Modifying a Commonly Expressed Endocytic Receptor Retargets Nanoparticles *in vivo*

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Nanoparticles are often target to receptors expressed on specific cells, but few receptors are both highly expressed on one cell type and involved in endocytosis. This creates a problem, since the number of potential idealized receptors on a cell is very limited. One untested alternative is to manipulate a common endocytosis gene (i.e., a receptor expressed on many cell types) after identifying two types of cells: those which (i) really depend on that gene and (ii) those that do not. In this way, manipulating a common gene may be used to promote delivery to one cell type over others.

This approach requires a commonly expressed endocytic gene to alter nanoparticle delivery in a cell type-dependent manner *in vivo*, but it remains unknown if this is feasible. Based on microenvironmental regulation, we hypothesized that Caveolin 1 (Cav 1) would exert cell type-specific effects on nanoparticle delivery. Fluorescence was not sensitive enough to investigate this question, as a result we created a platform which is $10^8$ times more sensitive than fluorescence named QUANT to study nanoparticle biodistribution. We measured how 226 lipid nanoparticles (LNPs) delivered nucleic acids to multiple cell types *in vivo* in wild types and Cav1 knockout mice. Cav1 knockout did not alter LNP delivery to lung and kidney macrophages but it substantially reduced LNP delivery in Kupffer cells (liver resident macrophages), showing that manipulating receptors expressed on multiple cell types can tune drug delivery.
Modifying a Commonly Expressed Endocytic Receptor Retargets Nanoparticles in Vivo

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ABSTRACT: Nanoparticles are often targeted to receptors expressed on specific cells, but few receptors are (i) highly expressed on one cell type and (ii) involved in endocytosis. One unexplored alternative is manipulating an endocytic gene expressed on multiple cell types; an ideal gene would inhibit delivery to cell type A more than cell type B, promoting delivery to cell type B. This would require a commonly expressed endocytic gene to alter nanoparticle delivery in a cell type-dependent manner in vivo; whether this can occur is unknown. Based on its microenvironmental regulation, we hypothesized Caveolin 1 (Cav1) would exert cell type-specific effects on nanoparticle delivery. Fluorescence was not sensitive enough to investigate this question, and as a result, we designed a platform named QUANT to study nanoparticle biodistribution. QUANT is $10^5$× more sensitive than fluorescence and can be multiplexed. By measuring how 226 lipid nanoparticles (LNPs) delivered nucleic acids to multiple cell types in vivo in wild-type and Cav1 knockout mice, we found Cav1 altered delivery in a cell-type specific manner. Cav1 knockout did not alter LNP delivery to lung and kidney macrophages but substantially reduced LNP delivery to Kupffer cells, which are liver-resident macrophages. These data suggest caveolin-mediated endocytosis of nanomedicines by macrophages varies with tissue type. These results suggest manipulating receptors expressed on multiple cell types can tune drug delivery.

KEYWORDS: DNA barcode, nanoparticle, drug delivery, Caveolin, Kupffer cell, ddPCR

Delivery of siRNAs to hepatocytes has treated disease in patients, 1−21 but delivery to other cell types remains challenging. 4 The liver exhibits physiological advantages that promote nanoparticle accumulation, 2,6 and as a result, rational approaches will be required to minimize unwanted liver delivery. An ideal approach would involve (i) synthesizing hundreds of nanoparticles with diverse chemical structures and (ii) analyzing them in vivo using an animal model that (iii) tests a specific biological hypothesis (e.g., gene X alters delivery) in vivo. However, the current gold standard is to study nanoparticles in vitro. Thousands of nanoparticles can be synthesized for nucleic acid delivery, but they are screened in vitro, 7−12 which can be a poor predictor of in vivo delivery. 13 Genes that alter nanoparticle delivery in vitro have been identified, 14−17 genes that affect systemic nanoparticle delivery in vivo remain much more difficult to study. Exceptions to this 18−21 have provided valuable insights but have focused on soluble factors in serum 18,19 or receptors on hepatocytes. 19−21 Whether a commonly expressed gene can exert cell type-specific effects on nanoparticle delivery in vivo remains unexplored.

Nanomedicines are often delivered using ligands that bind receptors expressed on target cells. 22 For example, the asialoglycoprotein receptor (ASGPR) have targeted ASGPR, 23 leading to delivery in animals and patients. Other receptors include epidermal growth factor (EGFR), 24 folate receptor, 25 transferrin receptor, 26 VCAM-1, 27 and ICAM-1. 28 Given that few receptors are (i) highly expressed on one cell type and (ii) induce nanomedicine endocytosis upon binding, we envisioned an alternative approach: manipulating an endocytosis receptor expressed on many cells. An ideal receptor would inhibit delivery to cell type A more than cell type B, promoting delivery to cell type B. This approach is timely. Our understanding of cell heterogeneity is progressing; RNA-seq 29 has revealed that gene expression varies with disease state 30 and within cell populations previously believed to be homogeneous. 31,32

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Large-scale approaches like the Human Cell Atlas33 are likely to uncover endocytic genes whose importance varies with cell type. Because hundreds of genes are involved in endocytosis,34 and many genes are regulated by disease- and microenvironment-derived cues,35,36 it is foreseeable that manipulating one gene could alter delivery in a tissue- or disease-specific manner. To test the hypothesis that manipulating a commonly expressed receptor can affect nanoparticle delivery in cell type-dependent manner, we focused on Caveolin 1 (Cav1), a gene involved in caveolin-mediated endocytosis.37 Caveolin can endocytose nanoparticles in vitro38 and in vivo.39 Its expression changes with fibrosis,40−43 lung disease,44 cancer,45−48 neurological disease,49,50 and other pathologies,51−53 demonstrating that its expression is regulated by microenvironmental signals and disease.

Results. Given that in vitro nanoparticle delivery can be a poor predictor of in vivo delivery13 and that gene expression can change when cells are cultured in vitro,54 we tested our hypothesis in vivo, eventually testing 226 chemically distinct lipid nanoparticles (LNPs). This approach is distinct from previous studies for two reasons. First, LNP studies typically evaluate many nanoparticles in vitro before selecting a small number to test in vivo.7−12 Second, most LNP studies focus on hepatocytes not macrophages.55−58 We used microfluidics55 to formulate a validated ionizable LNP that has delivered siRNAs in vivo.7,56−60 The LNP carried a single-stranded DNA (ssDNA) (Figure S1a) that was chemically modified with phosphorothioates to reduce exonuclease degradation and fluorescently tagged with Alexa-647. We chose Alexa-647 because it was significantly brighter than Alexa488 (Figure S1b). One hour after intravenously injecting wild-type (WT) or Cav1 deficient (Cav1−/−) mice with the clinically relevant dose of 0.5 mg/kg of DNA, we quantified Alexa-647 mean fluorescence intensity (MFI) in 13 cell types (Figure S1c) using flow cytometry (Figure S1d,e). In WT mice, >75% of the MFI signal was found in Kupffer cells or hepatic endothelial cells; we could not reliably quantify delivery in other cell types. The same was true for Cav1−/− mice, suggesting Cav1 did not change LNP biodistribution (Figure S1f).

We used microfluidics55 to formulate a validated ionizable LNP that has delivered siRNAs in vivo.7,56−60 The LNP carried a single-stranded DNA (ssDNA) (Figure S1a) that was chemically modified with phosphorothioates to reduce exonuclease degradation and fluorescently tagged with Alexa-647. We chose Alexa-647 because it was significantly brighter than Alexa488 (Figure S1b). One hour after intravenously injecting wild-type (WT) or Cav1 deficient (Cav1−/−) mice with the clinically relevant dose of 0.5 mg/kg of DNA, we quantified Alexa-647 mean fluorescence intensity (MFI) in 13 cell types (Figure S1c) using flow cytometry (Figure S1d,e). In WT mice, >75% of the MFI signal was found in Kupffer cells or hepatic endothelial cells; we could not reliably quantify delivery in other cell types. The same was true for Cav1−/− mice, suggesting Cav1 did not change LNP biodistribution (Figure S1f).

Given the role of Cav1 in nanoparticle endocytosis,61 the fact caveolin inhibitors affect this LNP in vitro,7 and the fact this LNP delivers siRNA and sgRNA to pulmonary and cardiovascular endothelial cells in vivo,7,56−60 we hypothesized that our Alexa-647 biodistribution data were inaccurate. Our hypothesis was recently strengthened by demonstrations that the fluorescent biodistribution of small molecules delivered by nanoparticles can change in ways that do not reflect delivery.62
Because nucleic acids are degraded by nucleases that cleave phosphodiester bonds, fluorophores are not, we reasoned the fluorescent signal may not track with the nucleic acid. To test this, we engineered a novel biodistribution assay named quantitative analysis of nucleic acid therapeutics (QUANT). QUANT utilizes digital droplet PCR (ddPCR), a technique used to quantify rare genomic events, to quantify the biodistribution of the nucleic acid itself with high sensitivity. This allows us to directly compare it to the biodistribution of the fluorescent readout. This is important because fluorescent biodistribution studies are ubiquitously used throughout to measure nucleic acid biodistribution.

Because ddPCR requires efficient DNA amplification, we rationally designed QUANT DNA barcodes to increase DNA polymerase access (Figure 1a,b, Figure S1a). We minimized DNA 2° structure on the forward and reverse primer sites and minimized G-quadruplex formation by separating our randomized seven nucleotide region into semirandomized NWNH and NWH sites. We flanked the primer sites with three additional phosphorothioate-modified nucleotides to reduce exonuclease degradation of the primer site. Finally, we identified universal primer binding sites that would not amplify mouse or human genomic DNA (gDNA). Specifically, we designed primers with similar melting temperatures (within 1 °C) and added them to human and mouse gDNA without barcode template (Figure S2a). We identified primers that did not amplify gDNA after 40 cycles (Figure S2b) but amplified barcode templates with 20 cycles. On the basis of these results, we added the “no gDNA background” primer sites to our barcodes. We then optimized the ddPCR protocol (Figure S2c−g) by varying annealing temperatures, primer concentrations, and probe concentrations. We increased the signal-to-noise ratio 14-fold compared to that of current standard protocols. As a control, we scrambled the ddPCR probe site; no signal was generated, demonstrating that the signal required specific barcode-probe interactions (Figure S2h).

Standard curve experiments revealed QUANT was highly sensitive. QUANT ddPCR signal was linear (with respect to the DNA added) when barcodes were diluted in Tris-EDTA buffer to a concentration between 750 aM and 12 fM ($R^2 = \ldots$)
Figure 3. QUANT biodistribution is more sensitive than fluorescence in vivo. (a) QUANT barcodes with (or without) a fluorophore were formulated into LNPs, injected intravenously, and isolated at different time points. Nanoparticle distribution was measured using QUANT or fluorescence. (b) Relative nanoparticle biodistribution (normalized to maximal signal in any cell type) 0.4, 0.75, 1.25, 12, 24, and 36 h after administration of an LNP carrying 647-QUANT barcode or QUANT barcodes at a dose of 0.5 mg/kg. Asterisk denotes a signal that was significantly different than that of PBS-treated mice. (c) Comparisons of area under the curve as measured by QUANT or fluorescence. Delivery to the lungs was underestimated by >3 fold by fluorescence. No fluorescent signal was detected in lung macrophages. ***p < 0.01, **p < 0.001 two-tailed t test. (d) Peak DNA delivery (normalized to liver ECs) as measured by QUANT and ddPCR. We compared samples immediately after completing the experiment to samples analyzed after storage at −20°C for 20 or 31 days. Readouts were consistent when performed by different individuals using different reagent stocks (Figure 1f). We hypothesized that fluorescent biodistribution would yield different results than QUANT. We formulated the same LNP with QUANT barcodes that were, or were not, fluorescently tagged with Alexa-647. One hour after intravenously administering 0.5 mg/kg, we isolated the same 13 cell types (Figure S1c) using FACS and quantified LNP delivery using Alexa-647 MFI or QUANT (Figure 2a); 87% of the Alexa-647 signal was found in liver cells, and the remaining 9 cell types only generated 13% of the total fluorescent signal (Figure 2b−d). QUANT biodistribution was different: only 56% of the ddPCR signal derived from the liver (Figure 2b−d). We compared delivery in all 13 cell types and found statistically significant differences in seven of them (Figure 2e). In Figure 2e, we normalized delivery to Kupffer cells, which readily clear nanoparticles.5,6 Notably, in all cases, fluorescence overestimated liver biodistribution. To exclude the possibility these results were due to a specific time point, we performed a pharmacokinetics experiment in five cell types: liver endothelial cells, Kupffer cells, hepatocytes, lung endothelial cells, and lung macrophages. We intravenously injected mice with 0.5 mg/kg of QUANT barcodes and sacrificed mice 0.4, 0.75, 1.25, 12, 24, and 36 h later (Figure 3a). At the 0.4, 0.75, and 1.25 h time points, fluorescent biodistribution was localized to liver cells; only one of six nonliver signals (two cell types, three time points) was statistically significant compared to untreated mice. At later time points, fluorescent biodistribution was not significantly above PBS-treated mice in any cell type. Out of 30 potential data points (five cell types, six time points), only six generated a statistically significant difference compared to untreated mice. As a control, we reduced the concentration to 30 aM (Figure 1c, d). As a control, we reduced the concentration to 30 aM (Figure 1c, d). As a control, we reduced the concentration to 30 aM (Figure 1c, d).
We investigated how robust QUANT readouts were across experiments. We compared the absolute ddPCR values from all five cell types in the first QUANT biodistribution experiment (Figure 2) and the pharmacokinetic experiment at similar time points (1 and 1.25 h, respectively). ddPCR readouts were reproducible ($R^2 = 0.98$) between experiments (Figure 3e). Figures 1–3 demonstrate that QUANT is a sensitive and repeatable method of quantifying nanoparticle biodistribution.

QUANT enabled us to measure LNP delivery with increased sensitivity; we took advantage of this to test the hypothesis Cav1 affects LNP delivery in a cell type-specific manner in vivo. To ensure our results were not specific to one LNP chemical structure, we exploited a second advantage of QUANT: it can be multiplexed so the distribution of >100 LNPs is analyzed at once. Multiplexed analysis of nanoparticle delivery has been reported by our group and others, but critically, these barcoding systems can only quantify delivery of LNP-1 relative to LNP-2 within the same sample; it cannot quantify absolute delivery. Without the ability to quantify absolute delivery, it is difficult to directly compare readouts (i) between different tissues, (ii) between different mouse models (e.g., WT and Cav1−/−), or (iii) between ddPCR and fluorescence.

We performed two high-throughput in vivo LNP screens. We formulated LNP-1 with chemical structure 1, so it carried QUANT barcode 1; we formulated LNP-N with chemical structure N to carry QUANT barcode N (Figure 4a–c). The 8-nucleotide barcode region on the QUANT DNA sequence, located in the center, can generate 65,536 unique barcodes; we designed 156 that were compatible with one another on Illumina sequencing machines (Figure S4a). Each barcode has a base distance of 3 or more, which means every 8-nucleotide barcode sequence is different from all other 8-nucleotide barcodes at 3 of the 8 positions (or more). We used microfluidics to formulate LNPs with QUANT barcodes. We analyzed the hydrodynamic diameter of each LNP individually using dynamic light scattering and pooled LNPs together if they met two inclusion criteria: (i) hydrodynamic...
diameters between 20 and 200 nm and (ii) an autocorrelation curve with 1 inflection point (Figure 4d, Figure S4b). We also included a naked DNA barcode, which served as a negative control. We selected these LNP criteria and the control based on experience with a different barcoding system.13 We studied two LNP libraries; as expected, the normalized delivery of the naked DNA barcode was lower than the normalized delivery for barcodes carried by LNPs in library 1 (Figure 4e,f) and library 2 (Figure S4c,d). Normalized delivery quantifies how well an LNP performs relative to all other LNPs in a given sample (Figure 4a, Figure S4e). It is analogous to counts per million in RNA-seq29 and can describe nanoparticle biodistribution.13,65

We intravenously administered the LNPs to WT and Cav1−/− mice at a total DNA dose of 0.5 mg/kg and used FACS to isolate 10 cell types 24 h after administering the LNPs (Figure S4f). We focused on endothelial cells and macrophages because they exist in every tissue; this allowed us to study cell- and tissue-level effects. In LNP library 1, we formulated 128 LNPs; 111 met our two inclusion criteria and were pooled together (Figure 4c,d, Figure S4g,h). Multiple lines of evidence suggested Cav1 influenced nanoparticle delivery in a tissue- and cell-type dependent way. First, the “total experimental” biodistribution, defined as the total ddPCR counts in the 10 tested cell types, was reduced in Cav1−/− mice, relative to that in WT mice (Figure 5a). In these pie charts, the area corresponds to total ddPCR counts. It is important to note this pie chart is not equal to the total clearance for the organ because we did not measure the clearance in every cell type, and the values are not weighted by the percentage of a given cell type within the organ. We chose not to weight the values because the percentage of each cell type within an organ, for all tested organs, was not available.

The decrease in ddPCR counts was not constant across different tissues; Cav1 exerted tissue-specific changes on LNP delivery. For example, the biodistribution to the liver was predominant in WT mice but was much less so in Cav1−/− mice (Figure 5a). The decrease in liver delivery was substantial; the total number of ddPCR counts in the liver of Cav1−/− mice decreased by 93%. The Cav1−/− counts in lung and kidney were reduced by 43 and 27%, respectively, relative to those of WT mice (Figure 5c,d). We then analyzed this effect at the cellular level in all three organs. In the liver, most of the decrease in Cav1−/− barcode readouts were due to decreased Kupffer cell delivery (Figure 5e). Delivery to hepatic endothelial cells also decreased, but delivery to hepatocytes was not impacted significantly (Figure 5b,e). The data above describe the average change in barcode counts for all LNPs. We then quantified how all 111 individual LNPs were affected by Cav1 expression. We multiplied the ddPCR readouts by the normalized delivery to calculate absolute delivery for each LNP. We then plotted absolute delivery for each LNP in WT and Cav1−/− mice for three cell

Figure 5. High-throughput QUANT studies reveal Caveolin1 affects delivery in a tissue- and cell-type dependent manner in vivo. (a) The total ddPCR counts in all tested cell types, which are equal to the area of the circle, were used to determine the “total experimental” biodistribution in WT and Cav1−/− mice. (b) The total ddPCR counts were determined in different cell types from the liver, (c) lung, and (d) kidney. Compared to cells isolated from wild-type mice, ddPCR counts from Cav1−/− decreased with the most dramatic effect in the liver. (e) Within the liver cell types, normalized library 1 nanoparticle biodistribution demonstrates that Kupffer cells in Cav1−/− uptake fewer nucleic acids when compared to Kupffer cells from wild-type mice. ****p < 0.0001 two-way ANOVA. (f) Combined sequencing data and ddPCR results shows the absolute delivery of 111 nanoparticles for each LNP in the liver in wild-type (blue) and Cav1−/− (red) mice, from library 1, in Kupffer cells, liver endothelial cells, and hepatocytes.

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types in the liver (Figure 5f). Delivery to Kupffer cells in Cav1−/− mice was visibly lower than delivery to Kupffer cells in WT mice even when plotted on a log10 scale (Figure 5f). Delivery to Kupffer cells was affected more than to endothelial cells and hepatocytes. Notably, the high-throughput analysis of absolute delivery has not been reported before; thus, it is not possible to generate these plots using previous LNP DNA barcoding technologies.

We repeated this experiment using a second LNP library. We rationally designed LNP library 2 to be similar to LNP library 1 with an important distinction: In library 1, LNPs contained the 7C1 lipid, cholesterol, and PEG. We omitted DSPC to exclude the possibility that the effect we observed did not require the phosphocholine group in the LNP's (Figure S5b,e). Delivery to hepatic endothelial cells and hepatocytes was decreased significantly (Figure S5e). Finally, we calculated the delivery for all 115 individual LNPs and were able to visually observe the differences in Kupffer cells more so than in hepatic endothelial cells and hepatocytes.

Given the consistent results in Kupffer cells, we investigated whether Cav1 similarly affected pulmonary and renal macrophages (Figure 6a). The total ddPCR counts in pulmonary macrophages did not change with Cav1 expression; renal macrophage delivery decreased but not significantly. When assessing absolute delivery in WT Kupffer cells as well as pulmonary and renal macrophages, we observed that average nanoparticle delivery was ~10-fold higher in Kupffer cells than in lung and renal macrophages (Figure 6b). To exclude the possibility the Kupffer cell reduction was due to (i) fewer Kupffer cells or (ii) differences in Kupffer cell phenotype in Cav1−/− mice, we measured the (i) number of Kupffer cells/total liver immune cells and (ii) the expression of CD86 and Cav1−/− Kupffer (CD68+ CD45+) cells were determined by MFI of (d) CD86 and (e) CD206.
QUANT is an important advance. More specifically, we found that a rationally designed ddPCR-based barcode system can quantify delivery with very high sensitivity. The results suggest that Cav1 plays a more prominent role in LNP clearance in Kupffer cells relative to other macrophage populations. These results are important for drug delivery systems given that macrophages clearly administer LNPs. Given that LNPs are lipid-like nanomaterials with compositions that can be similar to HDL, LDL, and VLDL, it will be interesting to evaluate whether the observations we made here extend to these “natural” nanoscale carriers. If so, these results could elucidate how cholesterol is trafficked to different tissues. Our results suggest macrophage uptake changes with tissue type. If future studies elucidate the cell signaling pathways that govern these differences, these cell signaling pathways could be manipulated to alter nanoparticle targeting.

**DISCUSSION**

Here, we show that Cav1 affects LNP delivery in a cell type-specific manner in vivo. Delivery to Kupffer cells was significantly altered, leading to changes in nanoparticle biodistribution. Interestingly, Kupffer cell delivery was affected more than delivery to lung or kidney macrophages. These results suggest that Cav1 impacts nanoparticle biodistribution between different cell types but could not be used to compare pathways that can be similar to HDL, LDL, and VLDL, it will be interesting to evaluate whether the observations we made here extend to these “natural” nanoscale carriers. If so, these results could elucidate how cholesterol is trafficked to different tissues. Our results suggest macrophage uptake changes with tissue type. If future studies elucidate the cell signaling pathways that govern these differences, these cell signaling pathways could be manipulated to alter nanoparticle targeting.

Independent of the Cav1 results, we believe the discovery of QUANT is an important advance. More specifically, we found that a rationally designed ddPCR-based barcode system can quantify delivery with very high sensitivity. Previous DNA barcode technologies designed to track nanoparticle biodistribution could only compare relative biodistribution within the same cell type but could not be used to compare biodistribution between different cell types. We anticipate future studies further improving the design of QUANT barcodes by incorporating different patterns of chemical modification. Moreover, given that QUANT was able to read out delivery to all tested cell types, we anticipate it will help scientists identify nanoparticles that target stem cells, immune cells, neurons, and other hard-to-target cell types.

We found that fluorescent biodistribution overestimated delivery to the liver compared to readouts of the nucleic acid itself. Our results were consistent over several experiments and echo results generated by scientists studying small molecule delivery. Given that the known mechanisms of nucleic acid degradation are different than the mechanisms that degrade fluorophores, we hypothesize that degradation of the nucleic acid is different from degradation of the fluorophore. Further studies will be required to confirm or disprove this hypothesis. If confirmed, our results will be important because most LNPs are thought to preferentially target the liver, often based on fluorescent biodistribution assays. These results could have important implications for nanoparticle discovery pipelines.

It is important to note the limitations of our work. First, we used only one nucleic acid size; changing the size of QUANT barcodes may better model different classes of nucleic acid drugs. Second, toxic or unstable LNPs will not work with QUANT. Nonetheless, QUANT enables new types of nanoparticle studies that will help elucidate the biological factors that affect LNP targeting and provides proof-of-principle data that manipulating one gene can be used to retarget nanomedicines.

**Materials and Methods. Nanoparticle Formulation.** Nanoparticles were formulated using a microfluidic device as previously described. Briefly, nucleic acid barcodes (DNA barcodes) were diluted in 10 mM citrate buffer (Teknova), whereas lipid-amine compounds, alkyl-tailed PEG, cholesterol, and helper lipids were diluted in ethanol. 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 flow rates of 600 and 200 μL/min, respectively.

**DNA Barcoding.** Each chemically distinct LNP was formulated to carry its own unique DNA barcode (Figure 1a,b). For example, LNP1 carried DNA barcode 1, and the chemically distinct LNP2 carried DNA barcode 2. Ninety-one nucleotide long single-stranded DNA sequences were purchased as ultramers from Integrated DNA Technologies (IDT). Three nucleotides on the 5′ and 3′ ends were modified with phosphorothioates to reduce exonuclease degradation and improve DNA barcode stability. To ensure equal amplification of each sequence, we included universal forward and reverse primer regions on all barcodes. Each barcode was distinguished using a unique 8-nt sequence. An 8-nt sequence can generate over 4^8 (65,536) distinct barcodes. We used 156 distinct 8-nt sequences designed to prevent sequence bleaching on the Illumina MiSeq sequencing machine. A 26-nt probe was purchased from IDT with 5′ FAM as the fluorophore, and internal Zen and 3′ Iowa Black FQ were used as quenchers. Fluorescent barcode was purchased from IDT with Alexa-Fluor647 or AlexaFluor488 conjugated to the 5′ end.

**In Vitro L2K.** Immortalized mouse aortic endothelial cells (IMAECs) were seeded at 10,000 cells per well in a 96-well plate. Twenty-four hours after seeding, QUANT barcodes or 200 nM LNP1 were delivered to liver, lung, heart, and kidney macrophages. Twenty-four hours after transfection, DNA was isolated from cells treated with QUANT barcodes, and ddPCR was performed as previously described. Each chemically distinct LNP was then calculated the barcode delivery in Cav1−/− mice (Figure S6c–e).

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**Nanoparticle Characterization.** LNP hydrodynamic diameter was measured using high-throughput dynamic light scattering (DLS) (DynaPro Plate Reader II, Wyatt). LNPs were diluted in sterile 1× PBS to a concentration of ~0.06 μg/mL and analyzed. For the use of unstable LNPs to be avoided and sterile purification using a 0.22 μm filter to be enabled, LNPs were included only if they met the criteria of monodisperse population with diameters between 20 and 200 nm. Particles that met these criteria were dialyzed with 1× phosphate-buffered saline (PBS, Invitrogen) and were sterile filtered with a 0.22 μm filter.

**Animal Experiments.** All animal experiments were performed in accordance with the Georgia Institute of Technology IACUC. C57BL/6J (#000664) and Caveolin1−/− (#007083) 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 = 3–5 group. Mice were injected intravenously via the lateral tail vein. The nanoparticle concentration was determined using NanoDrop (Thermo Scientific). For in vivo nanoparticle screens, mice were administered at a dose of 0.5 mg/kg.

**Cell Isolation and Staining.** Cells were isolated 24 (for screens) or 96 (for in vivo gene editing) hours after injection with LNPs unless otherwise noted. 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 50 rpm for 45 min. The digestive enzyme for heart and spleen included Collagenase IV.7,56,58 The cell suspension was filtered through 70 μm mesh, and red blood cells were lysed. Cells were stained to identify specific cell populations and sorted using the BD FacsFusion and BD FACS Aria III cell sorters in the Georgia Institute of Technology Cellular Analysis Core. For in vitro flow cytometry experiments, a BD Accuri C6 was used 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), anti-CD11b (M1/70, Biolegend), anti-CD206 (C068C2, Biolegend), and anti-CD86 (GL-1, Biolegend). Representative flow gates are located in Figure S1.

**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 μL of ddPCR with ddPCR Supermix for Probes (Bio-Rad), 1 μL of primer and probe mix (solution of 10 μM target probe and 20 μM reverse/forward primers), 1 μL of template/TE buffer, and 8 μL of water. Then, 20 μL of each reaction and 70 μL of Droplet Generation Oil for Probes (Bio-Rad) were loaded into DG8 Cartridges and covered with DG8 Gaskets. Cartridges were placed in the QX200 Droplet Generator to create water–oil emulsion droplets. Cycle conditions for PCR were as follows: 1 cycle of 95 °C for 10 min followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min, and 1 cycle of 95 °C for 10 min. Plates were stored at 4 °C until ran on the QX200 Droplet Digital PCR System. For each biological rep, three technical repetitions were completed. In all cases, technical reps were averaged. Technical reps were only excluded if they saturated the detection or showed inconsistent positive event amplitudes.

**PCR Amplification for Illumina Sequencing.** All samples were amplified and prepared for sequencing using a two-step, nested PCR protocol (Figure S1c). More specifically, 2 μL of primers (10 μM for base reverse/forward) were added to 5 μL of Kapa HiFi 2× master mix and 3 μL of template DNA/water. This first PCR reaction was run for 20–30 cycles. The second PCR, to add Nextera XT chemistry, indices, and i5/i7 adapter regions was run for 5–10 cycles and used the product from “PCR 1” as template. Dual-indexed samples were run on a 2% agarose gel to ensure that the PCR reaction occurred before being pooled and purified using BluePippin (Sage Science).

**Deep Sequencing.** Illumina sequencing was conducted in Georgia Institute of Technology’s Molecular Evolution core. Runs were performed on an Illumina Miniseq. Primers were designed based on Nextera XT adapter sequences.

**Barcode Sequencing Normalization.** Counts for each particle, per cell type, were normalized to the barcoded LNP mixture applied to cells or injected into the mouse.

**Endothelial RNA Interference.** C57BL/6J and Caveolin1−/− mice were injected with 7C1 carrying 1 mg/kg of siCTRL (siLuc) (AxoLabs). In all cases, siRNAs were chemically modified at the 2′ position to increase stability and negate immunostimulation. Seventy-two hours after injection, tissues were isolated, and protein expression was determined via flow cytometry. ICAM2MFI in siLuc-treated mice (for each background) was normalized to 100%.

**Data Analysis and Statistics.** Sequencing results were processed using a custom R script to extract raw barcode counts for each tissue. These raw counts were then normalized with an R script prior for further analysis. Statistical analysis was done using GraphPad Prism 7; more specifically, one-tail t test, paired two-tail t test, or one-way ANOVAs were used where appropriate. Data are plotted as mean ± standard error unless otherwise stated.

**Data Access.** The data, analyses, and scripts used to generate all figures in the paper are available upon request.

**ASSOCIATED CONTENT**

> Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.nanolett.8b03149.

QUANT barcode design, MFI comparison and barcodes, FACS sorting and representative gating, multi-step approach to optimizing the signal generated by ddPCR QUANT barcodes, Alexa-647 fluorescence results, 8-nt barcodes and DLS results, total ddPCR barcode counts, normalized nanoparticle biodistribution across two screens and QUANT results (PDF)

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C.D.S., M.P.L., and J.E.D. designed experiments, which were performed by C.D.S., M.P.L., G.N.L., N.D., N.S, A.V.B., and J.E.D. C.D.S., M.L., N.S, and J.E.D. analyzed data. C.D.S., M.P.L., and J.E.D. wrote the paper, which was reviewed by G.N.L., N.D., N.S, and A.V.B.

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**Notes**

The authors declare the following competing financial interest(s): C.D.S., M.P.L., and J.E.D. have filed intellectual property related to this publication.

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