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Lipid nanoparticle chemistry determines how nucleoside base modifications alter mRNA delivery

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ABSTRACT

Therapeutic mRNA has the potential to revolutionize the treatment of myriad diseases and, in 2020, facilitated the most rapid vaccine development in history. Among the substantial advances in mRNA technology made in recent years, the incorporation of base modifications into therapeutic mRNA sequences can reduce immunogenicity and increase translation. However, experiments from our lab and others have shown that the incorporation of base modifications does not always yield superior protein expression. We hypothesized that the variable benefit of base modifications may relate to lipid nanoparticle chemistry, formulation, and accumulation within specific organs. To test this theory, we compared IV-injected lipid nanoparticles formulated with reporter mRNA incorporating five base modifications (ψ, m1ψ, m5U, m5C/ψ, and m5C/s2U) and four ionizable lipids (C12–200, cKK-E12, ZA3-Ep10, and 200O_{i10}) with tropism for different organs. In general, the m1ψ base modification best enhanced translation, producing up to 15-fold improvements in total protein expression compared to unmodified mRNA. Expression improved most dramatically in the spleen (up to 50-fold) and was attributed to enhanced protein expression in monocytic lineage splenocytes. The extent to which these effects were observed varied with delivery vehicle and correlated with differences in innate immunogenicity. Through comparison of firefly luciferase and erythropoietin mRNA constructs, we also found that mRNA modification-induced enhancements in protein expression are limited outside of the spleen, irrespective of delivery vehicle. These results highlight the complexity of mRNA-loaded lipid nanoparticle drug design and show that the effectiveness of mRNA base modifications depend on the delivery vehicle, the target cells, and the site of endogenous protein expression.

1. Introduction

In vitro transcribed (IVT) mRNA has emerged as a powerful gene therapy platform for a wide range of therapeutic applications such as protein replacement [1,2], gene editing [3,4], vaccines [5–9], and immunotherapies [10,11]. Indeed, the lipid nanoparticle mRNA vaccines produced by BioNTech/Pfizer and Moderna have been effectively combating the SARS-CoV-2 pandemic since late 2020 [8,9,12]. While traditional vaccines typically require years of development, time scales are reduced for mRNA vaccines because mRNA synthesis is relatively quick [13,14] and mRNA therapeutics are inherently modular. These attributes confer mRNA therapeutics with theoretically unlimited clinical potential for existing and emerging viruses [15–17].

The FDA-approved and other clinically advanced mRNA vaccines and therapeutics rely on a lipid nanoparticle-based delivery vehicle to protect the mRNA cargo and deliver it to the cytosol of target cells [8,9]. Fortunately, numerous lipid nanoparticles have been discovered that potently deliver mRNA to select targets including muscle, immune cells, and the liver [3,4,18–22]. Despite these successes, many therapeutic applications are hindered by immunogenicity due to both the lipid carrier and the exogenous mRNA molecules.

While the lipid carrier interfaces with immune cells outside of the target cell, the mRNA is primarily exposed to intracellular components of the immune system, which defend against viral genetic material [23].

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Many cell types express toll-like receptors (TLRs) that, upon binding to mRNA, trigger a signaling cascade. This cascade produces a cytokine and interferon inflammatory response that stalls mRNA translation [24–26]. While TLR activation may enable vaccine immunogenicity and efficacy [10,27], it also hinders the potency of protein replacement therapies. Further, all applications of mRNA therapeutics are limited by similar inflammatory responses induced by cytoplasmic receptors such as RIG-I, PKR, and MDA5 upon binding exogenous mRNA [28].

These immunostimulatory mRNAs are unmodified – meaning that they contain only unmodified nucleosides – adenosine (A), cytosine (C), uridine (U), and guanosine (G). Such mRNAs are recognized as foreign because they lack post-transcriptional modifications (*e.g.* pseudouridylation and methylation) that are characteristic of endogenous mRNAs [29,30]. Karikó, Weissman, and others have found that the immunogenicity of exogenous mRNA can be mitigated through the incorporation of modified nucleosides [31–34]. These nucleosides, including pseudouridine (ψ), 5-methylcytidine (m5C), 5-methyluridine (m5U), and 2-thiouridine (s2U), reduce activation by TLRs and RIG-I [32,35–37]. Decreased immune recognition often results in an increase in mRNA translation. Early studies using ψ-modified mRNA achieved both greater mRNA translation and decreased inflammatory cytokine production in human dendritic cells and in mice following intraperitoneal administration relative to uridine-containing mRNA [37]. Later studies found that the modification 1-methylpseudouridine (m1ψ) outperforms ψ *in vitro* and in mice following intramuscular and intradermal injection [38] by decreasing immunogenicity and enhancing ribosome binding [35,38,39]. The success of this modification led to its incorporation into the BioNTech/Pfizer and Moderna SARS-CoV-2 vaccines [8,9]. Of note, while these clinically successful mRNA products used modified mRNA, there are also mRNA candidates without base modifications that have failed in clinical trials, including Translate Bio's cystic fibrosis treatment [40] and CureVac's SARS-CoV-2 vaccine [41].

Given the increasing interest in mRNA therapeutics, we anticipate a surge in related delivery research that will build upon previous discoveries of lipid nanoparticles that potently delivery mRNA. [3,4,10,18,19,21,38–49] Although mRNA base modifications are generally considered to improve efficacy, our lab and others have found that this is not always the case [54]. As such, the field would benefit from a better understanding of how delivery vehicles work together with mRNA base modifications to impact protein expression in different tissues. We hypothesized that the variable benefit of base modifications may relate to the chemistry and formulation of mRNA delivery vehicles, and, therefore, to the distribution or translation of the RNA within specific cell or tissue types. To test this idea, we systematically studied a series of lipid nanoparticles incorporating six types of mRNA and four ionizable amino lipids and evaluated efficacy in the liver, spleen, and lungs. Our results show that the utility of mRNA base modifications is not universal; instead, it depends on the delivery material, the biology of target cells, and on the site of endogenous protein expression. Further, we show that, as anticipated, effects relate to the immunogenicity of the mRNA-loaded lipid nanoparticles. Together, these data underscore the importance of tailoring the nucleoside modification to both the delivery system, the target cells, and the application.

2. Results and discussion

Our goal in this study was to elucidate the relationship between mRNA delivery vehicle, biological site of mRNA translation, and base modification performance. Therefore, we delivered a group of *in vitro* transcribed (IVT) mRNAs containing nucleoside modifications demonstrated to reduce immune activation and enhance translation using four lipid nanoparticles with differential tropism *in vivo*. Specifically, we tested unmodified mRNA along with five modified mRNAs (Fig. 1A), each of which contained either modified uridine (ψ, m1ψ, and m5U) or modified uridine and cytidine (m5C/ψ and m5C/s2U). Because previous studies have described the mechanisms by which these modifications reduce immunogenicity [32,55,56], we focused on how they affected protein expression *in vivo*. These six mRNAs were delivered using lipid nanoparticles formulated with the four lipids shown in Fig. 1B. These lipid nanoparticles were selected to allow comparison of tissue tropism as a function of mRNA modification. C12-200 and cKK-E12 (also known as MD-1) are five- and four-tailed ionizable lipids that predominately deliver mRNA to the liver [57,58]. The ionizable lipid 200O$_{i10}$, which has the same amino core as C12-200 but contains ester linkages next to the alkyl tails with a branch on the terminal carbon, induces protein expression in the spleen and the liver [19]. Finally, the zwitterionic

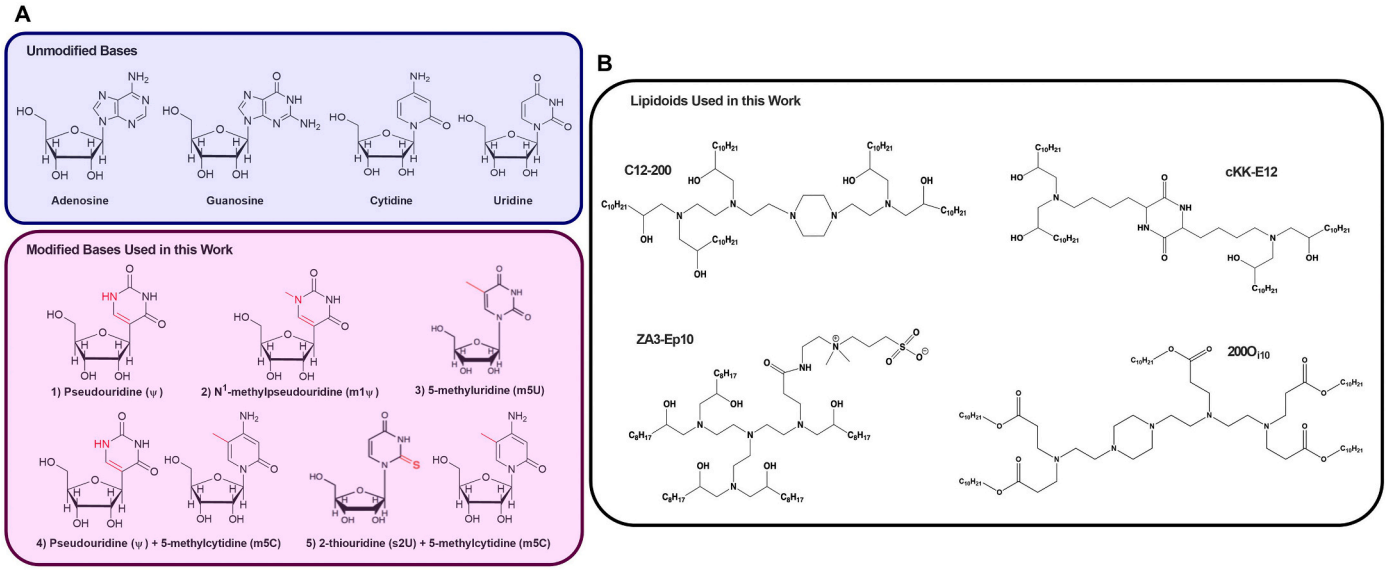

Fig. 1. Four unique ionizable amino lipids materials were formulated into lipid nanoparticles containing base-modified mRNA. (A) In addition to unmodified mRNA, we examined five modified mRNAs. The modifications were of either the uridine nucleoside (ψ, m1ψ, or m5U) or of both uridine and cytidine (m5C/ψ and m5C/s2U). The groups shown in red represent the difference between the modified and unmodified nucleoside. (B) The six mRNAs were formulated into nanoparticles using one of four different lipids: C12-200, cKK-E12, 200O$_{i10}$, and ZA3-Ep10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

amino lipid ZA3-Ep10 facilitates mRNA delivery predominantly to the lungs of mice [3].

2.1. Nanoparticle biodistribution does not correlate with protein expression

Before delivering modified mRNAs, we conducted a side-by-side comparison of the *in vivo* delivery properties of the four lipid nanoparticles. First, we examined the biodistribution by injecting mice intravenously with nanoparticles loaded with Cy5-labeled mRNA. One hour after injection, the mice were sacrificed, and the major organs were removed for *ex vivo* fluorescent imaging. This time point was chosen because our previous work demonstrates that lipid nanoparticles have a serum half-life of ~6 min with nearly 100% clearance by 1 h post-injection with immediate uptake by hepatocytes [59]. As shown in Fig. 2A and Fig. S1, most nanoparticles accumulated in the liver (>60%) and, to a lesser extent, the spleen, kidneys, and lungs.

Next, we assessed protein expression for each delivery vehicle, as the relationship between nanoparticle biodistribution and protein expression does not always correlate. To do this, we formulated nanoparticles with unmodified firefly luciferase-encoding mRNA (mLuc) and delivered them intravenously at a dose of 0.75 mg/kg. Six hours after injection, we sacrificed the mice and imaged the major organs for bioluminescence (Fig. 2B). This time point was chosen because our previous studies show that maximum luciferase expression occurs 6 h after LNP injection, which allows sufficient time for the mRNA to undergo translation and resultant protein to accumulate and maximize detection sensitivity [4]. Consistent with previous reports, C12–200 and cKK-E12 facilitated protein expression almost entirely in the liver (87 and 97%, respectively) [57,58]. Lipid nanoparticles formulated with $200O_{i10}$ induced a more diverse protein expression pattern (64% liver, 21% spleen, and 13% lungs). Also consistent with previous studies, ZA3-Ep10 nanoparticles produced protein mostly in the lungs (70%) [3].

Interestingly, despite cKK-E12 having the most diverse biodistribution profile (Fig. 2), it had the narrowest expression pattern with almost all translation occurring in the liver. We also noted that, despite having low accumulation in the spleen and lungs, $200O_{i10}$ had significant levels of protein expression in these organs. These discrepancies between biodistribution and protein expression are commonly observed in mRNA delivery studies [19,60]. While it isn't always clear why these discrepancies occur, we postulate that a complex combination of cell-specific differences in nanoparticle internalization, endosomal escape, and translational kinetics likely contribute to this. Based on these results, we categorized the lipid nanoparticles into three categories based on the organ(s) in which they facilitated protein expression: liver (C12–200 and cKK-E12), hybrid ($200O_{i10}$), and lung (ZA3-Ep10).

2.2. Base modifications improve translation in the spleen in a nanoparticle-dependent manner

To determine the influence of mRNA modifications on protein translation, we combinatorially formulated nanoparticles with the four lipids and five modified mRNAs encoding mLuc. Nanoparticles were injected intravenously at an mRNA dose of 0.75 mg/kg. Six hours later, mice were sacrificed, and their major organs were imaged for bioluminescence (left column, Figs. 3, S2). Luminescence was quantified in each organ using a region of interest analysis to determine relative protein expression (middle column, Figs. 3, S2). We also calculated the fold increase in expression resulting from mRNA modifications for each organ by normalizing the bioluminescence for that modification by the bioluminescence for unmodified mLuc in that organ (right column, Figs. 3, S2).

For nanoparticles incorporating C12–200, only ψ-, m1ψ-, and m5C/ψ-modified mRNAs significantly improved overall Luc expression (Figs. 3A-B, S2). These improvements were driven by enhanced translation in the spleen. Of the mRNAs, m1ψ-modified mLuc was the most effective. It produced a 6-fold increase in overall expression and a marked 54-fold increase in spleen expression compared to unmodified mLuc (Fig. 3C). Notably, the results for cKK-E12 differed from those for C12–200, even though these materials both deliver unmodified mRNA almost entirely to the liver. None of the five modified mLuc sequences resulted in a statistically significant increase in expression when delivered with cKK-E12 (Figs. 3D-E, S2). However, even though overall efficacy was not enhanced, the m1ψ and m5C/ψ modifications nonetheless increased spleen expression 8-fold and 7-fold, respectively, for this delivery vehicle.

We then examined $200O_{i10}$ lipid nanoparticles, which delivered unmodified mLuc to the liver, spleen, and lungs. As shown in Figs. 3G-H, S2, the m1ψ modification performed best, producing an 11-fold increase in overall Luc expression. As with C12-200, this enhancement was

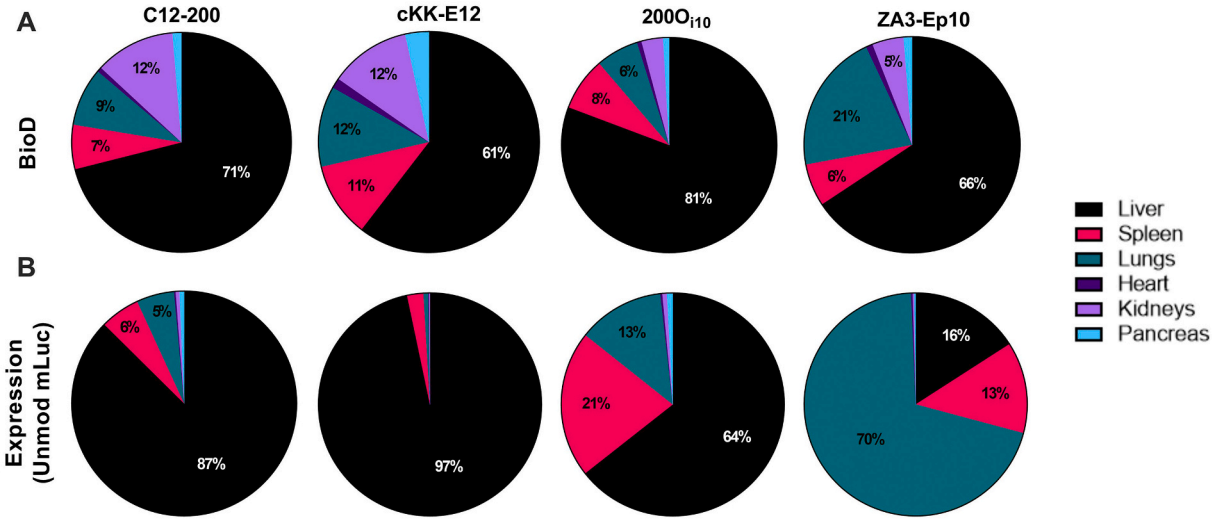

Fig. 2. mRNA biodistribution does not indicate sites of mRNA translation. (A) To assess biodistribution, mice received IV injections of LNPs carrying Cy5-labeled mRNA at a dose of 0.75 mg/kg. LNPs predominantly distributed to the liver and to the spleen, lungs, and kidneys to a lesser extent. (B) Protein expression was determined by injecting mice *via* tail vein with LNPs carrying unmodified luciferase mRNA at a dose of 0.75 mg/kg. C12–200 and cKK-E12 facilitated protein expression almost entirely in the liver. $200O_{i10}$ delivery resulted in a more diverse expression profile, as did ZA3-Ep10, with 70% of luciferase expression occurring in the lungs.

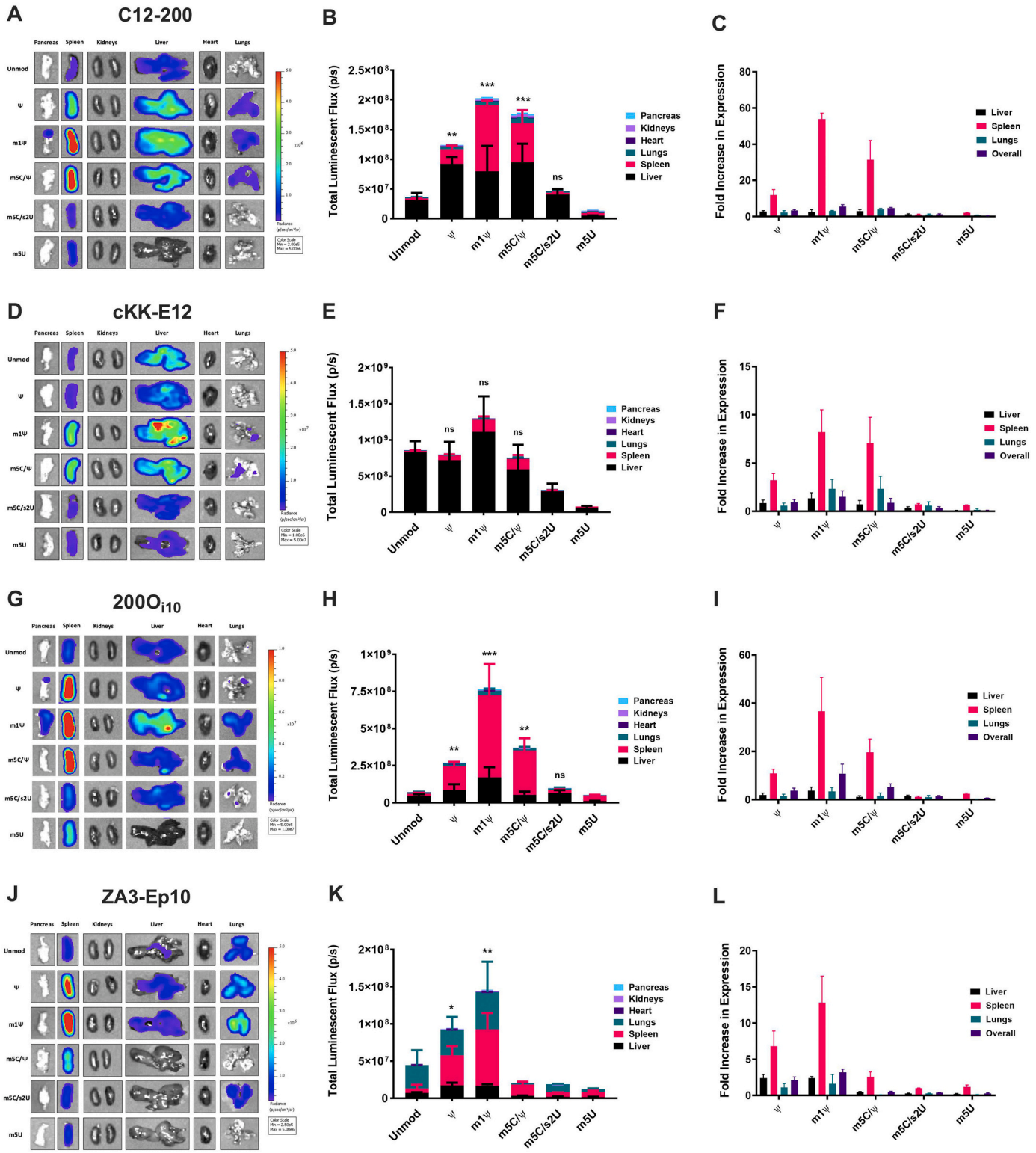

Fig. 3. The m1ψ mRNA modification best enhanced *in vivo* luciferase expression for all delivery vehicles, with greatest improvement in the spleen. Mice were IV-injected with LNPs carrying either unmodified or modified luciferase mRNA at a dose of 0.75 mg/kg. Six hours after injection, organs were harvested and imaged for bioluminescence by IVIS. (A-C) Protein expression resulting from C12-200 delivery was enhanced by the ψ, m1ψ, and m5C/ψ modifications, with the vast majority of the improvement in the spleen. (D—F) mRNA modifications did not improve overall expression for cKK-E12 mRNA delivery with statistical significance. Nonetheless, spleen expression improved with m1ψ and m5C/ψ modifications. (G-I) 200O$_{i10}$-mediated protein expression benefitted the most from nucleoside modifications, particularly with the m1ψ modification. Improvements in spleen expression were dramatic. (J-L) When formulated into ZA3-Ep10 LNPs, ψ and m1ψ modifications enhanced expression in the spleen, with very little improvement in the lungs. Error bars represent s.d. ($n = 3$; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; unpaired *t*-test relative to unmodified).

attributable to a substantial increase in spleen expression (37-fold over unmodified mRNA, Fig. 3I). Delivery of ψ- and m5C/ψ-modified mLuc with $200O_{i10}$ also resulted in significant improvements in overall Luc expression (4-fold and 5-fold, respectively).

As for the lung-targeting lipid nanoparticle ZA3-Ep10, the m1ψ modification, once again, enhanced protein expression most effectively in mice, with a 3-fold increase in overall Luc expression (Figs. 3J-K, S2). This, too, was driven by increased expression in the spleen (13-fold). Delivery of ψ-modified mLuc with this material also produced a 7-fold increase in spleen expression but no improvement in the lungs.

Although the performance of the IVT mRNAs varied significantly with delivery vehicle, there were several consistencies. For example, two of the modified mRNAs – m5C/s2U (25% substitution) and m5U – did not improve protein expression when incorporated into any of the four delivery vehicles. This confirms that there are many important factors in mRNA design, and that incorporations of base modifications known to decrease immune stimulation does not guarantee improved translation. The m5C/s2U modification had no impact on total expression when incorporated into C12–200 and $200O_{i10}$ nanoparticles, and it decreased expression for cKK-E12 and ZA3-Ep10. The m5U modification was always detrimental to efficacy, inducing up to 90% reductions in total Luc expression.

Another consistency across vehicles was that the m1ψ modification was always the most effective. This nucleoside modification enhanced overall efficacy 1.5-fold to 11-fold, depending on the delivery vehicle. Although the m1ψ modification offered modest improvements in liver and lung expression, improvements in Luc expression were driven by exceptional increases in spleen expression (8- to 54-fold), depending on the delivery vehicle. The increased expression in the spleen extended to the other two pseudouridine-containing modifications (ψ and m5C/ψ). A previous study noted a similar phenomenon in which ψ-modified mRNA delivered to mice with a commercially available transfection agent produced protein almost entirely in the spleen [61]. Together, the results in Fig. 3 provide an important takeaway: the choice of nucleoside modification should be tailored to the delivery vehicle, the target organ, and the degree of specificity required.

2.3. *m1ψ enhances mRNA translation in splenic monocytic lineage cells*

We next asked why m1ψ-modified mRNA improved protein expression when incorporated into some delivery vehicles but not others. We noted that $200O_{i10}$, which was the most spleen-tropic of the lipids tested with unmodified mRNA, benefited the most from nucleoside modifications. Conversely, the performance of the least spleen-directed material, cKK-E12, was not augmented by any modification. To better understand the effect of base modifications on protein expression in the spleen, we used flow cytometry to identify the splenic cell types being transfected. We used all four lipids to deliver unmodified and m1ψ-modified Cre recombinase-encoding mRNA (mCre) to Ai9 mice, which express tdTomato upon Cre-mediated recombination [62]. Importantly, this model reveals only which cells translate mCre, not the amount of protein expressed. Eighteen hours post-injection, spleens were removed, mechanically digested into single-cell suspensions, and analyzed by flow cytometry. The gating scheme for this experiment is shown in Fig. S3. Results were similar for all four lipid nanoparticles in that modified mRNA produced the best improvement in percent of cells transfected for monocytic lineage cells (Fig. 4). Generally, both modified and unmodified mRNA was delivered to splenic monocytes, macrophages, neutrophils, and dendritic cells, as evidenced by the fractions of tdTomato+ cells within these populations. Unmodified and modified mCre induced tdTomato activation in the highest percentage of neutrophils and macrophages, respectively, though splenic macrophages make up a

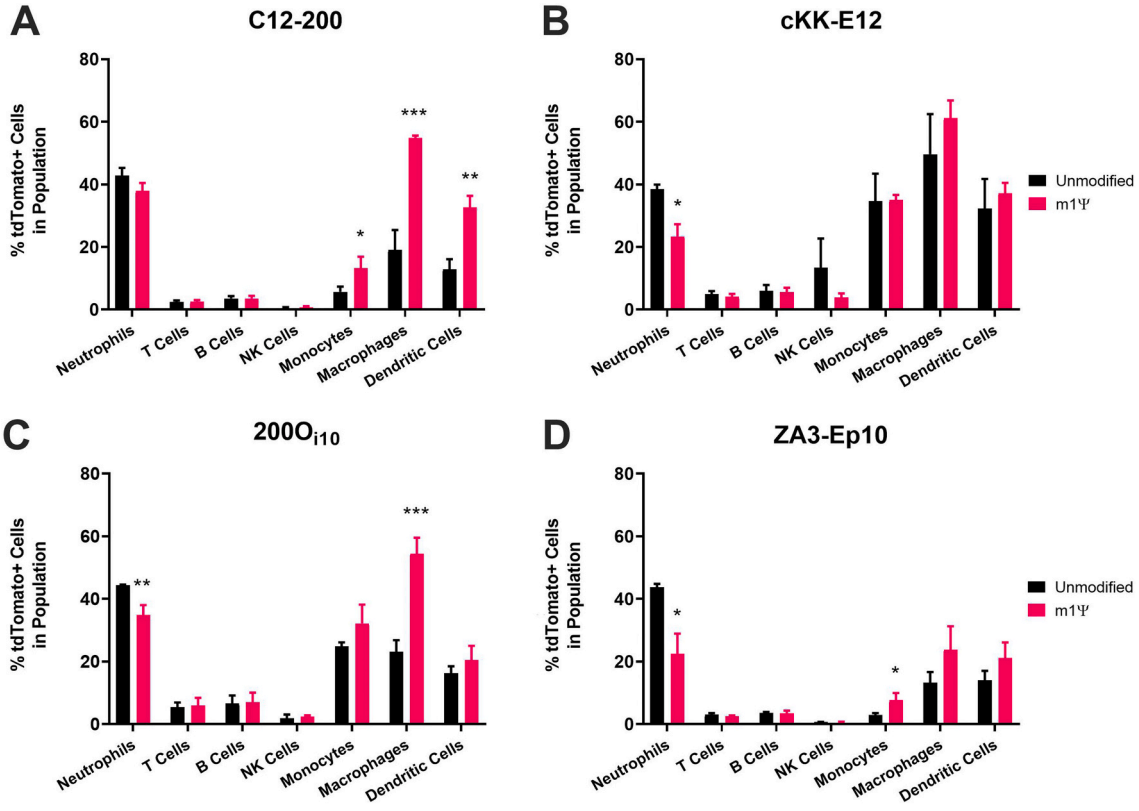

Fig. 4. m1ψ modifications, when effective, improved mRNA translation in monocytic lineage immune cells. Ai9 mice were injected with LNPs carrying unmodified or m1ψ-modifed Cre recombinase mRNA using either A) C12–200, B) cKK-E12, C) $200O_{i10}$, or D) ZA3-EP10 at a dose of 0.5 mg/kg. Cells were isolated from spleens 18 h post-injection and stained with antibodies to identify cell populations. Error bars represent s.d. ($n = 3$; $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; unpaired *t*-test relative to unmodified mRNA for each cell type).

considerably greater portion of the total splenocyte population than neutrophils. Overall, the data indicate that base modifications best improve transfection rates in monocytes and macrophages compared to other cell types in the spleen.

Because immune cells have evolved to recognize and degrade foreign nucleic acids, we hypothesized that the increased percentage of transfected monocytic splenocytes was due to the reduced immunogenicity of modified mRNAs [32,61]. We tested this hypothesis by conducting a reporter assay for immunogenicity in cultured macrophages. Specifically, we used RAW Blue™ cells, which express a secreted alkaline phosphatase (SEAP) reporter inducible by NF-κB and AP-1. NF-κB and AP-1 are transcription factors that mediate cellular inflammatory responses downstream of endosomal and cytosolic receptors including TLRs, RIG-I, and MDA-5. Therefore, cells that secrete higher levels of SEAP are undergoing an inflammatory response.

For all nanoparticles except cKK-E12, m1ψ-mRNA significantly reduced innate immunogenicity compared to unmodified mRNA (Fig. S4). Specifically, m1ψ decreased SEAP levels for 200O_{i10}, C12–200, and ZA3-Ep10 by 40, 30, and 70%, respectively. The lipid cKK-E12 was the only delivery material for which modifications did not significantly improve protein expression *or* reduce immunogenicity. These data suggest that improvements in splenic protein expression with modified mRNA correlate with reduced immunogenicity. While the SEAP reduction we observed in this study was significant, it was also fairly modest and may not completely account for the drastic increases in mRNA translation shown in Fig. 3. A simple explanation for this may be that RAW Blue cells are a useful model for interrogating innate immunogenicity *in vitro* but may not fully recapitulate the signaling endogenous to monocytic splenocytes. Further, one study showed that m1ψ-modified mRNAs exhibit increased ribosome density through eIF2α-dependent and independent mechanisms, indicating another potential mechanism by which modified mRNAs undergo enhanced translation independent of innate immune activation [39].

2.4. The site of endogenous protein expression affects the extent to which mRNA modifications enhance translation

Finally, we asked whether the results we observed for reporter proteins Luc and Cre recombinase also applied to mRNA encoding a therapeutic protein. As such, we delivered mRNA encoding murine erythropoietin (mEPO) synthesized with the same five modified nucleosides and delivered with the four lipid nanoparticles. EPO is produced predominantly in the kidneys and to a lesser extent in the liver. It is then secreted into the bloodstream to stimulate red blood cell production in the bone marrow [63]. Because EPO is secreted (unlike Luc and Cre), we measured only overall changes in serum EPO levels instead of expression in individual organs. Despite our observations that modifications are most impactful in the spleen, we hypothesized that the modification of mEPO would best improve protein expression in the liver because it is a site of endogenous EPO production.

As expected, EPO expression was greatest using liver-targeting nanoparticles (Fig. 5A). C12-200 lipid nanoparticles encapsulating m1ψ-modified mEPO produced a 2.6-fold increase in protein expression relative to unmodified mEPO. While this improvement did not match the overall increase in Luc expression for m1ψ (5.6-fold), it was equivalent to the enhancement in liver Luc expression for the m1ψ modification (2.5-fold). Results for cKK-E12 lipid nanoparticles were also consistent with mLuc delivery data. Indifferent to mRNA modification, this ionizable lipid nonetheless boasted the highest serum EPO concentrations of any nanoparticle given its exceptional potency in the liver. Next, 200O_{i10} nanoparticles benefitted the most from mRNA base modifications, with m1ψ-modified mEPO producing a 5.6-fold increase in serum EPO levels relative to unmodified mRNA. Improvements observed from the various mRNA modifications were more consistent with our Luc

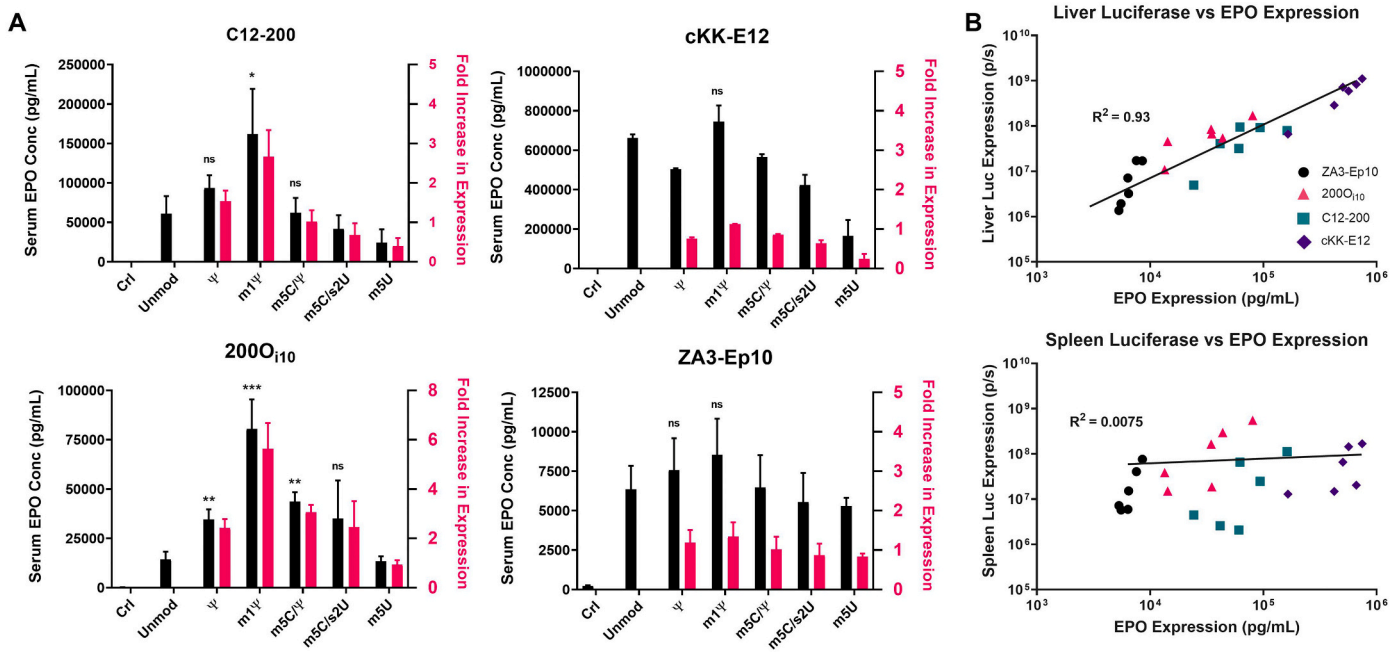

Fig. 5. The m1ψ modification best enhanced *in vivo* erythropoietin expression at the site of endogenous protein expression. A) Unmodified or modified erythropoietin mRNA was delivered using either C12–200, cKK-E12, 200O_{i10}, or ZA3-EP10 lipid nanoparticles at a dose of 0.75 mg/kg. Serum EPO levels were determined by ELISA. m1ψ-modified mRNA facilitated a statistically significant increase in EPO expression when delivered with C12–200 and 200O_{i10}. The vehicle cKK-E12 mediated the highest serum levels of EPO, with the m1ψ modification facilitating a small but non-statistically significant increase in EPO concentrations. There was no statistically significant increase in EPO liver production with the m1ψ modification using lung-targeting ZA3-Ep10 lipid nanoparticles. Error bars represent s.d. ($n = 3$; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; unpaired *t*-test relative to unmodified mRNA). B) The expression of erythropoietin (EPO) correlates strongly with the expression of luciferase in the liver but not in the spleen for the delivery of modified mRNAs for all LNPs. The six different mRNAs were delivered using the four lipid nanoparticles: purple diamonds = cKK-E12, teal squares = C12–200, pink triangles = 200Oi10, black circles = ZA3-Ep10. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression data from the liver than with overall Luc expression. Finally, ZA3-Ep10 nanoparticles facilitated the lowest serum EPO concentrations of any delivery vehicle due to its relatively low tropism for the liver. As expected for a lung targeting formulation, ZA3-Ep10 nanoparticle potency was not influenced by mEPO modifications that would be most influential in the liver (Fig. 5A).

With these data in hand, we compared EPO expression to the expression of Luc in the liver and in the spleen for all four delivery vehicles, using all six mRNAs. As shown in Fig. 5B, the EPO and liver Luc expression data collected for the 24 mRNA-lipid combinations correlated well ($R^2 = 0.93$), whereas the EPO and spleen Luc expression data did not ($R^2 = 0.0075$). This analysis indicates that EPO was produced predominantly in the liver. This is interesting because some of the delivery materials (*e.g.*, $200O_{i10}$) were expected to produce EPO in the spleen based on biodistribution and tropism data and, therefore, benefit significantly from mRNA modification. However, this was not the case – thus demonstrating that, at least for endogenous proteins, the site of endogenous protein expression must be considered in addition to delivery vehicle chemistry when assessing the potential benefits of modified mRNA inclusion in lipid nanoparticle formulations.

3. Conclusions

These results shed important light on the use of modified mRNAs to improve protein expression and decrease aberrant immunogenicity. While modified bases reduced inflammation in macrophages and improved protein translation in a variety of organs in an ionizable amino lipid nanoparticle-dependent manner, the greatest increases in translation occurred in monocytic lineage cells, which are plentiful in the spleen. However, in the case of proteins that are not endogenously produced in the spleen or in immune cells, mRNA modifications may increase splenic mRNA translation to a lesser extent. Together, our data indicate that the use and choice of modified mRNA in lipid nanoparticle formulations is a complex decision that must be made in the context of the native site of target protein expression (when applicable), the target cell type, and the delivery vehicle itself.

4. Materials and methods

4.1. Materials

Cy5-labeled mRNA, ψ- 5′-triphosphate, m1ψ- 5′-triphosphate, m5C-5′-triphosphate, s2U- 5′-triphosphate, and m5U- 5′-triphosphate were purchased from Trilink Biotechnologies (San Diego, CA). MEGAscript™ T7 Transcription Kit was purchased from Thermo Fisher Scientific (Waltham, MA). ScriptCap™ 2'-O-Methyltransferase Kit was purchased from CellScript (Madison, WI). Cholesterol and 1,2-epoxyhexadecane (C12) were purchased from Sigma Aldrich (St Louis, MO). The phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) and C14-PEG2000 were purchased from Avanti Polar Lipids (Alabaster, AL). DMG-PEG2000 was purchased from NOF America (White Plains, NY). The isodecyl acrylate (O_{i10}) amine and the 2[4–2(2-aminoethyl)amino) ethylpiperazine-1-YL)ethan-1-amine (200) were purchased from Sartomer (Colombes, France) and Enamine (Princeton, NJ), respectively. XenoLight D-Luciferin Potassium Salt was purchased from PerkinElmer (Waltham, MA). The lipid cKK-E12 was generously donated by the Anderson Lab at the Massachusetts Institute of Technology (Cambridge, MA). The Mouse Erythropoietin Quantikine ELISA Kit was purchased from R&D Systems (Minneapolis, MN).

4.2. mRNA synthesis

mRNAs were synthesized as previously described [64,65]. Linearized plasmids encoding firefly luciferase (pTEV-Luc-A101), Cre recombinase (pTEV-Cre-A101), mouse erythropoietin (pTEV-muEPO-A51), and eGFP (pTEV-eGFP-A101) were transcribed using the MEGAscript™ T7 Transcription Kit. IVT reactions were performed in the presence of 100% modified uridines (*i.e.* ψUTP, m1ψUTP, or m5UTP) except for the m5C/s2U modified mRNA where 5 methylcytosine and thiouridine were added at a 1:4 mol ratio (25%) in the NTP mixture to ensure efficient transcription and subsequent translation. mRNAs were capped using either the ScriptCap™ 2′-O-Methyltransferase Kit (eGFP, muEPO) or co-transcriptionally (mLuc, Cre) using the trinucleotide cap1 analog, CleanCap (TriLink). All mRNAs were purified by cellulose purification as described [66]. All mRNAs were analyzed by electrophoresis using agarose gels, endotoxin tested, and dsRNA content assessed using dot blot.

4.3. Lipid synthesis

To synthesize the ionizable lipid C12–200, the amine 2[4–2(2-aminoethyl)amino)ethylpiperazine-1-YL)ethan-1-amine (200) was combined with the tail 1,2-epoxyhexadecane (C12) in a glass scintillation vial at a molar ratio of 1: 5 and stirred for 3 days at 90 °C without solvent. To synthesize the lipid $200O_{i10}$, the amine 2[4–2(2 aminoethyl) amino)ethylpiperazine-1-YL)ethan-1-amine (200) was combined with the tail isodecyl acrylate (O_{i10}) in a glass scintillation vial at a molar ratio of 1: 5 and stirred for 3 days at 90 °C without solvent. The lipids were then purified using a Teledyne ISCO Chromatography system (Thousand Oaks, CA) to isolate the fully substituted lipid product. The zwitterionic amino lipid ZA3-Ep10 was synthesized as previously described [3].

4.4. Lipid nanoparticle formulations

The lipids cKK-E12, C12-200, and $200O_{i10}$ were formulated into lipid nanoparticles as previously described [19,20]. Specifically, ionizable lipid, DOPE, cholesterol, and C14-PEG2000 were combined at a molar ratio of 35: 16: 46.5: 2.5 in 100% ethanol. The mRNA was diluted in 10 mM sodium citrate buffer (pH 3). For cKK-E12 and C12–200, the solutions were combined in a microfluidic device (Precision Nanosystems) at a flow ratio of 1: 3 (ethanol: aqueous phase) with a total flow rate of 4 mL/min (cKK-E12) or 12 mL/min (C12–200). For $200O_{i10}$, the lipid and mRNA solutions were combined at 1: 1 volume ratio, and rapidly mixed by pulse vortexing. The final weight ratio of lipid: mRNA was 10: 1. The zwitterionic lipid ZA3-Ep10 was formulated as previously described [3], by combining ZA3-Ep10, cholesterol, and DMG-PEG2000 at a molar ratio of 50: 38.5: 0.5 in 100% ethanol. The mRNA was diluted in 10 mM sodium citrate (pH 3). The solutions were combined by microfluidic mixing at a flow ratio of 1: 3 (ethanol: aqueous phase) with a total flow rate of 12 mL/min. The final weight ratio of lipid: mRNA was 7.5: 1. All lipid nanoparticles were dialyzed against PBS for 1 h prior to use.

4.5. Biodistribution studies

All animal experiments were conducted using institutionally approved protocols (IACUC). Female C57BL/6 mice (Charles River Laboratories) were injected *via* tail vein with lipid nanoparticles carrying Cy5-labeled mRNA at a dose of 0.75 mg/kg (0.375 mg/kg for ZA3-Ep10). One hour after injection, the mice were sacrificed and the pancreas, spleen, liver, kidneys, heart, and lungs were harvested and imaged for Cy5 fluorescence at an excitation of 649 nm and an emission of 670 nm on an IVIS® imaging system (PerkinElmer). The total radiant efficiency was determined by a region of interest analysis using the Living Image® software (PerkinElmer). The percent biodistribution for each organ was determined by dividing the signal in that organ by the total signal.

4.6. In vivo luciferase mRNA delivery

Female C57BL/6 mice were injected *via* tail vein with each of the four lipid nanoparticles carrying either unmodified, ψ-modified, m1ψ-

modifed, m5C/ψ-modified, m5C/s2U-modifed, or m5U-modified firefly mLuc at a dose of 0.75 mg/kg. Six hours after injection, mice received an intraperitoneal injection of 130 μL D-luciferin at a concentration of 30 mg/mL. Fifteen minutes later, the mice were sacrificed, and the pancreas, spleen, liver, kidneys, heart, and lungs were harvested and imaged for bioluminescence by IVIS®. The total luminescent flux was determined by a region of interest analysis using the Living Image® software.

4.7. In vivo erythropoietin mRNA delivery

Female C57BL/6 mice were injected *via* tail vein with each of the four lipid nanoparticles carrying either unmodified, ψ-modified, m1ψ-modifed, m5C/ψ-modified, m5C/s2U-modifed, or m5U-modified mouse erythropoietin mRNA at a dose of 0.75 mg/kg. Six hours after injection, mice were sacrificed, and blood was collected by cardiac puncture. Serum was isolated by centrifuging blood samples in BD Microtainer Serum Separator Tubes at 14,000 RPM for 10 min. Serum samples were diluted either 1: 10 or 1: 100, and erythropoietin concentrations were determined using the Mouse Erythropoietin Quantikine ELISA Kit according to the manufacturer's instructions.

4.8. Flow cytometry

Female Ai9 mice received tail vein injections of lipid nanoparticles carrying either unmodified or m1ψ-modifed Cre mRNA at a dose of 0.5 mg/kg. Mice were sacrificed 18 h post-injection, and the spleens was harvested. Cells were isolated by mashing the spleen through a 70 μm nylon mesh cell strainer (Thermo Fisher), followed by treatment with red blood cell lysis buffer (Thermo Fisher). Cells were then pelleted by centrifuging at 400 x g for 5 mins, followed by resuspension in DMEM supplemented with 10% FBS. 2×10^6 cells were suspended in blocking buffer (PBS + 1% FBS + 10% Fc block; Miltenyi Biotec) and stained with antibodies (BioLegend) diluted 1: 100 against CD45, CD19, CD11c, CD11b, CD64, F4/80, CD31, Ly-6G, CD3, and MCHII for 30 min at 4 °C. Cells were washed by centrifugation, resuspended, and analyzed by flow cytometry using a NovoCyte 3000 (ACEA Biosciences). Flow cytometry data were compensated and analyzed using NovoExpress software (ACEA Biosciences).

4.9. In vitro immunogenicity

Raw Blue™ cells were obtained from InvivoGen and maintained at 37 °C, 5% CO_2 in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 100 μg/mL Normocin supplemented with Zeocin selective antibiotic according to the manufacturer's instructions. Cells were seeded at 100,000 cells/well in 96-well plates and treated with 40 nM (0.5 μg/mL) modified or unmodified mRNA using each of the four lipid nanoparticles investigated in this work. 48 h later, samples were analyzed for SEAP using a QUANTI-Blue™ assay (InvivoGen) performed according to the manufacturer's instructions. Data were collected using a Synergy H1 plate reader (Biotek), and absorbance values for treated samples were normalized to that of untreated control cells.

Declaration of Competing Interest

K.A.W. is an inventor on at least seven U.S. Patents that are related to the materials described here. D.J.S. is a co-founder of ReCode Therapeutics. D.W. and N.P. are named on U.S. patents describing the use of RNA containing modified nucleotides.

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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.2021.11.022.

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