Mild Innate Immune Activation Overrides Efficient Nanoparticle-Mediated RNA Delivery

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Understanding how physiology affects nanoparticle delivery in vivo is important to overcome some of the challenges of clinical mRNA delivery. Here we take LNPs that deliver mRNA as an example and study how toll-like receptor 4 (TLR4) activation and inhibition alter LNP-mediated mRNA delivery. Results show that TLR4 activation (i) blocks the translation of mRNA in all cell types and (ii) does not reduce LNP uptake. Blocking TLR4 or its downstream effector PKR improve LNP delivery. We also utilize DNA barcoding to discover a novel LNP that delivers mRNA to Kupffer cells at clinically relevant doses.

These data highlight the importance of understanding how inflammatory signaling influences delivery to both on- and off-target cells, especially as LNPs delivering mRNA are used to treat more diseases in patients.
Clinical mRNA delivery remains challenging, in large part because how physiology alters delivery in vivo remains underexplored. For example, mRNA delivered by lipid nanoparticles (LNPs) is being considered to treat inflammation, but whether inflammation itself changes delivery remains understudied. Relationships between immunity, endocytosis, and mRNA translation lead to hypothesize that toll-like receptor 4 (TLR4) activation reduced LNP-mediated mRNA delivery. Therefore, LNP uptake, endosomal escape, and mRNA translation with and without TLR4 activation are quantified. In vivo DNA barcoding is used to discover a novel LNP that delivers mRNA to Kupffer cells at clinical doses; unlike most LNPs, this LNP does not preferentially target hepatocytes. TLR4 activation blocks mRNA translation in all tested cell types, without reducing LNP uptake; inhibiting TLR4 or its downstream effector protein kinase R improved delivery. The discrepant effects of TLR4 on i) LNP uptake and ii) translation suggests TLR4 activation can “override” LNP targeting, even after mRNA is delivered into target cells. Given near-future clinical trials using mRNA to modulate inflammation, this highlights the need to understand inflammatory signaling in on- and off-target cells. More generally, this suggests an LNP which delivers mRNA to one inflammatory disease may not deliver mRNA to another. 

Nanoparticles interact with biomolecules that alter nanoparticle efficacy and safety.[1] Insights into biological pathways that affect how nanoparticles interact with the surface of target cells and how nanoparticles are endocytosed are valuable. In an example of (i), the ApoE-mediated mechanism by which lipid nanoparticles (LNPs) can be delivered to hepatocytes helped lead to a Food and Drug Administration (FDA)-approved siRNA drug.[2] Interactions between LNPs and serum ApoE were necessary for LNP to bind hepatocytes, and low-density lipoprotein receptor, a cell surface receptor for ApoE, increased nanoparticle uptake.[3] Reports have also shown that the nanoparticle corona affects delivery[4] by changing how nanoparticles interact with on- and off-target cells. In representative examples of (ii), authors used small molecules, RNAi, CRISPR, and knockout mice to reveal how nanoparticle uptake changes after endocytic genes are inhibited.[5,6] These studies and others[7] identified that clathrin, caveolin, and other now-canonical pathways impact nanoparticle uptake.

We reasoned that another step in the drug delivery process seemed underestimated. Specifically, we hypothesized that (iii) inflammation could alter the efficacy of a mRNA drug after the nanoparticle reaches the target cell by changing how the drug was translated. We tested this hypothesis by focusing on the gene toll-like receptor 4 (TLR4). We chose TLR4 for two reasons. First, understanding how TLR4 alters drug delivery is relevant to near-future mRNA therapies. LNP-mediated mRNA delivery[8] is proposed for myocardial infarction,[9] colitis,[10] and Fabry disease,[11] as well as vaccines targeting influenza,[12] Zika,[13] and others.[14] TLR4 signaling plays a role in these diseases.[15] In addition, adjuvants have been used in concert with mRNA vaccines, in order to improve their immunogenicity;[16] many adjuvants activate TLRs.[17] For example, authors found that delivering a TLR4 agonist in concert with mRNA encoding a vaccine did not improve vaccine efficacy in nonhuman primates, relative to the mRNA only.[18] We hypothesized this result could be explained by TLR4-driven reduction in mRNA delivery. The second reason we focused on TLR4 is its critical role dictating how pathogens are endocytosed and processed. This has been reviewed;[19] here we highlight three examples. In one example, scientists found cells exposed to lipopolysaccharide (LPS), which is an agonist for TLR4, led to TLR4 colocalization on endosomes. Imaging and biochemical assays led the authors to conclude that inflammation and endosomal trafficking were linked via TLR4.[20] In another example, by labeling TLR4, authors found it was present at the plasma...
membrane as well as intracellular vesicles that contained the endosome marker Rab5; the authors concluded TLR4 played a central role in endocytosis. In a third example, scientists found that pathogens evolved to evade recognition by the human immune system by preventing TLR4 recognition. This suggested reducing TLR4 activation could improve the survival of a foreign material in a human system.

To test the hypothesis that TLR4 activation could override LNP-mediated mRNA delivery, we quantified functional mRNA delivery (more specifically, gene editing mediated by a protein encoded by mRNA) in hepatocytes, liver endothelial cells, and Kupffer cells in the presence of LPS, which activated TLR4 recognition. This suggested reducing TLR4 activation could improve the survival of a foreign material in a human system.

To test the hypothesis that TLR4 activation could override LNP-mediated mRNA delivery, we quantified functional mRNA delivery (more specifically, gene editing mediated by a protein encoded by mRNA) in hepatocytes, liver endothelial cells, and Kupffer cells in the presence of LPS, which activated TLR4. TLR4 is expressed on hepatocytes, Kupffer cells and dendritic cells. In our follow-up mechanistic studies, we focused on Kupffer cells, which we selected for two reasons. First, upon TLR4 activation, macrophage gene expression changes. For example, TLR4 activation induces phosphorylation of protein kinase R (PKR), which reduces mRNA translation. We therefore reasoned TLR4 could reduce mRNA translation, even after mRNA was delivered into the cytoplasm. Second, Kupffer cells play a central role in nanoparticle clearance by removing nanoparticles from circulation.

Initially, we performed experiments with LNPs formulated with the compound cKK-E12. cKK-E12 LNPs have delivered RNA to hepatocytes at clinical doses in mice and non-human primates; they are being considered for clinical development. After synthesizing cKK-E12, we mixed it with a poly(ethylene glycol) (PEG)-lipid, cholesterol, and 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) in a microfluidic device, in order to formulate LNPs. The LNPs were small, had a low polydispersity, and a pKa of 6.2. We investigated whether GFP mRNA delivery changed when cells were exposed to LPS. First, we added LPS or phosphate-buffered saline (PBS) to murine macrophages (RAWs) in a 24 well plate. 6 h later, we added LNPs carrying GFP mRNA at a dose of 1 µg. 12 h later, we quantified GFP mean fluorescent intensity (MFI) using flow cytometry. Compared to cells pretreated with PBS, LPS pretreated cells exhibited a robust reduction in MFI.

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**Figure 1.** LPS impacts cKK-E12-mediated delivery of mRNA. A) cKK-E12 was formulated by combining the ionizable material cKK-E12, C14PEG2000, cholesterol, and DOPE. B) Characteristics of cKK-E12. C) GFP mean fluorescent intensity 12 h after cKK-E12 LNPs carrying GFP mRNA were administered. Data are normalized to GFP MFI of control cells that were not treated with LPS. ****p < 0.0001, One-way ANOVA. D) To quantify mRNA delivery in vivo, we formulated cKK-E12 LNPs with Cre mRNA and injected them into Ai14 mice. If Cre mRNA is functionally translated into Cre protein, cells become tdTomato+. E) The percentage of tdTomato+ cells in mice pretreated with PBS or 0.1 mg kg⁻¹ LPS, then injected with 0.3 mg kg⁻¹ Cre mRNA. Data are normalized to mice pretreated with PBS ****p < 0.0001, two-way ANOVA.
sufficient to reduce GFP expression by over 75% (Figure 1C). We reasoned that the reduced GFP MFI could be driven by overt cell death. We therefore determined the percent of live/dead cells in our flow cytometry analyses by assessing the size and granularity of the cell populations. We observed no significant decrease in populations after exposure to LPS, suggesting the effect was not caused by overt cell death (Figure S1A, Supporting Information).

We analyzed the effect of LPS on mRNA delivery in vivo. We formulated cKK-E12 LNPs to carry Cre mRNA, and intravenously injected them into Ai14 mice at the clinical dose of 0.3 mg kg\(^{-1}\) mRNA. Ai14 mice have a Lox-Stop-Lox-tdTomato construct driven by a cytomegalovirus IE enhancer and chicken β-actin (CAG) promoter. If Cre mRNA is delivered into the cytoplasm and translated into functional Cre protein, the stop site is edited out of the genome, leading to tdTomato\(^+\) cells (Figure 1D). The percentage of tdTomato\(^+\) cells is a validated way to quantify mRNA delivery in vivo.\(^{[31–33]}\) \(6\) h before injecting LNPs, we administered 0.1 mg kg\(^{-1}\) LPS via an intraperitoneal injection; this dose is considered low.\(^{[34]}\) 3 d after injecting the LNPs, we quantified the percentage of Kupffer cells (CD68\(^{-}\)CD45\(^{-}\)CD31\(^{+}\)), liver endothelial cells (CD45\(^{-}\)CD31\(^{-}\)), and hepatocytes (CD31\(^{-}\)CD45\(^{-}\)) that were tdTomato\(^+\) using flow cytometry. Compared to mice that were not treated with LPS, the percentage of tdTomato\(^+\) cells isolated from mice treated with LPS decreased to nearly 0% (Figure 1E). We reasoned the effects could be driven by overt cell death. However, four lines of evidence suggested otherwise. First, a 0.1 mg kg\(^{-1}\) LPS dose is considered low.\(^{[34]}\) Second, control- and LPS-treated mice experienced no significant weight loss during the experiment (Figure S2B, Supporting Information). Third, the data we plotted were the percentage of live hepatocyte, endothelial, or Kupffer cells that were also tdTomato\(^+\). Fourth, the size and granularity in cell populations cells isolated from the liver remained unchanged in LPS and control mice (Figure S2B, Supporting Information).

To ensure the data were not specific to one LNP, we repeated the in vivo experiment using a second clinically relevant LNP named MC3. MC3 has delivered RNA in mice, nonhuman primates, and led to an FDA-approved siRNA drug.\(^{[2]}\) The MC3 results recapitulated the cKK-E12 experiments. Specifically, i) the percentage of tdTomato\(^+\) hepatocytes, endothelial cells, and Kupffer cells decreased in mice treated with LPS compared to mice treated with PBS (Figure S2C, Supporting Information), and ii) we did not observe any signs of cell death isolated from mice (Figure S2D,E, Supporting Information).

We then investigated whether a novel LNP selected for Kupffer cell targeting could overcome the LPS-mediated reduction in mRNA delivery. To identify a novel LNP that preferentially delivers RNA to Kupffer cells (instead of hepatocytes) at a clinical dose, we performed a high throughput functional in vivo DNA barcoding screen (Figure S3A,B, Supporting Information). We have detailed this approach in previous work.\(^{[32,33]}\) Briefly, we formulated LNP-1, with chemical structure 1, to carry Cre mRNA and DNA barcode 1, and LNP-96, with chemical structure 96, carried Cre mRNA and DNA barcode 96. The formulation details for all 96 LNPs are detailed (Figure S3C, Supporting Information). We evaluated the diameter and polydispersity index of each individual LNP, and pooled together LNPs that were stable, monodisperse, and had a diameter less than 200 nm.\(^{[33,35]}\) Of the 96 LNPs, 82 met these quality control criteria (Figure 2A). These 82 LNPs were injected a total mRNA dose of 1.0 mg kg\(^{-1}\) (0.012 mg kg\(^{-1}\)/particle, on average). 3 d after administering the LNPs to Ai14 mice, we isolated 12 tdTomato\(^+\) cell types in order to evaluate the on-target delivery (Kupffer cells) as well as off-target delivery (11 cell types, Figure 2B; and Figure S3D, Supporting Information). We sequenced the tdTomato\(^+\) cells, thereby identifying barcodes colocalized with cells that were functionally transfected by Cre mRNA. We did not observe changes in mouse weight (Figure S3E, Supporting Information). We then analyzed previously described controls\(^{[33,35]}\) controls. We characterized the size and polydispersity of the 82 pooled LNPs, and found the pool was similar to the LNPs making up the pool (Figure 2C,D). Second, we included a “naked” DNA barcode as a negative control. Since DNA does not readily enter cells on its own, this DNA barcode was delivered into cells less efficiently than barcodes delivered by LNPs (Figure 2E). Third, we found the nanoparticle sequencing data were consistent across mouse replicates (Figure S3F, Supporting Information).

These controls led us to believe that it was appropriate to analyze the in vivo screening results. We quantified the percentage of live cells that were tdTomato\(^+\), using 12 cell types. We found that the LNP pool preferentially delivered Cre mRNA to Kupffer cells (≈80% tdTomato\(^+\)), followed by liver endothelial cells (≈30% tdTomato\(^+\)) and hepatocytes (≈15% tdTomato\(^+\)). We then sequenced all 82 barcodes and evaluated whether LNP size or chemical composition affected in vivo delivery. LNP delivery varied with the chemical composition more than the physical structure of the LNP. Specifically, LNPs formulated with epoxide-terminated fully saturated lipid tails consisting of 15 carbons were enriched in tdTomato\(^+\) cells, relative to LNPs made with other lipid tails (Figure 2F). Enrichment analysis is detailed (Figure S3G, Supporting Information). LNP diameter did not affect delivery within the tested range of 20–200 nm (Figure S3H–J, Supporting Information), nor did lipid molar ratios (Figure S3K, Supporting Information).

An important control for any high throughput screen is whether individual compounds perform as predicted. We quantified Cre mRNA delivery mediated by the best LNP from the screen, which we named LNP3 for simplicity (Figure 2G,H). 3 d after mice were systemically injected with 0.3, 0.1, or 0.03 mg kg\(^{-1}\) Cre mRNA formulated into LNP3, we observed mRNA delivery quantified by tdTomato\(^+\) cell percentage. LNP3 recapitulated the screening results: As the screen predicted, Kupffer cells were preferentially targeted, followed by liver endothelial cells and hepatocytes (Figure 2I). We did not observe delivery after a 0.03 mg kg\(^{-1}\) dose. Having identified a novel LNP that preferentially targeted Kupffer cells, we tested our hypothesis that LNP3 would overcome the LPS-dependent reduction in mRNA delivery. The data did not support this hypothesis; pretreating mice with 0.1 mg kg\(^{-1}\) LPS reduced mRNA delivery in all 3 cell types (Figure 2J). To confirm the effect was consistent across LPS batches, we purchased two additional batches from the same company and tested them. We observed a statistically significant reduction of mRNA delivery in all cell types in the liver (Figure S3L, Supporting Information).
Figure 2. In vivo barcoding identifies an LNP that preferentially delivers mRNA to Kupffer cells in vivo. A) 96 chemically distinct LNPs were formulated with Cre mRNA and DNA barcodes. After quality control, 82 LNPs were injected into Ai14 mice. FACS was used to measure mRNA delivery and isolate tdTomato+ cells. B) The percentage of cells that are tdTomato+ after pooled LNPs carrying DNA barcodes and Cre were administered. 12 different cell types were isolated; the pool preferentially delivered to Kupffer cells. ****p < 0.0001, **p < 0.01, two-way ANOVA. C) Hydrodynamic diameter and D) polydispersity index of all administered LNPs. E) Normalized delivery of all LNPs across all cell types. The naked barcode control performed poorly as expected. F) Enrichment of lipid variants in the top 10% compared to total formulated. cKK-E15 was the most enriched in Kupffer cells, Endothelial cells, and hepatocytes. G) LNP3 was formulated with ionizable material cKK-E15, C14PEG2000, unmodified cholesterol, and DOPE. H) Molar ratio, characteristics, and transmission electron microscopy image of cKK-E15. I) The percentage of cells that are tdTomato+ after injections of 0.03, 0.1, or 0.3 mg kg\(^{-1}\) Cre mRNA. ****p < 0.0005, ***p < 0.001, two-way ANOVA. J) LNP3 delivery of Cre mRNA at 0.3 mg kg\(^{-1}\) in mice pretreated with PBS or LPS. tdTomato+ cells decreased significantly. ****p < 0.0001, two-tailed t-test.
Having observed that mRNA delivery mediated by cKK-E12, MC3, and LNP3 was reduced by mild doses of LPS, we studied the biological mechanism, in vitro and in vivo. In order to deliver a mRNA drug that is translated inside the cell, an LNP must i) reach the target cell and ii) help the mRNA enter the cytoplasm. Then, the mRNA must iii) translated into protein (Figure 3A). Based on our data, we first hypothesized that (i) LPS reduced LNP uptake. We analyzed the biodistribution of LNP3 with QUANT, a sensitive system utilizing digital droplet PCR to quantify LNP biodistribution. [6] We formulated LNP3 to Adv. Mater. 2019, 1904905

Figure 3. LPS-mediated innate immune activation did not significantly decrease LNP uptake or endosomal escape. A) To functionally deliver a mRNA drug, an LNP must be taken up by a cell, enter the cytoplasm, and be translated into protein. B) To compare cellular uptake of LNP3 with and without LPS, LNP3 was formulated with QUANT DNA barcodes at 0.3 mg kg$^{-1}$ and intravenously injected into mice. 24 h later, biodistribution was assessed. Biodistribution, shown as barcode counts (AU), for LNP3 with and without LPS. Nucleic acid uptake increased significantly in Kupffer cells when treated with LPS. ****$P < 0.0001$, two-way ANOVA. C) Colocalization of fluorescently tagged mRNA formulated with LNP3 and endosomal markers in RAWs after treatment of LPS. Cy3B labeled IVT mRNA (red) formulated with LNP3 was delivered to RAWs untreated or treated with LPS. 6 and D) 24 h later, cells were stained for CD64, EEA1, and Rab11 (green) for early, intermediate and late endosomes, respectively. Scale bar represents 10 µm. Cropped regions are magnifications of white boxes in whole cell images with intensity profiles along the direction of the white arrow. E) Percent of free mRNA was determined at 3, F) 6, and G) 24 h. n≥30 cells per group. **$P<0.005$, two-tailed t-test.
carry a DNA barcode and injected mice at a dose of 0.3 mg kg\(^{-1}\) DNA. 6 h before injecting LNPs, we administered 0.1 mg kg\(^{-1}\) LPS; control mice were pretreated with PBS. 24 h after injecting the LNPs, we isolated Kupffer cells, liver endothelial cells, and hepatocytes using fluorescence activated cell sorting (FACS). When compared to mice pretreated with PBS, we observed increased Kupffer cell uptake in mice pretreated with LPS. We did not see significant changes in the other two cell types (Figure 3B). These data did not support the hypothesis that LPS reduced LNP uptake.

We then tested whether (ii) LPS reduced endosome escape. We formulated LNP3 with a fluorescently tagged and chemically modified mRNA encoding Cre. We administered these LNPs to RAWs in a 24 well plate at a dose of 1 \(\mu\)g mRNA/well; 6 h beforehand, we treated RAWs with 500 ng of LPS (or did not) 3, 6, or 24 h after administering the LNPs, we fixed cells for imaging. Cells were stained for the endosomal markers CD63, EEA1, and Rab11, which colocalize with early, and recycled endosomes, respectively (Figure 3C,D; and Figure S4, Supporting Information). We quantified the percentage of mRNA trapped in endosomal compartments using Mander’s overlap coefficient in 30 or more cells per condition as we previously described\(^{[36]}\) (Figure 3E-G). At 3 and 24 h, the Mander’s overlap coefficient was similar in PBS- and LPS-treated cells. At 6 h, the Mander’s overlap coefficient was statistically lower in LPS-treated cells, but the difference was not sufficient to explain the complete reduction in protein production. Based on these data, we concluded that LPS-mediated decrease in protein expression was not driven predominantly by reduced endosomal escape.

We reasoned that LPS caused a difference in cell signaling that affected (iii) mRNA translation after the LNP reached the target cell; we therefore studied the cell signaling that drove this effect. We treated RAW cells with 100 ng of LPS 6 h before administering LNP3 carrying fluorescently tagged mRNA. Cells were fixed for imaging one and 3 h after administering the LNPs, we fixed cells for imaging. Cells were stained for the endosomal markers CD63, EEA1, and Rab11. We observed vesicular localization of TLR4, and colocalization of TLR4 and LNP delivered mRNA (Figure S5, Supporting Information). We reasoned that TLR4 inhibition could rescue mRNA delivery. We pretreated RAWs in a 24 well plate with 100 \(\times\) 10\(^{-9}\) M of TAK-242, a TLR4 inhibitor. 1 h later, we administered 100 ng of LPS to cells; 6 h later, we delivered 1 \(\mu\)g GFP mRNA using LNP3. 24 h after GFP mRNA was delivered, we quantified GFP MFI, and found that delivery was rescued with TAK-242 (Figure 4A).

TLR4 can activate the downstream effector PKR, which can reduce protein translation.\(^{[25]}\) We therefore treated cells with varying doses of C16 (Figure 4B), 2AP (Figure 4C), and ISRIB (Figure 4D), three PKR inhibitors. C16 and 2AP PKR inhibitors improved GFP delivery, while the results from ISRIB were inconclusive. Finally, we evaluated whether it was possible to overcome TLR4-mediated inhibition of mRNA delivery by increasing the LNP dose. We injected LPS pretreated mice with 0.3 or 2.0 mg kg\(^{-1}\) Cre mRNA. Increasing the dose led to a non-significant increase in tdTomato\(^{+}\) cells (Figure 4E). These data led us to conclude that TLR4 was a potent inhibitor of mRNA delivery (Figure 4F).

We then evaluated whether other inflammatory signaling similarly affected delivery. We treated RAWs in a 24 well plate with 0.1, 1, or 5 \(\mu\)g of CpG or poly[I:C]. CpG and poly[I:C] activate TLR3 and TLR9, respectively. 6 h later, we delivered 1 \(\mu\)g GFP mRNA using LNP3. 24 h later, we quantified GFP MFI. CpG and poly[I:C] activation reduced mRNA delivery, but neither reduced delivery as potently as 1 ng of LPS (Figure 4G,H). Finally, to evaluate whether low-grade chronic inflammation reduced mRNA delivery, we injected mice subcutaneously with B16-F10 murine melanoma cells; this model is often used to evaluate immunotherapies.\(^{[37]}\) 10 d later, we intravenously injected LNP3 carrying Cre mRNA at a dose of 0.3 mg kg\(^{-1}\); as a control, we injected LNP3 into mice without tumors. 3 d later, we digested the liver and quantified the percentage of tdTomato\(^{+}\) Kupffer cells, liver endothelial cells, and hepatocytes. When compared to mice that did not have tumors, there was no significant decrease in percentage of tdTomato\(^{+}\) cells (Figure 4I). These data suggest that tumor-driven inflammation was not sufficient to reduce delivery to other organs in vivo. These data led us to conclude that although yet-to-be discovered inflammation pathways may also reduce mRNA delivery, TLR4 signaling is particularly potent. Since TLR4 is expressed on the cell membrane while TLR3 and TLR9 are primarily expressed intracellularly, we cannot exclude the possibility that the relative potency of TLR4 signaling was driven by the physical availability of the receptor.

The summation of these data led us to conclude that mild doses of the TLR4 agonist LPS reduce mRNA delivery in vitro and in vivo. Notably, reduced delivery was not caused by reduced cell uptake. Instead, the data suggest that the mechanism could be driven, in part, by TLR4-mediated reductions in endosomal escape and was likely driven by a TLR4-mediated reduction in mRNA translation. Independent of the relative importance of endosomal escape and mRNA translation, the data support the hypothesis that TLR4 activation can block effective mRNA delivery after the LNP reaches the target cell. We believe these results have implications for mRNA drugs delivered by LNPs. Specifically, they suggest that if an LNP delivers therapeutic mRNA to a given cell type in one disease state, it is not guaranteed to deliver mRNA to the same cell type in another disease. Thus, the on- and off-target delivery profiles of a given LNP could vary with disease state, not just with the LNP. These data demonstrate the need to understand how cell state impacts the safety and efficacy of a given nanoparticle. Historically, cell state has been difficult to study; however, recent advances in single cell RNA-sequencing\(^{[38]}\) make near-future experiments relating cell state to delivery feasible. We believe our data provide an impetus for these studies. We also found that a lead compound, LNP3, identified by a high throughput in vivo DNA barcoding screen performed as the screen predicted. Specifically, we generated and analyzed nearly 4000 in vivo data points, and analyzed these in vivo data points to study the relationship between LNP chemical structure and in vivo delivery. Interestingly, LNP3 efficiently delivered mRNA in vivo at clinically relevant doses. This LNP was small, uniform, and potent, and unlike most previously reported LNPs, did not preferentially target hepatocytes in vivo. If pooled in vivo LNP screens consistently predict the behavior of LNPs,\(^{[32,35,39]}\) we believe this will be important for the nanomedicine field, given that historically, in vivo nanoparticle behavior has been difficult to predict using in vitro assays.\(^{[40]}\)
It is important to acknowledge the limitations of this work. First, our studies were limited to mice. It is possible that the results are not replicated in nonhuman primates. Second, it is likely that other genes work in concert with TLR4 to mediate this effect. There are several proteins involved in LPS-mediated signaling; we hope to detail the genetic networks driving this.
phenotype in future work using technologies, such as RNA-seq.\(^{[41]}\) Third, our studies were limited to lipid-based nanoparticles. It is possible the results are not recapitulated with other nanoparticle classes. Fourth, the high throughput in vivo barcoding system will not work for all nanoparticles; it is critical to include the controls described herein when performing barcoding studies. Despite these limitations, we believe these data provide compelling evidence that inflammatory signaling can alter LNP delivery, and that high throughput in vivo barcoding can more efficiently identify nanoparticles that deliver drugs in vivo.

### Experimental Section

Nanoparticles were formulated in a microfluidic device by mixing nucleic acids and LNP components.\(^{[52]}\) Nucleic acids were diluted in citrate buffer and LNP components were diluted in ethanol. For screening, each LNP was formulated to carry a distinct barcode; LNPN carried Cre mRNA and DNA barcode N. LNP hydrodynamic diameter was measured using dynamic light scattering. LPS was administered via an intraperitoneal injection, whereas LNPs were administered intravenously. All animal experiments were performed in accordance with the Georgia Tech IACUC. Mice were aged 5–8 weeks, and a total of \(N = 6–10\) mice per group were used. Sequencing was performed on MiniSeq using Illumina protocols.

### Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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### Conflict of Interest

J.E.D. and C.D.S. are co-founders of Guide Therapeutics.

### Keywords

DNA barcoded nanoparticles, inflammation, lipid nanoparticles, LPS, mRNA delivery

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