximately 90% and 70% of LNP-associated LD001 and LD003, respectively. Little degradation was observed for LNP containing LD002, LD004 or LD005. As LD005 does not contain any biodegradable ester linkages, no degradation was expected; however, the lack of degradation of LD002 and LD004 suggests that these derivatives are less accessible to esterases. This could be due to the higher hydrophobicity of these compounds, which may cause the prodrug to partition more deeply into the hydrophobic LNP interior. To demonstrate that degradation can be attributed to esterase activity, LNP containing LD003 were incubated with purified porcine esterase (Fig. 2B). Appreciable degradation was observed after 4 h and this degradation was abolished by heat inactivation of the enzyme.

In order to assess whether the incorporation of prodrugs impact LNP tolerability, the effect of LNP treatment on HeLa cell viability and the ability of LNP to lyse red blood cells (RBC) were investigated. LNP were very well tolerated over the concentration range of 0.5 ng/mL to 5 mg/mL total lipid (Supplemental Fig. 1A). When 10 mol% of LD003 was incorporated into the LNP, no change in cell viability was observed, indicating that LNP containing hydrophobic prodrugs are well tolerated. Typically, < 50 μg/mL of lipid is necessary for LNP-siRNA mediated gene silencing in cultured cells [45]. Furthermore, at lipid concentrations as high as 2 mg/mL, no appreciable hemolysis was observed for LNP with or without LD003 (Supplemental Fig. 1B). A concentration of 2 mg/mL lipid is equivalent to a lipid dose of 140 mg/kg assuming a 25-g mouse has 70 mL/kg blood.

LNP-mediated immune stimulation is exacerbated when the cargo is highly immunogenic [3–5]. We have previously shown that ASO containing unmethylated cytosine-guanine dinucleotide motifs (CpG) and targeting the initiation codon region of the human/mouse c-myc proto-oncogene is highly immunostimulatory [5, 15], and can serve as an effective vaccine immune adjuvant when encapsulated in LNP (LNP-CpG) [26]. We investigated the ability of dexamethasone prodrugs to suppress LNP-induced immune stimulation by preparing LNP containing CpG ASO and various amounts and types of dexamethasone prodrugs. Mice were injected with LNP-CpG with or without dexamethasone prodrugs at a dose level of 10 mg CpG/kg body weight, and blood was collected after 2 and 4 h for plasma cytokine measurement. Dexamethasone (free drug) was also co-administered with LNP-CpG at doses of 5 and 20 mg/kg for comparison.

The ability of the dexamethasone prodrugs and free dexamethasone to ameliorate the immunostimulatory effects of LNP-CpG are shown in Fig. 3. Animals injected with LNP-CpG alone had elevated levels of plasma cytokines (IL-10, KC-GRO, TNFα, IL-1β, IL-6) 2 h post-administration. As shown in Fig. 3 A-D, incorporation of prodrugs at the equivalent free dexamethasone doses of 0.5 mg/kg and 2 mg/kg resulted in substantially reduced cytokine levels. For LD001 and LD003, as little as 0.5 mg/kg dexamethasone equivalent resulted in over 90% inhibition of KC-GRO, TNFα, IL-1β and IL-6 production. These levels are close to background levels (Fig. 3F). Equally impressive immunosuppressive effects were observed at 4 h (Supplemental Fig. 2). The immunosuppressive potencies of LD001 and LD003 are particularly notable when contrasted with the effects of free dexamethasone. As shown in Fig. 3E, a dose of dexamethasone at 20 mg/kg resulted in markedly less cytokine suppression as compared to 0.5 mg/kg LD001

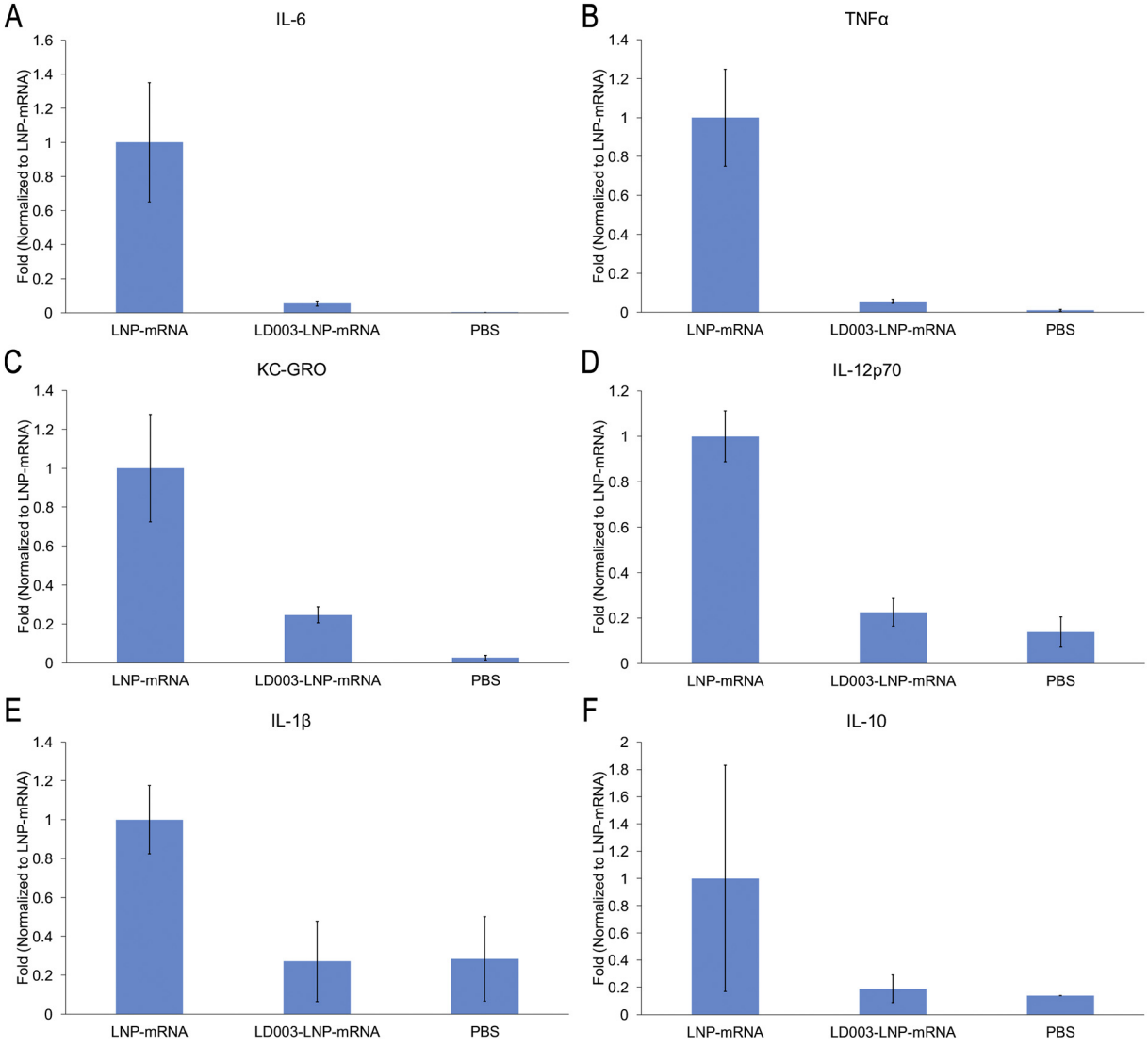

Fig. 5. Incorporation of LD003 in LNP-mRNA systems ameliorates LNP-mRNA mediated immune stimulation. LNP-mRNA with or without 10 mol% LD003 was formulated and injected i.v. at an mRNA dose of 3 mg/kg. Plasma was collected after 2 h and the amount of (A) IL-6, (B) TNFα, (C) KC-GRO, (D) IL-12p70, (E) IL-1β and (F) IL-10 was measured. Data is normalized to animals injected with LNP-mRNA and error bars represent the mean ± s.d. of four animals.

(Fig. 3A) or LD003 (Fig. 3C) incorporated into the LNP. This suggests that co-delivery of dexamethasone with the immune stimulating nucleic acid to the exact same cells is more effective than global immune suppression using free dexamethasone. As expected, incorporation of the non-degradable LD005 into LNP-CpG formulations did not result in immunosuppressive effects (Supplemental Fig. 3).

In contrast to CpG-containing ASO, which are known to stimulate the immune system, siRNA are usually chemically modified to evade immune detection [3–5]. However, despite having extensive chemical modifications, siRNA delivered by LNP can still elicit immune reactions [7, 22, 46–48]. In mice, we have shown that injection of 80 nm LNP containing modified siRNA in the interscapular space resulted in skin lesions forming at the injection site [32]. This local reaction was ascribed to an immune reaction to the LNP-siRNA, thus the use of immunosuppressants could possibly ameliorate this response. As shown in Fig. 4B, administration of dexamethasone prior to the injection of 80 nm LNP-siRNA in the interscapular space, did not reduce the visible irritation caused by LNP buildup as compared to control (Fig. 4A). In contrast, considerable improvements were observed when mice were injected with LNP-siRNA containing 10 mol% of LD003 (Fig. 4C left). In addition to the visible irritation, the loose interscapular space for animals injected with LNP became very rigid for animals injected with LNP-siRNA or LNP-siRNA with free dexamethasone while the skin remained loose for mice injected with LD003-LNP or PBS. Isolation, sectioning and staining of the injection site revealed that the cell density around the injection site was visibly higher for animals injected with LNP that did not contain prodrug (Fig. 4 A and B). This is likely a result of immune cell infiltration. The cell density is visibly reduced for animals injected with LD003-LNP (Fig. 4C right), although still greater than control animals injected with PBS (Fig. 4D right). In order to verify that the ability of LNP to deliver nucleic acids is not compromised by the addition of prodrugs, the *in vivo* efficacy of LNP-siRNA was tested using the well-established mouse FVII model [28, 31]. Inclusion of 10 mol% of either LD002 or LD003 did not impact the gene silencing ability of LNP-siRNA (Supplemental Fig. 4).

The immunosuppressive effects of LD003 for LNP containing short nucleic acids such as CpG ASO or siRNA suggest potential utility to render LNP formulations of nucleic acids less immunostimulatory. It is of interest to determine whether these immunosuppressive qualities extend to larger nucleic acid polymers such as pDNA or mRNA. LNP-mRNA formulations are showing considerable therapeutic promise [49], and the potential for avoiding a need for modifications to reduce

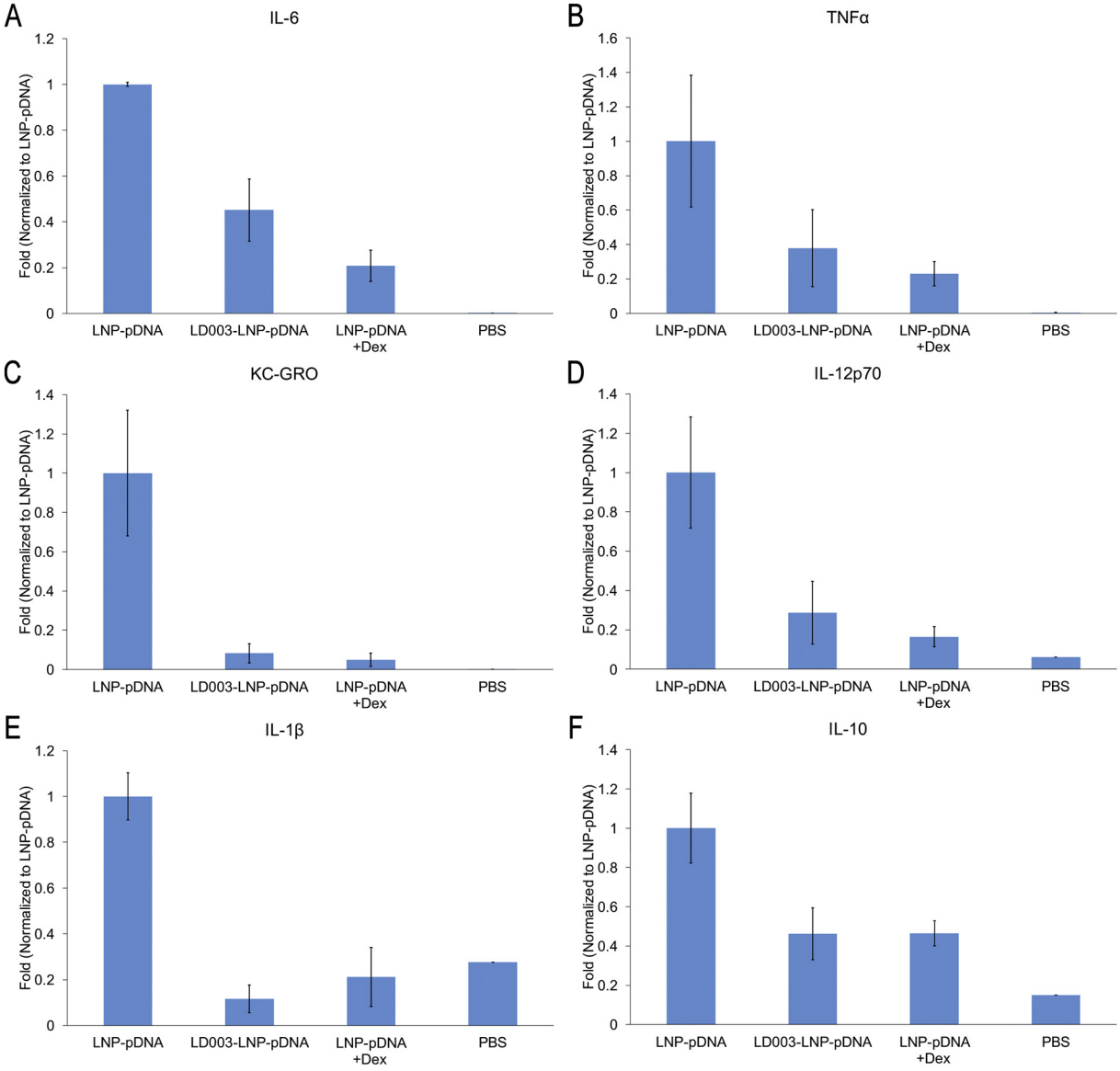

Fig. 6. Incorporation of LD003 ameliorates LNP-pDNA mediated immune stimulation. LNP-pDNA with or without 10 mol% LD003 was formulated and injected at a pDNA dose of 1 mg/kg. Free dexamethasone was administered at 5 mg/kg intraperitoneally 30 min prior to LNP injection. Plasma was collected after 4 h and the amount of (A) IL-6, (B) TNFα, (C) KC-GRO, (D) IL-12p70, (E) IL-1β and (F) IL-10 was measured. Data is normalized to animals injected with LNP-pDNA and error bars represent the mean ± s.d. of four animals.

the immunostimulatory potential [50–54] would significantly simplify mRNA synthesis. In order to test the effectiveness of dexamethasone prodrugs for preventing LNP-mRNA mediated immune activation, an unmodified 1.7 kb mRNA coding for firefly luciferase was formulated into LNP containing 0 or 10 mol% LD003 and injected into mice at an mRNA dose of 3 mg/kg. Blood was collected 2 h post-injection and plasma cytokine levels were determined. As shown in Fig. 5, animals that were treated with LNP-mRNA containing LD003 showed greatly reduced cytokine production (IL-6, TNFα, KC-GRO, IL-12p70, IL-1β, and IL-10) than animals treated with LNP-mRNA without prodrug.

In addition to mRNA, significant efforts are being made to employ pDNA for therapeutic use because of the inherent stability of double stranded DNA, durability of gene expression and the potential to use tissue-specific promoters. However, pDNA can be highly immunostimulatory, and this can be exacerbated by endotoxin contamination due to plasmid isolation from *E. coli* [55, 56]. LNP-pDNA are effective transfection agents in cultured cells and *in vivo* [33] but little attention has been given to the immune stimulatory potential of these systems. To test the effectiveness of dexamethasone prodrugs in preventing LNP-pDNA mediated immune activation, mice were injected with LNP systems encapsulating luciferase pDNA that contained 0 or 10 mol% of LD003. Administration of LNP-pDNA in mice at a pDNA dose of 1 mg/kg resulted in significantly elevated levels of IL-6, TNFα, KC-GRO, IL-12p70, IL-1β, and IL-10 compared to control animals injected with PBS (Fig. 6). When 10 mol% of LD003 was incorporated into the LNP-pDNA, plasma cytokine levels were reduced to levels similar to animals treated with dexamethasone prior to LNP-pDNA treatment.

To determine whether lower amounts of LD003 prodrug could suppress LNP-pDNA mediated immune stimulation, 0, 1, 5 and 10 mol% of LD003 was incorporated into LNP-pDNA and injected into mice at a pDNA dose of 1 mg/kg body weight (Fig. 7). We found that as little as 1 mol% of LD003 can suppress immune stimulation, while little additional benefit was observed when 5 or 10 mol% was used. This is consistent with the observation that low levels of dexamethasone prodrug are required to suppress LNP-CpG mediated immune stimulation and shows that co-entrapment of the immunosuppressant with the

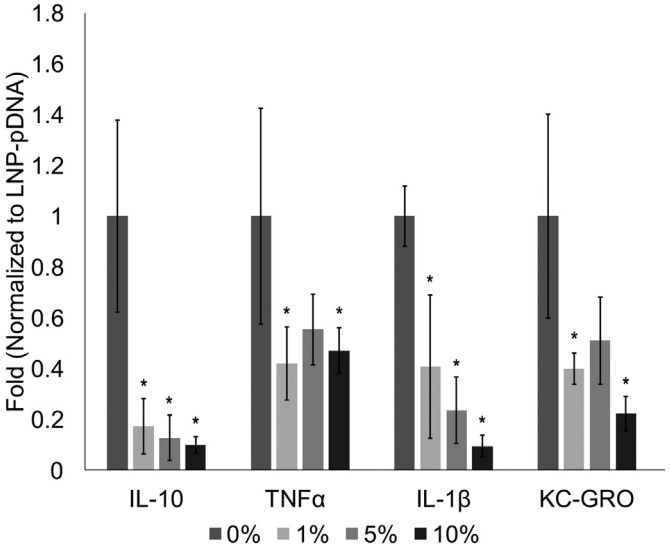

Fig. 7. Low amounts of LD003 can suppress immune stimulation by LNP-pDNA systems. LNP-pDNA systems containing 0–10 mol% of LD003 were formulated and injected at a pDNA dose of 1 mg/kg. Plasma was collected after 4 h and levels of representative cytokines (IL-10, TNFα, IL-1β, KC-GRO) were measured. Data is normalized to animals injected with LNP-pDNA that did not contain LD003 and error bars represent the mean ± s.d. of four animals. Statistical significance determined using multiple *t*-tests ($^{*}p < 0.05$).

immunostimulatory payload can result in greater immunosuppression as compared to free dexamethasone.

4. Conclusion

LNP are the most advanced non-viral systems available to enable gene therapies; however, their immunostimulatory properties remain a concern. Here we show that incorporation of a dexamethasone prodrug can greatly reduce the immune stimulation by LNP containing nucleic acid polymers. As little as 0.5 mg/kg of dexamethasone equivalent as a prodrug in LNP results in essentially complete suppression of pro-inflammatory cytokines such as KC-GRO, TNFα, IL-1β and IL-6 in the immunostimulatory LNP-CpG model (Fig. 3). The level of suppression observed is far superior compared to co-administration of 5 or 20 mg/kg free dexamethasone. Because such small quantities of dexamethasone are required in the form of the prodrugs, we expect that the adverse side effects associated with high doses of corticosteroids can also be avoided [57]. This enhanced potency is likely the result of preferential uptake of LNP systems by phagocytic immune cells [41, 42]. These findings extend to other nucleic acid payloads such as siRNA, mRNA and pDNA, suggesting broad utility for other nucleic acid based therapeutics as well as other immune stimulatory agents such as proteins.

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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.2018.07.026.

References

[1] H. Sakurai, K. Kawabata, F. Sakurai, S. Nakagawa, H. Mizuguchi, Innate immune response induced by gene delivery vectors, Int. J. Pharm. 354 (2008) 9–15.
[2] R. Barbalat, S.E. Ewald, M.L. Mouchess, G.M. Barton, Nucleic acid recognition by the innate immune system, Annu. Rev. Immunol. 29 (2011) 185–214.
[3] S.W. Dow, L.G. Fradkin, D.H. Liggitt, A.P. Willson, T.D. Heath, T.A. Potter, Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously, J. Immunol. 163 (1999) 1552–1561.
[4] M. Whitmore, S. Li, L. Huang, LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth, Gene Ther. 6 (1999) 1867–1875.
[5] B. Mui, S.G. Raney, S.C. Semple, M.J. Hope, Immune stimulation by a CpG-containing oligodeoxynucleotide is enhanced when encapsulated and delivered in lipid particles, J. Pharmacol. Exp. Ther. 298 (2001) 1185–1192.
[6] P.R. Cullis, M.J. Hope, Lipid nanoparticle Systems for Enabling Gene Therapies, Mol. Ther. 25 (7) (2017) 1467–1475 Jul 5.
[7] S.A. Barros, J.A. Gollob, Safety profile of RNAi nanomedicines, Adv. Drug Deliv. Rev. 64 (2012) 1730–1737.
[8] M.T. Abrams, M.L. Koser, J. Seitzer, S.C. Williams, M.A. Dipietro, W. Wang, A.W. Shaw, X. Mao, V. Jadhav, J.P. Davide, P.A. Burke, A.B. Sachs, S.M. Stirdivant, L. Sepp-Lorenzino, Evaluation of efficacy, biodistribution, and inflammation for a potent siRNA nanoparticle: effect of dexamethasone co-treatment, Mol. Ther. 18 (2010) 171–180.
[9] V. Kumar, J. Qin, Y. Jiang, R.G. Duncan, B. Brigham, S. Fishman, J.K. Nair, A. Akinc, S.A. Barros, P.V. Kasperkovitz, Shielding of lipid nanoparticles for siRNA delivery: impact on physicochemical properties, Cytokine induction, and efficacy, Mol. Ther. Nucleic Acids 3 (2014) e210.
[10] J. Szebeni, P. Bedocs, Z. Rozsnyay, Z. Weiszhar, R. Urbanics, L. Rosivall, R. Cohen, O. Garbuzenko, G. Bathori, M. Toth, R. Bunger, Y. Barenholz, Liposome-induced complement activation and related cardiopulmonary distress in pigs: factors promoting reactogenicity of Doxil and AmBisome, Nanomedicine 8 (2012) 176–184.
[11] J. Szebeni, Complement activation-related pseudoallergy: a stress reaction in blood triggered by nanomedicines and biologicals, Mol. Immunol. 61 (2014) 163–173.
[12] K.A. Whitehead, J.E. Dahlman, R.S. Langer, D.G. Anderson, Silencing or stimulation? siRNA delivery and the immune system, Annu. Rev. Chem. Biomol. Eng. 2 (2011) 77–96.
[13] M. Robbins, A. Judge, E. Ambegia, C. Choi, E. Yaworski, L. Palmer, K. McClintock, I. MacLachlan, Misinterpreting the therapeutic effects of small interfering RNA caused by immune stimulation, Hum. Gene Ther. 19 (2008) 991–999.
[14] A.D. Judge, V. Sood, J.R. Shaw, D. Fang, K. McClintock, I. MacLachlan, Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA, Nat. Biotechnol. 23 (2005) 457–462.
[15] S.C. Semple, T.O. Harasym, K.A. Clow, S.M. Ansell, S.K. Klimuk, M.J. Hope, Immunogenicity and rapid blood clearance of liposomes containing polyethylene glycol-lipid conjugates and nucleic acid, J. Pharmacol. Exp. Ther. 312 (2005) 1020–1026.
[16] T. Tagami, K. Nakamura, T. Shimizu, N. Yamazaki, T. Ishida, H. Kiwada, CpG motifs in pDNA-sequences increase anti-PEG IgM production induced by PEG-coated pDNA-lipoplexes, J. Control. Release 142 (2010) 160–166.
[17] T. Tagami, Y. Uehara, N. Moriyoshi, T. Ishida, H. Kiwada, Anti-PEG IgM production by siRNA encapsulated in a PEGylated lipid nanocarrier is dependent on the sequence of the siRNA, J. Control. Release 151 (2011) 149–154.
[18] H. Inaba, C.-H. Pui, Glucocorticoid use in acute lymphoblastic leukemia: comparison of prednisone and dexamethasone, Lancet Oncol. 11 (2010) 1096–1106.
[19] O. Teuffel, S.P. Kuster, S.P. Hunger, V. Conter, J. Hitzler, M.C. Ethier, P.S. Shah, J. Beyene, L. Sung, Dexamethasone versus prednisone for induction therapy in childhood acute lymphoblastic leukemia: a systematic review and meta-analysis, Leukemia 25 (2011) 1232–1238.
[20] W.W. Chatham, R.P. Kimberly, Treatment of lupus with corticosteroids, Lupus 10 (2001) 140–147.
[21] T. Rhen, J.A. Cidlowski, Antiinflammatory action of glucocorticoids — new mechanisms for old drugs, N. Engl. J. Med. 353 (2005) 1711–1723.
[22] T. Coelho, D. Adams, A. Silva, P. Lozeron, P.N. Hawkins, T. Mant, J. Perez, J. Chiesa, S. Warrington, E. Tranter, M. Munisamy, R. Falzone, J. Harrop, J. Cehelsky, B.R. Bettencourt, M. Geissler, J.S. Butler, A. Sehgal, R.E. Meyers, Q. Chen, T. Borland, R.M. Hutabarat, V.A. Clausen, R. Alvarez, K. Fitzgerald, C. Gamba-Vitalo, S.V. Nochur, A.K. Vaishnaw, D.W. Sah, J.A. Gollob, O.B. Suhr, Safety and efficacy of RNAi therapy for transthyretin amyloidosis, N. Engl. J. Med. 369 (2013) 819–829.
[23] R.L. Rungta, H.B. Choi, P.J. Lin, R.W. Ko, D. Ashby, J. Nair, M. Manoharan, P.R. Cullis, B.A. Macvicar, Lipid nanoparticle delivery of siRNA to silence neuronal gene expression in the brain, Mol. Ther. Nucleic Acids 2 (2013) e136.
[24] M. Jayaraman, S.M. Ansell, B.L. Mui, Y.K. Tam, J. Chen, X. Du, D. Butler, L. Eltepu, S. Matsuda, J.K. Narayanannair, K.G. Rajeev, I.M. Hafez, A. Akinc, M.A. Maier, M.A. Tracy, P.R. Cullis, T.D. Madden, M. Manoharan, M.J. Hope, Maximizing the potency of siRNA lipid nanoparticles for hepatic gene silencing in vivo, Angew. Chem. Int. Ed. Eng. 51 (2012) 8529–8533.
[25] A. Akinc, A. Zumbuehl, M. Goldberg, E.S. Leshchiner, V. Busini, N. Hossain,

S.A. Bacallado, D.N. Nguyen, J. Fuller, R. Alvarez, A. Borodovsky, T. Borland, R. Constien, A. de Fougerolles, J.R. Dorkin, K. Narayanannair Jayaprakash, M. Jayaraman, M. John, V. Koteliansky, M. Manoharan, L. Nechev, J. Qin, T. Racie, D. Raitcheva, K.G. Rajeev, D.W. Sah, J. Soutschek, I. Toudjarska, H.P. Vornlocher, T.S. Zimmermann, R. Langer, D.G. Anderson, A combinatorial library of lipid-like materials for delivery of RNAi therapeutics, Nat. Biotechnol. 26 (2008) 561–569.

[26] S. de Jong, G. Chikh, L. Sekirov, S. Raney, S. Semple, S. Klimuk, N. Yuan, M. Hope, P. Cullis, Y. Tam, Encapsulation in liposomal nanoparticles enhances the immunostimulatory, adjuvant and anti-tumor activity of subcutaneously administered CpG ODN, Cancer Immunol. Immunother. 56 (2007) 1251–1264.

[27] K.D. Wilson, S.G. Raney, L. Sekirov, G. Chikh, S.D. de Jong, P.R. Cullis, Y.K. Tam, Effects of intravenous and subcutaneous administration on the pharmacokinetics, biodistribution, cellular uptake and immunostimulatory activity of CpG ODN encapsulated in liposomal nanoparticles, Int. Immunopharmacol. 7 (2007) 1064–1075.

[28] S. Chen, Y.Y. Tam, P.J. Lin, M.M. Sung, Y.K. Tam, P.R. Cullis, Influence of particle size on the in vivo potency of lipid nanoparticle formulations of siRNA, J. Control. Release 235 (2016) 236–244.

[29] Q.S. Wang, H.H. Au, E. Jan, Methods for studying IRES-mediated translation of positive-strand RNA viruses, Methods 59 (2013) 167–179.

[30] L.B. Jeffs, L.R. Palmer, E.G. Ambegia, C. Giesbrecht, S. Ewanick, I. MacLachlan, A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA, Pharm. Res. 22 (2005) 362–372.

[31] S.C. Semple, A. Akinc, J. Chen, A.P. Sandhu, B.L. Mui, C.K. Cho, D.W. Sah, D. Stebbing, E.J. Crosley, E. Yaworski, I.M. Hafez, J.R. Dorkin, J. Qin, K. Lam, K.G. Rajeev, K.F. Wong, L.B. Jeffs, L. Nechev, M.L. Eisenhardt, M. Jayaraman, M. Kazem, M.A. Maier, M. Srinivasulu, M.J. Weinstein, Q. Chen, R. Alvarez, S.A. Barros, S. De, S.K. Klimuk, T. Borland, V. Kosovrasti, W.L. Cantley, Y.K. Tam, M. Manoharan, M.A. Ciufolini, M.A. Tracy, A. de Fougerolles, I. MacLachlan, P.R. Cullis, T.D. Madden, M.J. Hope, Rational design of cationic lipids for siRNA delivery, Nat. Biotechnol. 28 (2010) 172–176.

[32] S. Chen, Y.Y. Tam, P.J. Lin, A.K. Leung, Y.K. Tam, P.R. Cullis, Development of lipid nanoparticle formulations of siRNA for hepatocyte gene silencing following subcutaneous administration, J. Control. Release 196C (2014) 106–112.

[33] J.A. Kulkarni, J.L. Myhre, S. Chen, Y.Y.C. Tam, A. Danescu, J.M. Richman, P.R. Cullis, Design of lipid nanoparticles for in vitro and in vivo delivery of plasmid DNA, Nanomedicine 13 (2016) 1377–1387.

[34] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Can. J. Biochem. Physiol. 37 (1959) 911–917.

[35] A. Sehgal, A. Vaishnaw, K. Fitzgerald, Liver as a target for oligonucleotide therapeutics, J. Hepatol. 59 (2013) 1354–1359.

[36] U. Sahin, K. Kariko, O. Tureci, mRNA-based therapeutics–developing a new class of drugs, Nat. Rev. Drug Discov. 13 (2014) 759–780.

[37] H. Yin, R.L. Kanasty, A.A. Eltoukhy, A.J. Vegas, J.R. Dorkin, D.G. Anderson, Non-viral vectors for gene-based therapy, Nat. Rev. Genet. 15 (2014) 541–555.

[38] A.D. Judge, G. Bola, A.C. Lee, I. MacLachlan, Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo, Mol. Ther. 13 (2006) 494–505.

[39] Q. Ge, A. Dallas, H. Ilves, J. Shorenstein, M.A. Behlke, B.H. Johnston, Effects of chemical modification on the potency, serum stability, and immunostimulatory properties of short shRNAs, RNA 16 (2010) 118–130.

[40] J. Szebeni, F. Muggia, A. Gabizon, Y. Barenholz, Activation of complement by therapeutic liposomes and other lipid excipient-based therapeutic products: prediction and prevention, Adv. Drug Deliv. Rev. 63 (2011) 1020–1030.

[41] D.C. Litzinger, J.M. Brown, I. Wala, S.A. Kaufman, G.Y. Van, C.L. Farrell, D. Collins, Fate of cationic liposomes and their complex with oligonucleotide in vivo, Biochim. Biophys. Acta 1281 (1996) 139–149.

[42] G. Basha, T.I. Novobrantseva, N. Rosin, Y.Y. Tam, I.M. Hafez, M.K. Wong, T. Sugo, V.M. Ruda, J. Qin, B. Klebanov, M. Ciufolini, A. Akinc, Y.K. Tam, M.J. Hope, P.R. Cullis, Influence of cationic lipid composition on gene silencing properties of lipid nanoparticle formulations of siRNA in antigen-presenting cells, Mol. Ther. 19 (2011) 2186–2200.

[43] E.V. Rudakova, N.P. Boltneva, G.F. Makhaeva, Comparative analysis of esterase activities of human, mouse, and rat blood, Bull. Exp. Biol. Med. 152 (2011) 73–75.

[44] L.M. Berry, L. Wollenberg, Z. Zhao, Esterase activities in the blood, liver and intestine of several preclinical species and humans, Drug Metab. Lett. 3 (2009) 70–77.

[45] Y.Y. Tam, S. Chen, J. Zaifman, Y.K. Tam, P.J. Lin, S. Ansell, M. Roberge, M.A. Ciufolini, P.R. Cullis, Small molecule ligands for enhanced intracellular delivery of lipid nanoparticle formulations of siRNA, Nanomedicine 9 (2013) 665–674.

[46] K. Fitzgerald, M. Frank-Kamenetsky, S. Shulga-Morskaya, A. Liebow, B.R. Bettencourt, J.E. Sutherland, R.M. Hutabarat, V.A. Clausen, V. Karsten, J. Cehelsky, S.V. Nochur, V. Koteliansky, J. Horton, T. Mant, J. Chiesa, J. Ritter, M. Munisamy, A.K. Vaishnaw, J.A. Gollob, A. Simon, Effect of an RNA interference drug on the synthesis of proprotein convertase subtilisin/kexin type 9 (PCSK9) and the concentration of serum LDL cholesterol in healthy volunteers: a randomised, single-blind, placebo-controlled, phase 1 trial, Lancet 383 (2014) 60–68.

[47] O.B. Suhr, T. Coelho, J. Buades, J. Pouget, I. Conceicao, J. Berk, H. Schmidt, M. Waddington-Cruz, J.M. Campistol, B.R. Bettencourt, A. Vaishnaw, J. Gollob, D. Adams, Efficacy and safety of patisiran for familial amyloidotic polyneuropathy: a phase II multi-dose study, Orphanet J. Rare Dis. 10 (2015) 109.

[48] J. Tabernero, G.I. Shapiro, P.M. Lorusso, A. Cervantes, G.K. Schwartz, G.J. Weiss, L. Paz-Ares, D.C. Cho, J.R. Infante, M. Alsina, M.M. Gounder, R. Falzone, J. Harrop, A.C. White, I. Toudjarska, D. Bumcrot, R.E. Meyers, G. Hinkle, N. Svrzikapa, R.M. Hutabarat, V.A. Clausen, J. Cehelsky, S.V. Nochur, C. Gamba-Vitalo, A.K. Vaishnaw, D.W. Sah, J.A. Gollob, H.A. Burris 3rd, First-in-humans trial of an RNA interference therapeutic targeting VEGF and KSP in cancer patients with liver involvement, Cancer Discov. 3 (2013) 406–417.

[49] A. Thess, S. Grund, B.L. Mui, M.J. Hope, P. Baumhof, M. Fotin-Mleczek, T. Schlake, Sequence-engineered mRNA without chemical nucleoside modifications enables an effective protein therapy in large animals, Mol. Ther. 23 (2015) 1456–1464.

[50] K. Kariko, 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.

[51] B.R. Anderson, H. Muramatsu, B.K. Jha, R.H. Silverman, D. Weissman, K. Kariko, Nucleoside modifications in RNA limit activation of 2′-5′-oligoadenylate synthetase and increase resistance to cleavage by RNase L, Nucleic Acids Res. 39 (2011) 9329–9338.

[52] B.R. Anderson, H. Muramatsu, S.R. Nallagatla, P.C. Bevilacqua, L.H. Sansing, D. Weissman, K. Kariko, Incorporation of pseudouridine into mRNA enhances translation by diminishing PKR activation, Nucleic Acids Res. 38 (2010) 5884–5892.

[53] K. Kariko, 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.

[54] M.S. Kormann, G. Hasenpusch, M.K. Aneja, G. Nica, A.W. Flemmer, S. Herber-Jonat, M. Huppmann, L.E. Mays, M. Illenyi, A. Schams, M. Griese, I. Bittmann, R. Handgretinger, D. Hartl, J. Rosenecker, C. Rudolph, Expression of therapeutic proteins after delivery of chemically modified mRNA in mice, Nat. Biotechnol. 29 (2011) 154–157.

[55] M. Cotten, A. Baker, M. Saltik, E. Wagner, M. Buschle, Lipopolysaccharide is a frequent contaminant of plasmid DNA preparations and can be toxic to primary human cells in the presence of adenovirus, Gene Ther. 1 (1994) 239–246.

[56] I.P. Wicks, M.L. Howell, T. Hancock, H. Kohsaka, T. Olee, D.A. Carson, Bacterial lipopolysaccharide copurifies with plasmid DNA: implications for animal models and human gene therapy, Hum. Gene Ther. 6 (1995) 317–323.

[57] A.L. Buchman, Side effects of corticosteroid therapy, J. Clin. Gastroenterol. 33 (2001) 289–294.