Supporting Information

The transcriptional response to lung-targeting lipid nanoparticles *in vivo*

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Materials and Methods

Cre mRNA synthesis
mRNA was synthesized as described previously[39]. Briefly, the mRNA sequence was ordered as a DNA gBlock from Integrated DNA Technologies (IDT) containing a 5' UTR with Kozak sequence, a 3' UTR derived from the mouse alpha-globin sequence, and extensions to allow for Gibson assembly. The sequence was human codon-optimized using the IDT website. The sequences of the GPI anchor and nano-luciferase have been previously stated[39]. The gBlock was then cloned into a PCR-amplified pMA7 vector through Gibson assembly using NEB Builder with 3 molar excess of insert. All reaction transcripts were 0.8% agarose gel purified prior to assembly reaction. Subsequent plasmids from each colony were Sanger sequenced to ensure desired sequence fidelity. Plasmids were linearized with NotI-HF (New England BioLabs, NEB) overnight at 37 °C. Linearized templates were purified by ammonium acetate (Thermo Fisher Scientific) precipitation before being rehydrated with nuclease-free water. In vitro transcription (IVT) was performed overnight at 37 °C using the HiScribe T7 kit (NEB) following the manufacturer's instructions (N1-methyl-pseudouridine modified). RNA product was treated with DNase I (Aldevron) for 30 min to remove template and purified using lithium chloride precipitation (Thermo Fisher Scientific). RNA was 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 again. Concentrations were measured using a NanoDrop. mRNA stock concentrations were 3–5 mg/mL. Purified RNA products were analyzed by gel electrophoresis to ensure purity.

aVHH mRNA synthesis

mRNA was synthesized as previously described[29-31]. In summary, the GPI- anchored VHH sequence was ordered as a DNA gBlock from IDT containing a 3' UTR derived from the mouse alpha-globin sequence, 5' UTR with Kozak sequence, and extensions to allow for Gibson assembly. The sequence was human codon-optimized using the IDT website. The gBlock was then cloned into a PCR amplified pMA7 vector through Gibson assembly using NEB Builder with 3 molar excess of insert. Gibson assembly reaction transcripts were 0.8% agarose gel purified prior to assembly reaction. Subsequent plasmids from each colony were Sanger sequenced to ensure sequence identity. 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. IVT was performed overnight at 37 °C using the HiScribe T7 kit (NEB) following the manufacturer's instructions (full replacement of 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. NanoDrop was used to measure RNA concentration; mRNA stock concentrations were between 2 and 4 mg/mL and the mRNA stock was stored at -80°C. Purified RNA products were analyzed by gel electrophoresis to ensure purity.

Nanoparticle Formulation

Nanoparticles were formulated using a microfluidic device as previously described[40]. Cre mRNA and DNA barcodes were diluted in 10 mM citrate buffer (Teknova). DNA barcodes were purchased from IDT. cKK-E12 was purchased from Organix Inc. (O-8744). PEGs, cholesterol, and helper lipids were diluted in 100% ethanol and purchased from Avanti Lipids. Citrate and ethanol phases were combined in a microfluidic device by syringes (Hamilton Company) at a flow rate of 3:1.

Nanoparticle Characterization

The diameter and polydispersity of the LNPs were measured using 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 included only if they met 3 criteria: diameter >50 nm, diameter <200 nm, and correlation function with 1 inflection point. For screens, particles that met these criteria were pooled into respective groups. Particles were dialyzed in Slide-A-Lyzer G2 20 kD dialysis cassettes (Thermo Scientific) and then dialyzed in Float-A-Lyzer G2 100 kD dialysis cassettes (Millipore Sigma). The nanoparticle concentration was determined using NanoDrop (Thermo Scientific).

Encapsulation efficiency

Encapsulation was measured according to Precision NanoSystems RiboGreen assay protocol. In duplicates, 50 µL of 6 ng/µL LNP was added to 50 µL of 1X TE (Thermo Fisher) or 50 µL of

solution containing a 1:50 dilution of Triton X-100 (Sigma Aldrich). Following 10 minutes of incubation at 37 °C, 100 μL of 1:100 of RiboGreen reagent (Thermo Fisher) was added to each well. The fluorescence was quantified using a plate reader (BioTek Synergy H4 Hybrid) at an excitation wavelength of 485 nm and an emission wavelength of 528 nm.

Zeta Potential (ZP)

The zeta potential of LNPs was measured using a Malvern Zetasizer Nano Z. Eight hundred microliters of the particles were loaded into a Malvern disposable folded capillary cell and the following settings were executed: material refractive index of 1.4, absorbance of 0.01, dispersant viscosity of 0.882cp, refractive index of 1.33, and dielectric constant of 79.

TEM imaging

The lipid nanoparticles were received in an aqueous solution. Ten microliters of solution was dropped onto a copper TEM grid covered with Formvar support film and stabilizing carbon. The excess solution was blotted with filter paper and the negative stain was added. The particles were stained for 1-2 minutes, using either 2% phosphotungstic acid or UranyLess (Electron Microscopy Sciences, Hatfield, PA) then blotted and allowed to air dry for several minutes before examination in the TEM (JEOL, Tokyo, Japan) at 100 kV.

TNS Assay

The pKa of Cat-LNP, Neu-LNP, and An-LNP was measured as previously described[40]. A stock solution of 10 mM HEPES (Sigma Aldrich), 10 mM MES (Sigma Aldrich), 10 mM sodium acetate (Sigma), and 140 mM sodium chloride (Sigma Aldrich) was prepared and pH adjusted with hydrogen chloride and sodium hydroxide to the following pH: 3, 4, 5, 6, 7, 8, 9, 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 μg/mL). Five microliters of LNP was added to each well. After 5 minutes of incubation at 300 rpm, fluorescence absorbance was measured using excitation wavelengths of 325 nm and emission wavelength of 435 nm using a plate reader (BioTek Synergy H4 Hybrids)

Animal Experiments

All animal experiments were performed in accordance with the Georgia Institute of Technology's IACUC. All animals were housed in the Georgia Institute of Technology Animal Facility. Ai14s were bred at the Georgia Institute of Technology Animal Facility. C57BL/6J (B6/000664) were purchased from Jackson Laboratories. In all experiments, we used N = 2-4 mice/group, unless otherwise noted.

Cell Isolation & Staining

Cells were isolated 24 or 96 hours after injection with LNPs, unless otherwise noted. Mice were perfused with 20 mL of 1X 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 minutes. The digestive enzyme for heart included collagenase IV. For the spleen, 1X PBS without digestive enzymes was used. Cell suspension was filtered through 70 μm mesh, and red blood cells were

lysed. Fc receptors were blocked (TruStain fcX[TM] anti-mouse CD16/32, BioLegend) to avoid non-specific binding. Then, cells were stained to identify specific cell populations and sorted using the BD FACS Fusion cell sorters in the Georgia Institute of Technology Cellular Analysis Core. The antibody clones used were anti-CD31 (390, BioLegend), anti-CD45 (30-F11, BioLegend), anti-CD68 (FA11, BioLegend), anti-CD11b (M1/70, BioLegend), anti-CD11c (N418, BioLegend), CD3 (17A2, BioLegend), CD19 (6D5, BioLegend), LIVE/DEAD™ Fixable Far-Red Dead Cell Stain (Invitrogen), and PE anti-mCD47 (miap301, BioLegend). Representative flow gates are listed in **Supplementary Fig. 23**. PBS-injected Ai14 mice were used to gate tdTomato-positive populations.

PCR Amplification
All samples were amplified and prepared for sequencing using a one-step PCR protocol as previously described[20]. More specifically, 1 μL of primers (5 μM for Final Reverse / Forward, 0.5 μM for Base Forward) were added to 5 μL of Kapa HiFi 2X master mix and 4 μL template DNA/water. When the PCR reaction did not produce clear bands, the primer concentrations, DNA template input, PCR temperature, and number of cycles were optimized for individual samples.

Deep Sequencing
Illumina deep sequencing was performed on an Illumina MiniSeq[TM]. Primers were designed based on Nextera XT adapter sequences.

Data Normalization
Counts for each particle, per tissue, were normalized to the barcoded LNP mixture we injected into the mouse. This "input" DNA provided the DNA counts and was used to normalize DNA counts from the cells and tissues.

Data Analysis & Statistics
Sequencing results were processed using a custom Python-based tool to extract raw barcode counts for each tissue. These raw counts were then normalized with an R script prior to further analysis. Statistical analysis was done using GraphPad Prism 8. Data is plotted as mean ± standard error mean or mean ± standard deviation,

Cytokine Analysis
Six hours after LNP administration, blood was isolated from the heart. Serum was isolated and 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. The final panels were documented using a LI-COR Odyssey CLx Far Infrared Imager and relative concentrations were determined using ImageJ software by determining pixel intensities.

ddPCR
The QX200 Droplet Digital PCR System (Bio-Rad) was used to analyze all ddPCR results.

ddPCR 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. Once prepared, 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. Using the QX200 Droplet Generator, water−oil emulsion droplets were created. 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. For each biological replicate, three technical repetitions were completed. Unless stated otherwise, technical replicates were averaged. Technical replicates were only excluded if saturation was detected or there were inconsistent positive event amplitudes.

Tissue Immunostaining & Microscopy
Ai14 mice were treated with PBS or 1.3 mg/kg of Cre mRNA encapsulated in LNPs. Four days later, lung and liver tissues were dissected and fixed in 1 mL of 4% PFA for 24 hours. Cells were washed with 1X PBS. Tissues were cryoprotected in 30% sucrose for 48 hours at 4 °C. In tissue base molds, tissues were embedded in optimal cutting temperature compound (Tissue-Tek) and frozen. Blocks were stored at -80 °C. Tissues were sectioned through the Emory Winship Cancer Institute Cancer Tissue and Pathology Facility. Cells were stained with DAPI (Sigma). Images for a Zeiss Plan-Apo 20x 0.8 NA air objective. Images were captured and processed using Volocity software (PerkinElmer).

Cell isolation for scRNA-seq
One-hour post-injection, lungs were dissected from mice treated with Cat-LNP, Neu-LNP, An-LNP, and PBS. Lung cell suspensions were passed through 70 µm mesh and resuspended in RoboSep buffer (Stemcell Technologies) for further scRNA-seq processing.

Single-cell library preparation
Whole transcriptome analyses, using the BD Rhapsody Single-Cell Analysis System (BD, Biosciences), were performed on whole lung cells, following the manufacturer's protocol. Briefly, dead cells and red blood cells (RBCs) were depleted by using EasySep™ dead cell (Annexin V) and RBC (anti-TER119) removal kit (Stemcell Technologies) following the manufacturer's protocol. Cell viabilities and numbers were recorded for each sample and pooled at the same ratio. For a pooled sample, a BD Rhapsody cartridge was loaded with 40,000 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 the size distribution was measured using Experion™ automated electrophoresis system (Bio-Rad).

Processing of single-cell transcriptomics data
The data were processed using zUMIs (v 2.9.7) for the RNA mapping and counting and Salmon Alevin (v1.5.2) for the cell hashes[41, 42]. All samples were mapped to GRCm39, and only exonic regions were counted. All output files were loaded into Seurat (v 4.0.4), and in summary, cells were log normalized to a scale factor of 10,000, then scaled using a linear transformation[33].

DoubletFinder (v3) was used to identify doublets as previously described[43]. This was followed by PCA dimensional reduction and t-SNE clustering and then exported using rBCS for further analysis in BBrowser2 (v2.9.23). Once in BBrowser2, the cell search tool was used to identify the cell types within each cluster, and gene expression profiles were compared within cell types of interest. Reactome pathway analysis was performed using ReactomeGSA package[38] in R based on Reactome database 82. The pathway expression levels are shown as z-score normalized values.
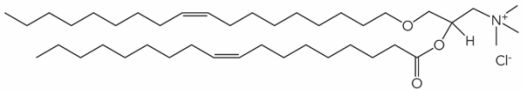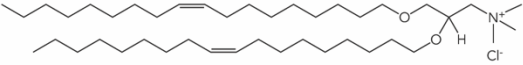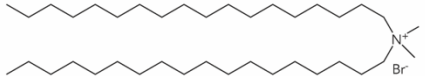
Supplementary figures table of contents

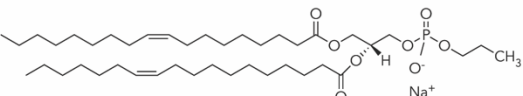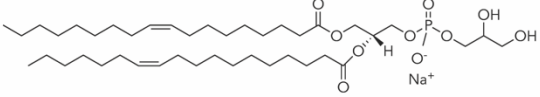| Molar Ratio				
Ionizable lipid	60	50	35	20
Cholesterol	26	35	18	50
PEG-Lipid	2	2.5	2.5	2.5
Helper Lipid	12	12.5	44.5	27.5
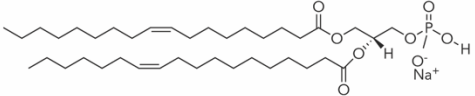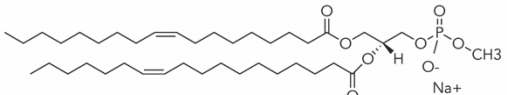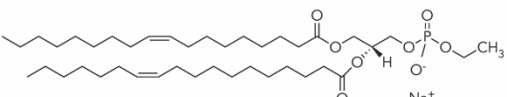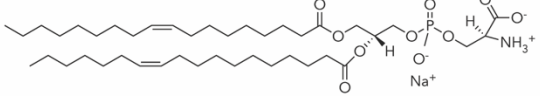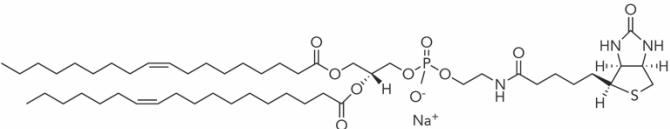
Supplementary Figure 1. Molar ratios used in the LNP screens.

a

Name	Charge	ID	Structure
DOTAP	Cationic	C1	
DOTMA	Cationic	C2	
18:0 DDAB	Cationic	C3	

Name	Charge	ID	Structure
18:1 Lyso PE	Neutral	N1	
18:1 Lyso PC	Neutral	N2	
DOPC	Neutral	N3	
DOPE	Neutral	N4	
18:1 Monomethyl PE	Neutral	N5	
18:1 Dimethyl PE	Neutral	N6	
18:0 PC	Neutral	N7	
18:1 Caproylamine PE	Neutral	N8	

b

c

Name	Charge	ID	Structure
18:1 PA	Anionic	A1	
18:1 Phosphati-dylmethanol	Anionic	A2	
18:1 Phosphati-dylethanol	Anionic	A3	
18:1 Phosphati-dylpropanol	Anionic	A4	
DOPG	Anionic	A5	
DOPS	Anionic	A6	
18:1 Biotinyl PE	Anionic	A7	

Supplementary Figure 2. Helper lipids used in the (a) cationic, (b) neutral, and (c) anionic libraries.
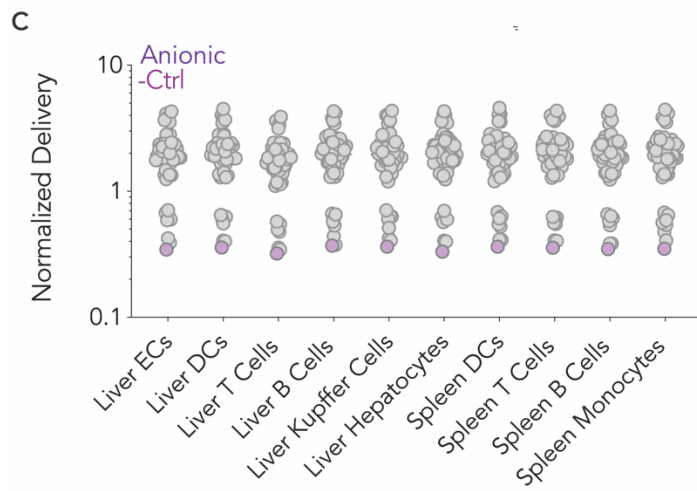
a

From mice → FACS cell types and sort functionally delivered cell types →

Cell type A

Sequence Barcodes →

Normalized delivery (%)

60

20   20
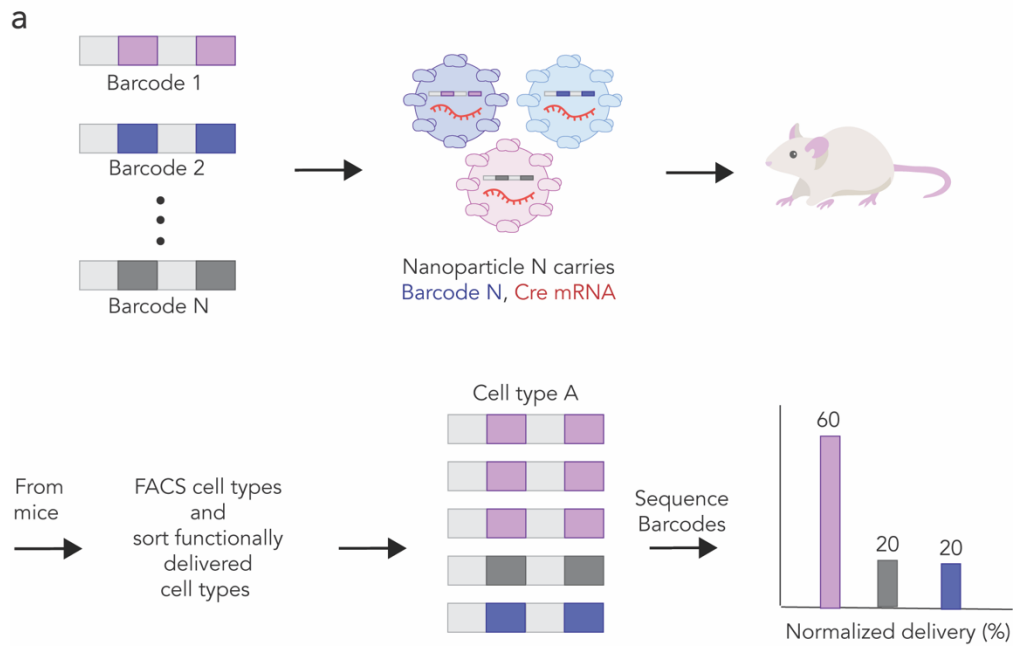
b

Cell Types	Flow Markers
Liver Hepatocytes	CD31- CD45-
Liver Endothelial Cells	CD31+ CD45-
Liver Dendritic Cells	CD31- CD45+ CD11c+
Liver T Cells	CD31- CD45+ CD3+
Liver B Cells	CD31- CD45+ CD19+
Liver Kupffer Cells	CD31- CD45+ CD68+
Lung Endothelial Cells	CD31+ CD45-
Lung Dendritic Cells	CD31- CD45+ CD11c+
Lung T Cells	CD31- CD45+ CD3+
Lung B Cells	CD31- CD45+ CD19+
Lung Monocytes	CD31- CD45+ CD11b+
Spleen Dendritic Cells	CD45+ CD11c+
Spleen T Cells	CD45+ CD3+
Spleen B Cells	CD45+ CD19+
Spleen Monocytes	CD45+ CD11b+
Heart Endothelial Cells	CD31+ CD45-
Heart Immune Cells	CD31- CD45+
Kidney Endothelial Cells	CD31+ CD45-
Kidney Immune Cells	CD31- CD45+

Supplementary Figure 3. (a) Example schematic of normalized delivery. (b) Cell type-specific markers for tdTomato expression measurement and sorting.

a

Cationic
-Ctrl

Normalized Delivery

Lung ECs, Lung DCs, Lung T cells, Lung B cells, Lung Monocytes, Lung Other, Liver ECs, Liver DCs, Liver T Cells, Liver B Cells, Liver Kupffer Cells, Liver Hepatocytes, Spleen DCs, Spleen T Cells, Spleen Monocytes

b

Neutral
-Ctrl

Normalized Delivery

Liver ECs, Liver DCs, Liver T Cells, Liver B Cells, Liver Kupffer Cells, Liver Hepatocytes, Spleen DCs, Spleen T Cells, Spleen B Cells, Spleen Monocytes

c

Anionic
-Ctrl

Normalized Delivery

Liver ECs, Liver DCs, Liver T Cells, Liver B Cells, Liver Kupffer Cells, Liver Hepatocytes, Spleen DCs, Spleen T Cells, Spleen B Cells, Spleen Monocytes

13

Supplementary Figure 4. Normalized delivery of all LNPs for various cell types. The negative control, unencapsulated DNA barcode, delivered less efficiently than barcodes delivered by LNPs in the (a) cationic, (b) neutral, and (c) anionic screens.
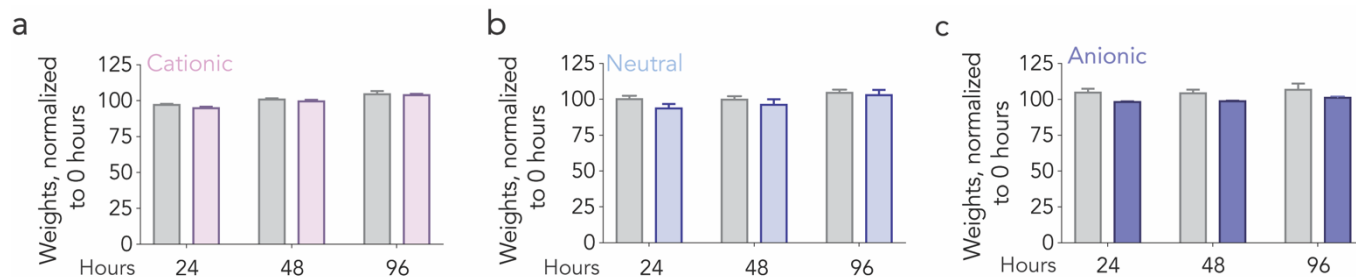
Supplementary Figure 5. Mouse weights measured 24, 48, and 96 hours after administration of PBS or (a) cationic, (b) neutral, and (c) anionic LNPs carrying Cre mRNA and DNA barcodes. Average +/- SEM.

a

| Barcodes	# LNP	Liver average	Spleen average	Average normalized delivery	Ionizable lipid	cholesterol	PEG	Helper lipid	Charge	Ionizable lipid molar ratio	Cholesterol Molar ratio	PEG molar ratio	Helper lipid molar ratio
AGGCGCTA	25	0.861818628	0.553721085	0.707769857	cKK-E12	Cholesterol	C14PEG2K	DOPE	Neutral	60	26	2	12
CTCCTTCG	26	2.68312333	4.300131754	3.491627542	cKK-E12	Cholesterol	C14PEG2K	DOPE	Neutral	50	35	2.5	12.5
GGCGCCAA	27	0.825177677	0.739277074	0.782227375	cKK-E12	Cholesterol	C14PEG2K	DOPE	Neutral	35	18	2.5	44.5
TATGCCTT	28	0.734607248	0.73938376	0.736995504	cKK-E12	Cholesterol	C14PEG2K	DOPE	Neutral	20	50	2.5	27.5
ATGGTAGA	30	0.649198486	0.51607998	0.582639233	cKK-E12	Cholesterol	C14PEG2K	DSPC	Neutral	50	35	2.5	12.5
TGGAGACG	31	0.55285347	0.85151389	0.70218368	cKK-E12	Cholesterol	C14PEG2K	DSPC	Neutral	35	18	2.5	44.5
CCGCAGAG	32	16.53876183	19.60354139	18.07115161	cKK-E12	Cholesterol	C14PEG2K	DSPC	Neutral	20	50	2.5	27.5
GATCTACC	33	0.721596127	0.404759536	0.563177832	cKK-E12	Cholesterol	C14PEG2K	18:1 CAP PE	Neutral	60	26	2	12
ACGCTAGC	34	4.068538713	5.875919068	4.97222889	cKK-E12	Cholesterol	C14PEG2K	18:1 CAP PE	Neutral	50	35	2.5	12.5
TAGATCCG	35	0.614616827	0.589923423	0.602270125	cKK-E12	Cholesterol	C14PEG2K	18:1 CAP PE	Neutral	35	18	2.5	44.5
GTAATTGC	36	0.685777659	0.548167358	0.616972508	cKK-E12	Cholesterol	C14PEG2K	18:1 CAP PE	Neutral	20	50	2.5	27.5
GAGAGTTG	37	0.666164327	0.614687633	0.64042598	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PC	Neutral	60	26	2	12
TCCAGGCG	38	0.569927146	0.609754876	0.589841011	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PC	Neutral	50	35	2.5	12.5
ATTAGGAC	39	0.681875772	0.643802241	0.662839007	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PC	Neutral	35	18	2.5	44.5
CTACTGAT	41	0.763248889	0.495121701	0.629185295	cKK-E12	Cholesterol	C14PEG2K	18:1 Dimethyl PE	Neutral	60	26	2	12
GCAGGACT	42	0.945473037	1.035068032	0.990270535	cKK-E12	Cholesterol	C14PEG2K	18:1 Dimethyl PE	Neutral	50	35	2.5	12.5
CGAGCAGC	43	0.784297957	0.664964075	0.724631016	cKK-E12	Cholesterol	C14PEG2K	18:1 Dimethyl PE	Neutral	35	18	2.5	44.5
GTCTCCGT	44	1.137339399	2.29912607	1.718232735	cKK-E12	Cholesterol	C14PEG2K	18:1 Dimethyl PE	Neutral	15	55	2.5	27.5
CATAATAG	45	0.770335336	0.899045726	0.834690531	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PE	Neutral	60	26	2	12
CTCAGCAT	46	0.558470732	0.677352235	0.617911483	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PE	Neutral	50	35	2.5	12.5
ATCAATTG	47	0.529532767	0.423949347	0.476741057	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PE	Neutral	35	18	2.5	44.5
TTATTAAT	48	1.020356455	0.93418106	0.977268758	cKK-E12	Cholesterol	C14PEG2K	18:1 Lyso PE	Neutral	15	55	2.5	27.5
TGATCTAT	49	0.956863938	0.637220531	0.797042235	cKK-E12	Cholesterol	C14PEG2K	18:1 Monomethyl PE	Neutral	60	26	2	12
ATTGCTCT	50	2.45632448	3.451525536	2.953925008	cKK-E12	Cholesterol	C14PEG2K	18:1 Monomethyl PE	Neutral	50	35	2.5	12.5
TAAGATGA	51	1.309864404	1.44747681	1.378670607	cKK-E12	Cholesterol	C14PEG2K	18:1 Monomethyl PE	Neutral	35	18	2.5	44.5
GGTCGGTC	55	0.598749306	0.353293591	0.476021448	cKK-E12	Cholesterol	C14PEG2K	DOPC	Neutral	35	18	2.5	44.5
AGGCTCAT	56	1.113919103	2.128677691	1.621298397	cKK-E12	Cholesterol	C14PEG2K	DOPC	Neutral	15	55	2.5	27.5
ATGAGATG	57	1.628513256	1.057576154	1.343044705	cKK-E12	Cholesterol	C18PEG2K	DOPE	Neutral	60	26	2	12
TACGCTCG	58	4.377292326	4.468662412	4.422977369	cKK-E12	Cholesterol	C18PEG2K	DOPE	Neutral	50	35	2.5	12.5
AGCTCGGA	59	0.88435095	0.543853807	0.714102379	cKK-E12	Cholesterol	C18PEG2K	DOPE	Neutral	35	18	2.5	44.5
AGTCCGGT	60	0.852707933	0.720587767	0.78664785	cKK-E12	Cholesterol	C18PEG2K	DOPE	Neutral	15	55	2.5	27.5
AAGTCTAG	61	0.674616596	0.770961456	0.722789026	cKK-E12	Cholesterol	C18PEG2K	DSPC	Neutral	60	26	2	12
AATGCTAC	62	1.084793827	0.572019213	0.82840652	cKK-E12	Cholesterol	C18PEG2K	DSPC	Neutral	50	35	2.5	12.5
TTGGATCC	63	1.656870446	1.416021919	1.536446183	cKK-E12	Cholesterol	C18PEG2K	DSPC	Neutral	35	18	2.5	44.5
TAGTACGT	64	5.779151285	7.457666771	6.618409028	cKK-E12	Cholesterol	C18PEG2K	DSPC	Neutral	15	55	2.5	27.5
GCGAATTC	65	0.743047533	0.429296234	0.586171884	cKK-E12	Cholesterol	C18PEG2K	18:1 CAP PE	Neutral	60	26	2	12
ACGCTCCA	66	2.4699494	4.046348488	3.258148944	cKK-E12	Cholesterol	C18PEG2K	18:1 CAP PE	Neutral	50	35	2.5	12.5
TAACCGAA	67	0.590231894	0.316801289	0.453516592	cKK-E12	Cholesterol	C18PEG2K	18:1 CAP PE	Neutral	35	18	2.5	44.5
TCCTGATG	68	0.801946901	0.736394821	0.769170861	cKK-E12	Cholesterol	C18PEG2K	18:1 CAP PE	Neutral	15	55	2.5	27.5
ATTCGAGA	69	0.462226071	0.420704907	0.441465489	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PC	Neutral	60	26	2	12
CGCGAGGC	70	0.440850955	0.317545278	0.379198117	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PC	Neutral	50	35	2.5	12.5
ATTGGTTC	71	0.353390538	0.257891876	0.305641207	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PC	Neutral	35	18	2.5	44.5
AATATACG	72	2.208160021	3.469532285	2.838846153	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PC	Neutral	15	55	2.5	27.5
GATTCCGG	73	1.370532068	1.083444048	1.226988058	cKK-E12	Cholesterol	C18PEG2K	18:1 Dimethyl PE	Neutral	60	26	2	12
CGGTCAAT	74	0.984142614	1.567468847	1.27580573	cKK-E12	Cholesterol	C18PEG2K	18:1 Dimethyl PE	Neutral	50	35	2.5	12.5
TATATCTA	75	11.46422587	0.783457322	6.123841597	cKK-E12	Cholesterol	C18PEG2K	18:1 Dimethyl PE	Neutral	35	18	2.5	44.5
ATCGTCTA	76	0.207095482	0.34433757	0.275716526	cKK-E12	Cholesterol	C18PEG2K	18:1 Dimethyl PE	Neutral	15	55	2.5	27.5
CTACCATT	77	0.47598737	0.382180081	0.429083725	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PE	Neutral	60	26	2	12
GTCGAAGT	78	0.410570173	0.429370102	0.419970137	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PE	Neutral	50	35	2.5	12.5
GATGGCCT	79	0.429523374	0.426689046	0.42810621	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PE	Neutral	35	18	2.5	44.5
CGATGCTT	80	2.033517386	2.938566917	2.486042152	cKK-E12	Cholesterol	C18PEG2K	18:1 Lyso PE	Neutral	15	55	2.5	27.5
ATAATATA	81	0.792989213	1.889577885	1.341283549	cKK-E12	Cholesterol	C18PEG2K	18:1 Monomethyl PE	Neutral	60	26	2	12
CGCCTATT	82	3.141775937	5.041427408	4.091601673	cKK-E12	Cholesterol	C18PEG2K	18:1 Monomethyl PE	Neutral	50	35	2.5	12.5
AAGAGGAT	83	1.355188536	0.649279953	1.002234244	cKK-E12	Cholesterol	C18PEG2K	18:1 Monomethyl PE	Neutral	35	18	2.5	44.5
GGACGTCC	84	0.894417467	0.491410264	0.692913866	cKK-E12	Cholesterol	C18PEG2K	18:1 Monomethyl PE	Neutral	15	55	2.5	27.5
GTTAGTCA	85	1.271284929	0.334964168	0.803124549	cKK-E12	Cholesterol	C18PEG2K	DOPC	Neutral	60	26	2	12
ACTATCTC	86	0.781952094	0.644871119	0.713411606	cKK-E12	Cholesterol	C18PEG2K	DOPC	Neutral	50	35	2.5	12.5
TTATAGCA	87	0.440182205	0.27199457	0.356088388	cKK-E12	Cholesterol	C18PEG2K	DOPC	Neutral	35	18	2.5	44.5
TGACCAGG	Naked Barcode	0.046855224	0.098606238	0.072730731									

b

Barcode	LNP #	Liver average	Spleen average	average normalized delivery	Ionizable lipid	Cholesterol	PEG	Helper lipid	Charge	Ionizable lipid molar ratio	Cholesterol molar ratio	PEG molar ratio	Helper lipid molar ratio
GACGCAAT	1	4.021957523	4.034426393	4.028191958	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylethanol	Anionic	60	26	2	12
GATTCAAC	2	2.378888101	2.364877116	2.371882609	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylethanol	Anionic	50	35	2.5	12.5
TCCTAAGA	3	2.592904106	2.590034791	2.591469449	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylethanol	Anionic	35	18	2.5	44.5
CATCATTA	4	2.312457781	2.312064813	2.312261297	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylethanol	Anionic	20	50	2.5	27.5
CAATCCGT	5	2.179047208	2.190960732	2.18500397	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylpropanol	Anionic	60	26	2	12
CTCAACTA	6	1.792971437	1.796966775	1.794969106	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylpropanol	Anionic	50	35	2.5	12.5
AGTTACCG	7	2.024783075	2.021071307	2.022927191	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylpropanol	Anionic	35	18	2.5	44.5
CGCTCCGG	8	1.613075135	1.610757019	1.611916077	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylpropanol	Anionic	20	50	2.5	27.5
GCGAGTAT	9	1.882683403	1.910537481	1.896610442	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylmethanol	Anionic	60	26	2	12
TACCTGCT	10	1.932287369	1.927959638	1.930123504	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylmethanol	Anionic	50	35	2.5	12.5
CAAGAAGG	11	2.726414256	2.725420218	2.725917237	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylmethanol	Anionic	35	18	2.5	44.5
TTGCAACT	12	0.406247919	0.413464049	0.409855984	cKK-E12	Cholesterol	C14PEG2K	18:1 Phosphatidylmethanol	Anionic	20	50	2.5	27.5
GGCGCTTG	13	1.383450969	1.381216911	1.38233394	cKK-E12	Cholesterol	C14PEG2K	18:1 (Δ9-Cis) PG	Anionic	60	26	2	12
CTTACGTC	14	2.406492122	2.397056946	2.401774534	cKK-E12	Cholesterol	C14PEG2K	18:1 (Δ9-Cis) PG	Anionic	50	35	2.5	12.5
GAGTATAC	15	1.891865534	1.895610456	1.893737995	cKK-E12	Cholesterol	C14PEG2K	18:1 (Δ9-Cis) PG	Anionic	35	18	2.5	44.5
ACTCAAGT	16	0.654826991	0.656881301	0.655854146	cKK-E12	Cholesterol	C14PEG2K	18:1 (Δ9-Cis) PG	Anionic	20	50	2.5	27.5
ACCTAATC	17	4.028778928	4.009837145	4.019308036	cKK-E12	Cholesterol	C14PEG2K	18:1 PA	Anionic	60	26	2	12
GCTAATCG	18	3.538539914	3.538066598	3.538303256	cKK-E12	Cholesterol	C14PEG2K	18:1 PA	Anionic	50	35	2.5	12.5
TAGAATTA	19	3.662188138	3.664231961	3.663210049	cKK-E12	Cholesterol	C14PEG2K	18:1 PA	Anionic	35	18	2.5	44.5
GTCCGTTA	21	1.412308546	1.408956732	1.410632639	cKK-E12	Cholesterol	C14PEG2K	18:1 PS (DOPS)	Anionic	60	26	2	12
TGAGTTCG	22	2.321968887	2.316624711	2.319296799	cKK-E12	Cholesterol	C14PEG2K	18:1 PS (DOPS)	Anionic	50	35	2.5	12.5
GGCAGTAG	23	1.661131669	1.655391318	1.658261493	cKK-E12	Cholesterol	C14PEG2K	18:1 PS (DOPS)	Anionic	35	18	2.5	44.5
CCGTTCGG	24	2.433786524	2.426291791	2.430039158	cKK-E12	Cholesterol	C14PEG2K	18:1 PS (DOPS)	Anionic	20	50	2.5	27.5
AGGCGCTA	25	2.332935032	2.329069744	2.331002388	cKK-E12	Cholesterol	C14PEG2K	18:1 Biotinyl PE	Anionic	60	26	2	12
CTCCTTCG	26	2.635473127	2.62850754	2.631990334	cKK-E12	Cholesterol	C14PEG2K	18:1 Biotinyl PE	Anionic	50	35	2.5	12.5
GGCGCCAA	27	2.761871407	2.753355469	2.757613438	cKK-E12	Cholesterol	C14PEG2K	18:1 Biotinyl PE	Anionic	35	18	2.5	44.5
TATGCCTT	28	1.888828973	1.900086833	1.894457903	cKK-E12	Cholesterol	C14PEG2K	18:1 Biotinyl PE	Anionic	20	50	2.5	27.5
GATCTACC	29	0.618966493	0.621760326	0.62036341	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylethanol	Anionic	60	26	2	12
ACGCTAGC	30	1.692227523	1.693133964	1.692680744	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylethanol	Anionic	50	35	2.5	12.5
TAGATCCG	31	1.242107808	1.247332471	1.24472014	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylethanol	Anionic	35	18	2.5	44.5
GTAATTGC	32	0.400936326	0.401517061	0.401226693	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylethanol	Anionic	20	50	2.5	27.5
GAGAGTTG	33	1.623050794	1.622838247	1.62294452	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylpropanol	Anionic	60	26	2	12
CGGTATCT	36	1.412293735	1.420897262	1.416595498	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylpropanol	Anionic	20	50	2.5	27.5
GCAGGACT	38	2.460927482	2.453570895	2.457249188	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylmethanol	Anionic	50	35	2.5	12.5
CGAGCAGC	39	1.975416712	1.969808923	1.972612817	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylmethanol	Anionic	35	18	2.5	44.5
GTCTCCGT	40	1.692249013	1.694979096	1.693614054	cKK-E12	Cholesterol	C18PEG2K	18:1 Phosphatidylmethanol	Anionic	20	50	2.5	27.5
CATAATAG	41	2.113006136	2.108318603	2.110662369	cKK-E12	Cholesterol	C18PEG2K	18:1 (Δ9-Cis) PG	Anionic	60	26	2	12
CTCAGCAT	42	0.53775489	0.537953146	0.537854018	cKK-E12	Cholesterol	C18PEG2K	18:1 (Δ9-Cis) PG	Anionic	50	35	2.5	12.5
ATCAATTG	43	1.845686044	1.843759497	1.844722771	cKK-E12	Cholesterol	C18PEG2K	18:1 (Δ9-Cis) PG	Anionic	35	18	2.5	44.5
TTATTAAT	44	1.401164973	1.414541708	1.407863341	cKK-E12	Cholesterol	C18PEG2K	18:1 (Δ9-Cis) PG	Anionic	20	50	2.5	27.5
TGATCTAT	45	1.963682303	1.954207477	1.95894489	cKK-E12	Cholesterol	C18PEG2K	18:1 PA	Anionic	60	26	2	12
ATTGCTCT	46	2.129405956	2.125857035	2.127631495	cKK-E12	Cholesterol	C18PEG2K	18:1 PA	Anionic	50	35	2.5	12.5
TAAGATGA	47	0.562905513	0.563076041	0.562990777	cKK-E12	Cholesterol	C18PEG2K	18:1 PA	Anionic	35	18	2.5	44.5
TCTAGAGT	49	2.321898341	2.32804315	2.324970745	cKK-E12	Cholesterol	C18PEG2K	18:1 PS (DOPS)	Anionic	60	26	2	12
TGCGTATA	50	1.776926588	1.779371818	1.778149203	cKK-E12	Cholesterol	C18PEG2K	18:1 PS (DOPS)	Anionic	50	35	2.5	12.5
AGGCTCAT	52	4.439148827	4.43943094	4.439289883	cKK-E12	Cholesterol	C18PEG2K	18:1 PS (DOPS)	Anionic	20	50	2.5	27.5
ATGAGATG	53	2.068792191	2.072694909	2.07074355	cKK-E12	Cholesterol	C18PEG2K	18:1 Biotinyl PE	Anionic	60	26	2	12
TACGCTCG	54	0.6219439	0.618805294	0.620374597	cKK-E12	Cholesterol	C18PEG2K	18:1 Biotinyl PE	Anionic	50	35	2.5	12.5
AGCTCGGA	55	2.512635853	2.518641331	2.515638592	cKK-E12	Cholesterol	C18PEG2K	18:1 Biotinyl PE	Anionic	35	18	2.5	44.5
AGTCCGGT	56	1.335397612	1.33884476	1.337121186	cKK-E12	Cholesterol	C18PEG2K	18:1 Biotinyl PE	Anionic	20	50	2.5	27.5
TGACCAGG	Naked Barcode	0.355311137	0.354670459	0.354990798									

Barcodes	# LNP	Lung average	Liver average	spleen average	average normalized delivery	onizable lipid	Cholesterol	PEG	Helper lipid	Charge	Ionizable lipid molar ratio	Cholesterol Molar ratio	PEG molar ratio	Helper lipid molar ratio
AGCATGCG	2	12.56256328	8.524294312	3.402668606	8.163175401	cKK-E12	Cholesterol	C14PEG2K	DOTAP	Cationic	50	35	2.5	12.5
TTGCGTTG	3	2.377185354	1.911603866	4.620508024	2.969765748	cKK-E12	Cholesterol	C14PEG2K	DOTAP	Cationic	35	18	2.5	44.5
ACGTCGAA	4	2.002639125	2.081597779	8.175763564	4.086666823	cKK-E12	Cholesterol	C14PEG2K	DOTAP	Cationic	20	50	2.5	27.5
CTACGAGG	5	3.461523684	2.550574912	1.425292919	2.479130505	cKK-E12	Cholesterol	C14PEG2K	DOTMA	Cationic	60	26	2	12
ATAGAATC	6	2.388169424	2.140902725	10.74356823	5.090880125	cKK-E12	Cholesterol	C14PEG2K	DOTMA	Cationic	50	35	2.5	12.5
GTCGCCTC	7	2.072582804	3.047458645	1.858859395	2.326300281	cKK-E12	Cholesterol	C14PEG2K	DOTMA	Cationic	35	18	2.5	44.5
GTCAATCT	8	1.496114241	1.40017483	4.971669107	2.622652726	cKK-E12	Cholesterol	C14PEG2K	DOTMA	Cationic	20	50	2.5	27.5
GACGCAAT	9	4.559002703	7.725292314	3.311535108	5.198610041	cKK-E12	Cholesterol	C14PEG2K	DDAB	Cationic	60	26	2	12
GATTCAAC	10	5.65587824	4.931457318	3.462286173	4.683207244	cKK-E12	Cholesterol	C14PEG2K	DDAB	Cationic	50	35	2.5	12.5
TCCTAAGA	11	10.70795163	6.787428278	3.698774055	7.064717988	cKK-E12	Cholesterol	C14PEG2K	DDAB	Cationic	35	18	2.5	44.5
CATCATTA	12	2.448116757	2.785822786	2.171807573	2.468582372	cKK-E12	Cholesterol	C14PEG2K	DDAB	Cationic	20	50	2.5	27.5
TACCTGCT	13	4.239008784	4.491167191	1.415055526	3.381743834	cKK-E12	Cholesterol	C18PEG2K	DOTAP	Cationic	50	35	2.5	12.5
CAAGAAGG	15	5.687825976	4.656207347	3.139577841	4.494537055	cKK-E12	Cholesterol	C18PEG2K	DOTAP	Cationic	35	18	2.5	44.5
TTGCAACT	16	2.328265292	1.905498327	2.021396178	2.085053266	cKK-E12	Cholesterol	C18PEG2K	DOTAP	Cationic	20	50	2.5	27.5
GGCGCTTG	17	4.739717288	3.938939394	4.660291473	4.446316052	cKK-E12	Cholesterol	C18PEG2K	DOTMA	Cationic	60	26	2	12
CTTACGTC	18	3.334529063	2.722239283	7.944338444	4.667035596	cKK-E12	Cholesterol	C18PEG2K	DOTMA	Cationic	50	35	2.5	12.5
GAGTATAC	19	4.255948685	3.024652113	2.68576884	3.322123213	cKK-E12	Cholesterol	C18PEG2K	DOTMA	Cationic	35	18	2.5	44.5
ACTCAAGT	20	5.750342045	3.341275375	3.008190304	4.033269242	cKK-E12	Cholesterol	C18PEG2K	DOTMA	Cationic	20	50	2.5	27.5
ACCTAATC	21	1.409544503	5.561151432	9.671625209	5.547440381	cKK-E12	Cholesterol	C18PEG2K	DDAB	Cationic	60	26	2	12
GCTAATCG	22	5.863723904	5.079908054	4.604695188	5.182775715	cKK-E12	Cholesterol	C18PEG2K	DDAB	Cationic	50	35	2.5	12.5
TAGAATTA	23	3.145606084	3.53427274	3.2043026	3.294727141	cKK-E12	Cholesterol	C18PEG2K	DDAB	Cationic	35	18	2.5	44.5
TCTAACTG	24	6.763484213	7.222256178	3.218984213	5.734908201	cKK-E12	Cholesterol	C18PEG2K	DDAB	Cationic	20	50	2.5	27.5
TTGCCGGT	Naked Barcode	0.11430871	0.163102562	0.033280231	0.103563834									
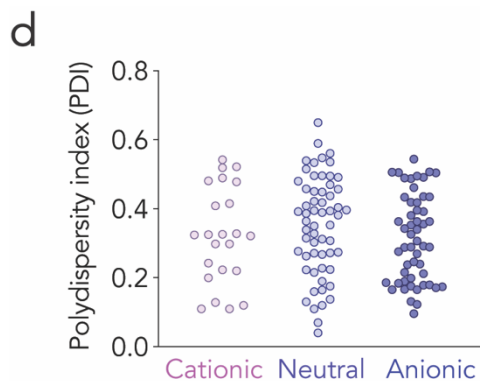
Supplementary Figure 6. Normalized delivery of LNPs in (a) neutral screen, (b) anionic screen, and (c) cationic screen. The lead LNPs (Neu-LNP, An-LNP, Cat-LNP in order) in each screen are shown in the red boxes. (d) Polydispersity index of all individual pooled LNPs.
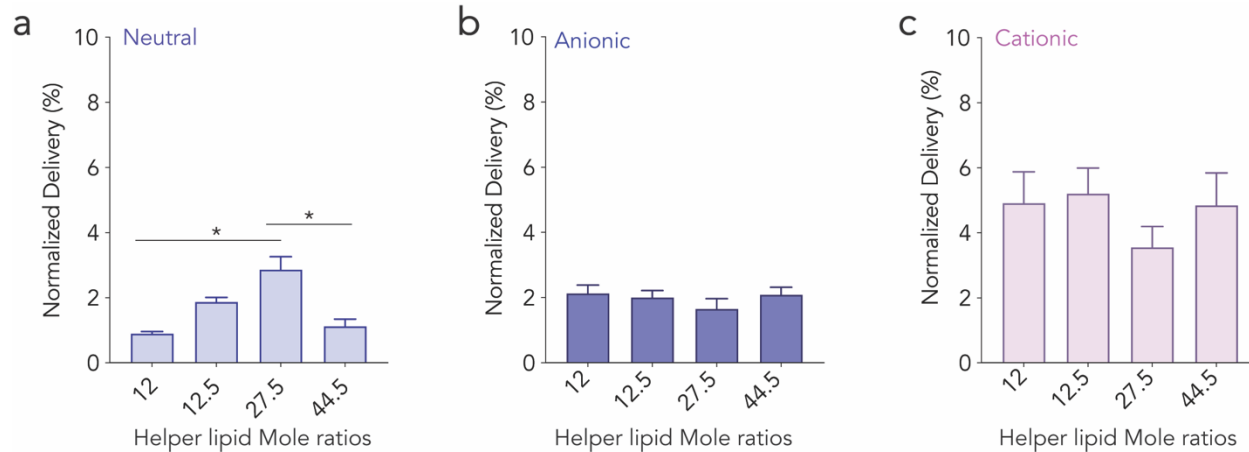
Supplementary Figure 7. (a) Normalized delivery of neutral LNPs was highest at 27.5% helper lipid mole ratio. (b, c) No relationship was found between helper lipid mole ratio and normalized delivery of anionic and cationic LNPs. *$P < 0.04$, one-way ANOVA, average +/- SEM.

Supplementary Figure 8. (a) Enrichment (fold) difference is calculated through the formula shown. Fold enrichment was calculated for cationic LNPs delivered to (b) lung cells, (c) liver cells, and (d) spleen cells. Average +/- SEM.
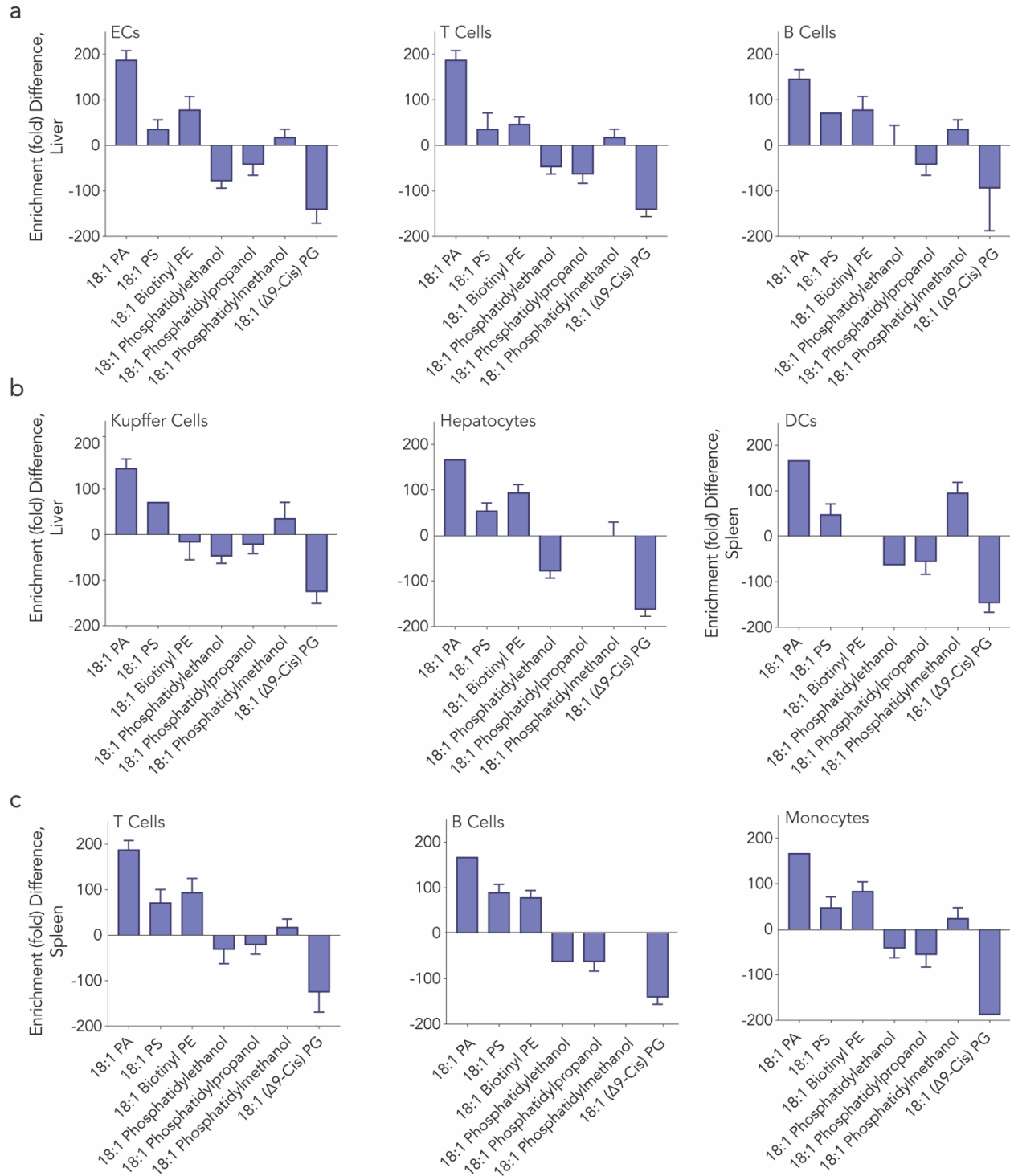
Supplementary Figure 9. (a, b) Fold enrichment was calculated for anionic LNPs delivered to liver cells and (c) spleen cells. Average +/- SEM.
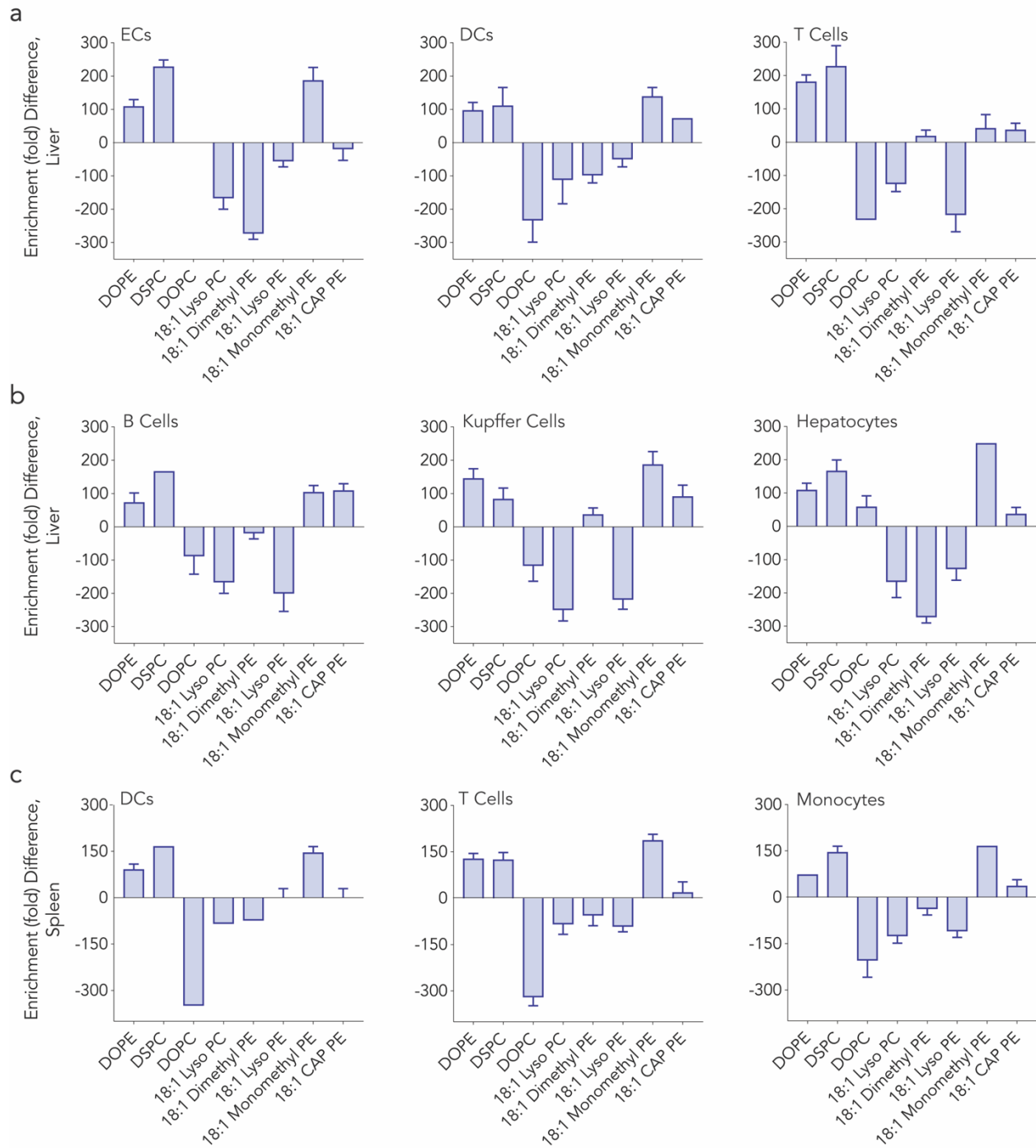
Supplementary Figure 10. (a, b) Fold enrichment was calculated for neutral LNPs delivered to liver cells and (c) spleen cells. Average +/- SEM.
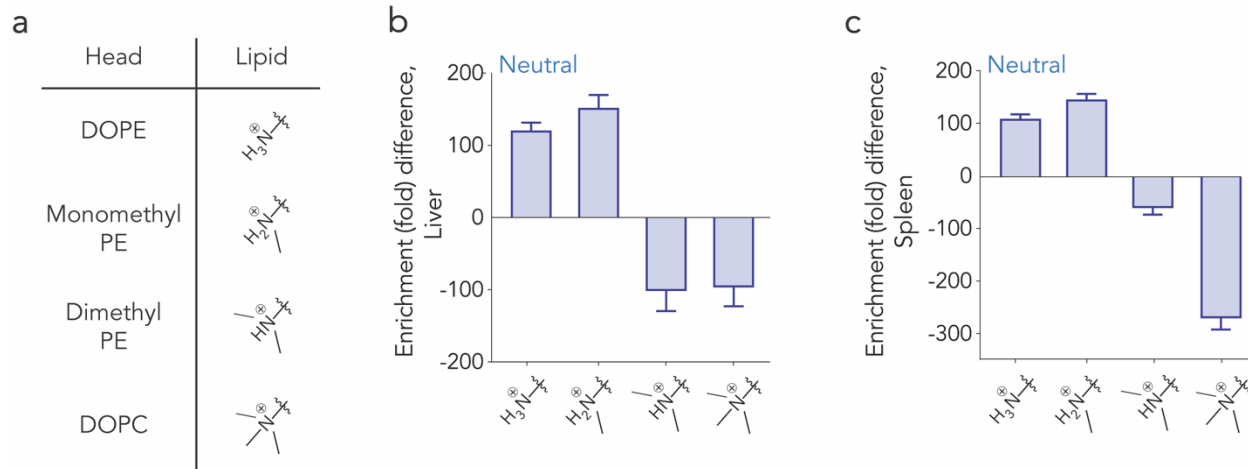
Supplementary Figure 11. (a) Fold enrichment in the neutral library was calculated for four different headgroups in the (b) liver and (c) spleen. Average +/- SEM.
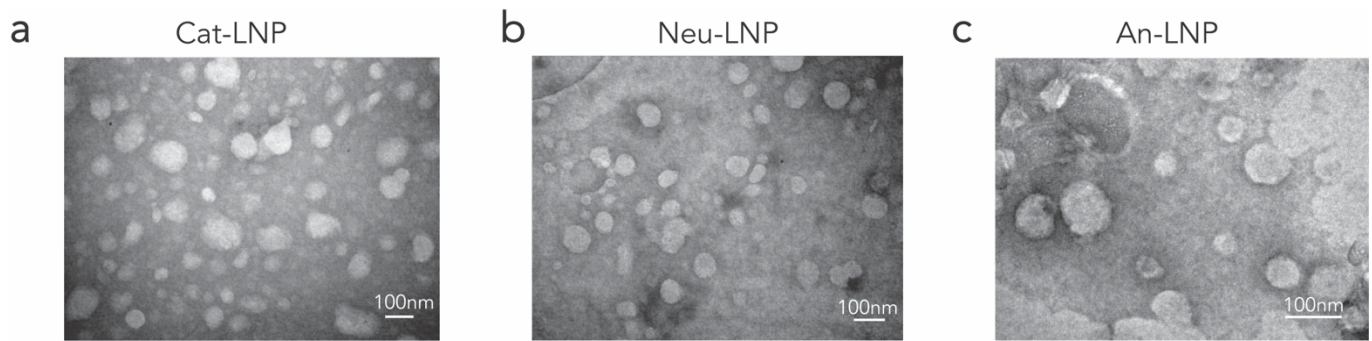
Supplementary Figure 12. TEM images of (a) Cat-LNP, (b) Neu-LNP, and (c) An-LNP.

a

PBS   Cat-LNP

b

PBS   Neu-LNP
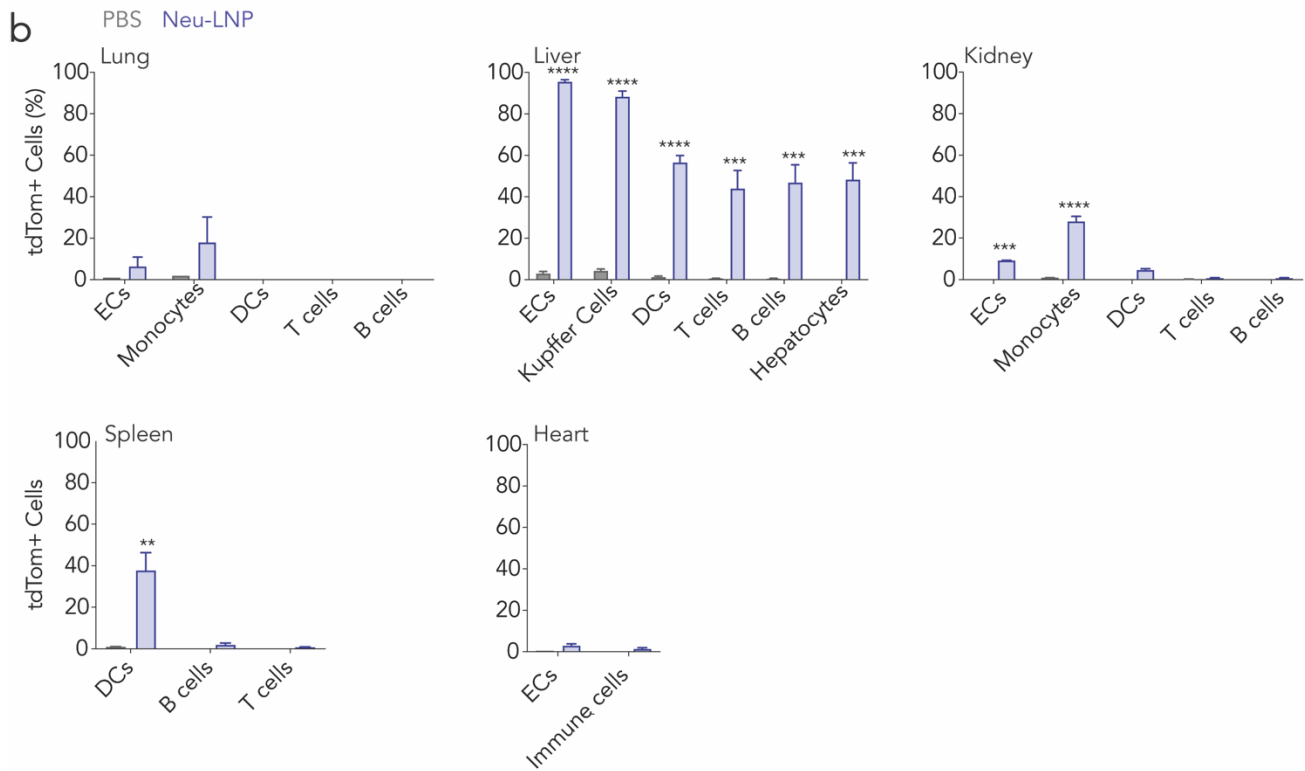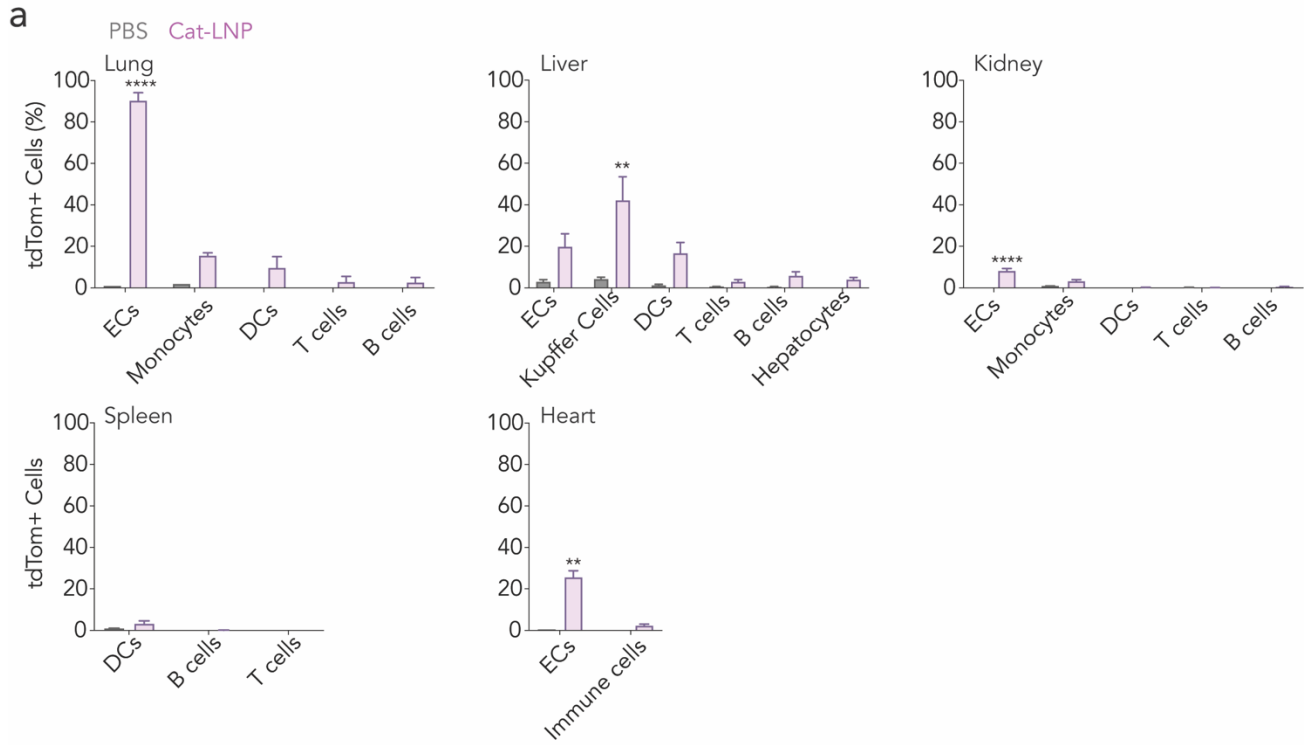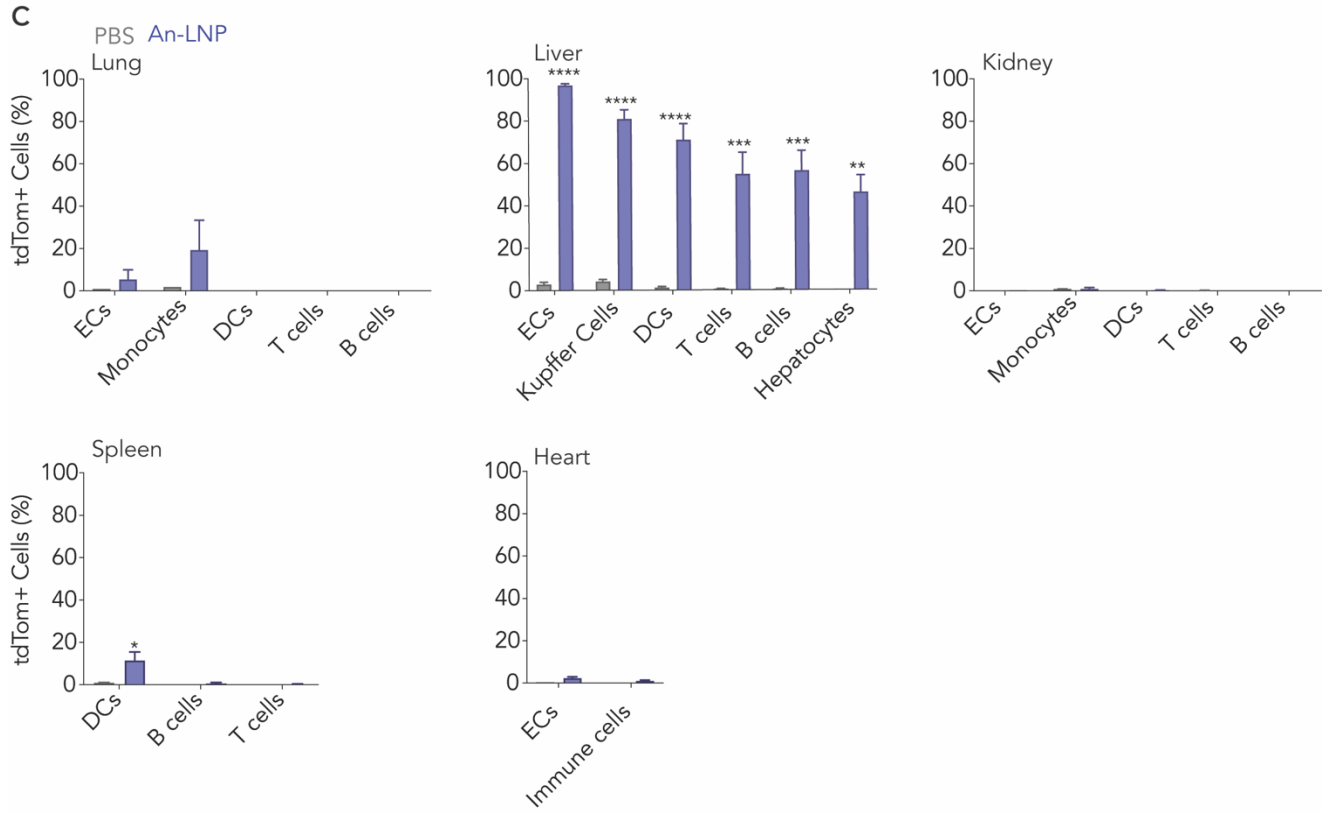
Supplementary Figure 13. (a) Cat-LNP enhanced functional non-liver:liver Cre mRNA delivery, with the highest delivery found in lung ECs. (b) Neu-LNP functionally delivered Cre-mRNA to liver cell types, with the most significant delivery to liver ECs and Kupffer cells. (c) An-LNP functionally delivered Cre-mRNA to liver cell types. ****P < 0.0001, ***P < 0.0008, **P < 0.006, *P = 0.035, two-way ANOVA, average +/- SEM.
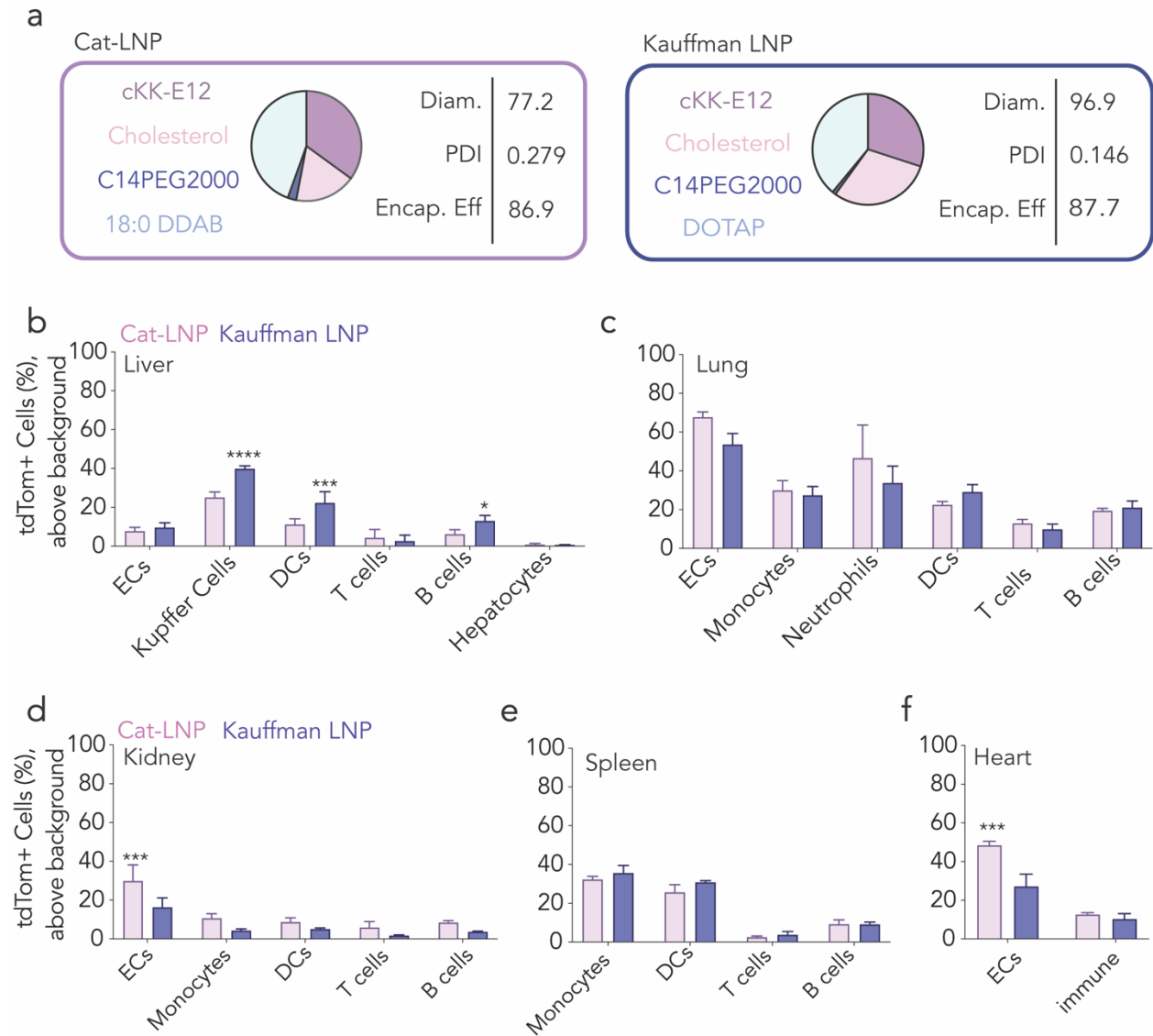
Supplementary Figure 14. (a) Cat-LNP and Kauffman LNP carrying Cre mRNA formed small, monodisperse particles. Diameters are in nm, and encapsulation efficiencies are in percentage. (b–f) Cat-LNP increased the ratio of non-liver to liver delivery as compared to Kauffman LNP. ****P < 0.0001, ***P < 0.0007, *P < 0.05, two-way ANOVA, average +/- SD.
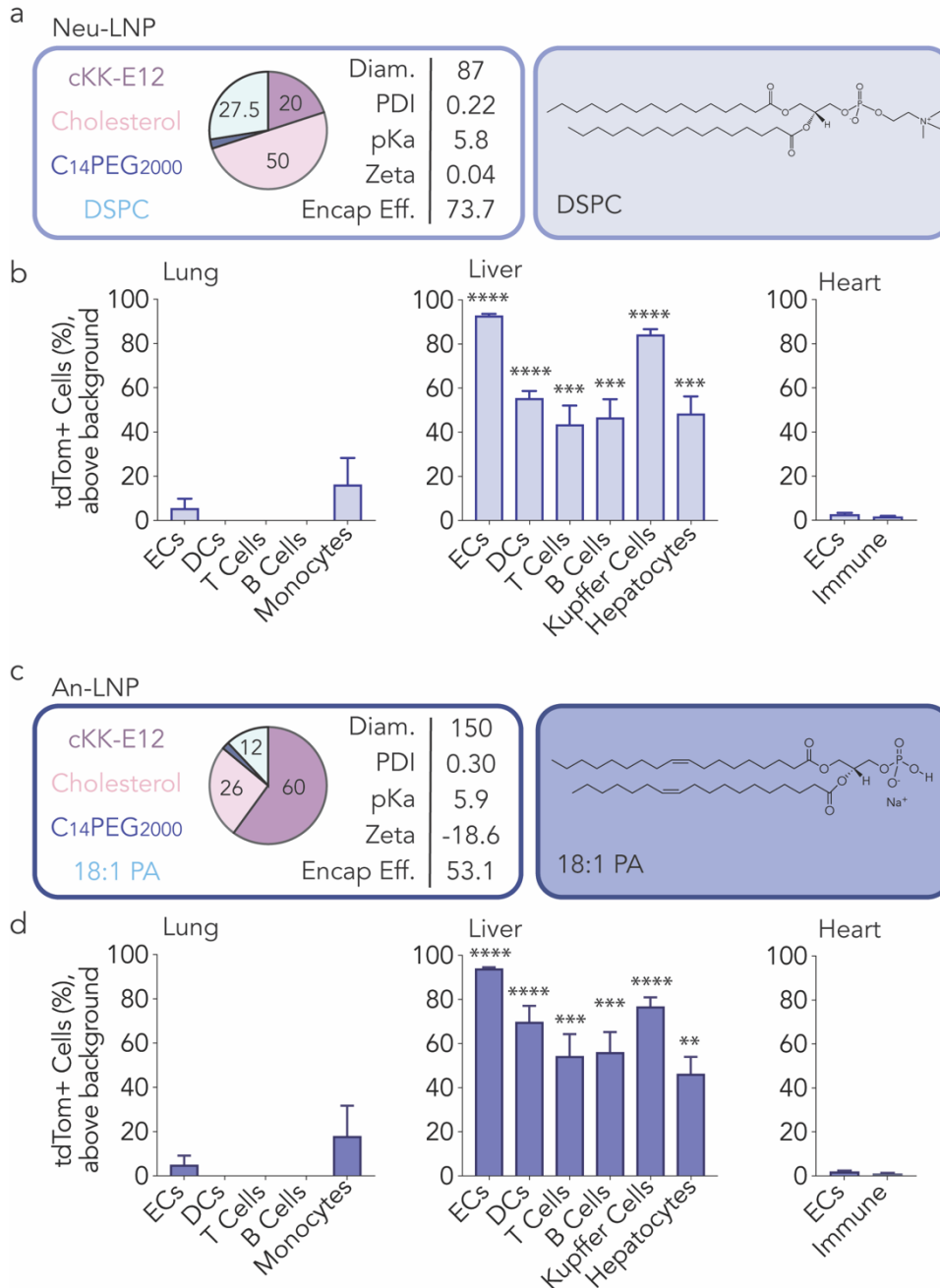
Supplementary Figure 15. Top LNPs from the neutral and anionic screens potently deliver mRNA to liver. (a) A lipid nanoparticle was identified from the neutral screen and named Neu-LNP. Using a neutral helper lipid, DSPC, a small and stable nanoparticle was formed. (b) The Neu-LNP was formulated with Cre mRNA and administered to Ai14 mice intravenously. Four days later, tdTomato signal was quantified. We found that the Neu-LNP delivered Cre mRNA significantly to all liver cell types. (c) An LNP was identified from the anionic screen and named An-LNP. Using an anionic helper lipid, 18:1 PA, a small and stable nanoparticle was formed. (d) The An-LNP was formulated with Cre mRNA and administered to Ai14 mice intravenously. Four days later, tdTomato signal was quantified. We found that the An-LNP delivered Cre mRNA significantly to all liver cell types. ****$P < 0.0001$, ***$P < 0.0008$, **$P = 0.0024$, two-way ANOVA, average +/- SEM. Diam.: Diameter in nm, Zeta: Zeta potential in mV, Encap Eff.: Encapsulation efficiency in %.
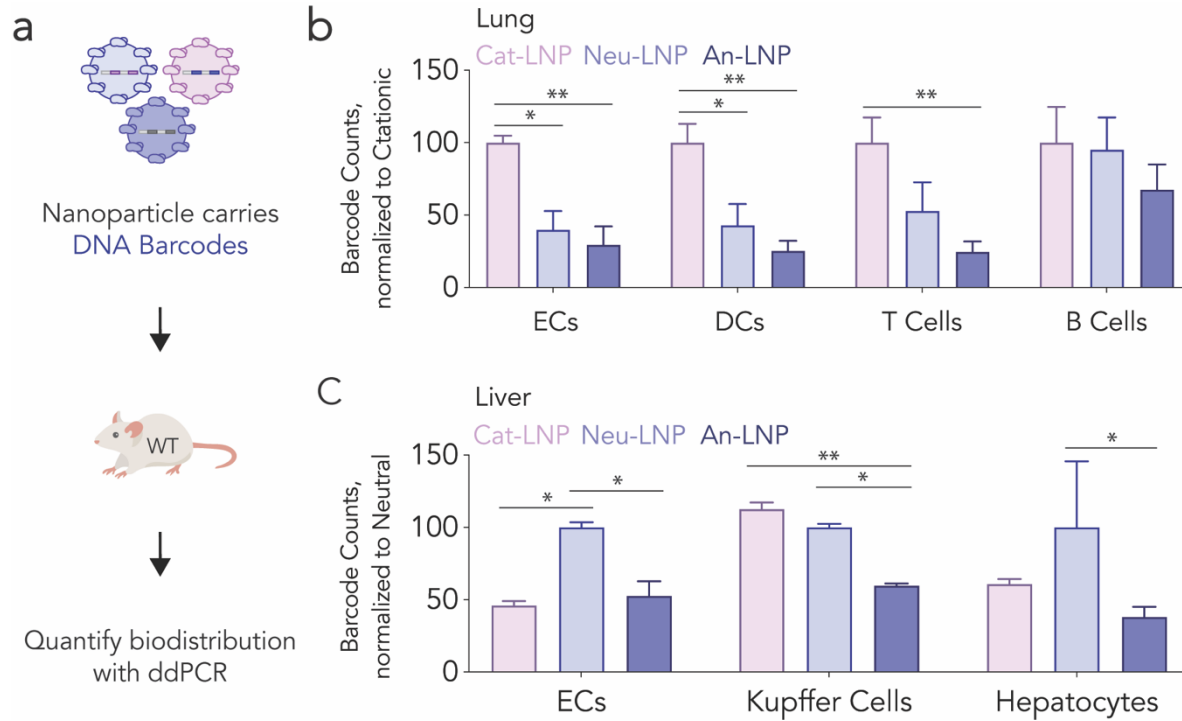
Supplementary Figure 16. (a) Biodistribution profile of Cat-LNP, Neu-LNP, and An-LNP. The Cat-, Neu-, and An-LNPs were individually formulated with DNA barcodes. Twenty-four hours later, distribution of each LNP was determined with ddPCR. (b, c) Biodistribution of the Cat-, Neu-, and An-LNPs was determined in (b) lung cell types and (c) liver cell types. **$P < 0.009$, *$P < 0.05$, two-way ANOVA, average +/- SEM.

h
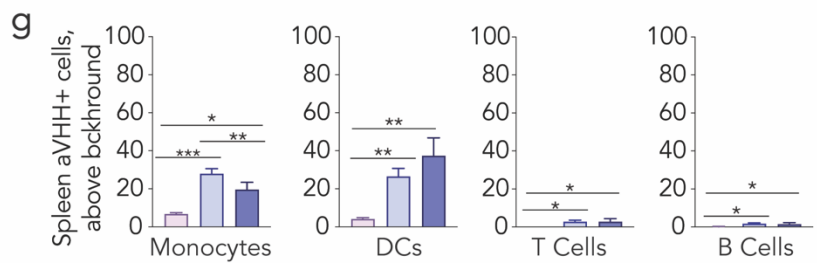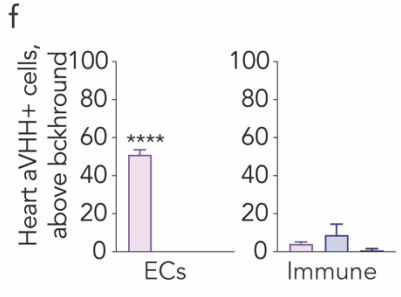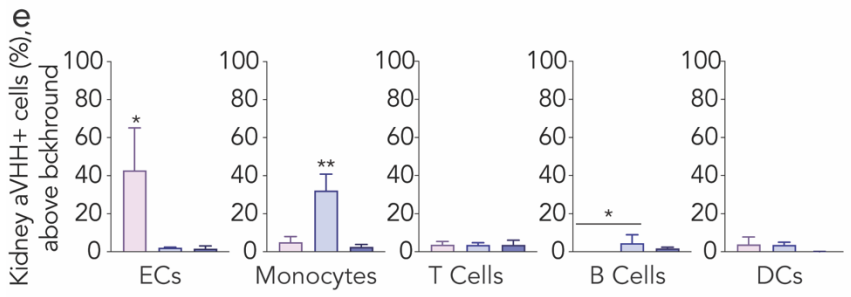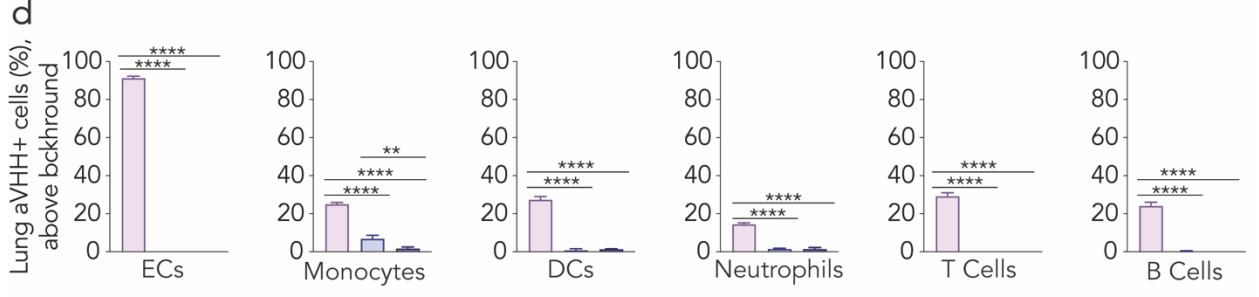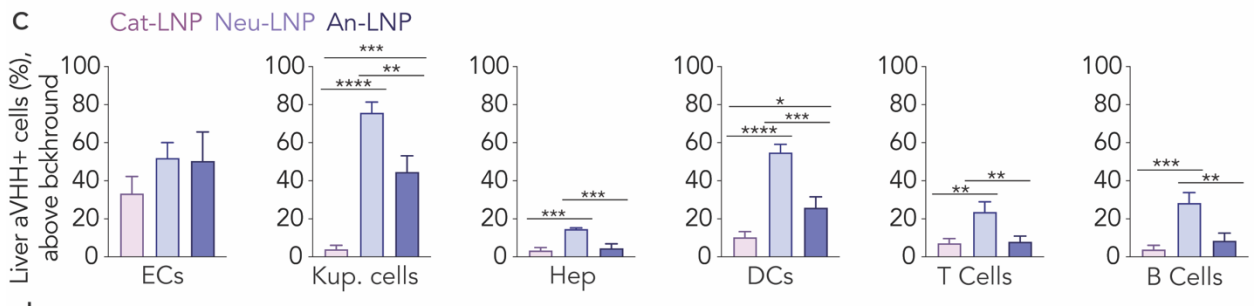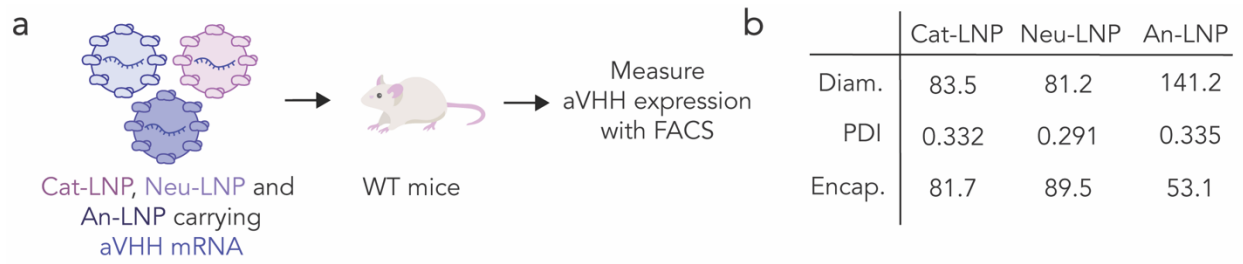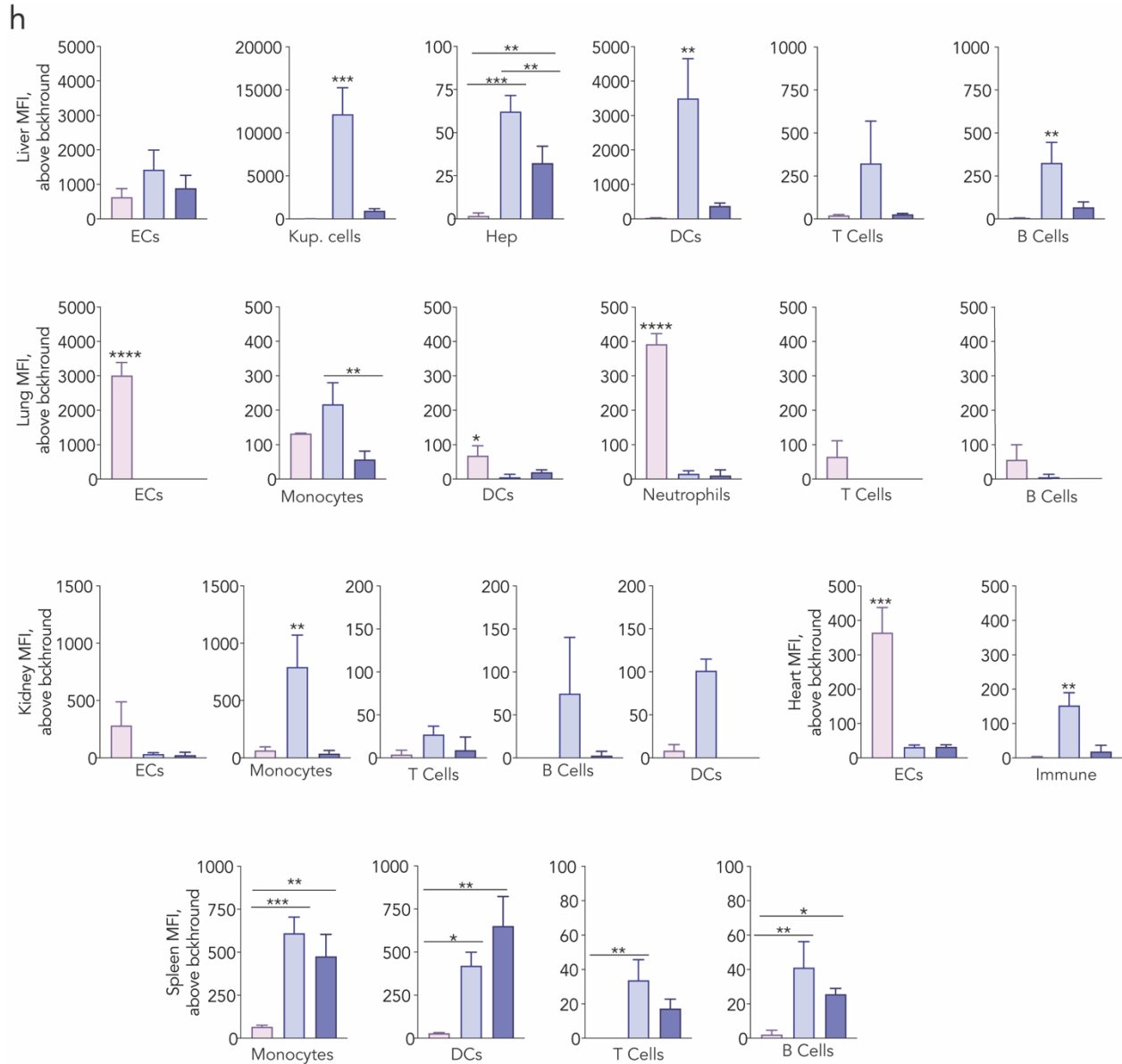
Supplementary Figure 17. (a) Cat-LNP, Neu-LNP and An-LNP were formulated with aVHH mRNA and administered through tail vein injection to C57BL/6 mice. Liver, lung, heart, spleen, and kidney were isolated 1 day post-injection (Diam.: Diameter (nm), PDI: polydispersity index, Encap.: Encapsulation efficiency (%)). (b) These LNPs formed small and monodisperse nanoparticles. (c) Cat-LNP de-targeted the liver, while Neu-LNP and An-LNP showed significantly higher liver tropism. (d) Cat-LNP functionally delivered aVHH mRNA to all lung cell types as compared to An-LNP and Neu-LNP. (e) Cat-LNP functionally delivered aVHH mRNA to kidney ECs. Neu-LNP showed significantly higher functional delivery to monocytes. (f) Cat-LNP significantly delivered aVHH mRNA to heart ECs. (g) Neu-LNP targeted spleen monocytes, and An-LNP targeted spleen DCs. (h) aVHH MFI measured in each cell type. ****$P < 0.0001$, ***$P <= 0.0009$, **$P <= 0.01$, *$P < 0.05$, one-way ANOVA, average +/- SD.
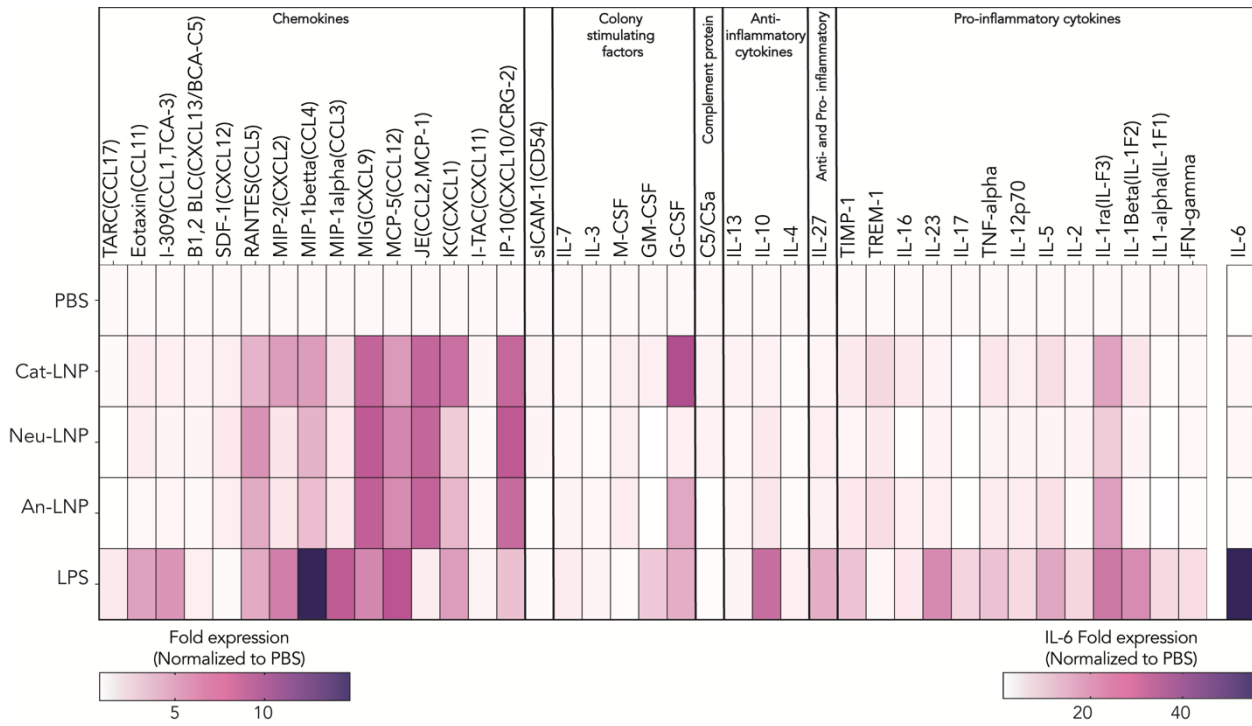
Supplementary Figure 18. Forty cytokines and chemokines were measured in blood serum at 6 hrs post-injection of Cat-LNP, Neu-LNP, An-LNP, PBS, and LPS at a dose of 1.3 mg/kg. Pixel intensities are normalized to PBS to report the fold expressions.
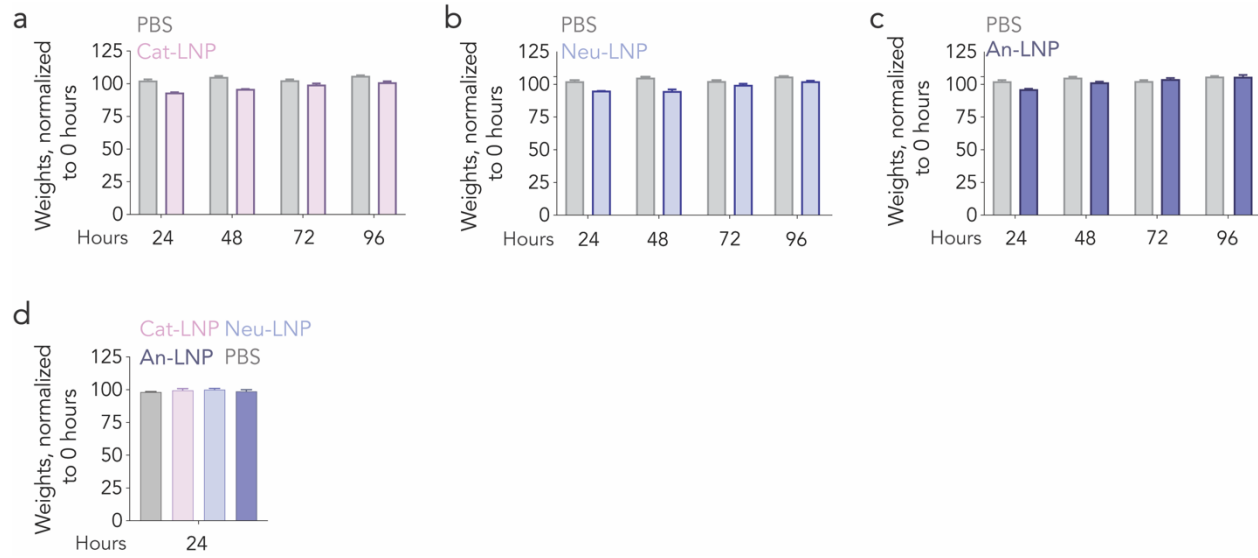
Supplementary Figure 19. (a–c) Mouse weights measured at 24, 48, and 96 hours after administration of PBS or Cat-, Neu-, and An-LNPs carrying Cre mRNA or (d) DNA barcodes. Average +/- SEM.
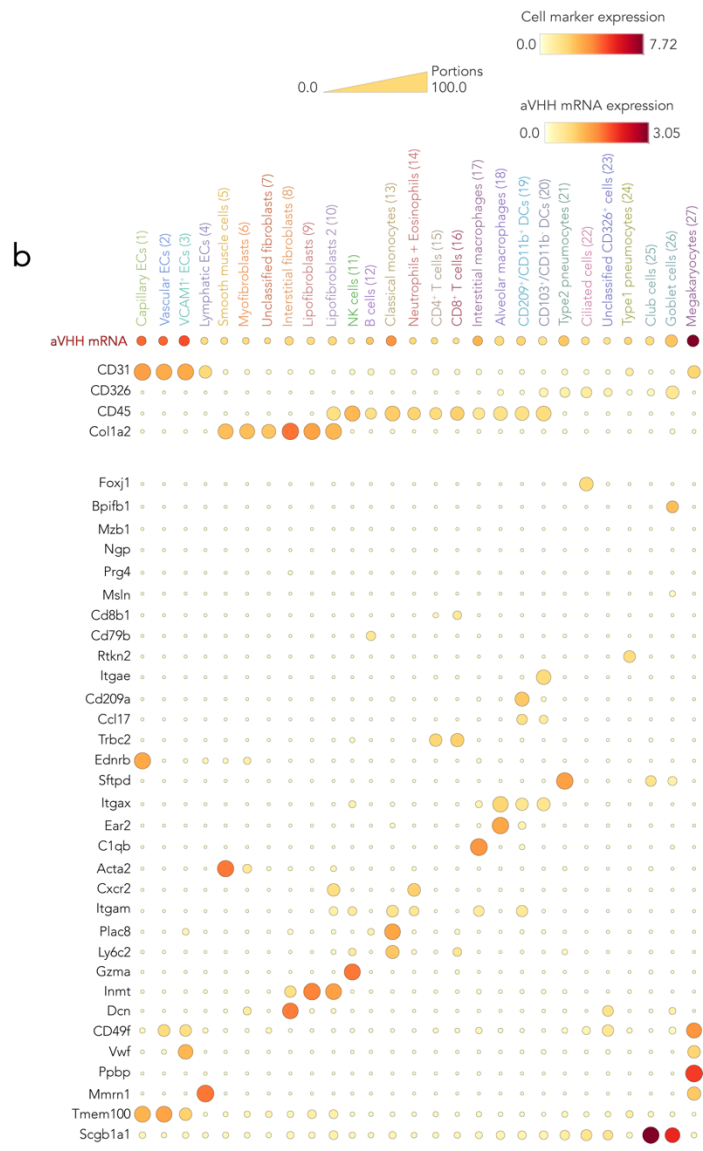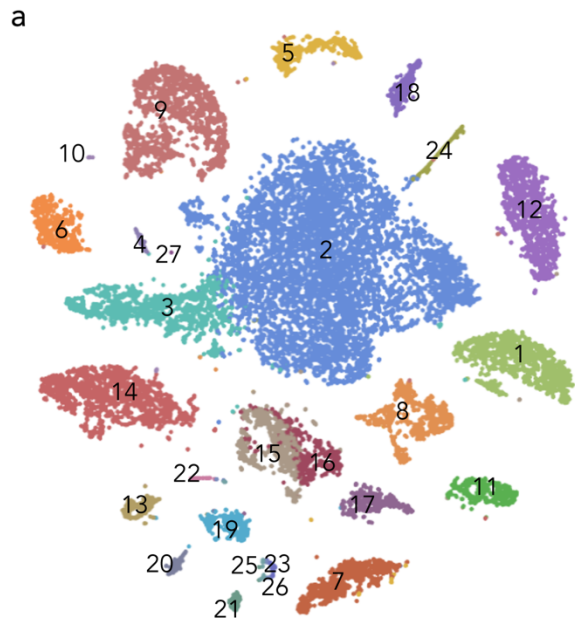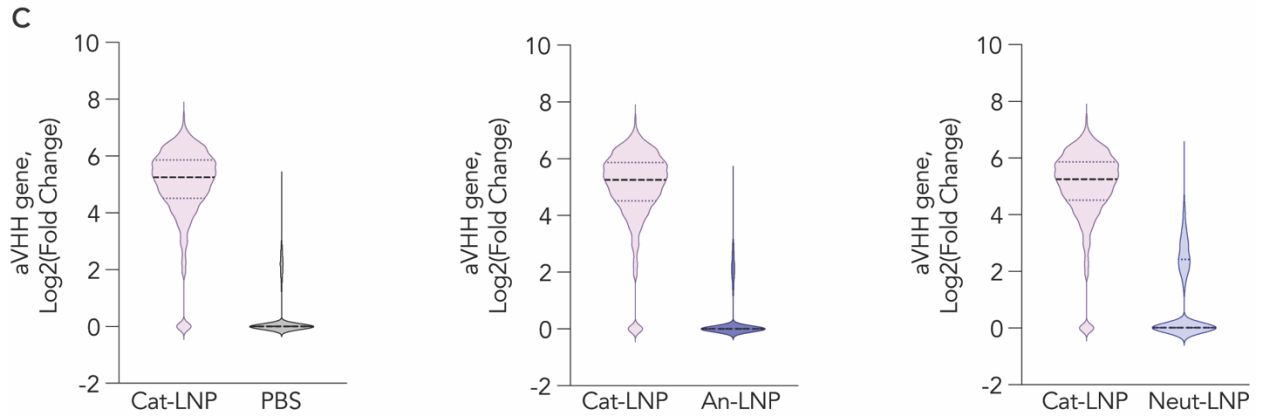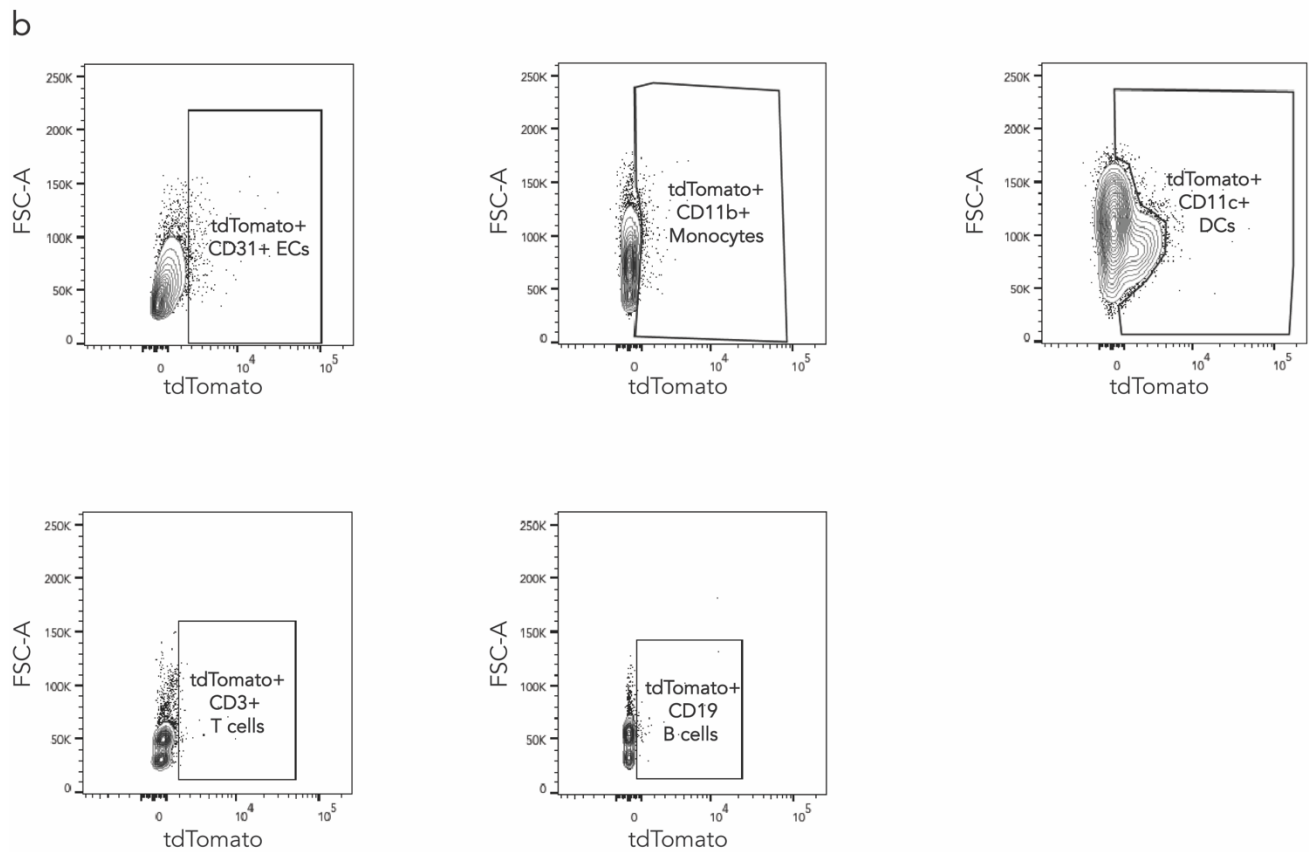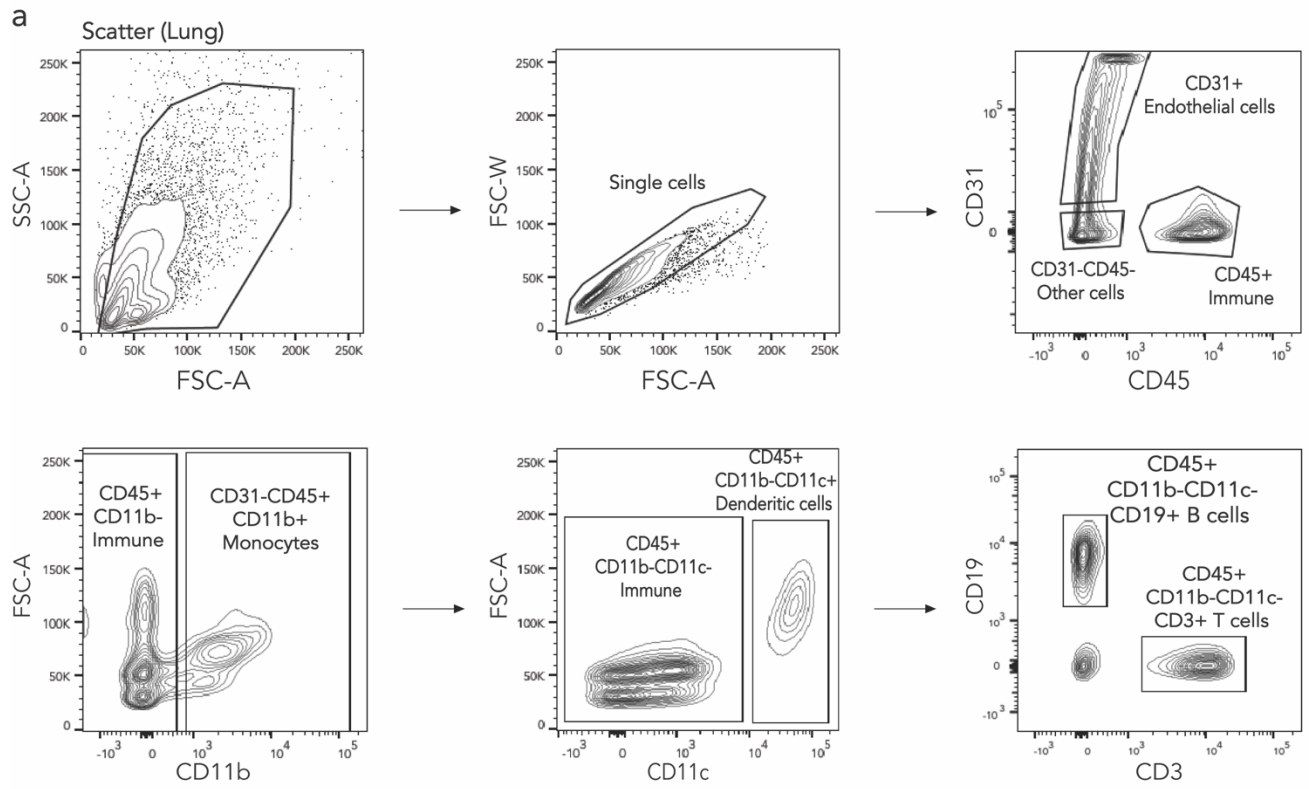
Supplementary Figure 20. (a) t-SNE plot showing the cells partitioned into 27 clusters. (b) Dot plot for cell marker and aVHH mRNA expressions in each cluster. (c) Cat-LNP showed about 32-fold higher aVHH mRNA expression compared to control groups (PBS, An-LNP, and Neu-LNP) in lung ECs.
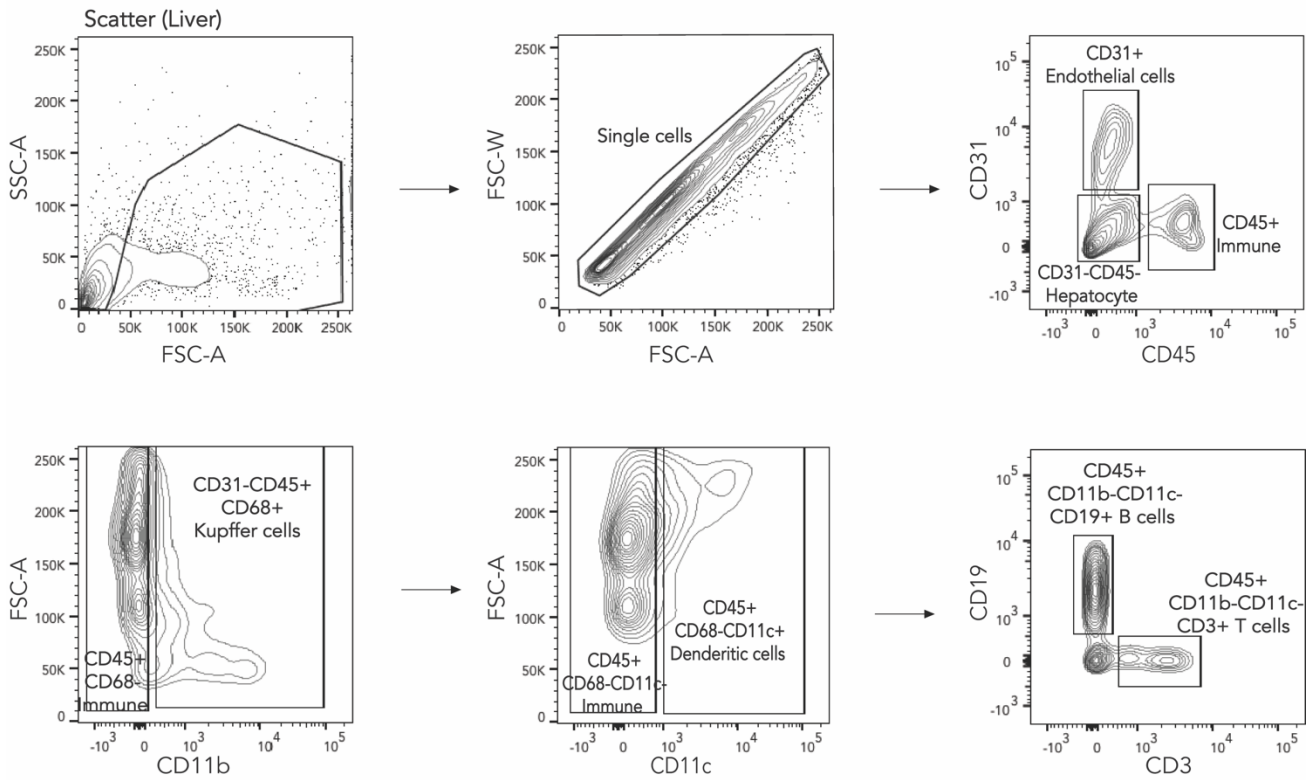
a   Cat-LNP vs PBS

b   Cat-LNP vs Neu-LNP

c   Cat-LNP vs An-LNP

35
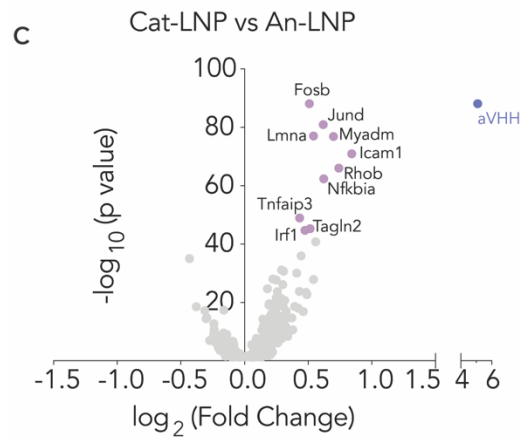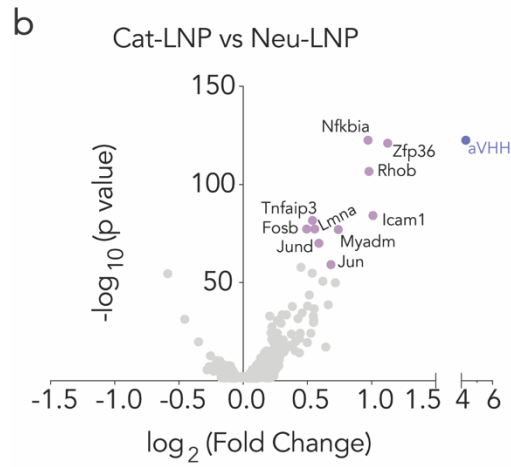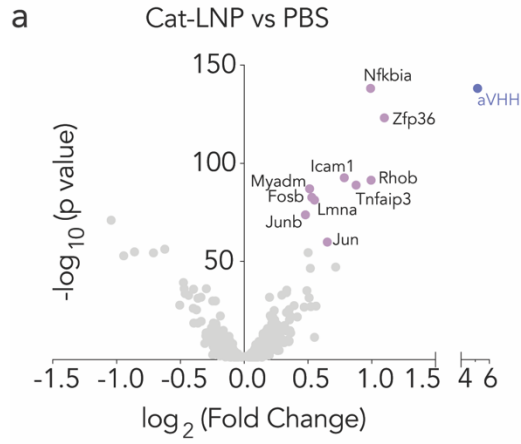
Supplementary Figure 21. Top 10 upregulated genes (purple) with Cat-LNP vs (a) PBS, (b) Neu-LNP, and (c) An-LNP P-value < 0.05.
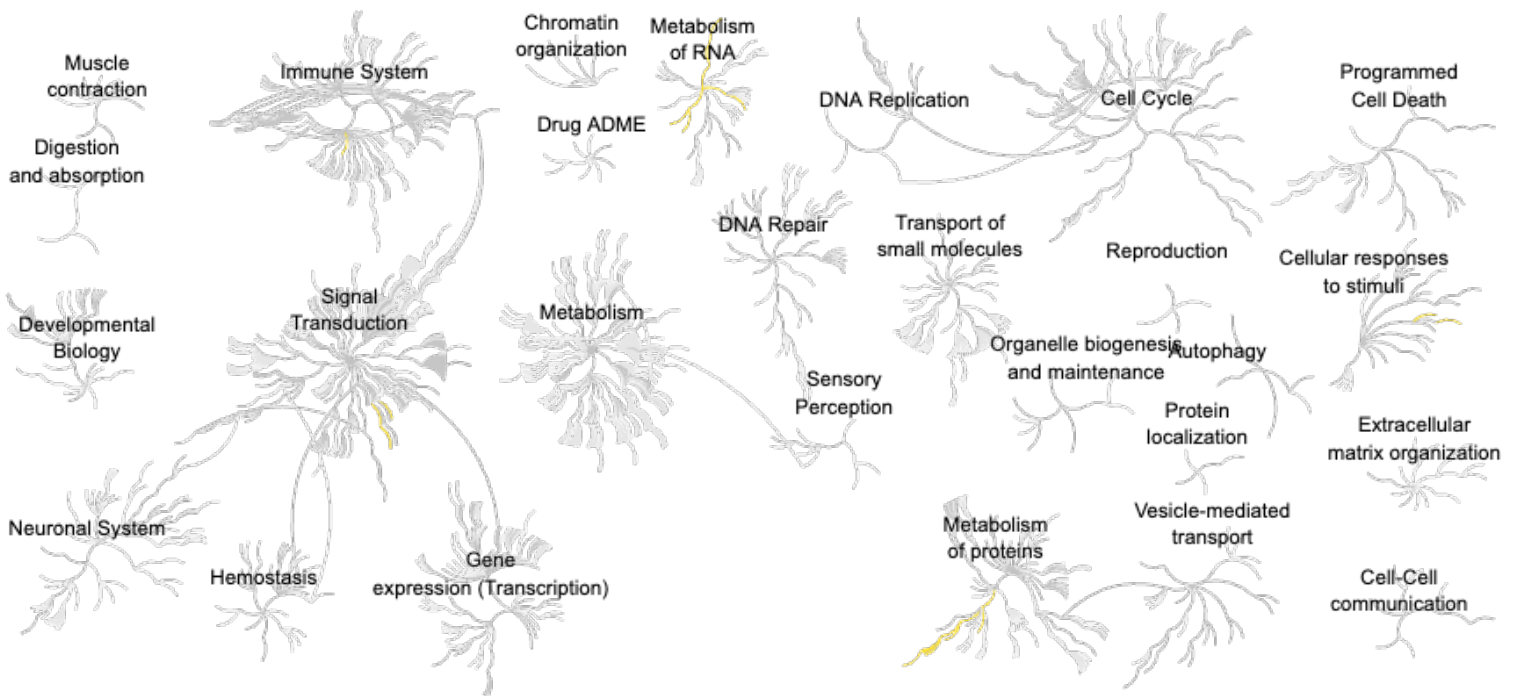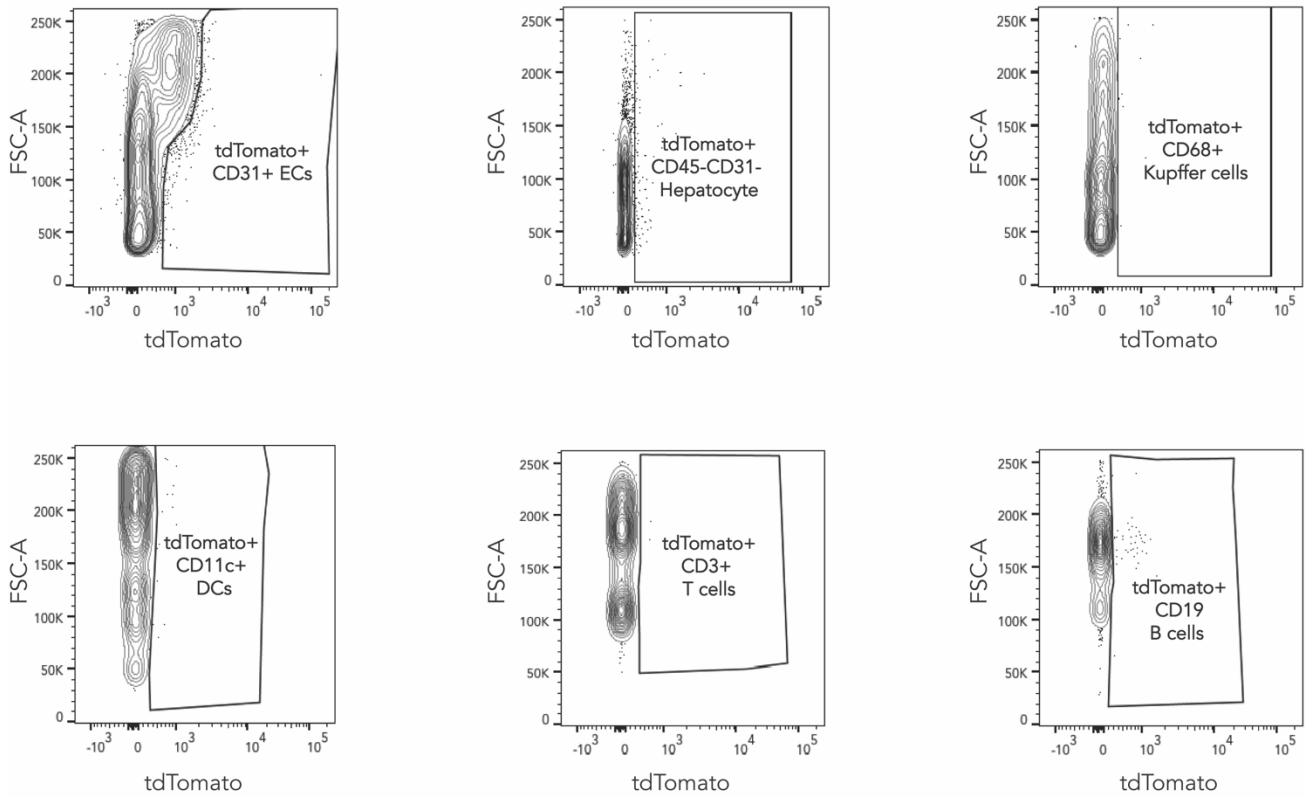

Supplementary Figure 22. Twenty-seven of 835 pathways were enriched in mice treated with Cat-LNP versus control groups (PBS, Neu-LNP, An-LNP), 19 of which were related to metabolism of RNA or protein, P-value < 0.001.
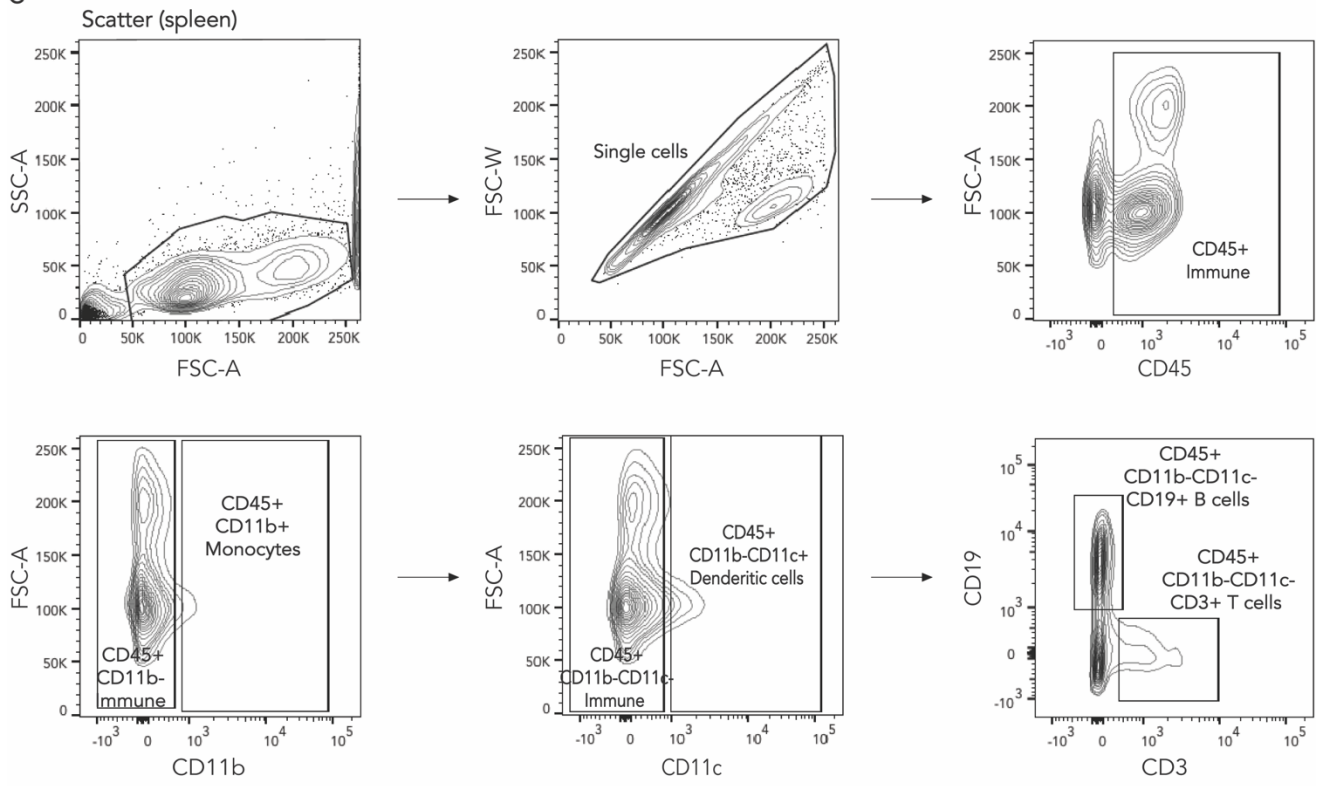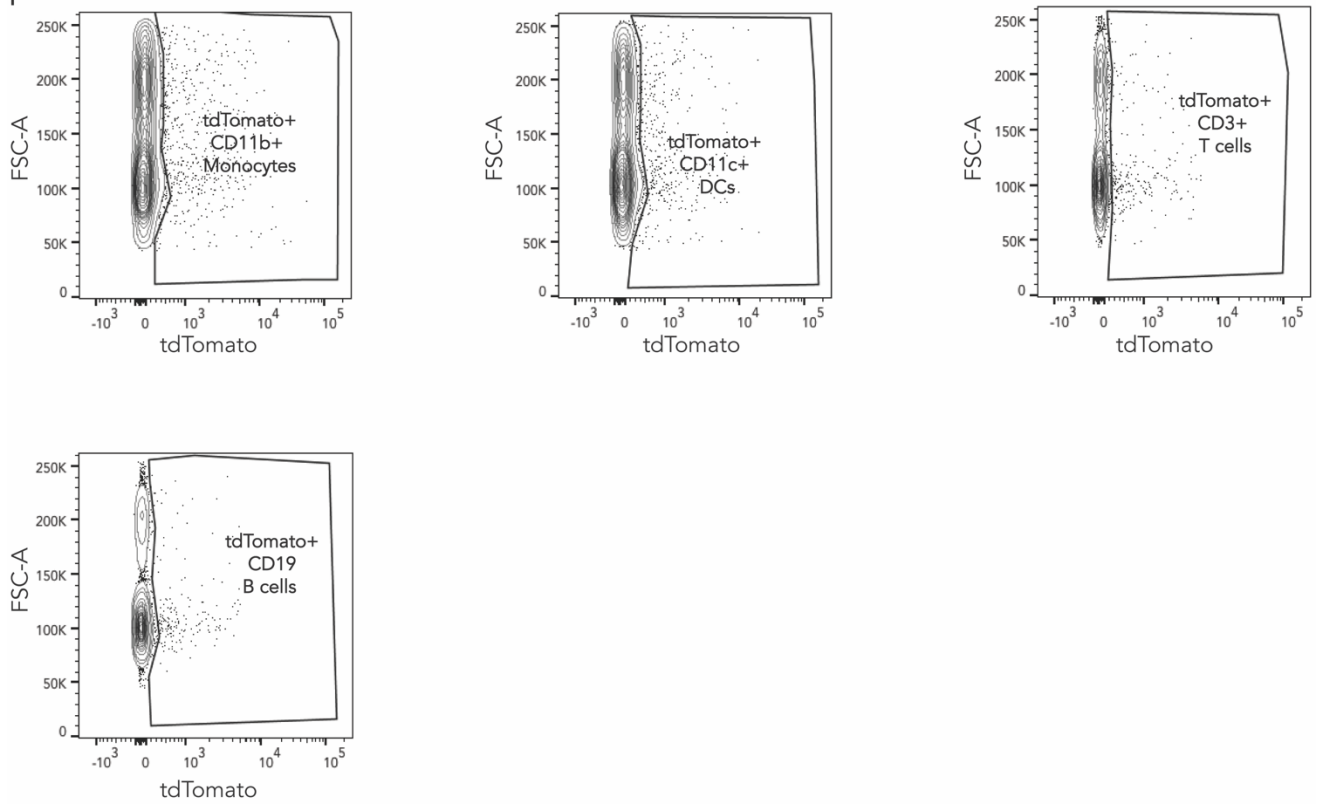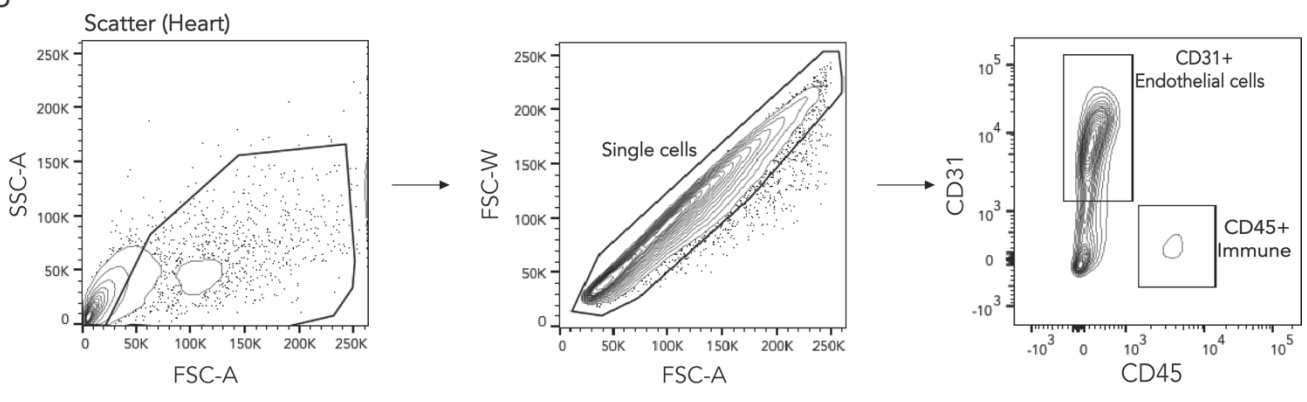
c

Scatter (Liver) — SSC-A vs FSC-A

Single cells — FSC-W vs FSC-A

CD31 vs CD45:
CD31+ Endothelial cells
CD31-CD45- Hepatocyte
CD45+ Immune

FSC-A vs CD11b:
CD31-CD45+ CD68+ Kupffer cells
CD45+ CD68- Immune

FSC-A vs CD11c:
CD45+ CD68-CD11c+ Denderitic cells
CD45+ CD68-CD11c- Immune

CD19 vs CD3:
CD45+ CD11b-CD11c- CD19+ B cells
CD45+ CD11b-CD11c- CD3+ T cells

d

FSC-A vs tdTomato: tdTomato+ CD31+ ECs

FSC-A vs tdTomato: tdTomato+ CD45-CD31- Hepatocyte

FSC-A vs tdTomato: tdTomato+ CD68+ Kupffer cells

FSC-A vs tdTomato: tdTomato+ CD11c+ DCs

FSC-A vs tdTomato: tdTomato+ CD3+ T cells
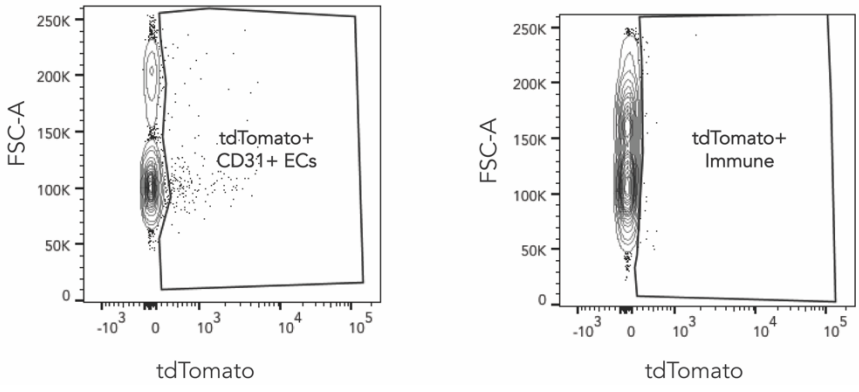
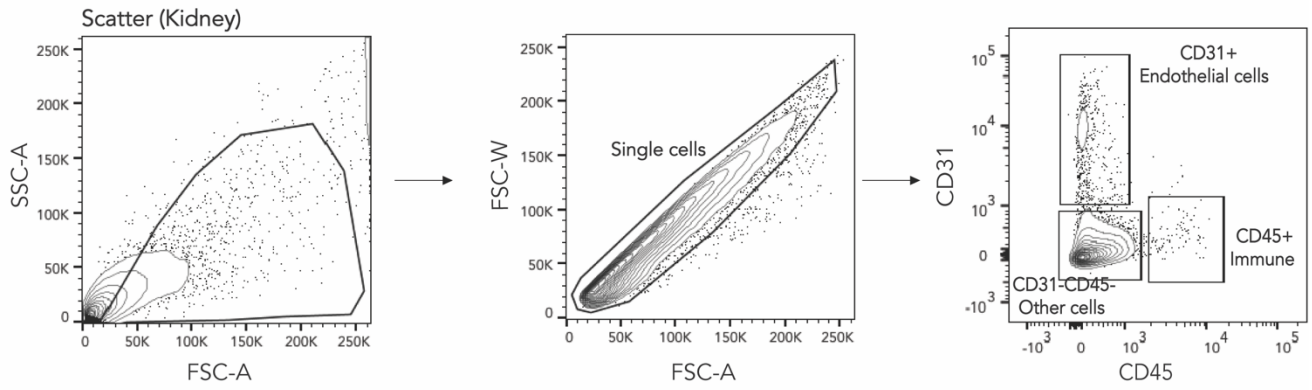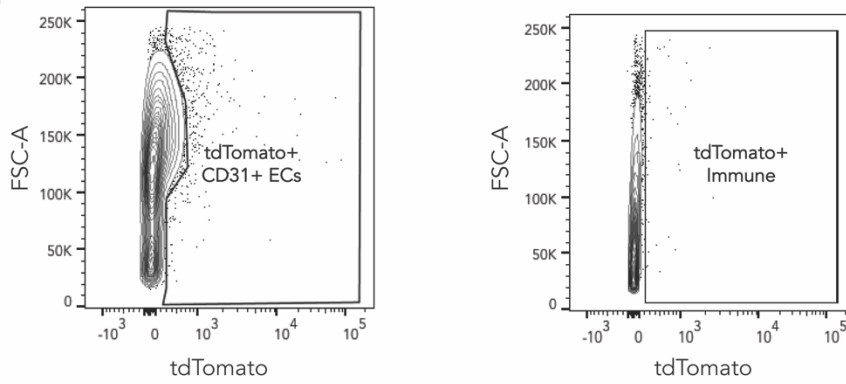FSC-A vs tdTomato: tdTomato+ CD19 B cells

g

Scatter (Heart)

Supplementary Figure 23. Representative gating strategies for FACS for cell types in the (a, b) lung, (c, d) liver, (e, f) spleen, (g, h) heart, and (i, j) kidney for the screens.