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Safety Evaluation of Lipid Nanoparticle–Formulated Modified mRNA in the Sprague-Dawley Rat and Cynomolgus Monkey

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Abstract

The pharmacology, pharmacokinetics, and safety of modified mRNA formulated in lipid nanoparticles (LNPs) were evaluated after repeat intravenous infusion to rats and monkeys. In both species, modified mRNA encoding the protein for human erythropoietin (hEPO) had predictable and consistent pharmacologic and toxicologic effects. Pharmacokinetic analysis conducted following the first dose showed that measured hEPO levels were maximal at 6 hours after the end of intravenous infusion and in excess of 100-fold the anticipated efficacious exposure (17.6 ng/ml) at the highest dose tested.[24] hEPO was pharmacologically active in both the rat and the monkey, as indicated by a significant increase in red blood cell mass parameters. The primary safety-related findings were caused by the exaggerated pharmacology of hEPO and included increased hematopoiesis in the liver, spleen, and bone marrow (rats) and minimal hemorrhage in the heart (monkeys). Additional primary safety-related findings in the rat included mildly increased white blood cell counts, changes in the coagulation parameters at all doses, as well as liver injury and release of interferon γ–inducible protein 10 in high-dose groups only. In the monkey, as seen with the parenteral administration of cationic LNPs, splenic necrosis and lymphocyte depletion were observed, accompanied with mild and reversible complement activation. These findings defined a well-tolerated dose level above the anticipated efficacious dose. Overall, these combined studies indicate that LNP-formulated modified mRNA can be administered by intravenous infusion in 2 toxicologically relevant test species and generate supratherapeutic levels of protein (hEPO) in vivo.

Keywords

modified mRNA, lipid nanoparticle, toxicology, pharmacokinetics, drug discovery

The promise of mRNA as a novel modality to deliver therapeutic proteins in humans is vast, as evidenced by the growth and success of recombinant human therapeutic proteins over the last 3 decades, such as recombinant human insulin for the treatment of diabetes mellitus.[17] Protein expression directed by exogenous mRNA offers many advantages over other nucleic acid–based concepts, as well as recombinant proteins. Potential advantages of mRNA over DNA-based technology include (1) no integration into the host genome thereby circumventing the risk of deleterious chromosomal changes, and (2) faster and more efficient expression with proper modifications, since mRNA therapeutics only require access to the cytoplasm. In comparison with recombinant proteins, mRNA would have lower manufacturing costs and could enable access to intracellular as well as cell membrane–bound therapeutic targets. The biggest challenges of mRNA technology are its potential for immunogenicity and its relatively poor in vivo stability. These challenges have been addressed through progress in chemistry and sequence engineering (eg, optimization of the 5′ cap, 5′-, and 3′-untranslated regions and coding sequences) and through the use of specific nucleotide modifications.[16,21,29]

Nucleotide-modified mRNA is nearly identical to naturally occurring mammalian mRNA, with the exception that certain nucleotides, normally present in mammalian mRNA, are partially or fully replaced with nucleosides, such as pyrimidine nucleosides—specifically, pseudouridine, 2-thiouridine,

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5-methyl cytosine, or N1-methyl-pseudouridine.[2,14,16,21,29] These naturally occurring pyrimidine nucleotides are present in mammalian tRNA, rRNA, and small nuclear RNAs.[20] Incorporation of these nucleotides in place of the normal pyrimidine base has been shown to minimize the indiscriminate recognition of exogenous mRNA by pathogen-associated molecular pattern receptors, such as toll-like receptors, retinoic acid–inducible gene 1, melanoma differentiation-associated protein 5, nucleotide-binding oligomerization domain-containing protein 2, and protein kinase R.[7]

Given the lability of a naked mRNA molecule, the development of mRNA therapeutics has been further hampered by the lack of appropriate formulations for delivery and potentially as a targeting mechanism to a diseased organ or tissue.[12] However, the application of lipid-based nanoparticle delivery systems, initially developed for the in vivo delivery of siRNA, has enabled systemic administration of modified mRNA.[22] Adequate delivery of mRNA with lipid nanoparticles (LNPs) has been demonstrated for mRNA-based vaccines, where intramuscular injection of low doses of mRNA formulated in either LNPs or nanoemulsion induced immune protection from influenza and respiratory syncytial virus in mice, as well as cytomegalovirus and respiratory syncytial virus in monkeys.[9] Furthermore, a single administration of modified mRNA-LNP complexes in mice by various routes resulted in high, sustained protein production.[19] Finally, Thess et al[25] reported that repeated administration of unmodified mRNA in combination with the nonliposomal polymeric delivery system (*Trans*IT) induced high systemic protein levels and strong physiologic responses in mice. These authors also noted similar observations following single-dose administration of erythropoietin (EPO)–mRNA in LNPs to pigs and monkeys.

LNPs have been reported to be clinically effective for the delivery of siRNA.[6] The LNP vehicle is currently in late-phase clinical trials of a synthetic siRNA in patients suffering from transthyretin amyloidosis and has been well tolerated in this population.[6] Therefore, considerable work has been done to understand the safety profile of systemic administration of siRNA-LNPs.[3] Here, we set out to describe, for the first time, the pharmacology and toxicologic effects of repeated administration of hEPO-mRNA in LNPs in male Sprague-Dawley rats and female cynomolgus monkeys.

Materials and Methods

Animals and Husbandry

The study plan and any amendments or procedures involving the care and use of animals in these studies were reviewed and approved by the Institutional Animal Care and Use Committee of Charles River Laboratories Preclinical Services (Montreal and Sherbrooke, Canada). During the study, the care and use of animals were conducted according to the guidelines of the US National Research Council and the Canadian Council on Animal Care.

Male Sprague-Dawley rats (Charles River Laboratories) were 11 to 12 weeks old and weighed between 390 and 497 g at dose initiation. Animals were group housed in polycarbonate bins and separated during designated procedures. The temperature of the animal room was kept between 19°C and 25°C, with humidity between 30% and 70%. The light cycle was 12 hours light and 12 hours dark, except during designated procedures. Animals were fed PMI Nutrition International Certified Rodent Chow No. 5CR4 (14% protein) ad libitum throughout the in-life studies, except during designated procedures. Municipal tap water treated by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system. Environmental enrichment was provided to animals per standard operating procedures of Charles River Laboratories (Montreal, Canada), except during study procedures and activities.

Female cynomolgus monkeys (Charles River Laboratories) were 1.5 to 6 years old and weighed 2.5 to 5.1 kg at the initiation of dosing. Animals were housed in stainless-steel cages and separated during designated procedures. The temperature of the animal room was kept between 20°C and 26°C, with humidity between 30% and 70%. The light cycle was 12 hours light and 12 hours dark except during designated procedures. Animals were fed PMI Nutrition International Certified Primate Chow No. 5048 (25% protein). Municipal tap water treated by reverse osmosis and ultraviolet irradiation was freely available to each animal via an automatic watering system. Psychological and environmental enrichment was provided to animals per standard operating procedures of Charles River Laboratories (Montreal, Canada) except during study procedures and activities.

Control, Test, and Reference Items

An 850-nucleotide messenger RNA was prepared by in vitro transcription from a linearized DNA template with T7 RNA Polymerase. The DNA template encoded the T7 promoter, a 5′ untranslated region, the 579-nucleotide open reading frame encoding human EPO (hEPO) mature protein with signal sequence, a 3′untranslated region, and a polyadenylated tail. The in vitro transcription was performed with the canonical nucleotides adenosine triphosphate and guanosine triphosphate and the modified nucleotides 1-methylpseudouridine triphosphate and 5-methylcytidine triphosphate. The mRNA contains a 5′ Cap 1 structure, which consisted of 7-methylguanosine linked to the 5′ nucleoside of the mRNA chain through a 5′–5′ triphosphate bridge and 2′-O-methyl group present on the first nucleotide of the mRNA.[23] The messenger RNA was purified and buffer exchanged into low ionic strength buffer for formulation.[18] The final mRNA had a calculated molecular weight of 277 786 Da.

The mRNA-loaded LNPs were generated via stepwise ethanol dilution, with an approach adapted from previously demonstrated methods.[13,30] The LNP formulation was prepared by dissolving the lipids (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3),

Table 1. Experimental Design for Safety Study: Rat.[a]

Group No.	Test Material	Dose Level, mg/kg[b]	Intravenous Administration	Dose Concentration mg/ml
1	PBS	0	10 min infusion, 2×/wk	0
2	mRNA EPO	0.03	10 min infusion, 2×/wk	0.006
3	mRNA EPO	0.1	10 min infusion, 2×/wk	0.02
4	mRNA EPO	0.3	10 min infusion, 2×/wk	0.06
5	mRNA EPO	0.3	10 min infusion, 1×/wk	0.06
6	Empty LNP	0.3	10 min infusion, 2×/wk	0.06

Abbreviations: EPO, erythropoietin; LNP, lipid nanoparticle; PBS, phosphate-buffered saline.
[a]No. of males per group, *n* 24. Dose volume per group, 5 ml/kg. Dose rate per group, 30 ml/kg/h.
[b]Dose levels in terms of mRNA content. For group No. 6, the dose level is listed in terms of the same amount of lipid:mRNA ratio (by weight).

Table 2. Experimental Design for Safety Study: Monkey.[a]

Group No.	Test Material	Dose Level (mg/kg)[b]	Intravenous Administration	Dose Concentration (mg/mL)
1	PBS	0	60 min infusion (2×/wk)	0
2	mRNA EPO	0.03	60 min infusion (2×/wk)	0.006
3	mRNA EPO	0.1	60 min infusion (2×/wk)	0.02
4	mRNA EPO	0.3	60 min infusion (2×/wk)	0.06
5	mRNA EPO	0.3	60 min infusion (1×/wk)	0.06
6	Empty LNP	0.3	60 min infusion (2×/wk)	0.06

Abbreviations: EPO, erythropoietin; LNP, lipid nanoparticle; PBS, phosphate-buffered saline.
[a]No. of females per group, *n* 3. Dose volume per group, 5 ml/kg. Dose rate per group, 5 ml/kg/h.
[b]Dose levels in terms of mRNA content. For group No. 6, the dose level is listed in terms of the same amount of lipid to mRNA ratio (by weight).

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC), cholesterol, and 1,2-dimyristoyl-rac-glycerol, methoxypolyethylene glycol (PEG2000-DMG) in ethanol. The 4 lipids were prepared as a combined stock, with a total concentration of 12.5 mM (molar ratio of 50:10:38.5:1.5, MC3:DSPC:cholesterol: PEG2000-DMG). In brief, the solution containing lipids was mixed with an acidic aqueous buffer containing mRNA (0.18 mg/ml, pH 4.0) in a T-mixer device. The resulting LNP dispersion was diluted and subsequently purified and concentrated by tangential flow filtration. The formulation was filtered through a clarification filter (0.8/0.2 μm nominal). Prior to storage, the formulation was additionally filtered through 2 in-line sterile filters (0.2 μm) and aseptically filled into sterilized vials, stoppered, and capped. Empty LNPs were generated with a similar approach, whereby mRNA was excluded from the process.

The final LNP lipid concentration was determined with an ultraperformance liquid chromatography system with online charged aerosol detection. The total concentrations of lipids in the final mRNA-LNPs and empty LNPs were 22.0 and 13.1 mg/ml, respectively. The final mRNA content in hEPO LNPs was quantified by ultraviolet analysis, resulting in an mRNA concentration of 1.2 mg/ml. Measured lipid and mRNA concentration values enabled dilution with phosphate-buffered saline (PBS) to target levels for dosing (Tables 1, 2). Particle hydrodynamic diameters were determined by dynamic light scattering. Resulting diameters for mRNA and empty LNPs were 81 nm (0.08 polydispersity index) and 61 nm (0.10 polydispersity index), respectively. Total mRNA encapsulation was quantified with the Ribogreen assay (ThermoFisher Scientific). The final value for hEPO-mRNA in LNP encapsulation was 97%. Additional information for the control, test, and reference items is provided in Supplemental Table 1.

Male Rat Study Design

Only male rats were used for this study, as there was no expected sex-specific differences in metabolism, distribution, or toxicity. The negative control, test, or reference items were administered over the course of 2 weeks in a 10-minute intravenous (IV) infusion via a caudal vein at a dose level, dose volume, and frequency listed in Table 1. Dose levels for each study were based on previous pharmacology data demonstrating production of efficacious levels of hEPO in the rat and cynomolgus monkey at doses $\leq$0.03 mg/kg of mRNA. Based on pharmacokinetic (PK) data indicating predictable increases in protein expression with dose, the mid- and high doses for these studies were selected to achieve significant multiples of the efficacious dose level. Since PK behavior and physiologic consequences are well defined for EPO therapy, we employed a similar approach in our study of hEPO-mRNA in LNPs.[15,16,25] Each infused dose was administered with a temporary indwelling catheter inserted in a caudal vein connected to an injection set and infusion pump. The animals were temporarily restrained for the dose administration and not sedated. The dose volume for each animal was based on the most recent body weight measurement. The first day of dosing was designated as day 1. Six males per group were used for toxicity assessment, 12 males per group for immunology assessment, and 6 males per group for PK / pharmacodynamic (PD) assessment. The following end points were evaluated: clinical signs (including observations of the infusion sites), body weights, food consumption, PK/PD, clinical pathology (hematology, coagulation, and clinical chemistry), macro- and microscopic examination of tissues, and immunotoxicology markers: histamine, interleukin 6 (IL-6), interferon γ–induced protein 10 (IP-10), tumor necrosis factor α (TNF-α), interferon α (IFN-α), and complement (C3).

Blood samples were collected from nonfasted animals and analyzed for hematology on day 9 and from fasted animals for hematology, coagulation, and clinical chemistry on day 16 (at necropsy). For PD (hEPO) or PK (hEPO-mRNA), blood samples were collected and processed to plasma prestudy and at 2, 6, 24, and 48 hours after the end of injection/infusion on days 1 and 15. After processing, the plasma samples were stored in a freezer set to maintain −80°C until analyzed. For cytokines (ie, IL-6, IP-10, TNF-α), histamine, and complement (C3) analysis, blood samples were collected prestudy and at 5 minutes and 2,

6, and 24 hours after the end of injection/infusion on days 1 and 15 in K_3EDTA tubes and processed to plasma or serum (no anticoagulant) for IFN-α analysis.

Female Monkey Study Design

Female monkeys were used for this study, as there was no expected sex-specific differences in metabolism, distribution, or toxicity. The negative control, test, and reference items were administered over the course of 2 weeks in a 60-minute IV infusion via an appropriate peripheral vein (eg, saphenous or brachial) at the dose level, dose volume, and frequency listed in Table 2. The dose volume for each animal was based on the most recent body weight measurement. The animals were temporarily restrained (on a sling or a chair) for the dose administration and not sedated. Each infused dose was administered with a temporary indwelling catheter inserted in a peripheral vein connected to an injection set and infusion pump. The first day of dosing was designated as day 1. The end points in this study included clinical signs (including observation of the infusion sites), body weights, food consumption, PK/PD, clinical pathology (hematology, coagulation, and serum chemistry), macro- and microscopic examination of tissues, and selected cytokines (interleukin 1 β [IL-1β], IL-6, TNF-α, and IP-10) and complement (C3a and C5b-9).

Blood samples were collected from overnight-fasted animals for hematology, coagulation, and clinical chemistry parameters at predose (baseline) and on day 16. Additionally, blood was analyzed on day 8 for hematology parameters only. For PK/PD assessments, blood samples were collected and processed to plasma at the following time points: predose; 2, 6, 24, and 48 hours after the first dose; and 6 hours after subsequent dosing occasions. After processing, the plasma samples were stored in a freezer set to −80°C until analyzed. Blood samples were collected in K_3EDTA tubes and processed to plasma for analysis of cytokines (ie, IL-1β, IL-6, TNF-α) and complement (ie, C3a and C5b-9) or to serum for analysis of IFN-α and IP-10 at the following time points for all groups: predose; at 2, 6, and 24 hours after the end of infusion on day 1; and at 2, 6, and 24 hours after the end of infusion on day 15. Additionally, for complement analysis only, blood samples were collected 2, 6, and 24 hours after the end of infusion on day 4 (groups 1–4 and 6).

Clinical Pathology

Hematology parameters were measured with Bayer Advia 120 Automated Hematology Analyzer (Siemens Healthcare). Standard coagulation parameters were measured on a START 4 Compact Stago Analyzer (Diagnostica Stago). Standard clinical chemistry parameters were measured with Modular Analytics (Roche/Hitachi).

Histamine, Cytokine and Complement Levels

Histamine levels in the rat plasma were determined with the Histamine EIA Kit (IM-2015; Immunotech). Serum levels of IFNα were determined with the Rat IFNα ELISA Kit (KT-60242; Kamiya Biomedical Company) and the Human IFNα Multi-subtype ELISA Kit (41105-1 or 41105-2; PBL Biomedical Laboratories). IL-6, IP-10, and TNFα in rat plasma were determined with the Rat Cytokine/Chemokine Magnetic Panel Kit (RECYMAG-65K; Millipore). IL1β, IL-6, and TNFα in monkey plasma were determined with the Non-Human Primate Cytokine/Chemokine Magnetic Panel Kit (PRCYTOMAG-40K; Millipore). IP-10 in the monkey serum was determined with the Monkey IP-10 Singleplex Magnetic Kit (LHB0001; Invitrogen). C3 levels in the rat plasma were determined with the Rat C3 ELISA Kit (GWB-A8B8AF; Genway). C3a levels in the monkey plasma were determined with the Human C3a EIA Kit (A031; Quidel). C5b-9 levels in the monkey plasma were determined with the Human C5b-9 ELISA Kit (558315; BD Bioscience).

Histopathology

Representative samples of the following tissues from all animals were preserved in 10% neutral buffered formalin: bone marrow (sternum), heart, infusion site (last dose), kidney, liver, lung, spleen, and thymus. Tissues were embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin. The histopathologic evaluation was internally peer reviewed.

hEPO bDNA

The bioanalysis of plasma samples for quantification of hEPO-mRNA levels was conducted at AxoLabs according to the bDNA method for mRNA detection developed by QuantiGene (Affymetrix).[26] Briefly, plasma samples were directly diluted in lysis buffer. On each bDNA plate, including a customized assay-specific set of probes, a dilution curve was pipetted with spiked standards into untreated plasma. Signal amplification was carried out with oligonucleotides bound to the enzyme alkaline phosphatase. The calculated amount in picograms was normalized to the amount of plasma in the lysate and to the amount of lysate applied to the plate. Since measurements in the PBS-treated control group were within the background level range, cross-reactivity of hEPO-mRNA to rat or monkey EPO mRNA was considered negligible.

hEPO ELISA

hEPO levels were measured with a human EPO Sandwich ELISA Kit (01630; Stemcell Technologies). For this assay, the lower and upper limits of quantitation were 12 and 800 pg/ml, respectively. Since predose measurements were within the background level range, cross-reactivity of hEPO to rat or monkey EPO was considered negligible.

Data Analysis and Reporting

The toxicokinetic parameters of human modified hEPO-mRNA and its expressed protein in plasma were calculated with a noncompartmental approach in WinNonlin Phoenix 64, version 6.3 (Pharsight). Dose-normalized maximum serum concentration (C_{max}/dose) and area under the curve (AUC/dose) were determined by dividing the respective parameters by dose and calculated by either WinNonlin or Excel. The mean, standard deviation, and percentage coefficient of variation of the toxicokinetic parameters were calculated in WinNonlin. All reported values were rounded to either 3 significant figures or 1 decimal place (time to reach maximum serum concentration [T_{max}], half-life [$t_{1/2}$]).

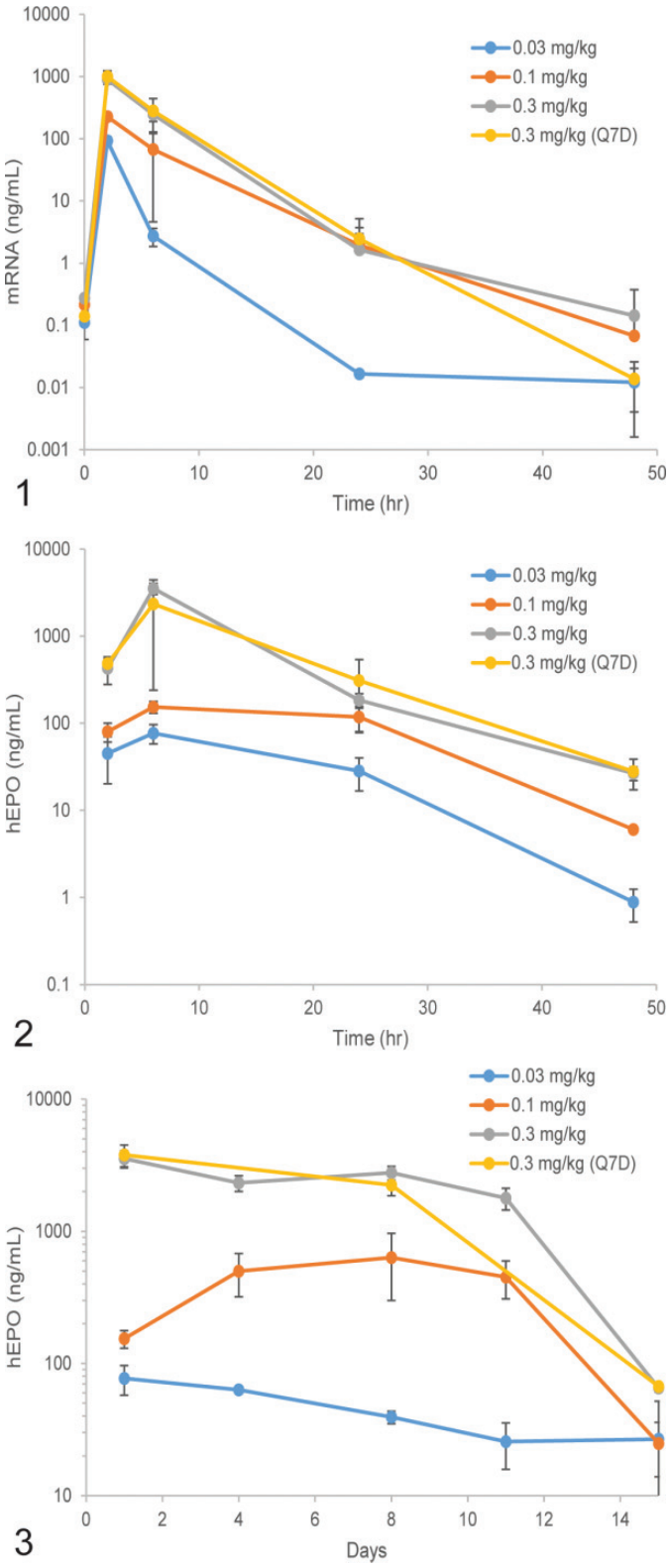
Results

Administration of hEPO-mRNA in LNP Results in Detection of Significant Serum hEPO Levels and Corresponding PD Effects in the Rat and Monkey

Toxicokinetic analysis in the rat revealed that hEPO-mRNA had a moderate half-life (2.9–5.7 hours) and low clearance (49.0–97.2 ml/h/kg; Fig. 1, Table 3). The C_{max}/dose values were consistent among the 4 dose groups, ranging from 2270 to 3320 ng/ml/mg/kg (Table 3). Measured hEPO levels were maximal approximately 6 hours after the 10-minute infusion (Fig. 2, Table 4). The AUC values (for hEPO-mRNA and hEPO) increased in more than a dose-proportional manner, between 0.03 and 0.3 mg/kg (Table 4). Plasma samples collected at 6 hours after each dose indicated that hEPO levels were constant at C_{max} at all dose levels until day 15, when measured hEPO levels were significantly decreased in the mid- and high-dosed groups (Fig. 3). Consistent with literature data,[1] peak reticulocytosis (PD marker described later) was observed by day 9, and levels remained elevated during the 15-day period. Overall, these results indicate that plasma concentrations of hEPO were mostly consistent throughout the study and exhibited greater-than-dose-proportional increases in AUC after IV administration.

Significant increases were noted in red blood cell and associated parameters (hemoglobin, hematocrit) in all male rat groups dosed with hEPO-mRNA in LNPs as compared with the PBS group and the group dosed with empty LNPs. Interestingly, the changes in red blood cell parameters (except mean corpuscular volume) were similar across all hEPO-mRNA-dosed groups and did not seem to be dose related (Fig. 4, Suppl. Fig. 1). In addition, dose-dependent increases in platelet counts and reticulocytes were noted, particularly at the highest doses administered twice weekly (Suppl. Fig. 1). Overall, these results indicate that repeated administration of hEPO-mRNA in LNPs achieves physiologically relevant and persistent hEPO levels that result in significant changes in precursor cells and mature red blood cell count at doses as low as 0.03 mg/kg.

Like in male rats, toxicokinetic findings in female monkeys indicated that the total exposure (AUC) to hEPO-mRNA and



Figures 1–3. Plasma concentration of hEPO mRNA (ng/ml; Fig. 1) and hEPO (ng/ml; Figs. 2, 3) in rats. Graphs represent mean values ($n = 6$); error bars indicate SD. Following a 10 minute infusion, peak plasma concentrations of hEPO mRNA appear to occur at approximately 2 hours, while peak plasma concentrations of hEPO are approximately at 6 hours. Note that hEPO levels appear constant at all dose levels until day 15. hEPO, human erythropoietin; Q7D, 1 dose per week.

Table 3. Toxicokinetic Values for hEPO mRNA in the Rat.[a]

Dose, mg/kg	$t_{1/2}$, h	Cmax, ng/ml	Cmax/Dose, ng/ml	AUC, h × ng/ml	AUC/Dose, h × ng/ml	Cl, ml/h/kg
0.03	5.7	92.6	3090	309	10,300	97.2
0.1	4.3	227	2270	1460	14,600	68.5
0.3	4	902	3010	5450	18,200	55.1
0.3[b]	2.9	995	3320	6120	20,400	49

Abbreviations: AUC, area under the curve; Cl, clearance; Cmax, maximum serum concentration; hEPO, human erythropoietin; $t_{1/2}$, half-life; Tmax, time to reach maximum serum concentration.
[a]Tmax per dose, 2 hours.
[b]One dose per week.

Table 4. Toxicokinetic Values for hEPO in the Rat.[a]

Dose, mg/kg	Cmax, ng/ml	AUC, h × ng/ml	$t_{1/2}$, h
0.03	77.1	1590	6.4
0.1	154	4480	8.7
0.3	3540	44 400	6.1
0.3[b]	2340	34 100	6.6

Abbreviations: AUC, area under the curve; Cmax, maximum serum concentration; hEPO, human erythropoietin; $t_{1/2}$, half-life; Tmax, time to reach maximum serum concentration.
[a]Tmax per dose, 6 hours.
[b]One dose per week.

hEPO increased in a dose-related manner after IV administration of 0.1 to 0.3 mg/kg/d of hEPO-mRNA in LNPs (Figs. 5, 6; Tables 5, 6). There was a notable difference in hEPO-mRNA exposure after the first dose between the groups administered 0.3 mg/kg twice and once per week, which could be due to the small sample size and large intragroup variability (Table 5). In addition, the results showed that hEPO-mRNA had a relatively long half-life (5.9–9.3 hours) and low clearance (9.03–27.0 ml/h/kg; Table 5). After a 60-minute infusion of hEPO-mRNA in LNPs, maximum plasma concentration of hEPO was estimated to occur between 6 and 24 hours (Table 6). The delay in estimated T_{max} observed in group 5 could be a consequence of

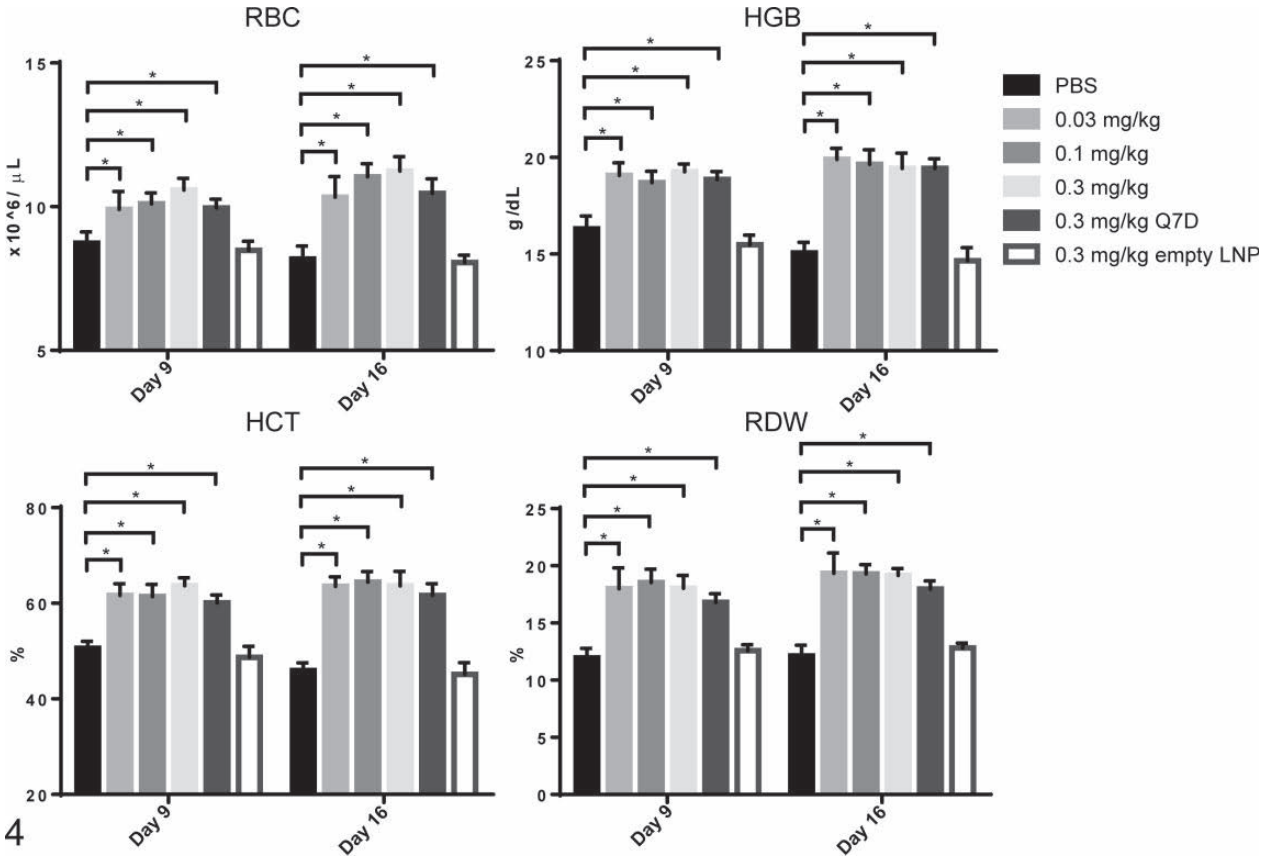
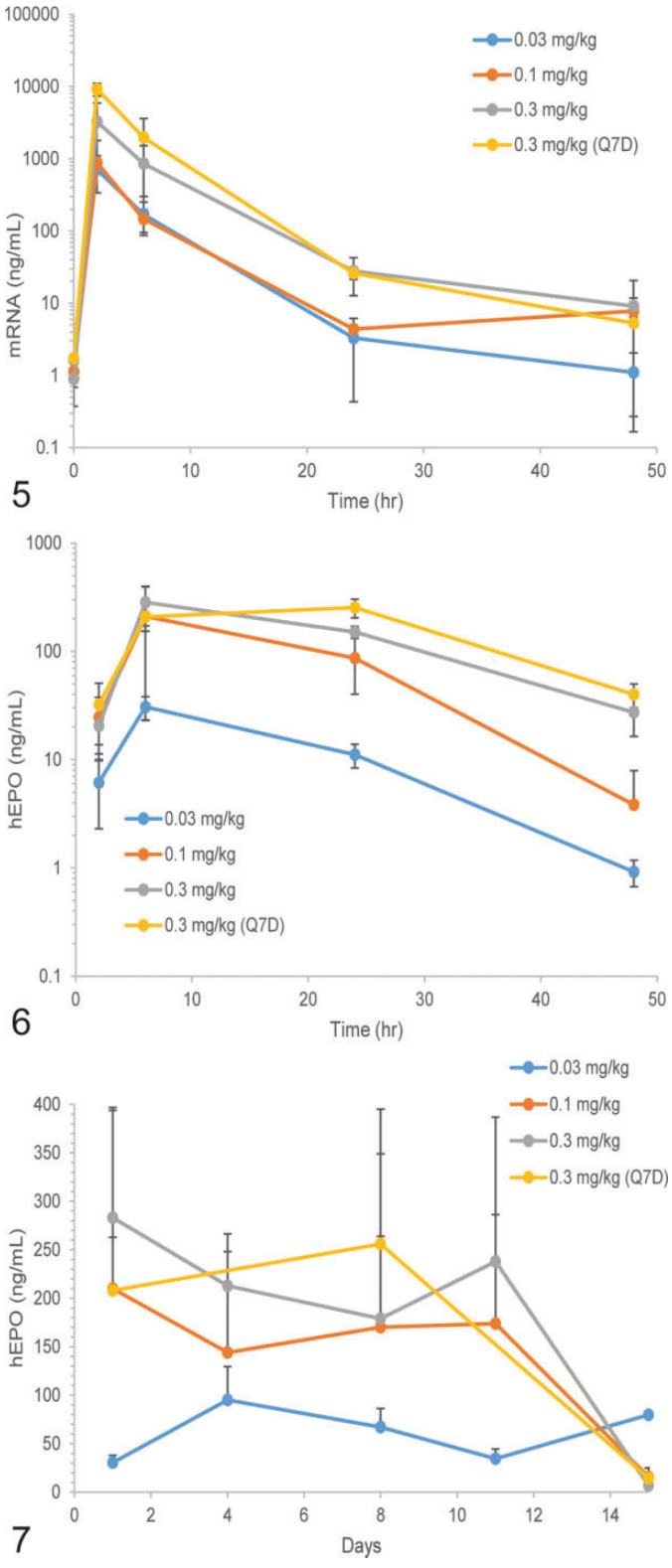


Figure 4. Red blood cell (RBC) mass parameters in rats: RBCs ($\times 10^6$ cells/µl), hemoglobin (HGB; g/dl), hematocrit (HCT; %), and erythrocyte distribution width (RDW; %). Graphs represent mean values ($n = 6$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. *$P < .05$. LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.



Figures 5–7. Plasma concentrations of hEPO mRNA (ng/ml; Fig. 5) and hEPO (ng/ml; Figs. 6, 7) in monkeys. Graphs represent mean values ($n = 3$); error bars indicate SD. Following a 60 minute infusion, peak plasma concentrations of hEPO mRNA appear to be at 2 hours, while peak plasma concentrations of hEPO are approximately at 6 hours. Note that hEPO levels appear relatively constant at all dose levels until day 15. hEPO, human erythropoietin; Q7D, 1 dose per week.

increased hEPO-mRNA exposure in this particular group or, as mentioned before, could be an artifact of high variability within that group. Also, as in male rats, serum hEPO concentrations at C_{max} were well maintained at all dose levels throughout the study until day 15, when measured hEPO levels significantly decreased in the mid- and high-dose groups (Fig. 7).

As expected, hEPO expression in monkeys led to significant changes in red blood cell mass. By day 8 of the study, absolute reticulocyte counts increased in all dose groups (Fig. 8). By the end of the study, a significant spike was noted in all other red blood cell parameters, such as absolute red blood cells, hematocrit, hemoglobin, and erythrocyte distribution width, whereas reticulocytes returned to baseline levels. Like in rats, the increases in red blood cell parameters were similar across all groups and did not seem to be dose related. These changes were not observed in the group dosed with empty LNPs.

Tolerability of hEPO-mRNA in LNPs in Male Rats

There were no notable clinical observations or body weights/food consumption changes in any group (data not shown). Slight changes were observed in coagulation parameters. Specifically, activated partial thromboplastin time was prolonged in all animals dosed with hEPO-mRNA in LNPs, and prothrombin time was prolonged in all animals dosed with hEPO-mRNA twice per week. In addition, fibrinogen levels were elevated for animals receiving 0.3 mg/kg of hEPO-mRNA once and twice per week (Suppl. Fig. 2).

Assessment of hematologic parameters on days 9 and 16 of the study indicated that white blood cells increased at doses $\geq$0.1 mg/kg of hEPO-mRNA in LNPs given twice weekly. Consistent with this, neutrophil, monocyte, and atypical lymphocyte counts were elevated across all groups dosed with hEPO-mRNA in LNPs. Interestingly, administration of hEPO-mRNA in LNPs once weekly or empty LNPs did not elicit the same increase in white blood cells (Fig. 9).

In addition, histamine release and serum levels of cytokines were evaluated. IP-10 was elevated 6 to 24 hours postdose on days 1 and 15 only in the groups dosed with 0.3 mg/kg of hEPO-mRNA in LNPs once and twice weekly (Fig. 10). No changes in IP-10 or histamine were seen in the group given empty LNPs (Fig. 10 and data not shown). No changes in other cytokines (IL-6, TNF-α, IFN-α) or complement (C3) were observed in any study group (data not shown).

Administration of hEPO-mRNA in LNPs at doses $\geq$0.03 mg/kg resulted in several macro- and microscopic findings in the spleen, bone marrow, liver, lungs, and stomach. Primary findings were considered to be related to increased hEPO expression and included an increase in extramedullary hematopoiesis in the spleen, liver, and bone marrow (Figs. 11–16).[1] In addition, macroscopic enlargement of the spleen was noted. These findings correspond to the hematologic changes (reticulocyte counts, red blood cells, and red cell mass parameters). Minimal hemorrhage in the lung and glandular stomach was noted at all doses of hEPO-mRNA in LNPs, which corresponds to macroscopic observations of dark foci in these tissues.

Table 5. Toxicokinetic Values for hEPO mRNA in the Monkey.[a]

	Cmax, ng/mL			AUC, h × ng/ml			$t_{1/2}$, h		Cl, mL/h/kg	
Dose, mg/kg	Mean	SD	Mean Cmax/Dose, ng/mL	Mean	SD	Mean AUC/Dose, h × ng/ml	Mean	SD	Mean	SD
0.03	715	379	23 800	4090	1870	136 000	5.89	0.554	9.03	5.57
0.1	882	904	8820	4440	3140	44 400	9.31	8.12	27	13
0.3	3270	2620	10 900	19 900	15 400	66 300	6.99	1.22	21.3	12.8
0.3[b]	9240	1860	30 800	49 900	13 600	166 000	7.36	6.51	6.33	1.87

Abbreviations: AUC, area under the curve; Cl, clearance; Cmax, maximum serum concentration; hEPO, human erythropoietin; $t_{1/2}$, half-life; Tmax, time to reach maximum serum concentration.
[a]Mean Tmax per dose, 2 hours.
[b]One dose per week.

Table 6. Toxicokinetic Values for hEPO in the Monkey.

		Cmax, ng/ml		AUC, h × ng/ml	
Dose, mg/kg	Mean Tmax, h	Mean	SD	Mean	SD
0.03	6	30.6	7.42	600	39.8
0.1	6	210	187	4240	2530
0.3	6	283	111	6660	915
0.3[a]	24	253	49.4	8170	1550

Abbreviations: AUC, area under the curve; Cmax, maximum serum concentration; hEPO, human erythropoietin; Tmax, time to reach maximum serum concentration.
[a]One dose per week.

Additional findings observed in groups dosed with hEPO-mRNA in LNPs or empty LNPs included increased mononuclear cell infiltration and a minimal to moderate extent of single-cell necrosis in the liver at doses ≥0.1 mg/kg (Figs. 17, 18). At 0.3 mg/kg per dose of hEPO-mRNA, minimal to mild hypertrophy/hyperplasia of the sinusoidal endothelial cells was observed in the liver. These liver findings were accompanied with mild elevations in alanine aminotransferase (ALT) and aspartate aminotransferase (AST; Fig. 19). Overall, these data suggest that doses ≥0.1 mg/kg result in minor liver injury that seems to be primarily driven by the vehicle (LNPs).

Tolerability of hEPO-mRNA in LNPs in Female Monkey

There were no clinical signs or effects on body weights and food consumption related to hEPO-mRNA in LNPs (data not shown). Mild decreases in phosphorus and albumin levels were noted only in animals given 0.3 mg/kg of hEPO-mRNA in LNPs once or twice weekly (Suppl. Fig. 3) and were likely related to the mild proinflammatory changes observed.

There were no changes in leukocytes when compared with PBS in all groups throughout the duration of the study (data not shown). No significant changes in cytokine release (IL-1β, IL-6, TNF-α, IP-10) were observed (data not shown). However, complement activation (C3a, C5b-9) was detected in the mid- and high-dose groups given hEPO-mRNA in LNPs once and twice a week. The magnitude of complement change appears to increase with repeat dosing; significant changes were observed at 2 to 6 hours postdosing on day 1, 6 hours postdosing on day 4, and at 2 to 6 hours postdosing on day 15. By day 15 (2–6 hour postdosing), a mild but not statistically significant trend in complement activation was observed in all groups dosed with hEPO-mRNA in LNPs as well as empty LNPs (Fig. 20).

Macro- and microscopic pathology findings were minimal and mainly present in the heart and spleen. Minimal hemorrhage was noted in the heart at doses ≥0.1 mg/kg and was consistent with suprapharmacologic effects of hEPO.[1] Findings in the spleen were primarily related to administration of the drug product (hEPO-mRNA in LNPs) once weekly at 0.3 mg/kg and included minimal lymphoid depletion in the periarteriolar lymphoid sheaths in the white pulp of the spleen in 3 animals, as well as mild multifocal white pulp necrosis and decreased cellularity of the red pulp in only 1 animal (data not shown).

Discussion

The ability to deliver therapeutic levels of proteins with modified mRNA opens the door to potential life-saving therapies in various indications. The modified mRNA platform has the potential to enable the development of single as well as combination therapeutic agents. However, like siRNA, mRNA is a labile biological molecule and therefore requires the use of protective delivery systems to effectively harness its potential. Indeed, systemic administration of unformulated and unmodified RNA molecules leads to degradation by RNAses, rapid renal clearance, and potential stimulation of an immune response, resulting in very short half-lives (<5 minutes postdosing).[5,11,16,27] Therefore, LNPs were utilized in our studies to improve the PK profile and cellular uptake of mRNA.[10]

In our studies, plasma AUC of hEPO-mRNA increased in a dose-proportional manner in monkeys and in a greater-than-dose-proportional manner in rats. In addition, hEPO-mRNA had moderate to long half-life and relatively low clearance in both species. Finally, hEPO expression resulted in supratherapeutic levels (~100-fold the projected clinically efficacious dose) in rats and monkeys.[24] The protein produced had expected PK and PD properties ($t_{1/2}$) as evidenced by exaggerated pharmacologic effects associated with supratherapeutic

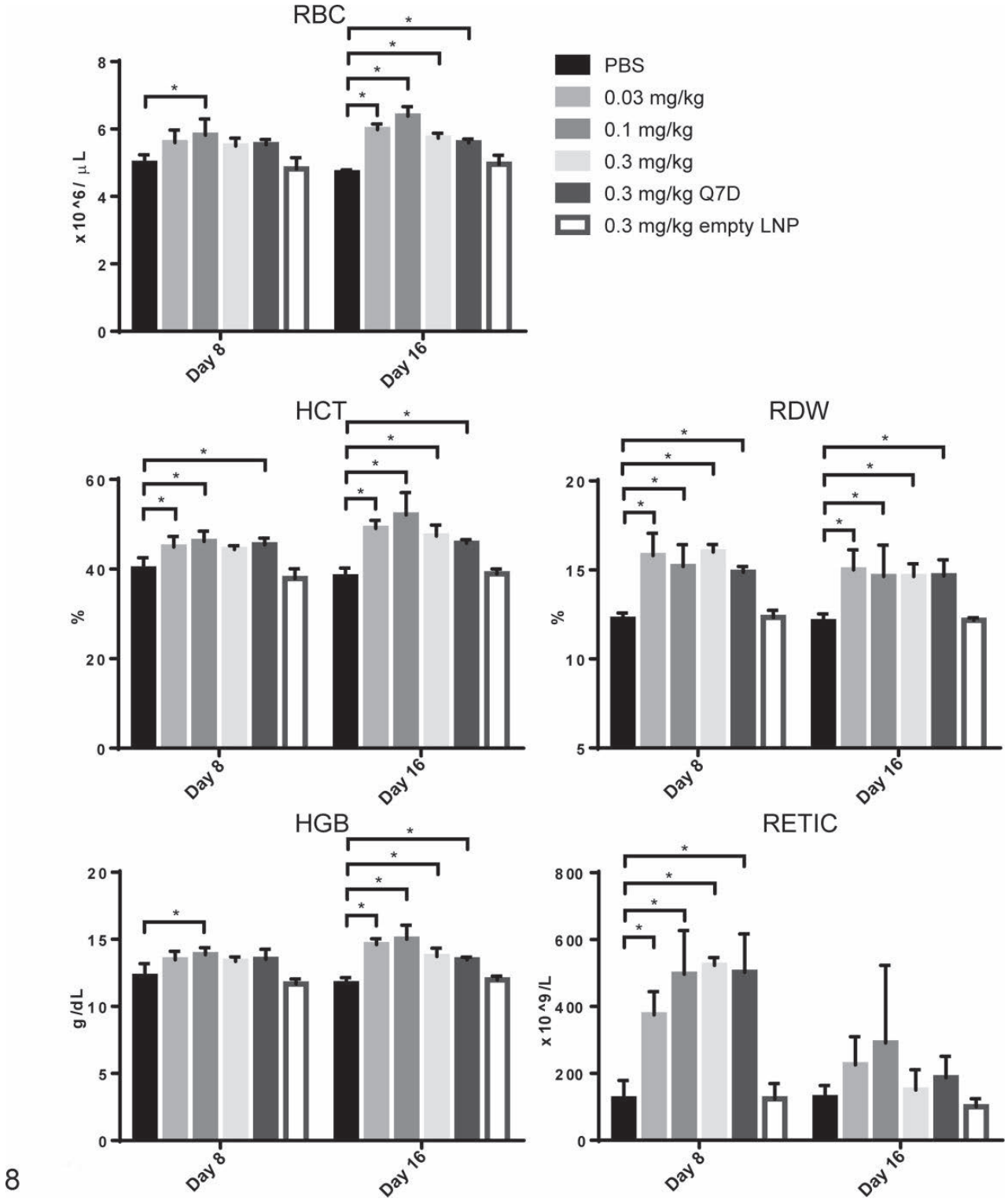


Figure 8. Red blood cell (RBC) mass parameters in monkeys: RBCs ($\times 10^6$ cells/µl), hemoglobin (HGB; g/dl), hematocrit (HCT; %), erythrocyte distribution width (RDW; %), and reticulocytes (RETIC; $\times 10^9$ cells/µl). Graphs represent mean values ($n = 3$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. **P* < .05. LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.

doses of hEPO observed in both species (increased red blood cell mass parameters; mild hemorrhages in the heart, stomach, and lungs; extramedullary hematopoiesis in the liver and spleen; and increased hematopoiesis in bone marrow). These data suggest that hEPO-mRNA (and putatively modified mRNA in general) administered in LNPs has acceptable

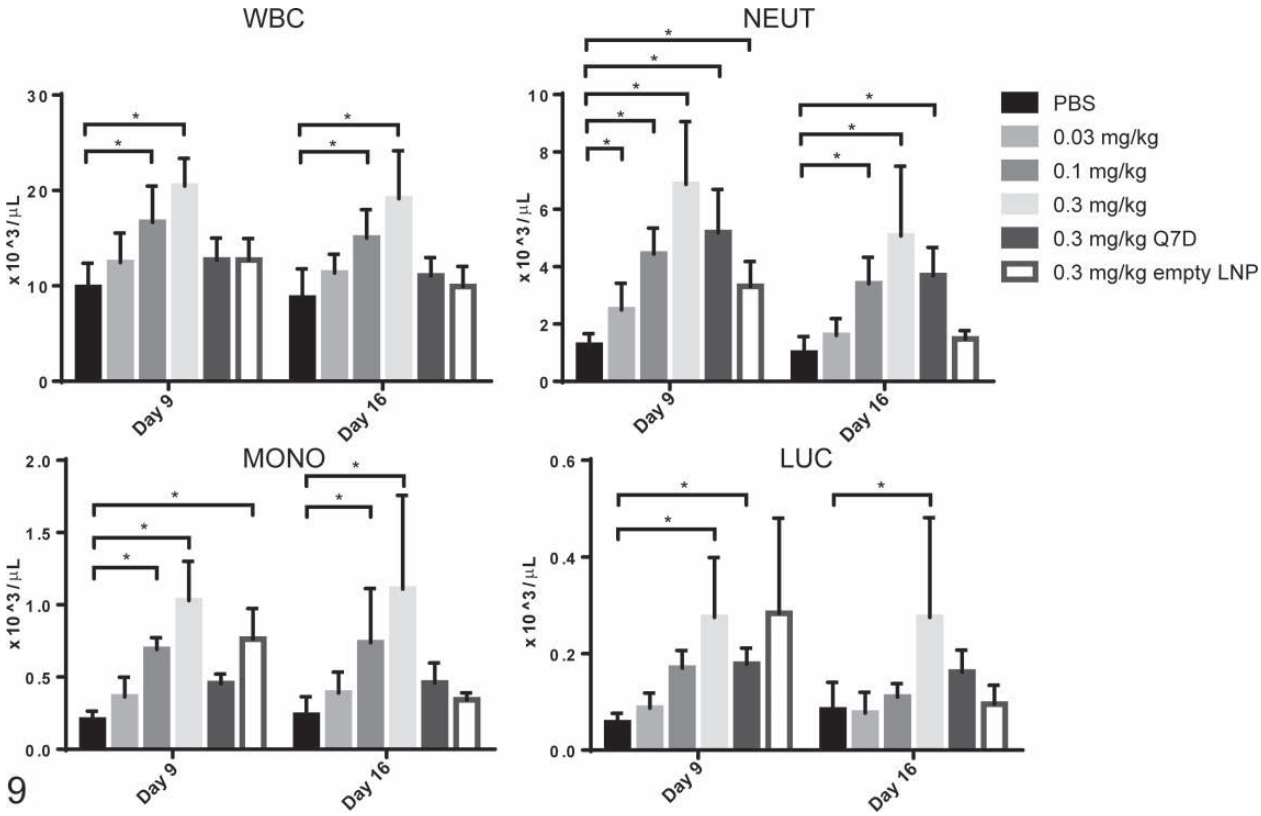


Figure 9. Leukocyte parameters in rats: white blood cells (WBC; $\times 10^3$ cells/µL), neutrophils (NEUT; $\times 10^3$ cells/µL), monocytes (MONO; $\times 10^3$/µL), and large unstained cells (LUC; $\times 10^3$/µL). Graphs represent mean values ($n = 6$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. **P* < .05. LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.

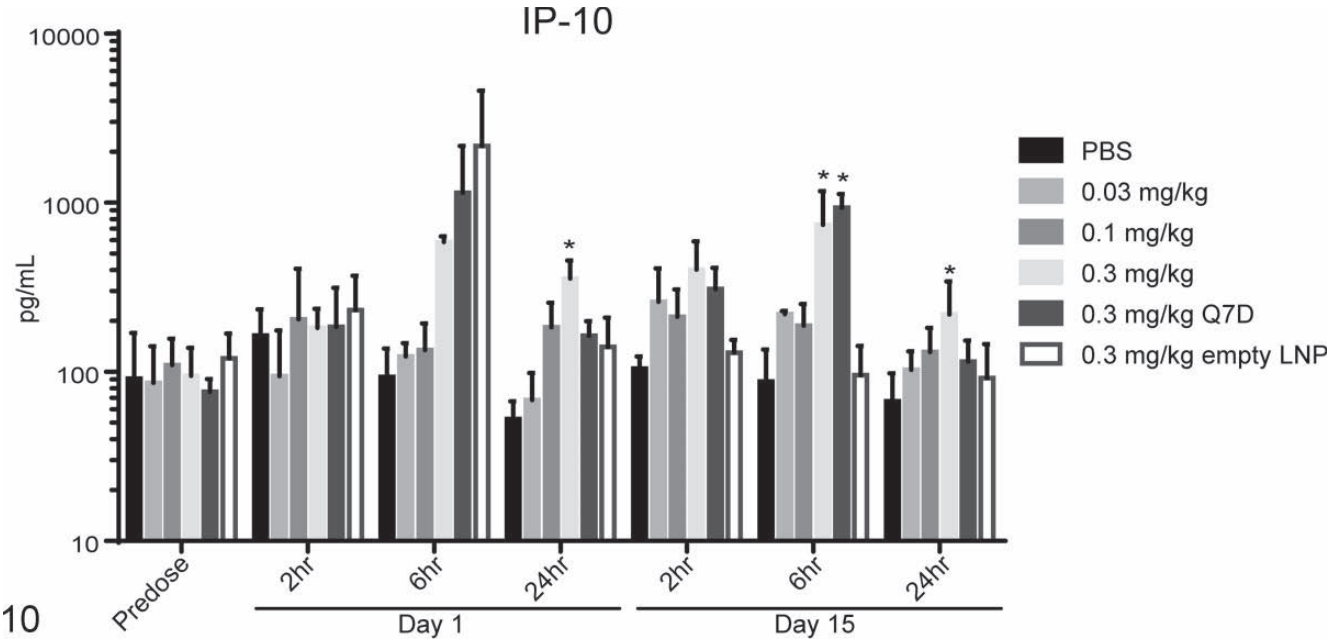
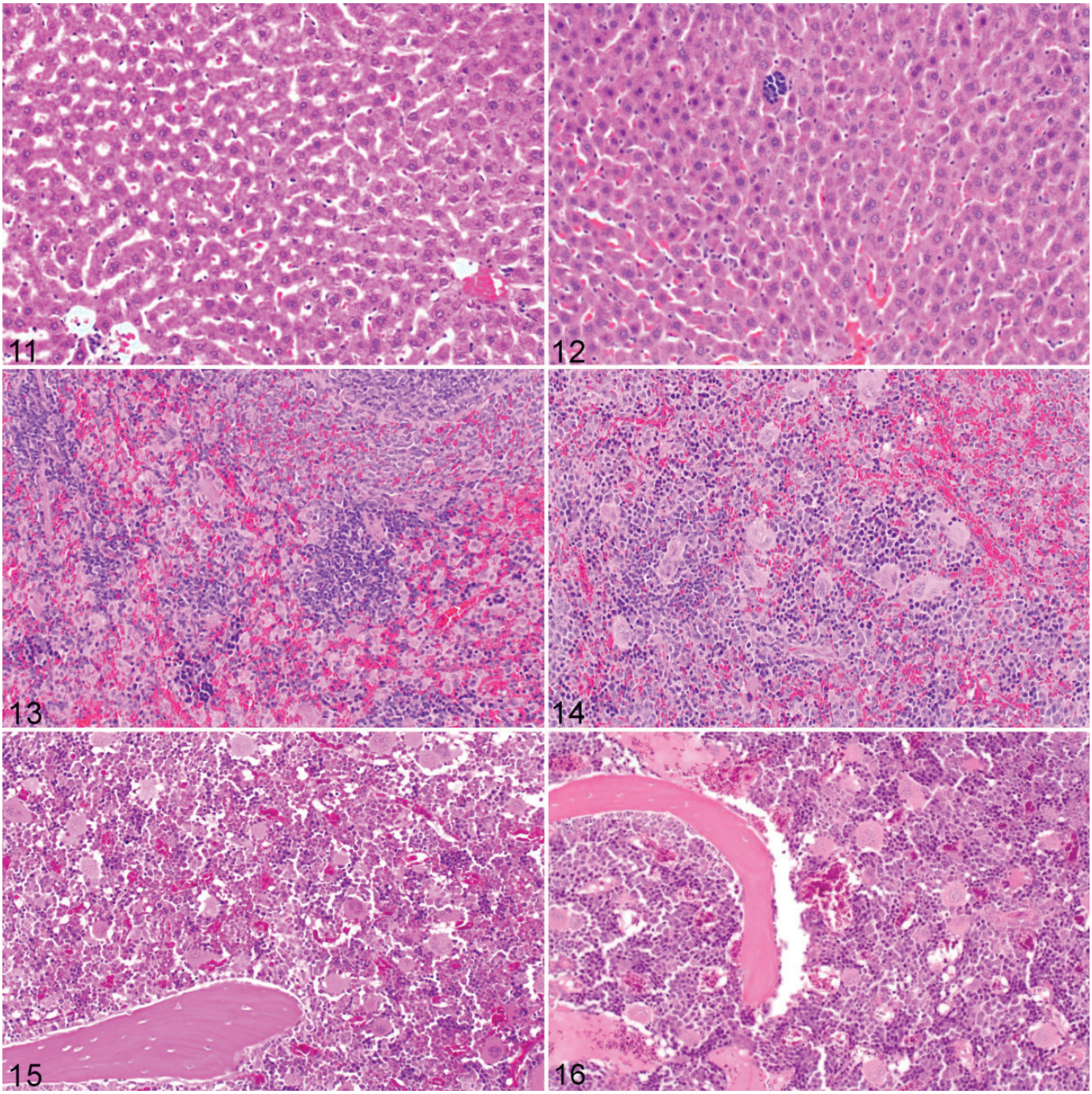


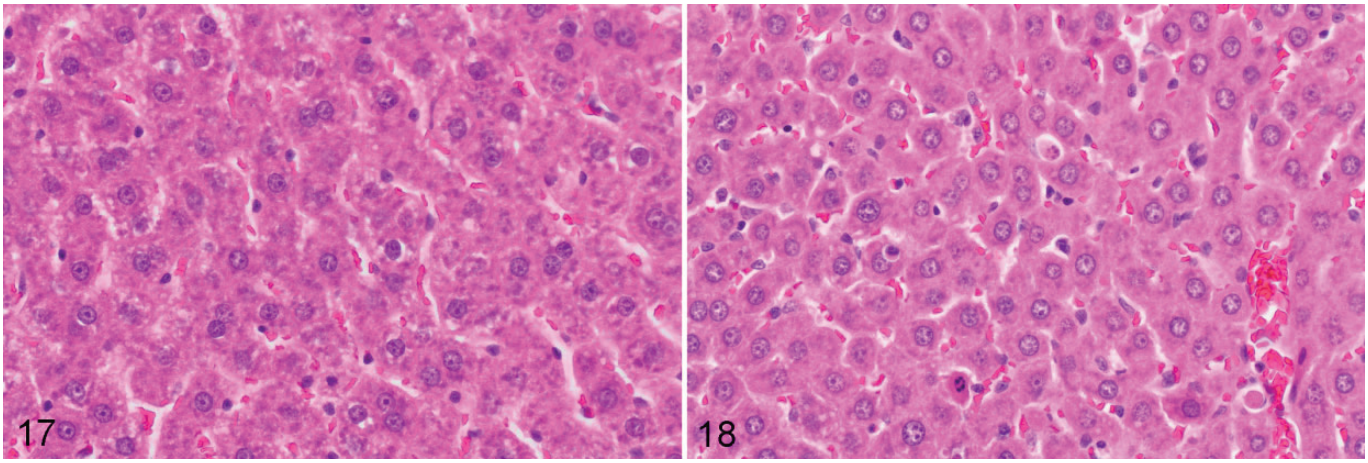
Figure 10. Plasma interferon γ–inducible protein 10 (IP 10) levels in rats. Graph represents mean values ($n = 6$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. **P* < .05. LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.



Figures 11–16. Histologic findings in control rats and in rats dosed with 0.3 mg/kg of hEPO mRNA twice a week. Hematoxylin and eosin. **Figure 11.** Liver, control rat. **Figure 12.** Liver, rat dosed with hEPO mRNA. There is extramedullary hematopoiesis. **Figure 13.** Spleen, control rat. **Figure 14.** Spleen, rat dosed with hEPO mRNA. There is increased extramedullary hematopoiesis. **Figure 15.** Bone marrow (sternal), control rat. **Figure 16.** Bone marrow (sternal), rat dosed with hEPO mRNA. There is increased hematopoiesis. hEPO, human erythropoietin.

properties for a drug product at least when administered over a 2-week period.

A marked decrease of the plasma concentration of hEPO was observed by day 15 in the mid- and high-dose groups in both species. This reduction in hEPO levels may have been mediated by antibodies against the expressed protein since animals were exposed to a nonhomologous protein.[4,8] To further investigate for the presence of antiprotein antibodies, we have subsequently developed appropriate bioanalytical methods, and the data collected from subsequent in vivo studies exploring the levels of species-specific anti-hEPO antibodies support this hypothesis (data not shown).



Figures 17 and 18. Histologic findings in control rat and in rat dosed with 0.3 mg/kg of empty lipid nanoparticles twice a week. **Figure 17.** Liver, control rat. **Figure 18.** Liver, rat dosed with empty empty lipid nanoparticles. There is single cell necrosis of hepatocytes.

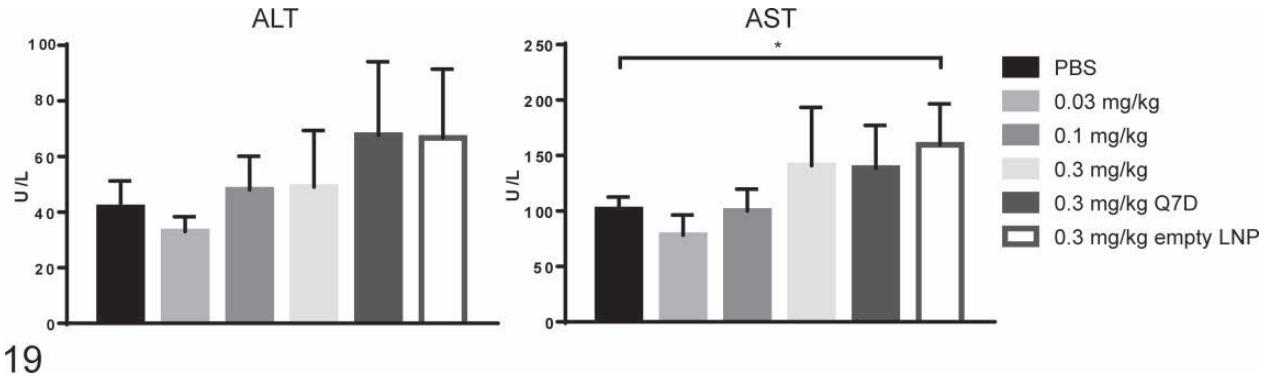


Figure 19. Serum levels of liver enzymes in rats. Graphs represent mean values ($n = 6$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. *$P < .05$. ALT, alanine aminotransferase; AST, aspartate aminotransferase; LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.

From a safety perspective, modified mRNA delivered in LNPs is a complex molecule that requires the assessment of safety of the delivery system (LNPs), mRNA, and the translated protein product. Overall, administration of 0.3 mg/kg of LNPs alone via IV infusion was well tolerated. In male rats, increases in neutrophils and monocytes were observed, and microscopic changes in liver were noted that correlated with mild and monitorable increases in liver enzymes (ALT and AST). In female monkeys, only minimal and transient complement activation was induced by LNPs alone. Similar changes have been reported for LNP vehicle.[3]

IV infusion of hEPO-mRNA in LNPs was well tolerated in both species. A mild and reversible proinflammatory profile was noted that consisted of elevations in IP-10 (rat) and detection of complement cleavage products (monkey). These findings correlated with slight changes in both species in terms of hematology parameters (white blood cell counts, lymphocytes, neutrophils, and monocytes) and coagulation parameters (activated partial thromboplastin time, prothrombin time, and fibrinogen), although more consistently observed in the rat. In the rat, liver microscopic changes with elevations of serum ALT and/or AST were similar to those observed in the group dosed with empty LNPs. In addition, changes in the spleen (depletion in the periarteriolar lymphoid sheaths and splenic necrosis) were observed in monkeys. These findings suggest that the administration of mRNA in LNPs at doses as high as 0.3 mg/kg per dose induces a mild to moderate and reversible proinflammatory response. Furthermore, it is evident that this immune activation can be mitigated by either lowering the dose or decreasing the frequency of dosing.

These findings (serum complement activation, cytokine elevation, and potential liver and spleen effects) are in agreement with those previously reported for LNPs loaded with siRNA in nonclinical and clinical studies.[3,6] Comparable toxicologic profiles of modified siRNA and mRNA in LNPs suggest that the toxicologic effects and possibly the distribution properties (primarily liver and monocyte phagocytic system) of the drug product are predominantly vehicle driven. Note that an empty LNP may have slightly different surface properties than the one loaded with mRNA; thus, small differences in magnitude or

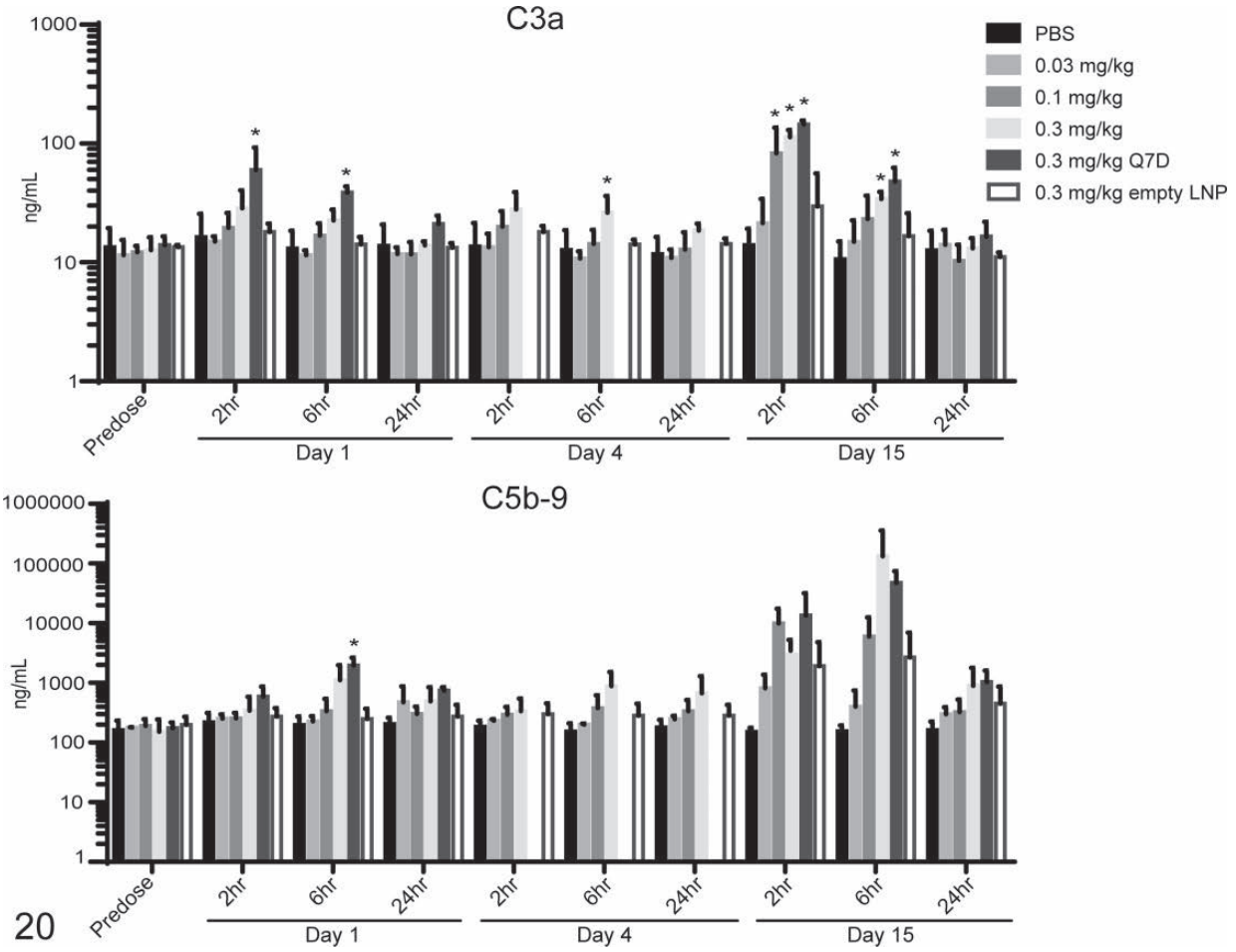


Figure 20. Plasma levels of complement components (C3a and C5b 9) in monkeys. Graphs represent mean values ($n = 3$); error bars indicate SD. Ordinary one way analysis of variance (multiple comparisons) was used to calculate the *P* values. *$P < .05$. LNP, lipid nanoparticle; PBS, phosphate buffered saline; Q7D, 1 dose per week.

effects may occur.[28] Therefore, a preferred control instead of the empty LNPs would be LNPs loaded with nonsense mRNA with equivalent base modifications.

In conclusion, the PK, PD, and toxicologic properties of modified mRNA loaded in LNPs are generally consistent between species. The primary toxicologic findings of hEPO-mRNA in LNPs are related to the supratherapeutic exposure to hEPO and inflammatory findings that are mainly LNP driven. Given the similarities in LNP-related toxicities between the rat and the monkey, it is likely that similar effects will translate to the clinic and be monitorable with the parameters identified in nonclinical species. Although the studies were not conducted to support advancement of a clinical candidate, they suggest that the mRNA-in-LNP approach might be feasible to safely deliver therapeutic levels of an exogenous protein. This would enable novel therapies for a variety of indications. Future work will be geared toward evaluating different routes of administration, the effects of chronic dosing, and the risk to juvenile animals, as juveniles may be particularly important in the setting of rare disease.

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Declaration of Conflicting Interests

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