Cell Subtypes Within the Liver Microenvironment Differentially Interact with Lipid Nanoparticles

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(Received 19 February 2019; accepted 2 May 2019; published online 8 May 2019)

Associate Editor Michael R. King oversaw the review of this article.

Abstract

Introduction—Lipid nanoparticles (LNPs) tend to accumulate in the liver due to physiological factors. Whereas the biological mechanisms that promote LNP delivery to hepatocytes have been reported, the mechanisms that promote delivery to other cell types within the liver microenvironment are poorly understood. Single cell profiling studies have recently identified subsets of Kupffer cells and hepatic endothelial cells with distinct gene expression patterns and biological phenotypes; we hypothesized these subtypes would differentially interact with nanoparticles.

Methods—To test the hypothesis, we quantified nucleic acid (i) biodistribution and (ii) functional mRNA delivery within

the liver microenvironment using two clinically relevant LNPs *in vivo*.

Results—We found that these LNPs distribute nucleic acids distribute to Kupffer cells and liver endothelial cells as efficiently as they distribute to hepatocytes, yet result in more functional mRNA delivery to endothelial cells. Additionally, we found these LNPs differentially accumulate in Kupffer and endothelial cell subsets.

Conclusions—These data suggest subsets of liver microenvironmental cells can differentially interact with nanoparticles *in vivo*, thereby altering LNP delivery. More generally, the data suggest that nucleic acid biodistribution is not sufficient to predict functional nucleic acid delivery *in vivo*.

Keywords—siRNA, Drug delivery, Microenvironment, Kupffer cell, Endothelial cell.

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James E. Dahlman is an Assistant Professor in the Wallace H. Coulter Department of Biomedical Engineering at Georgia Tech and Emory. His lab works at the interface of drug delivery, nanotechnology, genomics, and gene editing. James has designed nanoparticles that deliver RNA to blood vessels in the heart and lung; these nanoparticles have been validated by > 20 labs and have been licensed for clinical development. James also uses molecular biology to design the genetic drugs he delivers. He designed ‘dead’ guide RNAs to turn on genes using active Cas9. Similarly, using his background in nanoparticle chemistry, *in vivo* RNA delivery, and genomics, his lab has designed a series of increasingly sensitive DNA barcoding systems that can measure how > 200 nanoparticles target cells 30 different cell types at once, directly *in vivo*. James’ nanoparticle barcoding work led to his placement on the MIT Tech Review TR35 list. James has won scientific awards at every stage of his career, including the NSF, NDSEG, NIH OxCam, Whitaker, and LSRF Fellowships, and the Weintraub Graduate Thesis Award. He has been named a young / leading investigator by Bayer, the Parkinson’s Disease Foundation, and the *Journal of Materials Chemistry B*. At the age of 32, his research has been published in *Science*, *Cell*, *Nature Nanotechnology*, *Nature Biotechnology*, *Nature Cell Bio*, *Science Translational Medicine*, *PNAS*, *Advanced Materials*, *JACS* and other leading journals. He has given > 75 invited talks on drug delivery, gene editing, and nanoparticle DNA barcoding across the world, and is a co-founder of GuideRx.

This article is part of the 2019 CMBE Young Innovators special issue.

INTRODUCTION

In 2018, the first siRNA therapy was approved by the FDA for the treatment of hereditary ATTR amyloidosis. In this therapy, administering a lipid nanoparticle (LNP) carrying siTTR at a dose of 0.3 mg/kg every 3 weeks reversed symptoms in an

otherwise fatal disease.¹ The LNP is comprised of the ionizable lipid MC3, cholesterol, a PEG-lipid, as well as 1,2-distearylglycero-3-phosphocholine (DSPC); like many other LNPs, it preferentially delivers siRNA hepatocytes in the liver.^{5,11,23,24}

Clinically relevant functional delivery of siRNAs or other RNAs to cell types other than hepatocytes—even within the liver—remains a significant challenge.¹⁰ Yet several lines of evidence suggest other cells within the liver microenvironment can be targeted at low doses. Nanomedicines tend to accumulate in the liver due to discontinuous vasculature, decreased blood flow rates,²⁷ and an abundance of phagocytic cell types lining the hepatic sinusoids.²⁶ For example, it was recently demonstrated that decreased flow rates within the liver led to accumulation of inorganic nanomedicines in the liver, especially to Kupffer and endothelial cells.²⁷ Interestingly, the authors observed relatively little biodistribution of inorganic nanoparticles in hepatocytes. The same group subsequently demonstrated that depleting Kupffer cells from the liver *via* clodronate liposomes decreased the liver accumulation of gold nanoparticles from 80 to 20% of the injected dose.²⁶ This evidence generated with inorganic nanoparticles is supported by recently reports that (organic) LNPs can deliver RNAs to hepatic endothelial cells¹⁶ and Kupffer cells¹⁷ at doses as low as 0.05 mg/kg.

Our ability to understand which cell types interact with nanoparticles *in vivo* stands to benefit greatly from advances in RNA sequencing. Cells that were previously thought to be uniform have been shown to cluster into many distinct subtypes^{25,28,30}; these subtypes express different genes and subsequently exhibit different phenotypes. A recent example demonstrated that both Kupffer cells and endothelial cells within the liver microenvironment can be divided into subtypes based on the marker CD74 and CD32, respectively.¹³ The authors found that CD74^{High} Kupffer cells exhibited inflammatory phenotypes, whereas CD74^{Low} Kupffer cells exhibited tolerogenic phenotypes. Liver endothelial cells that were CD32^{High} were primarily localized on the central venous zone, whereas CD32^{Low} cells were located in the periportal zone. The evidence that (i) nanoparticles directly interact with cells in the liver microenvironment and (ii) different subsets of cells exist in the microenvironment led us to two hypotheses. First, that LNPs currently described as hepatocyte targeting may deliver nucleic acids to additional cell types within the liver microenvironment. Second, that these LNPs may differentially interact with the CD74 and CD32 subtypes. To test these hypotheses, we focused on LNPs formed with lipids that deliver siRNA and mRNA to hepatocytes at low doses. The first lipid—named MC3—was origi-

nally reported in 2010, and has safely delivered RNA in mice, non-human primates, and humans.^{1,24} The second—named cKK-E12—was reported in 2014 and has safely delivered RNA in mice and non-human primates.⁵

We first evaluated *in vivo* biodistribution within the liver microenvironment using QUANT,²⁰ which is a highly sensitive readout of nucleic acid copy number based on digital droplet PCR. We then quantified the functional delivery of Cre mRNA *in vivo* using Ai14 reporter mice,²¹ which contain cells that fluoresce when Cre mRNA is translated into functional Cre protein. Interestingly, we found biodistribution and functional delivery to all tested cell types within the liver; distribution was highest in Kupffer cells, whereas functional mRNA delivery was highest in endothelial cells. We also found that the amount of nanoparticle biodistribution changed with the CD74 or CD32 subtype. These data—which support the hypothesis that LNPs differentially interact with subtypes of cells within the liver—have implications for future studies that seek to understand how biological signaling governs nanomedicine safety and efficacy *in vivo*. They also provide evidence that the biological pathways active within a cell can affect nanoparticle targeting *in vivo*.

RESULTS AND DISCUSSION

We first quantified LNP biodistribution within the liver microenvironment *in vivo*. To do so, we formulated LNPs to carry QUANT DNA sequences using microfluidics (Fig. 1). We recently developed QUANT DNA in order to perform highly sensitive *in vivo* biodistribution experiments after we found that fluorescent biodistribution experiments did not generate sufficiently consistent or sensitive results.²⁰ By comparing the doses at which a linear dose response was observed after nanoparticle delivery *in vitro*, we found that QUANT readouts are roughly one billion-fold more sensitive than fluorescence. QUANT DNA barcode sensitivity is achieved by reducing DNA secondary structure, including a binding site for a digital droplet PCR probe, and including 5 phosphorothioated deoxy nucleotides on both the 5' and 3' termini²² (Fig. 1a). We included the chemically modified DNA after finding that they can improve the stability of nucleic acids, even when the nucleic acids are delivered by LNPs.

We purchased the ionizable lipid MC3 and synthesized/purified cKK-E12 as previously described^{5,17} (Fig. 1b). To create LNPs, we diluted either MC3 or cKK-E12 with cholesterol, poly(ethylene glycol) linked to 2 saturated alkyl tails (C₁₄PEG₂₀₀₀) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) in a syr-

FIGURE 1. QUANT DNA sequences for sensitive *in vivo* readouts of nanoparticle biodistribution. (a) QUANT barcodes contain a ddPCR probe site flanked by forward and reverse primer sites. (b) Structure of ionizable lipid (MC3 or cKK-E12), cholesterol, PEG-lipid, and DSPC used in each LNP formulation. (c) Molar ratio of each LNP. (d) Nanoparticle biomaterials and QUANT barcodes are formulated into stable LNPs by microfluidic mixing. (e) Hydrodynamic diameter, measured by dynamic light scattering, of MC3 and cKK-E12 LNPs. $N = 3/\text{group}$ (f) Polydispersity index (PDI) of MC3 and cKK-E12 LNPs. $N = 3/\text{group}$. Error bars are reported as standard error.

inge containing 100% EtOH (Fig. 1c). We then mixed the contents with QUANT DNA diluted in 10 mM citrate buffer in a microfluidic device³ (Fig. 1d). Finally, we characterized the hydrodynamic diameter of all the LNPs using dynamic light scattering. We recapitulated the previous reports, finding that microfluidic formulation resulted in nanoparticles with diameters between 50–75 nm and low polydispersity index (Figs. 1e and 1f). After dialysis and sterile filtration, the LNPs were intravenously administered at a dose of 0.3 mg/kg QUANT barcode.

The liver is a complex tissue consisting of several major cell types, including Kupffer, endothelial cells, and hepatocytes. Kupffer cells are resident macrophages lining the liver sinusoid, while liver endothelial

cells form a discontinuous vasculature that enables LNPs to extravasate into the Space of Disse, where they are exposed to hepatocytes (Fig. 2a). Previous work has demonstrated that blood flow rates decrease within the sinusoid, and that this reduction in flow rates can lead to increased nanoparticle accumulation, at least for inorganic nanoparticles.²⁷ We first analyzed the biodistribution of MC3 and cKK-E12 LNPs to liver endothelial cells, Kupffer cells, and hepatocytes. Six hours after injecting mice with 0.3 mg/kg QUANT DNA, we utilized fluorescent activated cell sorting (FACS) to isolate endothelial cells ($\text{CD31}^+\text{CD45}^-\text{CD68}^-$), Kupffer cells ($\text{CD31}^-\text{CD45}^+\text{CD68}^+$), and hepatocytes ($\text{CD31}^-\text{CD45}^-$). We observed that cKK-E12 facili-

FIGURE 2. Biodistribution of MC3 and cKK-E12 within the liver microenvironment. (a) As LNPs travel through the liver, they encounter Kupffer cells, endothelial cells and hepatocytes. (b) DNA delivery as mediated by cKK-E12 to endothelial cells, hepatocytes, and Kupffer cells. * $p < 0.05$, One-way ANOVA with Tukey's Multiple Comparison. (c) DNA delivery as mediated by MC3 to endothelial cells, hepatocytes, and Kupffer cells. (d) Normalized DNA delivery (to hepatocytes) by cKK-E12 and MC3 reveals similar intra-hepatic biodistribution patterns. (e) Comparison of DNA delivery by cKK-E12 and MC3 to major liver cell types. * $p < 0.05$, Two-way ANOVA. Error bars are reported as standard error.

tated DNA accumulation in all 3 cell types; the amount of DNA taken up per endothelial cells was less than Kupffer cells (Fig. 2b). MC3 LNPs behaved similarly; less DNA was measured per cell in endothelial cells, relative to the other cell types; these results were not statistically significant (Figs. 2c and 2d). However, we observed significantly lower amounts of DNA accumulation in all three cell types on an absolute scale with cKK-E12, compared to MC3 (Fig. 2e). These data suggested that both cKK-E12 and MC3 distribute broadly to all 3 tested cell types within the liver.

After confirming that nanoparticles distributed to different cells inside the liver, we investigated whether biodistribution varied in the recently reported subsets of Kupffer cells and hepatic endothelial cells. To do so, we first confirmed these subsets existed within the mouse liver using flow cytometry. As reported, we

observed distinct populations of $CD74^{\text{High}}$ and $CD74^{\text{Low}}$ Kupffer cells and $CD32^{\text{High}}$ and $CD32^{\text{Low}}$ endothelial cells (Fig. 3a). Given that these subsets have only recently been identified, the description of their phenotypes may change; however, the phenotypes of $CD74^{\text{High}}$ and $CD74^{\text{Low}}$ Kupffer cells are currently classified as inflammatory and tolerogenic, respectively (Fig. 3b). By contrast, the $CD32^{\text{High}}$ and $CD32^{\text{Low}}$ subsets of endothelial cells likely describes their location within the liver, either in the central venous zone and periportal zone, respectively (Fig. 3c). We observed noticeable (but not statistically significant) decreases in cKK-E12 biodistribution in $CD74^{\text{High}}$ cells, relative to $CD74^{\text{Low}}$ Kupffer cells (Fig. 3d). The same trends (this time, significant) were observed with MC3 biodistribution (Fig. 3e). These results suggest that result macrophage phenotype is related to the

FIGURE 3. LNP biodistribution varies within newly reported cellular subsets. (a) FACS markers for two Kupffer and two endothelial cells subsets. (b) Kupffer cells subsets and reported phenotype. (c) Endothelial cell subsets and reported intra-hepatic location. (d) DNA delivery as mediated by cKK-E12 to Kupffer cell subsets. (e) DNA delivery as mediated by MC3 to Kupffer cell subsets. (f) DNA delivery as mediated by cKK-E12 to endothelial cell subsets. (g) DNA delivery as mediated by MC3 to endothelial cell subsets. *** $p < 0.001$, **** $p < 0.0001$, Two-Tail T-Test. Error bars are reported as standard error.

degree with which the cells interact with systemically administered LNPs.

We then analyzed the biodistribution within CD32 endothelial cell subsets. We made several observations. First, we found that—in general—the biodistribution in endothelial cells was lower than the biodistribution to Kupffer cells. Second, cKK-E12 biodistribution in CD32^{Low} endothelial cells on a per cell basis was higher than CD32^{High} endothelial cells (Fig. 3f). While this could be due to changes in gene expression, one important limitation is that we cannot exclude the

possibility that LNPs were interacting with CD32^{Low} cells first. More specifically, since the LNPs were administered intravenously, they interacted with the periportal (CD32^{Low}) endothelial cells before they interacted with the central venous (CD32^{High}) endothelial cells. Interestingly, MC3 biodistribution was low in both CD32^{High} and CD32^{Low} endothelial cells (Fig. 3g).

Having observed extensive biodistribution to the primary three cell types in the liver (Fig. 2), as well as associated subsets (Fig. 3) we next sought to

FIGURE 4. Measuring functional delivery of Cre mRNA at the cellular level *in vivo*. (a) Upon the expression of Cre protein (as mediated by the delivery and expression of Cre mRNA by LNP), the Cre-reporter mice express tdTomato. (b) Percentage tdTomato⁺ cells of each major cell types after a 1.0 mg/kg administration of cKK-E12 and MC3. (c) Percentage tdTomato⁺ cells of each major cell types after a 0.3 mg/kg administration of cKK-E12 and MC3. ***p* < 0.01, ****p* < 0.001, Two-way ANOVA. Error bars are reported as standard error.

understand how nucleic acids were functionally delivered into the cytoplasm of target cells by MC3 and cKK-E12. We selected mRNA as our molecule, given it can be used for protein replacement, vaccines,^{2,18} and gene editing drugs.^{6,7,14,29} Notably, the delivery of mRNA to the liver is often measured *via* luminescence¹⁹ or a secreted protein⁸; however, neither methodology can be used to quantify delivery in different cell types within the organ. We utilized Ai14 ‘Cre-reporter’ mice; these mice have been used by our lab and others to quantify mRNA delivery at the cellular level^{9,17,21} (Fig. 4a). The Ai14 mice contain a Lox-Stop-Lox-tdTomato transgene under the control of a promoter that results in ubiquitous expression. As a result, cells in these mice only become tdTomato⁺ when Cre mRNA is (i) delivered into the cytoplasm and (ii) translated into Cre protein, which then (iii) translocates into the nucleus and (iv) excises the ‘Stop’ construct from genomic DNA. By quantifying the percentage of cells that are tdTomato⁺ after treating mice with Cre mRNA, it is possible to quantify the efficiency with which mRNA has been functionally delivered. In these experiments, as we previously reported,^{17,21} we utilized chemically modified Cre; chemically modified mRNA reduces inflammation and can increase the stability of mRNA contained within LNPs.

We administered Cre mRNA at a dose of 1.0 mg/kg using cKK-E12 and MC3. Three days later, we sacrificed the mice, and quantified the number of tdTomato⁺ cells, relative to a PBS-treated Ai14 control (Fig. 4b). More specifically, we isolated cells with flow cytometry, and quantified the percentage of hepatocytes, endothelial cells, or Kupffer cells that were tdTomato⁺. At this dose, we observed high levels of tdTomato⁺ across all three cell types. Interestingly, despite the fact the biodistribution to endothelial cells was lower than Kupffer cells or hepatocytes, the functional delivery to endothelial cells was higher than Kupffer cells or hepatocytes, both for mice treated with MC3, and for mice treated with cKK-E12, albeit not significantly. In order to exclude the possibility these results were an artifact of the 1.0 mg/kg dose we used, we repeated the experiment at a lower dose—0.3 mg/kg Cre mRNA. We observed the expected decrease in percent tdTomato⁺ cells for both cKK-E12 and MC3 at 0.3 mg/kg, compared to the 1.0 mg/kg dose (Fig. 4c). At 1.0 mg/kg, cKK-E12 performed slightly better than MC3; at 0.3 mg/kg, cKK-E12 performed delivery was statistically higher than MC3. At 0.3 mg/kg, liver endothelial and Kupffer cells had a statistically significant higher percent of tdTomato⁺ cells than hepatocytes.

Taken together, our biodistribution and functional delivery data led us to several conclusions. First, we concluded that both MC3 and cKK-E12 interact with different cell types within the liver microenvironment. This conclusion is important, since in many mRNA delivery assays, the delivery is often assumed to primarily occur in hepatocytes. In some cases (e.g., Cas9 gene editing), the final calculated percentage of mutations is based explicitly on this assumption. Our data therefore suggest that—when possible—tissue level delivery readouts should be replaced by cell-level delivery readouts. Second, we found evidence to support our original hypothesis: that subsets of liver microenvironmental cells differentially interact with nanoparticles. In particular, we found that biodistribution to Kupffer cells and endothelial cells seemed to change within the CD74 and CD32 subsets, respectively. Our results substantiate *in vitro* results from primary human macrophages and Kupffer cells, including the aspect that M2-like polarized cells uptake more nanoparticles.¹² The importance of targeting cellular subsets is likely to depend on the cells as well as the specific disease. For example, it may be critical in a disease driven by a specific subset, and relatively less important when the disease is caused by all the subsets. By demonstrating the LNPs can interact with subsets of cells *in vivo*, we hope to lay the foundation for future studies that evaluate the disease-specific importance of this effect. In addition, we acknowledge that future studies will need to uncover the cell signaling pathways that govern these changes, particularly whether direct re-polarization *in vivo* can be used to modulate LNP uptake. It remains to be seen whether the functional delivery of siRNA, mRNA, and other therapeutic RNAs changes within these cells. Finally, our data provide evidence that biodistribution data is not sufficient to predict functional delivery. Previous reports have identified endosomal escape as a limiting step in mRNA therapeutic efficacy, with some ionizable lipids facilitating a five-fold higher efficiency in endosomal escape compared to MC3 LNPs.¹⁹ This, in turn, suggests that internal cell signaling can alter the fate of RNA drugs after they have reached the target cell. These results are substantiated by data that found nanoparticle biodistribution does not necessarily predict nanoparticle functional delivery of siRNA⁴ as well as data demonstrating that intracellular metabolic signaling can alter the fate of delivered mRNA.¹⁵ Notably, biodistribution data are still critical for many studies, including those used to understand pharmacokinetics and off-target effects. However, biodistribution data may not be able to consistently predict the cells in which intracellular drugs have an active cellular effect. Together, these conclusions help provide evidence that cellular signaling with the liver microenvi-

ronment can have a direct role on the biodistribution and efficacy of nucleic acid therapies.

MATERIALS AND METHODS

Nanoparticle Formulation

Nanoparticles were formulated using a microfluidic device.⁸ Nucleic acids (DNA barcodes) were diluted in 10 mM citrate buffer (Teknova) while lipid-amine compounds, alkyl tailed PEG, cholesterol, and helper lipids were diluted in ethanol at a concentration of 10 mg/mL. MC3 was purchased from MedKoo LLC. All PEGs, cholesterol, and helper lipids were purchased from Avanti Lipids. Citrate and ethanol phases were combined in a microfluidic device by syringes (Hamilton Company) at a flow rate of 600 and 200 μ L/min, respectively. Specifically, lipids were formulated at a 50:38.5:1.5:10 molar ratio of ionizable lipid:cholesterol:PEG-Lipid:DSPC and a 10:1 mass ratio of lipid to nucleic acid.

QUANT DNA

For biodistribution experiments, each LNP was formulated to carry a DNA suitable for ddPCR analysis. 95 nucleotide long single stranded DNA sequences were purchased from Integrated DNA Technologies (IDT). Three nucleotides on the 5' and 3' ends were modified with 5 phosphorothioates to reduce exonuclease degradation and improve DNA stability. We included universal forward and reverse primer regions on all barcodes. A 26nt probe was purchased from IDT with 5' FAM as the fluorophore, while internal Zen and 3' Iowa Black FQ were used as quenchers.

Nanoparticle Characterization

LNP hydrodynamic diameter was measured using high throughput dynamic light scattering (DLS) (Wyatt). LNPs were diluted in sterile 1 $\times$ PBS to a concentration of $\sim$ 0.06 μ g/mL, and analyzed. Particles were dialyzed with 1 $\times$ phosphate buffered saline (PBS, Invitrogen), and were sterile filtered with a 0.22 μ m filter.

Animal Experiments

All animal experiments were performed in accordance with the Georgia Institute of Technology IACUC. C57BL/6J (#000664) and Ai14 (#007914) mice were purchased from The Jackson Laboratory and used between 5 and 8 weeks of age. In all *in vitro* and *in vivo* experiments, we used $N = 3$ –4 per group. Mice were injected intravenously *via* the lateral tail vein. The

nanoparticle concentration was determined using NanoDrop (Thermo Scientific).

Cell Isolation and Staining

Mice were perfused with 20 mL of 1× PBS through the right atrium. Tissues were finely cut, and then placed in a digestive enzyme solution with Collagenase Type I (Sigma Aldrich), Collagenase XI (Sigma Aldrich) and Hyaluronidase (Sigma Aldrich) at 37 °C at 550 rpm for 45 min. Cell suspension was filtered through 70 μ m mesh and red blood cells were lysed. Cells were stained to identify specific cell populations and sorted using the BD FACS Fusion in the Georgia Institute of Technology Cellular Analysis Core. The antibody clones used were: anti-CD31 (390, BioLegend), anti-CD45.2 (104, BioLegend), anti-CD68 (FA-11, Biolegend), and anti-CD74 (ln1/CD74, Biolegend), and anti-CD16/32 (93, BioLegend).

ddPCR

The QX200™ Droplet Digital™ PCR System (Bio-Rad) was used to prep and analyze all ddPCR results. All PCR samples were prepared with 10 μ L ddPCR with ddPCR™ Supermix for Probes (Bio-Rad), 1 μ L of primer and probe mix (solution of 10 μ M of target probe and 20 μ M of Reverse/Forward Primers), 1 μ L of template/TE buffer, and 8 μ L water. 20 μ L of each reaction and 70 μ L of Droplet Generation Oil for Probes were loaded into DG8™ Cartridges and covered with gaskets. Cartridges were placed in the QX200™ Droplet Generator to create water–oil emulsion droplets. Plates were stored at 4 °C until run on the QX200™ Droplet Digital™ PCR System. For each biological rep, 3 technical repetitions were completed. In all cases, technical reps were averaged. Technical reps were only excluded if they saturated the detection limit or showed inconsistent positive event amplitudes.

Functional mRNA Delivery

Nanoparticles were formulated using a microfluidic device.⁸ Nucleic acids (Cre mRNA, TriLink Biotechnologies) were diluted in 10 mM citrate buffer (Teknova) while lipid-amine compounds, alkyl tailed PEG, cholesterol, and helper lipids were diluted in ethanol. MC3 was purchased from MedKoo LLC. All PEGs, cholesterol, and helper lipids were purchased from Avanti Lipids. Citrate and ethanol phases were combined in a microfluidic device by syringes (Hamilton Company) at a flow rate of 600 and 200 μ L/min, respectively. Ai14 mice were dosed with 0.3 or 1.0 mg/kg Cre mRNA delivered by either cKK-E12 or MC3. Three days after administration,

transfection of the liver (percent tdTomato⁺ cells) was analyzed *via* flow cytometry. Critically, we used untreated Ai14 mice—not untreated C57BL/6 mice—as gating controls, since we previously demonstrated that Ai14 mice do have autofluorescence in the tdTomato channels, relative to BL/6 mice.

Data Analysis and Statistics

Statistical analysis was performed in GraphPad Prism 7. More specifically, 2-tail *T* test, or One-way ANOVAs were used where appropriate. Data is plotted as mean $\pm$ standard error mean unless otherwise stated.

Data Access

The data used to generate all figures in the paper are available upon request to james.dahlman@bme.gatech.edu.

AUTHOR CONTRIBUTIONS

C.D.S. and J.E.D. designed experiments, performed experiments, and analyzed data. B.Z.K., M.P.L., Z.G. performed experiments. C.D.S. and J.E.D. wrote the paper, which was reviewed by all other authors.

ACKNOWLEDGMENTS

The authors thank Sommer Durham and the Georgia Tech Cellular Analysis and Cytometry Core. Additionally, the authors thank Dalia Arafat and the Genome Analysis Core. J.E.D. thanks Jordan Cattie and Taylor E. Shaw.

CONFLICT OF INTEREST

Cory D. Sago is co-founder of Guide Therapeutics and an employee at Guide Therapeutics. James E. Dahlman is a co-founder of Guide Therapeutics and consultant for Guide Therapeutics. Brandon R. Krupczak is an employee at Guide Therapeutics. Melissa P. Lokugamage declares no conflict of interest. Zubao Gan declares no conflict of interest.

ETHICAL APPROVAL

All animal studies were carried out in accordance with the institutional and national guidelines, following an animal protocol approved by the Georgia Institute of Technology IACUC committee. No human studies were performed as part of this research.

FUNDING

C.D.S. and J.E.D. were funded by Georgia Tech startup funds (awarded to J.E.D.). C.D.S. was funded by the NIH-sponsored Research Training Program in Immunoengineering (T32EB021962). M.P.L. was funded by the NIH-sponsored Research Training Program in Computational Biology and Predictive Health Genomics (T32GM105490). This content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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