Supporting Information

The time course of *in vivo* cellular responses to LNPs

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Methods

Cre and aVHH mRNA Synthesis. mRNA was synthesized as described previously[1]. Briefly, the mRNA sequences were ordered as a plasmid from GeneArt (Thermo Fisher Scientific) containing a 5' UTR with Kozak sequence, a 3' UTR derived from the mouse alpha-globin sequence, and the ORF sequence, which was human codon optimized using the GeneArt website. Plasmids were digested into a linear template using NotI-HF (New England BioLabs) overnight at 37 °C. Linearized templates were purified by ammonium acetate (Thermo Fisher Scientific) precipitation before being resuspended with nuclease free water. *In vitro* transcription was performed overnight at 37 °C using the HiScribe T7 kit (NEB) following the manufacturer's instructions (full replacement or uracil with N1-methyl-pseudouridine). RNA product was treated with DNase I (Aldevron) for 30 min to remove template and purified using lithium chloride precipitation (Thermo Fisher Scientific). RNA transcripts were heat denatured at 65 °C for 10 min before being capped with a Cap1 structure using guanylyl transferase (Aldevron) and 2'-O-methyltransferase (Aldevron). Transcripts were then polyadenylated enzymatically (Aldevron). mRNA was then purified by lithium chloride precipitation, treated with alkaline phosphatase (NEB), and purified a final time. Concentrations were measured using a Nanodrop and mRNA stock concentrations were between 2–5 mg / mL. mRNA stocks were stored at -80 °C. Purified RNA products were analyzed by gel electrophoresis to ensure purity.

Nanoparticle Formulation. Nanoparticles were formulated as previously described[2]. Briefly mRNA was diluted in 10mM citrate buffer (Teknova). Lipid - amine compound cKK-E12 (Oragnix Inc., O-8744), $C_{14}PEG_{2000}$ (Avanti, 880150), 20α-hydroxycholesterol, and dioleoylphosphatidylethanolamine were diluted in 100% ethanol. Both phases were loaded into separate syringe pumps. The citrate and ethanol phase were mixed in a microfluidic device. All PEGs and cholesterol were purchased from Avanti Lipids.

Nanoparticle Characterization. LNP hydrodynamic diameter was measured using high throughput dynamic light scattering (DLS) (DynaPro Plate Reader II, Wyatt). LNPs were diluted in sterile 1X PBS and analyzed. To avoid using unstable LNPs, and to enable sterile purification using a 0.22 μm filter, LNPs were used only if they met 3 criteria: diameter >20 nm, diameter <200 nm, and correlation function with 1 inflection point. Particles that met these criteria were dialyzed with 1X PBS.

Animal Experiments. All animal experiments were performed in accordance with the Georgia Institute of Technology's IACUC. All animals were bred in the Georgia Institute of Technology Animal Facility. C57BL/6J (#000664) were purchased from The Jackson Laboratory. LSL-Tomato/Ai14 (#007914) were purchased from The Jackson Laboratory for breeding proposes. In all experiments, we used N=4 mice / group. Mice were injected intravenously via the lateral tail vein at a dose of 0.3 mg / kg unless otherwise noted. Dexamethasone (MedChem Express) was administered with an injection through the peritoneum. The nanoparticle concentration was determined using NanoDrop (Thermo Scientific).

Cell Isolation & Staining. Cells were isolated 1, 3, 6 and 24 hours after injection with LNPs for sequencing library preparation. For functional delivery of Cre mRNA, cells were isolated 72

hours after LNP injection, and for functional delivery of aVHH mRNA, cells were isolated 16 hours after LNP injection. Mice were perfused with 20 mL of 1X PBS through the right atrium. Liver tissues were finely minced, and then placed in a digestive enzyme solution with collagenase type I (Sigma Aldrich), collagenase XI (Sigma Aldrich) and hyaluronidase (Sigma Aldrich) at 37ºC at 550 rpm for 45 minutes[3, 4]. 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 IIIu cell sorters in the Georgia Institute of Technology Cellular Analysis Core. The antibody clones used were the following: anti-CD31 (390, BioLegend), anti-CD45.2 (104, BioLegend), anti-CD68 (FA11, BioLegend), anti-CD11c (N418, BioLegend), and PE anti-mCD47 (miap301, BioLegend). PBS-injected mice were used to gate cell populations.

Low-input bulk RNA-Seq library construction and sequencing. Samples were isolated and prepared as described above. The SMART-Seq v4 Ultra Low Input RNA method (Takara Bio, Japan) was employed for library construction, according to the manufacturer's instructions. The amplified cDNA was purified by the Agencourt AMpure XP Beads (Beckman Coulter, USA) with 150 pg being processed further using Illumina's Nextera XT DNA library construction method according to the manufacturer's instructions (Illumina, USA). The quantity and quality of the libraries were examined by Qubit DNA BR Assay Kit, Qubit™ 3.0 Fluorometer (Life Technologies) and Agilent 2100 Bioanalyzer (Agilent Technologies). Library sequencing was performed on an Illumina NextSeq platform to create paired-end reads with a length of 75 base pairs. The sequenced reads were trimmed and aligned to the mouse genome (*mm10*) using Isas analysis software 3.19.1.12, SAMtools 0.1.20, STAR aligner STAR_2.6.1a, Salmon quantification software 0.11.2, and Strelka Variant Calling software 2.9.9. Differential expression analysis was determined using DESeq2[5]. Only genes with an adjusted P value (false discovery rate) or less than 0.01 and a fold change greater than 2 were included within the subsequent GO and KEGG pathway analysis. To understand the functions of the differentially expressed genes, KEGG pathway analysis was carried out by KEGGMapper (www.kegg.jp/).

Cytokine Assay. Cytokines were measured using Proteome Profiler Mouse Cytokine Array Kit, Panel A (R&D Systems) as previously described[7].

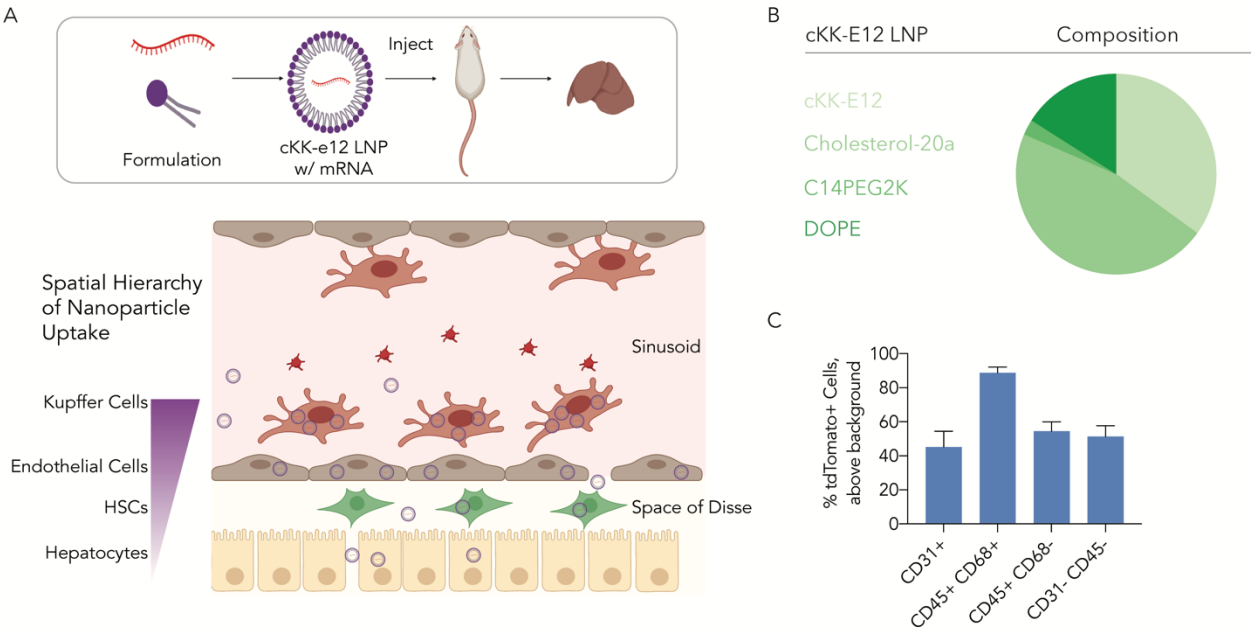
A

Sample	Total Number of Reads	% of Mappable Reads	Sample	Total Number of Reads	% of Mappable Reads
PBS 1hr A	25,515,252	88.78	20a 1hr A	9,829,135	69.72
PBS 1hr B	22,364,292	86.07	20a 1hr B	20,595,861	82.71
PBS 1hr C	18,145,017	86.44	20a 1hr C	16,158,968	78.85
PBS 3hr A	23,679,958	85.39	20a 3hr A	35,501,491	93.93
PBS 3hr B	4,567,133	55.87	20a 3hr B	29,283,332	93.89
PBS 3hr C	16,970,718	82.42	20a 3hr C	26,716,738	91.64
PBS 6hr A	11,938,683	68.07	20a 6hr A	24,862,563	89.10
PBS 6hr B	23,610,403	84.70	20a 6hr B	29,234,924	86.50
PBS 6hr C	8,721,433	72.87	20a 6hr C	10,891,048	81.14
PBS 24hr A	24,153,681	89.94	20a 24hr A	17,087,529	82.08
PBS 24hr B	5,929,954	85.21	20a 24hr B	6,326,581	81.45
PBS 24hr C	25,881,660	87.21	20a 24hr C	4,760,590	69.63

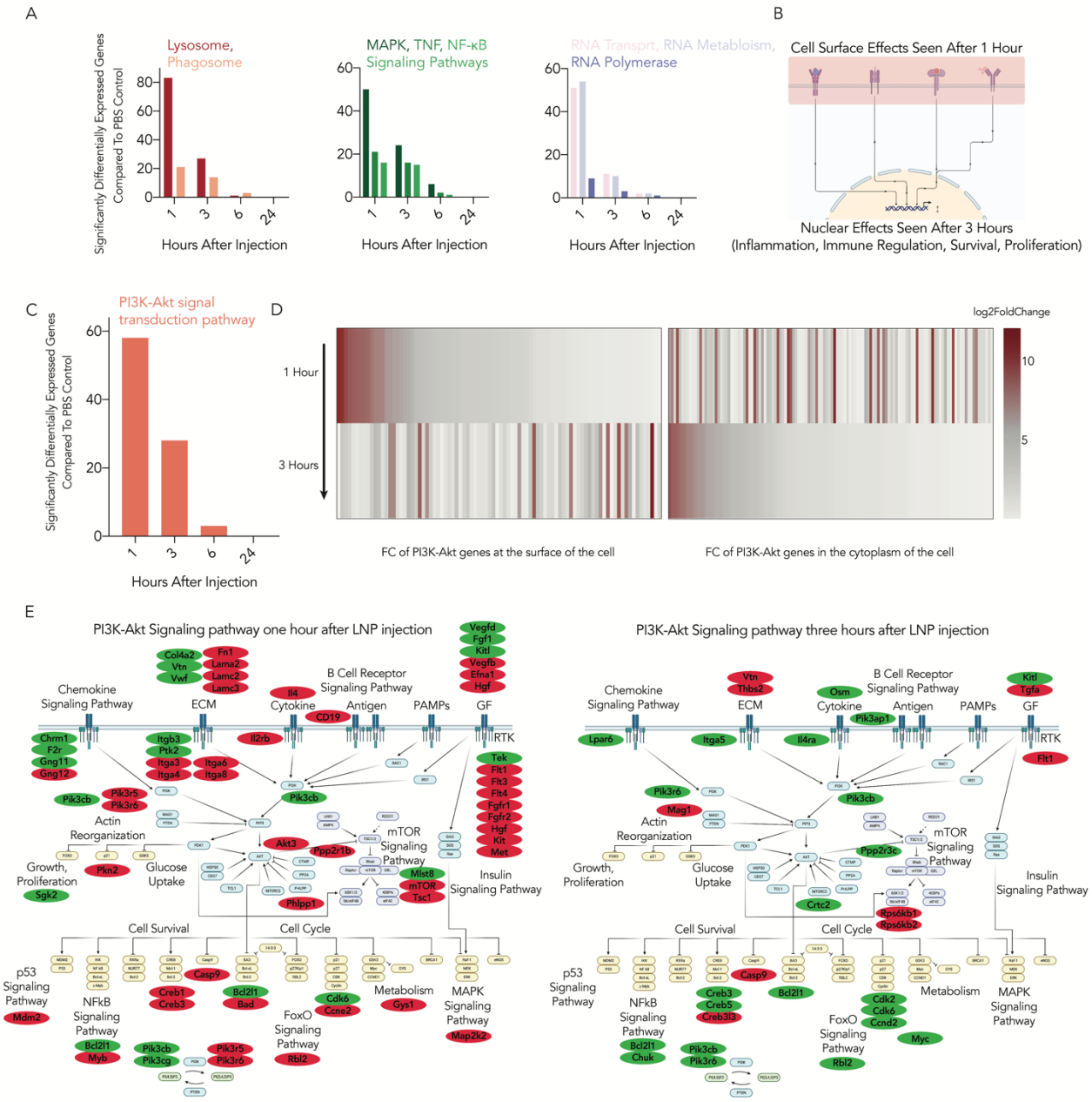
Supplementary Table 1. Mapping stats for the low-input SMART-seq v4 experiment for the PBS- and LNP-exposed conditions after 1, 3, 6, and 24 hours.

Significantly dysregulated ligand-receptor pairs one hour after injection		Significantly dysregulated ligand-receptor pairs three hours after injection	Significantly dysregulated ligand-receptor pairs six / twenty four hours after injection
Edn1-Ednrb	Vtn-Itga8	Edn1-Ednrb	None
Dcn-Met	Vtn-Itga3	Ccl7-Ccr1	
Ccl7-Ccr2	Lamc3-Itga6	Fgg-Itga5	
Ccl7-Ccr1	Lamc3-Itga3	Ccl3-Ccr1	
Ccl7-Ccr3	Bmp6-Acvr1	Rarres2-Cmklr1	
Lamc2-Itga6	Bmp6-Acvr2b	Rarres2-Ccrl2	
Lamc2-Itga3	Fgf12-Fgfr1	L1cam-Itga5	
Apob-Lrp8	Fgf12-Fgfr2	Col18a1-Itga5	
Apob-Itgam	Inhba-Eng	Tnf-Slc5a11	
Apob-Lrp6	Inhba-Bambi	Ccl4-Ccr1	
Cyr61-Itgam	Inhba-Acvr1	Cd34-Sell	
Cyr61-Itgb3	Inhba-Acvr2b	Cd34-Selp	
F2-F2r	Tnfsf13b-Tnfrsf13b	Vtn-Itga5	
F2-Gp9	Il15-Il2rb	Plg-Flt1	
Mmp9-Itgam	Vegfb-Flt1	Fga-Itga5	
Nrtn-Gfra2	Fgf1-Fgfr1	Hp-Asgr1	
Ctf1-Lifr	Fgf1-Fgfr2		
Fn1-Itga6	Fgf1-Fgfrl1		
Fn1-Nt5e	Adam15-Itgb3		
Fn1-Mag	Il1rn-Il1rl2		
Fn1-Itgb3	Lama3-Sdc2		
Fn1-Itga4	Cxcl10-Ccr3		
Fn1-Itga8	Vwf-Selp		
Fn1-Flt4	Hgf-Met		
Fn1-Itga3	Hgf-Sdc2		
Fn1-Tmprss6	Fga-Itgam		
Fn1-Sdc2	Fga-Itgb3		
F10-Itgam	Hp-Itgam		
Cd34-Selp	Fgb-Itgam		
Vtn-Itgb3	Qrfp-P2ry14		

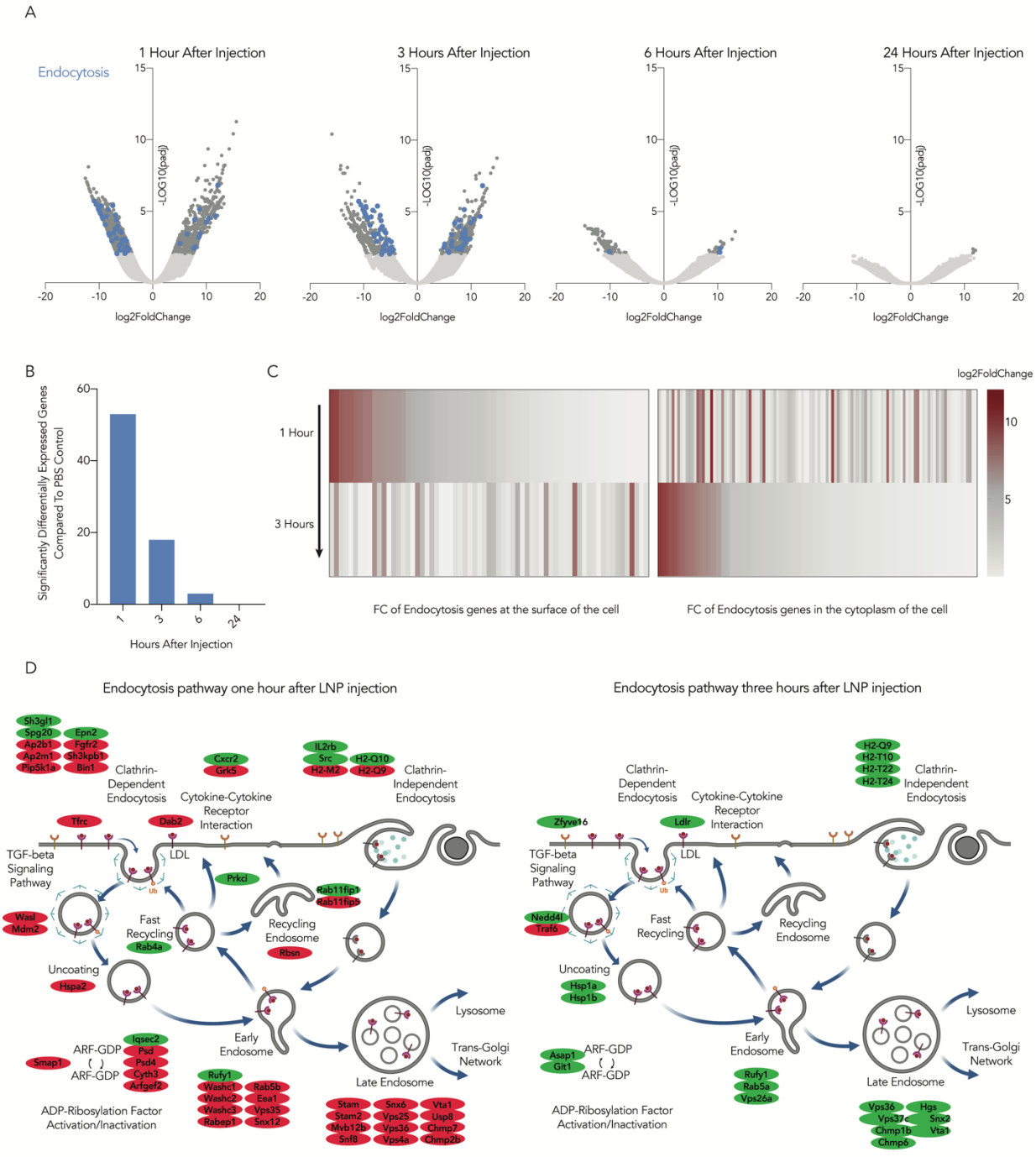
Supplementary Table 2. Comparing significantly differentially expressed genes at each timepoint to lists surveyed from published data outlining ligand-receptor pairs[8, 9]***. At 1 hour, 60 unique ligand-receptor pairs were dysregulated. After 3 hours, 16 ligand-receptor pairings were significantly differentially expressed that the LNP might act on and respond to. After 6 hours and after 24 hours, no published receptor-ligand pairs were dysregulated. Three dysregulated ligand-receptor pairs were present at both the 1- and 3-hour timepoints: Ccl7-Ccr1, Cd34-Selp and Edn1-Ednrb.***



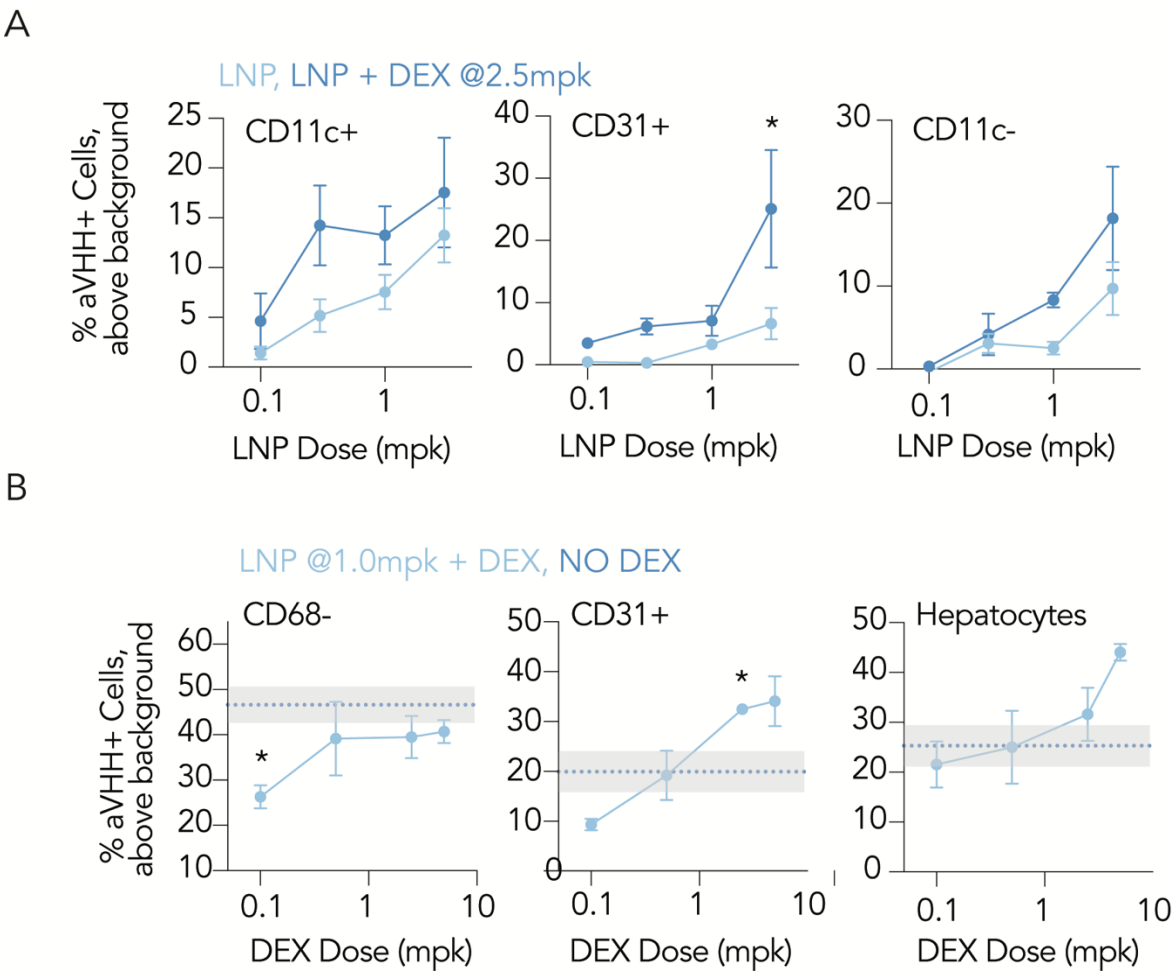
Supplementary Figure 1. (A) cKK-e12 LNP was formulated with Cre mRNA and injected into mice. Liver was harvested 1, 3, 6 and 24 hours later. Kupffer cells were isolated using FACS, and the cellular transcriptome was measured using low-input SMART-seq v4. (B) Molar ratios of different lipid components in the injected cKK-E12 LNP. (C) In parallel, tdTomato expression in the liver above background was measured using flow cytometry, +/- SD.



Supplementary Figure 2. (A) The number of DE genes assigned to different pathways as highlighted by KEGG analysis (B). In a number of these pathways, a temporal cascade effect can be seen whereby genes are colocalizing at the membrane level 1 hour post injection before the effects are then seen in the nucleus 3 hours post injection. (C) The number of DE genes assigned to the PI3K-Akt signaling pathway by KEGG analysis peaks after 1 hour. (C) Heatmap representing the Log2 fold change of genes for the PI3K-Akt signaling pathway and where they spatially occur within the cell, relative to PBS, 1 and 3 hours after injection. (D) Overlaying and colocalizing the DE genes along the PI3K-Akt signaling pathway after 1 and 3 hours, with upregulated genes in green and downregulated in red.



Supplementary Figure 3. (A) Volcano plots of differentially expressed genes compared to the PBS control after 1, 3, 6, and 24 hours. The genes related to endocytosis are highlighted in blue and quantified in (B). Again, the number of DE genes peaks after 1 hour. (C) Heatmap representing the Log2 fold change of genes for endocytosis at the surface and within the cell, relative to PBS 1 and 3 hours after injection. (D) Overlaying and colocalizing the DE genes along the endocytosis pathway after 1 and 3 hours, with upregulated genes in green and downregulated in red, showing the temporal transition of the DE genes as the LNP moves from receptor-mediated endocytosis through early-, recycling-, and late-endosomal maturation.



Supplementary Figure 4. (A) Pretreatment of 2.5 mg/kg dexamethasone 1 hour prior to LNP injection slightly increases aVHH expression in the liver in dendritic cells, endothelial cells and other immune cells at different LNP doses (0.1–3.0 mg/kg). (B) Varying the dexamethasone pretreatment dose (0.1–5.0 mg/kg) does not always show an increase in delivery in immune cells, endothelial cells, and hepatocytes. Two-way ANOVA, *P<0.05, average +/- SEM.

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