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Lipid nanoparticle-mediated mRNA delivery to CD34⁺ cells in rhesus monkeys

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Abstract

Transplantation of ex vivo–engineered hematopoietic stem cells (HSCs) can lead to robust clinical responses but carries risks of adverse events from bone marrow mobilization, chemotherapy conditioning, and other factors. HSCs have been modified in vivo using lipid nanoparticles (LNPs) decorated with targeting moieties, which increases manufacturing complexity. Here, we screen 105 lipid nanoparticles (LNPs) without targeting ligands for effective homing to the bone marrow in mouse. We report an LNP named LNP⁶⁷ that delivers mRNA to murine HSCs in vivo, primary human HSCs ex vivo, and CD34⁺ cells in rhesus monkeys (*Macaca mulatta*) in vivo at 0.25 and 0.4 mg/kg doses. Without mobilization and conditioning, LNP⁶⁷ can mediate delivery of mRNA to HSCs and HSPCs as well as the liver in rhesus monkeys and without serum cytokine activation.

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Competing Interests M.Z.C.H., H.N., and J.E.D. have filed a provisional patent related to this manuscript (U.S. patent application number 63/632,354). J.E.D. is an advisor to GV, Readout, Edge Animal Health, and Nava Therapeutics, and P.J.S. is a co-founder of Tether Therapeutics. None of these companies provided any financial support for this work. All other authors declare no competing interests.

These data support the hypothesis that *in vivo* delivery to hematopoietic stem and progenitor cells in nonhuman primates is feasible without targeting ligands.

Main Text

Lipid nanoparticles (LNPs) have delivered RNA in Food and Drug Administration (FDA)-approved drugs^{1–3} and early-stage clinical trials^{4–6}. The clinical impact of these LNP-RNA therapies supports exploring nanoparticles that deliver RNA to hematopoietic stem and progenitor cells (HSPCs)⁷. Since most blood cells are derived from hematopoietic stem cells (HSCs), their dysfunction drives pathologies including sickle cell disease⁸, β -thalassemia⁹, anemias¹⁰, immunodeficiencies¹¹, and metabolic disorders¹². Four HSC-based therapies have recently been FDA approved^{13–16}, all of which require *ex vivo* HSC manipulation. In this process, cells are mobilized¹⁷ from the bone marrow or removed from the bloodstream. While the cells are modified in the lab, the patient undergoes chemotherapy that depletes the immune system to make space for the manipulated cells to engraft after they are re-administered.

The morbidity that may result from this procedure¹⁸ has spurred interest in developing *in vivo* HSC therapies that do not require mobilization or conditioning. To this end, LNPs decorated with antibodies targeting cKit (CD117) have been shown to deliver mRNA to the bone marrow in mice^{19–21}. One potential limitation of this work is that human cKit expression is high in the respiratory system and low in bone marrow²². Separately, adenovirus-based delivery systems with active targeting to CD46 led to efficient delivery in nonhuman primates²³; this required cell mobilization and chemotherapy. Overall, LNP delivery in nonhuman primates using clinically relevant^{3–6} doses without active targeting ligands or any other interventions has proven difficult. Here, we report an LNP named LNP⁶⁷ that delivers mRNA to CD34⁺ cells in rhesus monkeys at doses as low as 0.25 mg/kg.

LNP⁶⁷ delivers mRNA to bone marrow HSPCs in mice

All three FDA-approved LNP-RNA drugs^{1–3} use nanoparticles with four components. These components are an ionizable lipid, which complexes the anionic phosphodiester backbone of RNA during microfluidic formulation; a poly(ethylene glycol) (PEG)-lipid, which creates a hydrophilic barrier around the LNP and affects the *in vivo* pharmacokinetics²⁴; cholesterol, which stabilizes the LNP and affects its fluidity²⁵; and a helper lipid, which further stabilizes the LNP (Ref 26) and can affect tropism^{27–30}.

All four components can alter LNP behavior *in vivo*, but predicting LNP tropism *in vivo* is difficult using cell culture³¹. Given the rarity of HSCs and subsequent difficulty isolating enough cells to sequence using flow cytometry, we used Single Cell Nanoparticle Targeting-sequencing (SENT-seq)³² to evaluate how well 105 LNPs delivered RNA *in vivo*. We formulated LNP-1, with chemical composition 1, to carry DNA barcode 1 as well as mRNA encoding a glycosylphosphatidylinositol (GPI)-anchored camelid VHH antibody (anchored VHH, aVHH)^{32–34}. We repeated this process 128 times, so that LNP-128, with chemical composition 128, carried DNA barcode 128 as well as mRNA encoding aVHH. We used 16 combinations of helper lipids and PEG-lipids, and to control for the fact that the molar ratio

of these components can affect delivery, we formulated eight molar ratios to generate 128 chemically distinct LNPs (Fig. 1a, Supplementary Fig. 1). In all cases the mRNA:barcode mass ratio was 10:1, and in all cases we used a proprietary ionizable lipid. To reduce the amount of total drug administered, the LNP:nucleic acid mass ratio was also 10:1. We then analyzed the hydrodynamic diameter and polydispersity of all 128 LNPs. Monodisperse LNPs with diameters less than 200 nm were pooled together; 105 met these inclusion criteria. As a control, we measured the hydrodynamic diameter and polydispersity of the pool and found them to be within the range of LNPs composing the pool (Fig. 1b).

We intravenously (IV) injected C57BL/6J mice with these 105 LNPs at a total dose of 1.7 mg/kg (i.e., 0.016 mg/kg/particle, on average), then isolated single-cell suspensions from the bone marrow using magnetic-activated cell sorting (MACS, Supplementary Fig. 2) and liver using fluorescence-activated cell sorting (FACS) (Fig. 1c). We next used single-cell RNA sequencing (scRNA-seq)³⁵ to define stem and progenitor cell populations from the bone marrow (Fig. 1d,e, Supplementary Fig. 3) and plotted the single cells using uniform manifold approximation and projection (UMAP)³⁶. We also used oligo-tagged anti-VHH antibodies to quantify aVHH protein and defined these reads as a pseudogene, then overlaid DNA barcode and aVHH reads onto the single cells (Fig. 1f, Supplementary Fig. 4). We observed barcode and aVHH delivery in transcriptionally defined HSC populations, multipotent progenitors (MPPs), and oligopotent progenitors (OPPs)³⁷. To identify relationships in LNP formulations that may improve bone marrow tropism, we quantified normalized delivery in aVHH⁺Lin⁻ bone marrow cells as a function of LNP molar ratios, PEG-lipid content, helper lipid content, molar percentage of a charged helper lipid, and molar percentage of the ionizable lipid (Supplementary Fig. 5). We did not observe any statistically significant relationships, which suggests that larger or more diverse chemical libraries will be needed to rationally improve tropism.

To complement these on-target data, we sequenced barcodes in liver cells, thereby quantifying delivery in a key off-target tissue³⁸ (Fig. 1c, Supplementary Fig. 6). We then ranked LNPs based on their on-/off-target delivery ratios and selected three lead LNPs, which we named LNP⁶⁷, LNP⁹⁵, and LNP¹⁰⁸, for further evaluation (Fig. 1g, Supplementary Fig. 7).

High-throughput screens that suggest that an LNP will target a cell type require individual confirmation of lead candidates. We therefore formulated the three lead LNPs with mRNA encoding aVHH, then individually injected mice IV at a dose of 0.5 mg/kg. As a control, we also administered an established LP01-based liver-targeting LNP (Ref 39). To generate a detailed on-/off-target delivery profile, we quantified aVHH protein 16 hours post-administration in 24 cell types from the bone marrow, liver, spleen, lung, heart, and kidney. We defined murine HSCs as Lin⁻Sca-1⁺cKit⁺CD48⁻CD150⁺ (LSK/SLAM⁴⁰); the other 23 cell populations are shown in Supplementary Fig. 8. LNP⁶⁷ had the most favorable bone marrow:liver delivery profile; by contrast, the LP01 LNP targeted the liver as expected (Fig. 2a, Supplementary Fig. 9).

These data led us to focus on LNP⁶⁷ (Fig. 2b). We found it formed spherical particles (Fig. 2c) with 80 nm hydrodynamic diameters (Fig. 2d), a zeta potential of -2.6 mV (Fig. 2e),

and a pKa of 6.4 (Fig. 2f). We then compared LNP structures for LNP⁶⁷ and liver-targeting control LP01 LNPs using cryo-TEM. We found that both LNPs had a solid core, a similar mean circularity, and a similar mean diameter (Supplementary Fig. 10). These data do not support the hypothesis that overt biophysical changes affected LNP⁶⁷ tropism. We then investigated serum protein interactions that may drive tropism to HSCs. We mixed LNP⁶⁷ or the LP01 control LNPs at 37°C with mouse blood plasma for 15 minutes at a 1:1 volumetric ratio. We then spun down the mixture on a sucrose cushion and analyzed the molecules adsorbed to the LNP using mass spectrometry. We identified 66 biomolecules in the corona (PSM >=4). Among these, nine were over twofold more abundant in the LNP⁶⁷ protein corona, relative to the LP01 LNP protein corona (Supplementary Fig. 11).

Compared to phosphate-buffered saline (PBS)-treated mice, mice injected with a 1.5 mg/kg dose did not demonstrate any differences in liver histopathology 24 hours after administration (Fig. 2g). Similarly, we did not observe robust changes in serum cytokines six or 24 hours post-administration (Fig. 2h, Supplementary Fig. 12). We then formulated LNP⁶⁷ to carry mRNA encoding aVHH and performed a dose response study. Sixteen hours after IV administration, we observed dose-dependent delivery in eight HSC subtypes (Fig. 2i), including delivery of up to 80% of MPPs.

LNP⁶⁷ delivers mRNA to transcriptionally defined HSCs *in vivo*

To complement the flow cytometry aVHH readouts, we performed single-cell multiomics to quantify delivery in transcriptionally defined HSCs. We formulated LNP⁶⁷ to carry aVHH mRNA, then intravenously injected mice with 2.0 mg/kg. Twenty-four hours later, we enriched lineage-negative cells from the bone marrow using MACS. We then treated these cells with DNA-conjugated anti-VHH antibodies to quantify aVHH protein and overlaid these reads onto the single-cell transcriptomes. We analyzed 36,142 single cells and divided them into 21 clusters based on differentially expressed gene sets (Supplementary Fig. 13a). Using an established scRNA-seq database⁴¹, we annotated 16 subsets of Lin⁺ cells: six lymphoid, nine myeloid, and one unclassified (Fig. 3a). Since clusters that contain true HSCs can include other cell types³⁷, we also manually annotated five subsets of Lin⁻ cell types.

Next, we overlaid aVHH protein expression onto the t-SNE (Fig. 3a). aVHH-positive cells were observed in all clusters, and the number of aVHH⁺ cells from the LNP⁶⁷-treated group were significantly higher than the background signal from the PBS-treated group in 18 out of 21 clusters (Fig. 3b, Supplementary Fig. 13b). We then focused on the clusters that contained HSCs (Fig. 3c). Approximately 50% of the LSK cells (both CD48⁻ and CD48⁺) showed aVHH protein expression, with significantly higher expression in CD48⁻ cells (HSCs and MPPs) compared to CD48⁺ cells (OPPs) (Supplementary Fig. 13c). Similarly, Lin⁻ cell types showed significantly higher aVHH expression compared to Lin⁺ cell types. Since previous reports used LNPs decorated with cKit antibodies to target HSCs, we measured aVHH in cKit⁺ cells and observed delivery (Fig. 3c). These data suggest LNP⁶⁷ can deliver mRNA to transcriptionally defined HSCs without cKit-targeting ligands.

LNP⁶⁷ delivers mRNA to HSCs in young rhesus monkeys *in vivo*

The results supported the exploration of LNP⁶⁷ in rhesus monkeys. However, nonhuman primate studies often require LNPs to function after they are cryopreserved, shipped to another site, and thawed prior to administration. We therefore optimized excipients and freeze-thaw conditions (Supplementary Fig. 14) and found that 10% sucrose in PBS resulted in particles that could be frozen and thawed without changes to the LNP hydrodynamic diameter and polydispersity index (Fig. 4a). We then compared the *in vivo* activity of LNP⁶⁷ in mice at a 1.0 mg/kg dose, administered after being frozen and thawed, in eight HSC subtypes. We found no differences in delivery when compared to fresh (i.e., never frozen) LNPs (Fig. 4b). These data led us to conclude that LNP⁶⁷ forms small, stable formulations that can deliver mRNA after freeze-thaw.

The relationships between LNP delivery in mice and LNP delivery in nonhuman primates is poorly understood. This is particularly true for non-liver tissues³⁸. To address the relative tropism of LNP⁶⁷ in rhesus monkeys, we conducted a study to quantify delivery in the bone marrow and liver. We formulated LNP⁶⁷ to carry an mRNA encoding an anchored nanoluciferase (aNLuc) (Supplementary Fig. 15), cryopreserved the LNPs, and shipped them from Georgia to California. The LNPs were then thawed, tested for potential contaminants (e.g., endotoxin, sterility), then immediately prepared for injection IV in young rhesus monkeys (N=7; 7–8 months of age, 1.5–1.8 kg; four males and three females) at either 0.25 mg/kg (N=4) or 0.4 mg/kg (N=3) doses. One control animal was also included (8 months; 1.7 kg; male). Animals selected for the study were pre-screened to ensure they were seronegative for PEG antibodies⁴². Approximately 24 hours later, blood samples (23 serum markers) and tissues (liver, bone marrow) were collected. Cells were immunoselected from the bone marrow aspirates (CD34⁺ and CD34[−] fractions) and liver cell suspensions prepared (Fig. 4c). These studies showed a dose-dependent delivery in the liver and both bone marrow cell populations (Fig. 4d,e). LNP⁶⁷ delivery also led to similar luciferase expression in CD34⁺ and liver cell types at both the 0.25 and 0.4 mg/kg doses. To confirm delivery in CD34⁺ and liver cells, we fluorescently imaged cells with anti-nanoluciferase antibodies (Fig. 4f,g).

We evaluated the serum cytokine profiles and did not observe differences in treated animals compared to the control animal (Fig. 4h). Many LNP studies in nonhuman primates²³ and humans⁴³ use an anti-inflammatory pre-treatment (e.g., antihistamine and steroid) to improve LNP tolerability. We did not use any pre-treatment in this study and observed no adverse findings. These data provide preliminary evidence that LNP⁶⁷ was well tolerated in young rhesus monkeys at the doses studied. Finally, we evaluated how well LNP⁶⁷ transfected primary human HSCs *ex vivo* and observed dose-dependent delivery in all six cell subtypes (Fig. 5, Supplementary Fig. 16). Functional delivery was more significant in hematopoietic stem and progenitor cells compared to non-stem (CD34[−]) cells (Supplementary Fig. 17).

Conclusions

The FDA has published guidelines for clinical trials using RNA-, LNP-, or gene therapy-based therapeutics⁴⁴. These guidelines highlight the value of a clear relationship between mutation and disease. In the case of HSCs, *ex vivo* clinical trials have clearly linked genes to their associated disorders. These de-risked gene targets, combined with existing clinical trial sites familiar with these patient populations, support efforts to deliver RNA to HSCs *in vivo*.

Here we present evidence that LNP⁶⁷ can mediate mobilization- and conditioning-free delivery to HSCs and HSPCs in mice *in vivo* and human primary cells *ex vivo*. However, the translational relevance of an *in vivo* LNP-mRNA drug is largely determined by its behavior in nonhuman primates⁴⁵. We found that LNP⁶⁷ delivered mRNA to CD34⁺ cells in nonhuman primates approximately as well as it delivered to the liver. While many preclinical studies and FDA-approved products pre-treat patients with a steroid and antihistamine⁴³, we observed no adverse findings in these studies either immediately after dosing or in the post administration period. We anticipate that future studies with LNP⁶⁷ could use pre-treatments to increase its therapeutic window. One open question is the tolerability required for *in vivo* HSC therapies, given that the alternative option (specifically, an *ex vivo* regimen) can result in morbidity.

We believe the nonhuman primate delivery at 0.25 and 0.4 mg/kg is a good starting point for future work. Intellia used doses up to 1.5 mg/kg⁶ assuming a 50 kg patient, and patisiran is administered to patients at a 0.3 mg/kg dose³. We anticipate that subsequent LNP optimization could lead to HSC delivery at lower doses. This optimization is likely to require significant effort, but a tenfold improvement from the current potency of LNP⁶⁷ may be worth it.

It is important to acknowledge limitations of this work. First, LNP⁶⁷ was discovered using an *in vivo* screen in mice. Given the unknown relationship between mRNA delivery in mice and mRNA delivery in nonhuman primates³⁴, it is likely that a distinct LNP⁶⁷-like formulation may be more potent in rhesus monkeys. One obvious but technically challenging solution is to screen dozens of LNPs in nonhuman primates. Another limitation is that the nonhuman primate data used luciferase, which makes it difficult to quantify the percentage of cells we transfected. Now that we have data suggesting LNP⁶⁷ can deliver mRNA in nonhuman primates, we anticipate future studies using other mRNA payloads. For example, it will be important to show that cells treated with mRNA encoding gene editors can lead to long-term engraftment in animals. Relatedly, given that we observe non-zero liver delivery, we anticipate incorporating mRNAs with modified UTRs to reduce unwanted expression. A third uncertainty is whether delivery in rhesus will precisely predict delivery in humans. To date, LNPs have performed as well³ or better⁴ in humans when compared to nonhuman primates, but these results were in the liver. Other cell types, including HSCs, may be more challenging. Despite these challenges, we believe these data justify additional exploration of LNP⁶⁷ and provide evidence that non-liver delivery can be achieved in rhesus monkeys at relatively low doses.

Materials and Methods

mRNA Synthesis.

Sequences of GPI-anchored VHH and nanoluciferase^{33, 47} was ordered as an IDT DNA gBlock. The sequences are shown in Supplementary Table 1. The gBlock was cloned into a PCR amplified pMA7 vector using a three-molar excess of insert. The resulting transcripts were then purified using a 0.8% agarose gel and confirmed using Sanger sequencing. In vitro transcription was performed overnight at 37°C using HiScribe T7 (New England Biolabs), per the manufacturer's instructions. The template was removed by treatment with DNase I. A Cap1 structure was added to the RNA transcripts after denaturing at 65°C for 10 min, and transcripts were enzymatically polyadenylated. Purified RNA products were analyzed by gel electrophoresis to ensure purity.

Nanoparticle Formulation.

LNPs were formulated with aVHH or aNLuc as follows^{34, 35, 48}; Nucleic acids were diluted in 25 mM sodium acetate buffer, while the remaining components were diluted in 100% ethanol. DNA barcodes (Integrated DNA Technologies, sequences listed in Supplementary Table 1) were mixed in RNA nucleic acid phase at 1:10 ratio of DNA barcodes: mRNA. The phases were then microfluidically mixed^{49, 50} using NanoAssemblr Spark or Ignite (Precision Nanosystems) at a flow rate of 3:1 (RNA:lipid phases). LNPs were dialyzed in sterile 1X PBS in 20 kDa microdialysis plate (ThermoFisher) at 4°C overnight. Then, the LNPs were sterile-filtered with 0.22-µm filter before administration or downstream analysis. The LNP composition and its pKa is described in Fig. 2 and the ionizable lipid is described in U.S. patent application number 63/632,354.

LNP Characterization.

Hydrodynamic diameters of LNPs were measured using high throughput dynamic light scattering (DynaPro Plate Reader II, Wyatt). DYNAMICS software (v8.3.1.1145, Wyatt) was used for data collection. In the case of the barcoding assays, LNPs were only included if they had a hydrodynamic diameter between 20 and 200 nm as well as a correlation function with 1 inflection point. Endotoxin levels were measured with PierceTM Chromogenic Endotoxin Quant Kit (Thermo Scientific) before administration to mice and cryopreservation and shipping (Supplementary Fig. 15d). The standard range of 0.01–0.1 EU/ml was used following the manufacturer's protocol. Encapsulated mRNA and encapsulation efficiency were evaluated using Quant-iTTM RiboGreenTM RNA assay kit (Thermo Fisher Scientific). Briefly, an equal volume of 6 ng/µL LNP and 1X Tris-EDTA (TE; Thermo Fisher) or 2% Triton X-100 (Sigma Aldrich) in 1X TE were mixed. Following incubation at 37°C for 10 min, an equal volume of 1:100 of RiboGreen reagent (Thermo Fisher) was added to each well. The fluorescence was quantified using a plate reader (PerkinElmer Victor X4 Microplate Reader) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Perkin Elmer 2030 Workstation software 4.0 (v4.00.0.15) was used for data collection. Zeta potential was measured using a Malvern Zetasizer Nano Z. ZetaSizer software (v8.02) was used for data collection. The measurement was done with the refractive index of 1.4, dispersant viscosity of 0.882 cp, and refractive index of 1.33.

Freeze-Thawing of LNPs.

Sucrose (NF, EP, JP, ChP, high purity, low endotoxin, Fisher Scientific) was dissolved in 1X PBS at 55% (w/v). LNPs in 1X PBS were mixed with 55% (w/v) sucrose solution at 10:2.2 (LNP:sucrose) for the final concentration of sucrose at 10% (w/v). LNPs were incubated in dry ice-ethanol bath for flash freezing. Hydrodynamic diameter, PDI, and encapsulation efficiency were measured before and after freeze-thawing. Frozen LNPs were stored at $\leq -80^{\circ}\text{C}$ before experiments were initiated.

pKa Assay.

A stock solution of 10 mM HEPES (Sigma Aldrich), 10 mM MES (Sigma Aldrich), 10 mM sodium acetate (Sigma Aldrich), and 140 mM sodium chloride (Sigma Aldrich) was prepared, and pH adjusted with hydrogen chloride and sodium hydroxide (pH 3–10). Using four replicates for each pH, 140 μL pH-adjusted buffer was added to a 96-well plate, followed by adding 5 μL of 2-(p-toluidino)-naphthalene-6-sulfonic acid (60 $\mu\text{g}/\text{mL}$). LNPs (5 μL) were added to each well. After 5 minutes of incubation at 300 rpm, fluorescence (ex. 325 nm, em. 435 nm) was measured using a plate reader.

TEM Imaging of LNPs.

For TEM imaging, 10 μL of LNP-containing solution was deposited onto a copper TEM grid covered with Formvar support film and stabilizing carbon. After blotting the excess solution with filter paper, the samples were negatively stained for 2 minutes, using either 2% phosphotungstic acid or UranylLess (Electron Microscopy Sciences, Hatfield, PA). The blotted and air-dried samples were analyzed with JEOL 100XC II TEM at the acceleration voltage of 100 kV. MaxIM DL software (v3, Diffraction Limited) was used for image collection.

Characterization of LNPs by Cryo-TEM.

LNP samples were placed on 300-mesh copper grids, with a holey carbon substrate (1.2 μm holes spaced by 1.3 μm ; C-flat, Electron Microscopy Sciences). Grids were glow-discharged (negative charge) for 15 seconds using a GloQube Plus glow discharge system (Quorum Tech, Laughton, UK). Samples (3 μL) were blotted with filter paper for one second at room temperature and 100% humidity and plunge-frozen into liquid ethane using a Vitrobot Mark IV (Thermo Scientific). Cryo-EM grids were stored in liquid nitrogen until cryo-TEM data acquisition. Cryo-EM grids were loaded under liquid nitrogen temperatures, using a Gatan 914 cryo-TEM sample holder, into a JEOL JEM-2200FS TEM (JEOL, Japan) operating at 200 keV. Micrographs were acquired under low dose conditions using the Serial EM software (v4.1.6)⁵¹ with a DE20 direct electron detector device (Direct Electron, San Diego, CA), at nominal magnifications of 40kx or 80kx, yielding pixel sizes of 1.4 and 0.8 Angstrom per pixel, respectively.

Isolation of Plasma Proteins Adsorbed to LNP⁶⁷ and LP01 LNP.

Following the previously published protocol,⁵² LNPs were diluted to a final lipid concentration of 1 g/L with 1 $\times$ PBS. C57BL/6J plasma was added to each LNP solution at a 1:1 volume ratio and incubated for 15 min at 37 $^{\circ}\text{C}$. A 0.7-M sucrose solution was

prepared by dissolving solid sucrose in MilliQ water. The LNP/plasma mixture was loaded onto a 0.7-M sucrose cushion of equal volume to the mixture and centrifuged at 15,300 g and 4°C for 1 h. The supernatant was removed, and the pellet was washed with 1× PBS. Next, the pellet was centrifuged at 15,300 g and 4°C for 5 min, and the supernatant was removed. Washing was performed twice more for a total of three washes. Following the final wash, the pellet was resuspended in RIPA buffer (Thermo Scientific). The concentration of protein in each sample was quantified by BCA assay (Thermo Scientific).

Preparation of Plasma Protein Samples for Mass Spectrometry.

Plasma proteins isolated from the surface of LNPs were loaded onto a Bolt™ 4–12% Bis-Tris Plus Gel (Invitrogen) at a volume of 35 µL and run into the gel 1 cm at 200 V. The gel was stained with SimplyBlue Safe Stain (Invitrogen) for 1 h to fix and visualize the proteins. After destaining for 1 h, the protein bands were excised using a sterile razor blade. The bands were added to a 1.5-mL tube stored at 4°C until being submitted to the Emory Integrated Proteomics Core for mass spectrometry analysis. The identified proteins (PSM >=4) were ordered based on their abundance/Mw.

Mouse Experiments.

All mouse experiments were performed in accordance with the approval of the Georgia Institute of Technology's and Emory University School of Medicine's Institutional Animal Care and Use Committee (IACUC). All mice were housed in the Georgia Institute of Technology or Emory University animal facilities in conventional cages with a 12:12 hour light-dark cycle and ad libitum access to food and water. Vivarium was kept at 23°C with 50% humidity. C57BL/6J mice were purchased from Jackson Laboratory. Mice (N=3) were injected intravenously using the lateral tail vein.

Mouse Cell Isolation and Staining.

Mice were euthanized by CO₂ asphyxiation, followed by cervical dislocation and perfusion with 5 mL of 1X PBS through the right atrium. Liver, lung, kidney, and heart tissues were finely minced, and then placed in a digestive enzyme solution including collagenase type I (Sigma Aldrich), collagenase XI (Sigma Aldrich) and hyaluronidase (Sigma Aldrich). The mixture was then incubated at 37°C, 550 rpm for 45 min^{53, 54}. For bone marrow, both ends of the femur were cut and 5 ml of RPMI1640 (Sigma Aldrich) per femur was flushed using a 25-gauge needle. Spleen were finely minced and placed in RPMI1640. Cell suspensions were filtered through a 70-µm mesh. Cells were stained to identify specific cell populations and sorted using the BD FACS Aria II Cell Sorter. The anti-mouse antibody clones used were the following: anti-CD31 (390, BioLegend, dilution 1:500), anti-CD45.2 (104, BioLegend, dilution 1:500), anti-CD19 (6D5, BioLegend, dilution 1:500), anti-CD68 (FA-11, BioLegend, dilution 1:500), anti-CD3 (17A2, BioLegend, dilution 1:500), anti-CD11c (N418, BioLegend, dilution 1:500), anti-CD11b (M1/70, BioLegend, dilution 1:500), anti-Ly6A/E (Sca-1; D7, BioLegend, dilution 1:500), anti-CD117 (cKit; ACK2, BioLegend, dilution 1:500), anti-CD326 (EpCAM; G8.8, BioLegend, dilution 1:500), anti-lineage cocktail (anti-mouse CD3ε, clone 145–2C11; anti-mouse Ly-6G/Ly-6C, clone RB6–8C5; anti-mouse CD11b, clone M1/70; anti-mouse CD45R/B220, clone RA3–6B2; anti-mouse TER-119/erythroid cells, clone Ter-119, BioLegend, 20 µl per test), CD48 (HM48–1,

BioLegend, dilution 1:500), and CD150 (TC15–12F12.2, BioLegend, dilution 1:500). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, dilution 1:5000) was used for live cell gating. Anti-camelid VHH antibody (clone 96A3F5, GenScript, dilution 1:500) was used for detecting aVHH expression.

Flow Cytometry.

Flow cytometry staining panels and gating strategies are shown in Supplementary Fig. 19. In all cases, mice injected with PBS were used as gating controls. FACS Diva software (v9.1, BD) was used for data collection on BD FACS ARIA II and BD FACSymphony A5.

Mouse Cytokine Analysis.

Blood was collected at 6 and 24 hours post-administration from terminal cardiac puncture after intravenous administration of PBS, LNP⁶⁷, or LPS (1 mg/kg, positive control). After incubating at room temperature for 30 min for clotting, samples were centrifuged at 2,000 x g for 15 min. The resulting supernatant was collected. Blood sera were pooled (N=3) for each experimental condition, and cytokine levels were detected using the Mouse Cytokine Profiler Array Panel A (R&D Systems) following the manufacturer's instructions. Pixel intensities were determined using Image Studio software (v5.2.5, LI-COR) on Odyssey CLx Far Infrared Imager (LI-COR) and relative concentrations were determined using ImageJ2 software (v2.14.0/1.54f).

Histology.

Livers were harvested from mice at 24 hours after intravenous administration of PBS or LNP, and fixed using 10% formalin solution. After incubating overnight at 4°C, tissues were shipped to HistoWiz, where they were paraffin-embedded, sectioned, stained with hematoxylin and eosin (H&E). Whole slide scanning (40x) was done with Scanner Console software (v102.0.7.5, Leica Biosystems) on Aperio AT2 (Leica Biosystems).

Single-Cell Multiomics Preparation.

For cell capture beads provided by BD Rhapsody Single Cell Analysis System were used. The custom DNA barcode capture site was added at the end of the poly T site as follows³⁵: The beads were washed once in PCR grade water and resuspended in 4.5 ml of 1× Kapa HF master mix. Then, 45 µl of this suspension was aliquoted into a 96-well PCR plate. Next, 5 µl of a mixture of poly-A (50 µM, 5'-AAAAAAAAAAAAAAAAAAAAA-3') and poly-A:barcode capture site (5 µM, 5'-GTGGTTAGTCGAGCAGAGACTAGAAAAAAAAAAAAAAAAAAAAA-3') oligonucleotides was added to each well. Then, PCR program was run as follows: 95°C for 5 min, five cycles of 95°C for 15 s, 50°C for 4 min, 72°C for 4 min and a final 4°C hold. The beads were then pooled and washed twice with water. After the PCR, the beads were pooled, washed twice in water and denatured in denaturation solution composed of 150 mM sodium hydroxide solution containing 0.01% Tween 20 for 10 min at room temperature with rotation. The beads were then washed twice in denaturation solution followed by three washes with a neutralization solution containing 100 mM Tris (pH 8.0), 1 mM EDTA and 0.01% Tween 20. The final beads were stored in 1× TE containing 0.01% Tween 20 at 4°C.

After the removal of dead cells and red blood cells (RBCs) using EasySep™ dead cell (Annexin V) and RBC (anti-TER119) removal kit (StemCell Technologies) following the manufacturer's protocol, the cells were stained with FITC-conjugated anti-mouse lineage cocktail. The lineage negative cells were enriched using EasySep™ Release Mouse FITC Selection Kit (StemCell Technologies) following the manufacturer's protocol. Then, TotalSeq-A anti-mouse Hashtag antibodies (BioLegend) and oligo-tagged anti-camelid VHH antibodies (5'-

CCTTGGCACCCGAGAATTCCAAAGTATGCCCTACGABAAAAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAA*A*A-3' chemically conjugated to MonoRab™ Rabbit Anti-Camelid VHH Antibody, mAb, GenScript. Star (*) denotes phosphorothioate bond) were added. After washing the labeled cells twice with PBS, the cell viability and numbers were recorded for each sample. The cells were then pooled at the same ratio and a BD Rhapsody cartridge was loaded with 60,000 cells. cDNA libraries were prepared using the BD Rhapsody Whole Transcriptome Analysis Amplification Kit following the BD Rhapsody System mRNA Whole Transcriptome Analysis (WTA) and Sample Tag Library Preparation protocol (BD Biosciences). The final libraries were quantified using a Qubit Fluorometer and sent to Novogene for sequencing. After the assessment of library quality using Bioanalyzer (Agilent), sequencing was performed by Illumina NovaSeq Plus PE150 at Novogene.

Processing of Single-Cell Multiomics Sequencing Data.

The data were processed using STARsolo (v2.7.9a)⁵⁵. All samples were mapped to GRCm39, and only exonic regions were counted. All output files were loaded into Seurat (v5.0.1). Global-scaling normalization was used for the LNP barcode assay in a Seurat object. The process normalizes the feature expression measurements for each cell by the total expression, multiplies this by a scale factor 10,000, and log-transforms the result^{32, 56}. DoubletFinder (v3) was used to identify doublets⁵⁷. This was followed by PCA dimensional reduction and UMAP/t-SNE clustering. The cell types were identified, and the clusters were annotated from marker genes in mouse bone marrow. aVHH oligo-tag and LNP barcode counts were combined with RNA counts in Seurat and treated similarly to other multimodal datasets such as cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq)⁵⁸. Visualization of gene, barcode, and aVHH expression levels was performed using R/Seurat commands (FeaturePlot, and DotPlot) and R/Nebulosa package (v1.12.0). For UMAP representation in Fig. 1e, cell clusters were manually annotated in Seurat with potency-specific marker genes listed in Fig. 1d. Similarly, for tSNE representation in Fig. 3a, LSK CD48⁻, LSK CD48⁺, Lin⁻Sca1⁺, and Lin⁻Sca1⁻ populations were manually annotated. 16 other lineage-positive clusters were annotated automatically by BioTuring Browser X cell type prediction database⁴¹.

Barcode Counts in Liver.

For FACS-sorted aVHH⁺ liver ECs (CD31⁺CD45⁻) and immune cells (CD31⁻CD45⁺), DNA barcodes were isolated by QuickExtract DNA Extraction Solution (Lucigen) following the manufacturer's protocol. The samples were then amplified using KAPA HiFi HotStart Ready Mix (Roche), following the manufacturer's protocol. Next-generation sequencing runs were performed using multiplexed runs on Illumina MiniSeq^{48, 59}. Briefly, the results were processed using a custom Python-based tool to extract raw barcode counts for each cell

type. These raw counts were then normalized using R prior to further analysis. Counts for each particle were normalized to the barcoded LNP mixture injected into mice.

Rhesus Monkey Studies.

All animal procedures conformed to the requirements of the Animal Welfare Act and protocols were approved prior to implementation by the UC Davis IACUC. Rhesus monkeys with a history of prior conceptions were time-mated according to standard protocols and pregnancy was identified by ultrasound early in gestation⁶⁰. Maternal blood samples were collected for screening purposes. Dams were selected for the study (N=8) using standardized assays. Newborns were delivered by cesarean section at term and raised in a nursery then moved to juvenile housing. At approximately 7–8 months of age selected animals were screened and confirmed for study initiation. The LNPs were thawed, screened (e.g., sterility, endotoxin), then aliquoted for injection IV under aseptic conditions in young rhesus monkeys (N=7; 7–8 months of age, 1.5–1.8 kg; 4 males and 3 females) comparing the two doses (0.25 versus 0.4 mg/kg); one control animal was included (8 months; 1.7 kg; male). Animals were sedated with telazol (5–8 mg/kg IM), the 0 timepoint blood sample collected, and a slow IV injection of LNPs performed using aseptic technique. CBCs and clinical chemistry panels were evaluated pre- and post-administration and found to be within normal limits. Animals were sedated 24 hours post-administration, weighed, final blood samples collected (serum, plasma, peripheral blood mononuclear cells), and euthanized (overdose of pentobarbital). Tissues were collected and weighed including the liver (cell suspensions) and bone marrow, which was collected from all long bones for immunoselection (CD34⁺ and CD34[−] fractions) according to established protocols⁶¹. Cells were isolated using the mini-MACS immunomagnetic separation system (Miltenyi Biotec) and cryopreserved in multiple aliquots, then stored in liquid nitrogen until assay.

Volumetric Light-field Imaging of Rhesus Cells.

Cells were seeded in glass-bottom 24-well plate. Primary antibodies were used as follows: For bone marrow cells, rabbit anti-CD34 (1:200, polyclonal, Invitrogen) and mouse anti-NanoLuc (3 µg/ml, monoclonal, Promega). For liver, rabbit anti-ASGR1 (1:200, polyclonal, Proteintech) and anti-NanoLuc (3 µg/ml). Secondary antibodies were used as follows: For CD34 and ASGR1, goat anti-rabbit Alexa Fluor 488 (4 µg/ml, Invitrogen). For NanoLuc, goat anti-mouse Alexa Fluor 647 (4 µg/ml, Invitrogen). Cells were incubated for 30 min at room temperature with 10% normal goat serum (Cell Signaling Technology) for blocking. Primary antibodies were diluted in PBS and treated to cells. After washing three times with PBS, secondary antibodies in PBS were added. After 1 hour incubation at room temperature, cells were washed three times with PBS for imaging. Images were acquired using a high-resolution Fourier light-field microscope^{62, 63}. The system was built upon an epi-fluorescence microscope (Eclipse Ti2-U, Nikon Instruments) with a 100× objective lens (NA = 1.45, CFI Plan Apochromat Lambda 100× Oil, Nikon Instruments). A customized microlens array (RPC Photonics) was used to partition the light field at the conjugated Fourier plane. The fluorescence was collected using a quadband dichroic mirror (ZT405/488/561/647, Chroma) and an emission filter (ZET405/488/561/647m, Chroma). Images were captured by a sCMOS camera (ORCA-Flash4.0 V3, Hamamatsu). For the post-processing, the light-field images went through rolling-ball background subtraction⁶⁴

and ACsN denoising algorithms⁶⁵ to improve image signal-to-noise ratio (SNR). After that, a circular mask was applied to highlight each elemental image and 3D reconstructions performed by Richardson-Lucy deconvolution. Fifty iterations were used to reconstruct each high-resolution 3D volume. ImageJ2 (v.2.14.0/1.54f) software was used to show orthogonal cut and z-projection of the images with max intensity.

IVIS Imaging of Rhesus Cells.

CD34⁺ and CD34⁻ fractions of bone marrow and whole liver cell suspensions (1×10^5 cells) in RPMI1640 were mixed with an equal volume of 2X Nano-Glo Luciferase Assay Substrate (Promega) and incubated at room temperature for 5 min before imaging. Luminescence was measured using an IVIS imaging system (PerkinElmer, Waltham, MA) and quantified using Living Image software (v4.7.3.20616, PerkinElmer).

Rhesus Cytokine Analysis.

Rhesus monkey sera were sent to IDEXX BioAnalytics and tested on the Milliplex MAP Non-Human Primate Cytokine Magnetic Bead Panel (Millipore, Cat No. PCYTMG-40K-PX23) according to the manufacturer's protocol. Data were collected by xPONENT[®] 4.3 (Luminex) and data analysis was completed using BELYSA[®] 1.1.0 software. The data collected by the instrument software are expressed as Median Fluorescence Intensity (MFI). Analyte standards, quality controls, and sample MFI values were adjusted for background. Calibrator data were fit to either a five-parameter logistic (5PL) or four parameter logistic (4PL) model depending on best fit to produce accurate standard curves for each analyte. Quality control and sample data were interpolated from the standard curves and then adjusted according to dilution factor to provide calculated final concentrations of each analyte present in the sample. Qualified assay ranges are shown in Supplementary Fig. 18.

Ex vivo Testing of LNP⁶⁷ with Human Primary Bone Marrow CD34⁺ Cell.

Primary human CD34⁺ cells isolated from bone marrow mononuclear cells using positive immunomagnetic separation reagents purchased from StemCell Technologies. Donor status was confirmed as 'normal' with characterization criteria of cell count, viability, purity, donor virus testing (HIV-1 and 2, hepatitis B, and hepatitis C), age, sex, ethnicity, weight, height, and smoking status from distributor's information. Cells were maintained in StemSpan SFEM II supplemented with StemSpan CD34⁺ Expansion Supplement for 4 days before the experiment according to the manufacturer's protocol. Cells were seeded at 1×10^5 cells/ml in 24-well plate. At 24 hours after seeding, cells were treated with LNP⁶⁷ carrying aVHH mRNA (0.25, 0.5, 1.0, 2.0, or 4.0 μ g/ml). At 24 hours after treatment, cells were analyzed by flow cytometry. The anti-human antibody clones used were the following: anti-CD34 (561, BioLegend, dilution 1:600), anti-CD45RA (HI100, BioLegend, dilution 1:600), anti-CD38 (HIT2, Invitrogen, dilution 1:600), and anti-CD90 (5E10, BioLegend, dilution 1:600). LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, dilution 1:5000) was used for live cell gating. Flow cytometry staining panels and gating strategies are shown in Supplementary Fig. 16.

Statistics and Reproducibility.

For in vivo and in vitro experiments, a minimum of three biological replicates were analyzed for quantification, except the control rhesus monkey (N=1). For the representative TEM image (Fig. 2c), 8 wide-field images were collected from one batch of LNP⁶⁷ that have the representative size distribution. All data are presented as the mean $\pm$ standard deviation or standard error mean. Statistical analysis between groups was performed using GraphPad Prism (v.9.5 and v.10). For data with multiple groups, the statistically significant differences were assessed using two-way ANOVA with Tukey's multiple-comparison test. For comparisons between two groups, unpaired two-tailed Student t-tests were used. The sample sizes (biological replicates), specific statistical tests and main effects of our statistical analyses for each experiment are detailed in each figure legend. A *p* value of less than 0.05 was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data Availability

scRNA-sequencing raw data have been deposited to the NCBI Sequence Read Archive (SRA) database (SRR28416440, SRR28416441, SRR28416442, SRR28417638, and SRR28417639) under BioProject ID PRJNA1090779⁶⁶. The mouse genome GRCm39 can be accessed at https://www.ncbi.nlm.nih.gov/datasets/genome/GCF_000001635.27/. All other data are shown.

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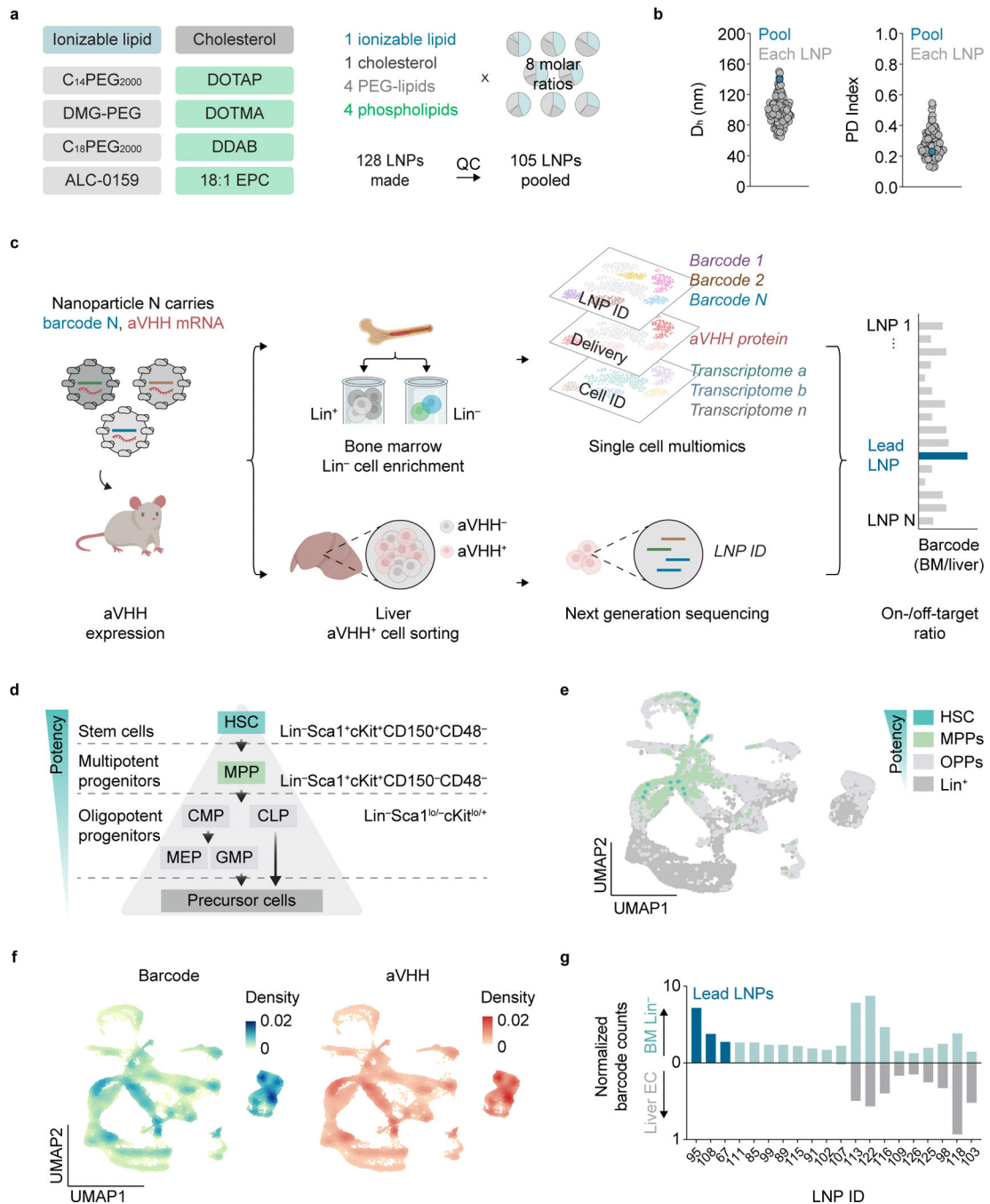


Figure 1. Barcoded LNPs deliver mRNA to transcriptionally defined HSCs *in vivo*.

a, Chemical composition of the 128 formulated LNPs. Of these, 105 LNPs met quality control (QC) criteria. **b**, The hydrodynamic diameter and polydispersity of the LNP pool were contained within the range of the LNPs composing the pool. **c**, After injecting 105 LNPs, we isolated the lineage (Ter119, CD45, CD3e, ITGAM, and Gr-1)-negative (Lin⁻) population from bone marrow and quantified LNP (barcode), functional delivery (aVHH protein), and the cell identity (transcriptome) in single cells, then overlaid the data. Functional delivery to liver endothelial (CD31⁺CD45⁻) and immune cells (CD31⁻CD45⁺)

was also analyzed by DNA barcode sequencing. This figure was generated in part using graphics created by [Biorender.com](https://biorender.com). **d**, Bone marrow cells were subdivided as MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte-erythrocyte progenitor; and GMP, granulocyte-monocyte progenitor. **e**, UMAP representation of 21,618 single cells within the bone marrow. **f**, Barcode counts and aVHH density distribution overlaid on the UMAP. **g**, Delivery mediated by the top 20 LNPs in aVHH⁺ cells in the transcriptionally defined Lin⁻ cell subtypes in bone marrow. Off-target delivery to liver endothelial cells (ECs) is also shown in gray.

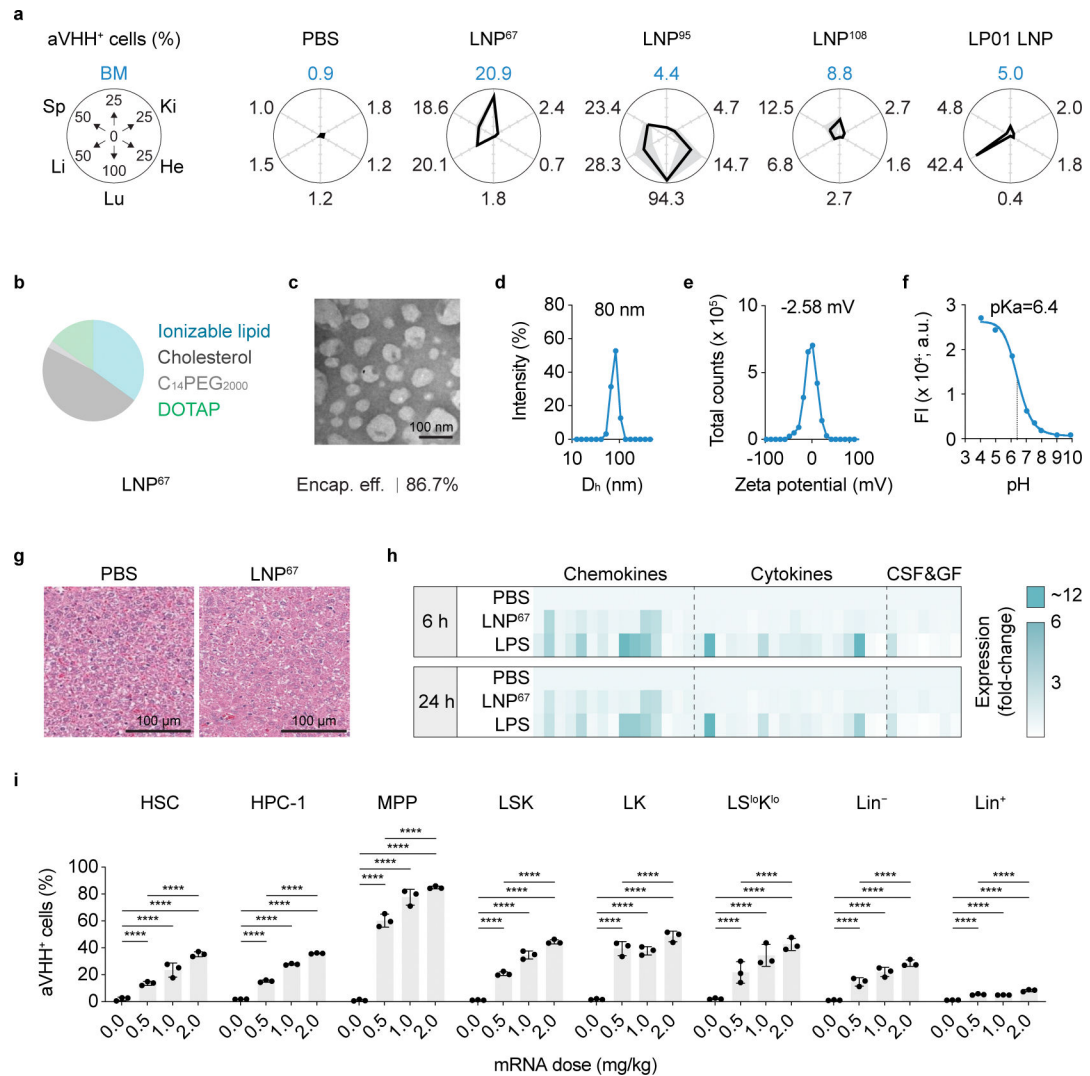


Figure 2. LNP⁶⁷ delivers mRNA to cells in mouse bone marrow.

a, Radar plot showing the percentage of aVHH-positive cells after PBS, or LNP⁶⁷, LNP⁹⁵, and LNP¹⁰⁸, or LP01 LNP carrying aVHH mRNA were administered to C57BL/6J mice. LSK for bone marrow (BM), immune cells (CD45⁺CD31⁻) for spleen (Sp), and endothelial cells (CD31⁺CD45⁻) for the liver (Li), lung (Lu), heart (He), and kidney (Ki) are shown. Axis scales are 0% to 25% (BM, Ki, and He), 50% (Sp and Li), or 100% (Lu) toward corresponding directions. Data are presented as mean (black line) $\pm$ SD (gray) (N=3, biological replicates). **b**, LNP⁶⁷ comprises an ionizable lipid, cholesterol, C₁₄PEG₂₀₀₀, and DOTAP. **c-e**, Characteristics of LNP⁶⁷. **c**, TEM image, **d**, hydrodynamic diameter, and **e**, zeta potential. **f**, TNS fluorescence intensity between pH 4 and 10 reveals the pKa of LNP⁶⁷. **g**, Representative hematoxylin and eosin (H&E) images of liver from PBS- or LNP⁶⁷-treated mice. N=3 (biological replicates) for both groups were analyzed, and the representative images are shown. **h**, Chemokines (CCL1, CCL2, CCL3, CCL4, CCL5, CCL11, CCL12, CCL17, CXCL1, CXCL2, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13), cytokines (TNF- α , IL-6, IL-12p70, IL-1 β , IFN- γ , IL-2, IL-17, IL-5, IL-16, TIMP-1, TREM-1, IL-23, IL-10, IL-1ra, IL-13, IL-4), colony-stimulating factors and growth factors (CSF

and GF; G-CSF, GM-CSF, M-CSF, IL-3, IL-7), and soluble cell adhesion molecules (CD54 and complement component 5a) were analyzed in serum from PBS-, LNP⁶⁷-, or lipopolysaccharide (LPS, 1 mg/kg)-treated mice at 6- or 24-hour timepoints. Data show mean fold-change compared to PBS group (N=3, biological replicates were pooled for measurement). Raw values are shown in Supplementary Fig. 12. **i**, The percentage of aVHH-positive cells in bone marrow cell subsets at 16 hours after administration of LNP⁶⁷ at 0.5, 1.0, and 2.0 mg/kg. Data are presented as mean $\pm$ SD (N=3, biological replicates). **** $p < 0.0001$, analyzed by two-way ANOVA with Tukey post-hoc test for multiple comparisons.

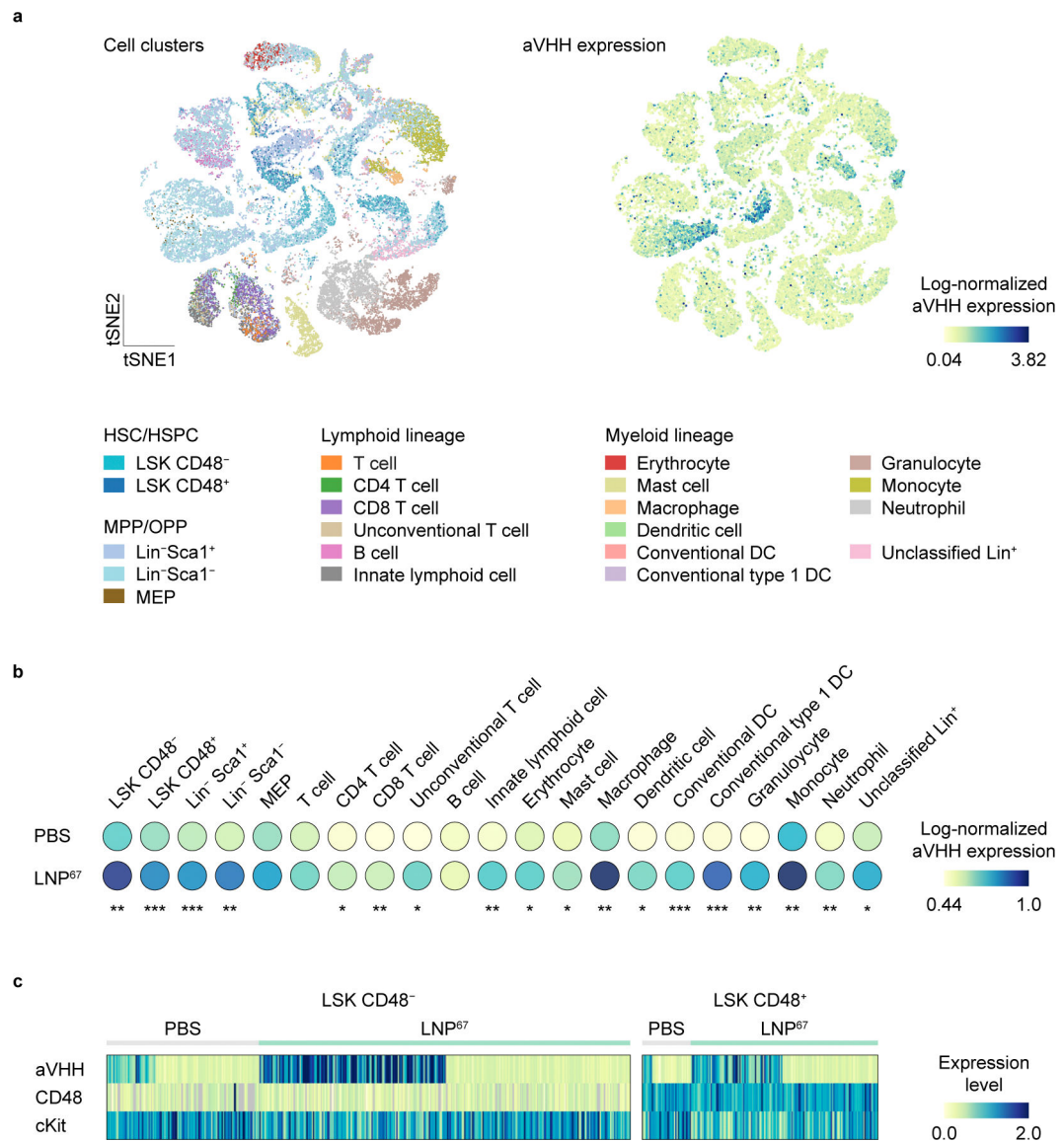


Figure 3. LNP⁶⁷ delivers mRNA to transcriptionally defined HSCs *in vivo*.

a, t-SNE clustering of 21 cell types in the bone marrow and their aVHH protein expression with single-cell resolution, overlaid on the t-SNE (36,142 cells). **b**, aVHH protein expression in for all 21 clusters. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ analyzed by two-tailed unpaired student's t-test (2 mg/kg LNP⁶⁷ compared to PBS, N=3). **c**, aVHH protein, CD48 mRNA, and cKit (CD117) mRNA expression levels in LSK cells with or without CD48 expression.

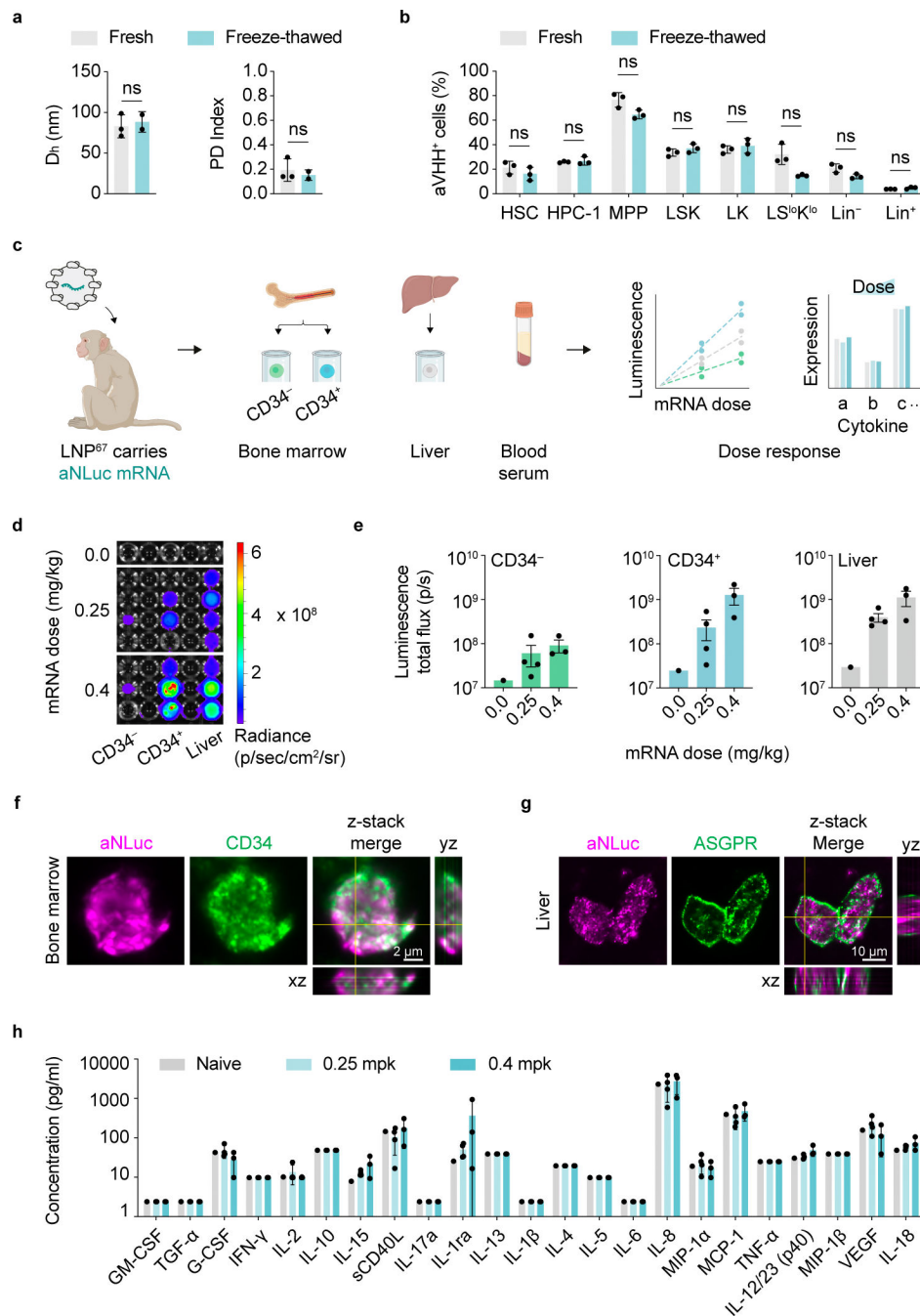


Figure 4. LNP⁶⁷ delivers mRNA to CD34⁺ cells in rhesus monkeys.

a, Hydrodynamic diameter (D_h) and polydispersity index (PD index) of LNP⁶⁷ before (N=3, independent batches) and after freeze-thawing (N=2, independent batches). Data are presented as mean $\pm$ SD. ns = not significant, analyzed by two-tailed unpaired student's t-test. **b**, aVHH expression did not show significant differences in mice treated with fresh LNP⁶⁷ or freeze-thawed LNP⁶⁷ in bone marrow. Data were analyzed by two-way ANOVA with Tukey post-hoc test for multiple comparisons. All data are presented as mean $\pm$ SD (N=3, biological replicates). **c**, aNLuc mRNA-carrying LNP⁶⁷ was administered

intravenously to young rhesus monkeys at 0.25 or 0.4 mg/kg. Bone marrow cells were immunoselected (CD34⁻ and CD34⁺ fractions) and liver cell suspensions prepared. Luminescence intensities were evaluated after adding the luciferase substrate luciferin to address the dose response. This figure was generated in part using graphics created by [Biorender.com](https://www.biorender.com). **d-e**, IVIS imaging of CD34⁻ and CD34⁺ fractions from the bone marrow and liver cell suspension from untreated (N=1) or LNP⁶⁷ (0.25 mg/kg, N=4; or 0.4 mg/kg, N=3)-treated rhesus. **d**, raw images after adding luciferin and **e**, the signal quantification. Data are presented as mean $\pm$ SEM. **f-g**, z-projection of Fourier light-field fluorescence microscopy images of **f**, rhesus bone marrow CD34⁺ and **g**, liver cell suspensions from animals administered LNP⁶⁷ (0.4 mg/kg). The cells were stained with anti-NanoLuc and anti-CD34 (bone marrow, **f**) or anti-NanoLuc and anti-ASGPR (liver, **g**). **h**, 23 rhesus monkey serum cytokines were analyzed from untreated (N=1) or LNP⁶⁷ (0.25 mg/kg, N=4; or 0.4 mg/kg, N=3)-treated rhesus collected at 24 hours post administration. Raw values and qualified assay ranges are shown in Supplementary Fig. 18.

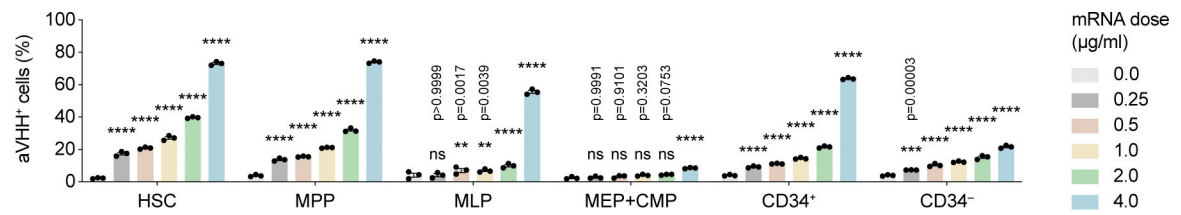


Figure 5. LNP⁶⁷ delivers mRNA to HSCs to primary human HSCs ex vivo. aVHH expression from LNP⁶⁷-treated primary human stem and progenitor cells. MLP, multilymphoid progenitor. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = not significant, compared to matched untreated group, analyzed by two-way ANOVA with Tukey post-hoc test. All data are presented as mean $\pm$ SEM (n=3).