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

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Table of Contents

Supplementary Figures	1
Supplementary Fig. 1. Compositions for 128 LNPs and formulation parameters	1
Supplementary Fig. 2. Enrichment of lineage-negative cells from mouse bone marrow using magnetic-activated cell sorting (MACS)	3
Supplementary Fig. 3. UMAP representation of 21,618 single cells within the bone marrow	4
Supplementary Fig. 4. Single-cell multiomics-based screening of LNPs	5
Supplementary Fig. 5. Normalized delivery of LNPs to aVHH ⁺ Lin ⁻ bone marrow cells in mice.	6
Supplementary Fig. 6. Lead LNP selection	7
Supplementary Fig. 7. Compositions of LNP ⁶⁷ , LNP ⁹⁵ , LNP ¹⁰⁸ , and LP01 LNPs	9
Supplementary Fig. 8. aVHH expression in 24 cell types from the bone marrow, liver, lung, spleen, kidney, and heart after administration of LNP ⁶⁷ , LNP ⁹⁵ , or LNP ¹⁰⁸	10
Supplementary Fig. 9. aVHH expression in 21 cell types from the bone marrow, liver, lung, spleen, kidney, and heart after administration of LP01 LNP	13
Supplementary Fig. 10. Cryo-TEM images of LNP ⁶⁷ and LP01 LNPs carrying mRNA	15
Supplementary Fig. 11. Sixty-six serum plasma biomolecules adsorbed to the surface of LNP ⁶⁷ and LP01 LNPs	16
Supplementary Fig. 12. Serum chemokine, cytokine, and acute-phase protein expression after LNP ⁶⁷ administration	17
Supplementary Fig. 13. LNP ⁶⁷ delivers mRNA to transcriptionally defined HSCs in vivo ..	20
Supplementary Fig. 14. Optimization of excipients and freeze-thawing conditions	23
Supplementary Fig. 15. Physical characteristics of LNP ⁶⁷ with different cargo mRNAs and endotoxin testing of LNP ⁶⁷	24
Supplementary Fig. 16. Cell types in human bone marrow cells and flow cytometry gating strategy for quantifying aVHH expression.....	25
Supplementary Fig. 17. Mean aVHH expression from LNP ⁶⁷ -treated primary bone marrow cells.....	28
Supplementary Fig. 18. Serum cytokine responses from rhesus macaque after administration of LNP ⁶⁷	30
Supplementary Fig. 19. Flow cytometry gating strategy for quantifying aVHH expression in mouse cells	31
Reference	36

Supplementary Figures

a 128 LNP library design

8 different molar ratios

Ratio	1	2	3	4	5	6	7	8
Ionizable lipid	50	45	35	50	45	45	35	30
Cholesterol	35.0	37.5	47.5	17.5	22.5	17.5	18.0	30.0
PEG-lipid	5.0	2.5	2.5	2.5	2.5	2.5	2.5	1.5
Helper lipid (mol%)	10	15	15	30	30	35	44.5	38.5

4 different PEG-lipids

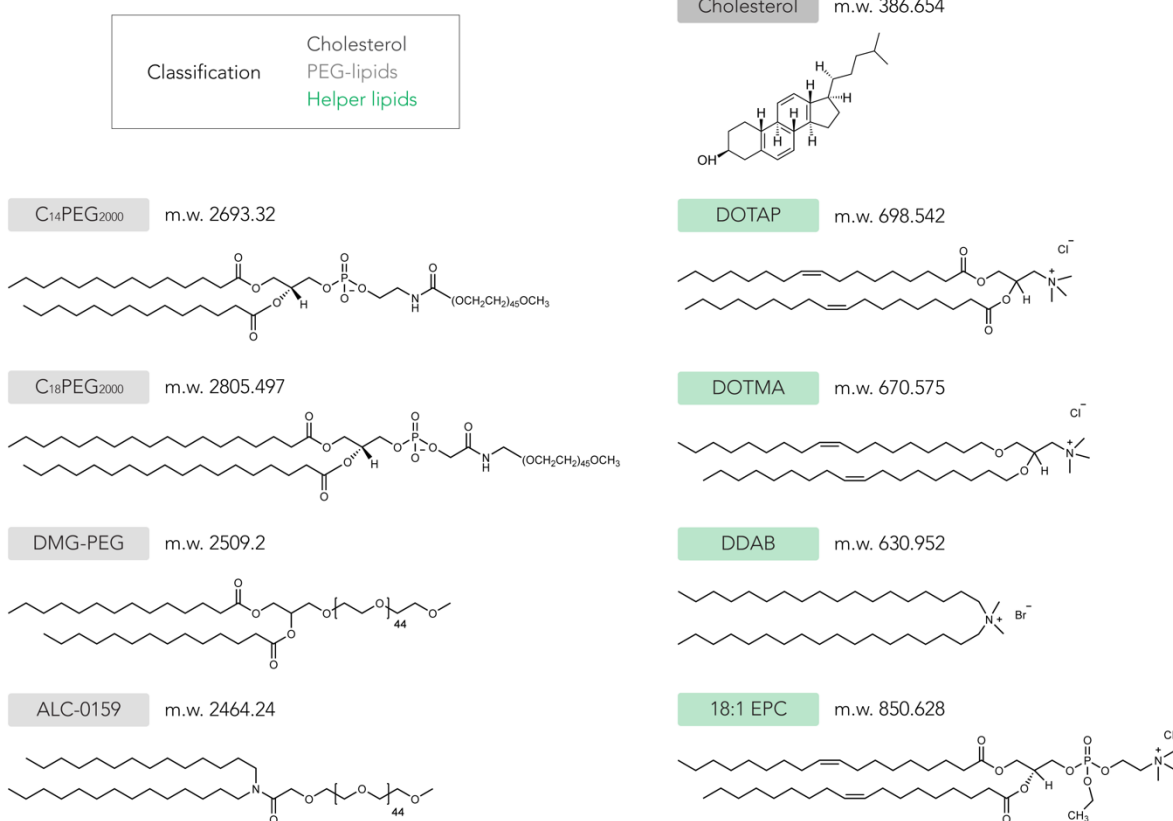
PEG ID	PEG
1	C ₁₄ PEG ₂₀₀₀
2	C ₁₈ PEG ₂₀₀₀
3	DMG-PEG
4	ALC-0159

4 different helper lipids

HL ID	HL
1	DDAB
2	DOTMA
3	DOTAP
4	18:1 EPC

Ratio	LNP ID															
1	1	9	17	25	33	41	49	57	65	73	81	89	97	105	113	121
2																
3																
4																
5	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓	↓
6																
7																
8	8	16	24	32	40	48	56	64	72	80	88	96	104	112	120	128
PEG ID	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
HL ID		1				2				3				4		

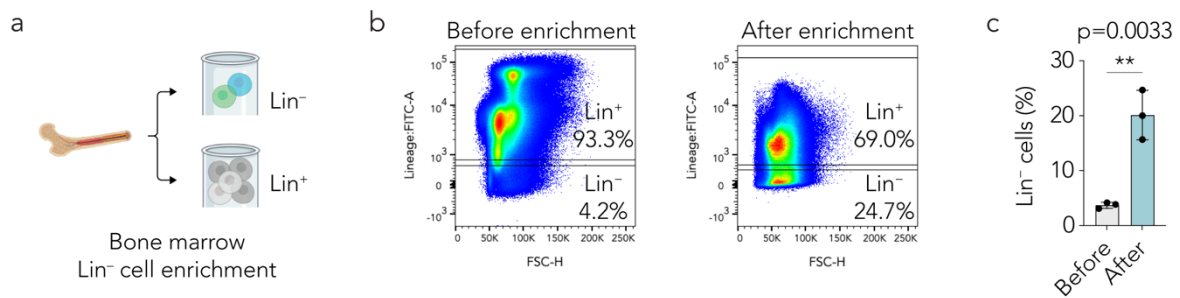
b Chemical structures and molecular weights



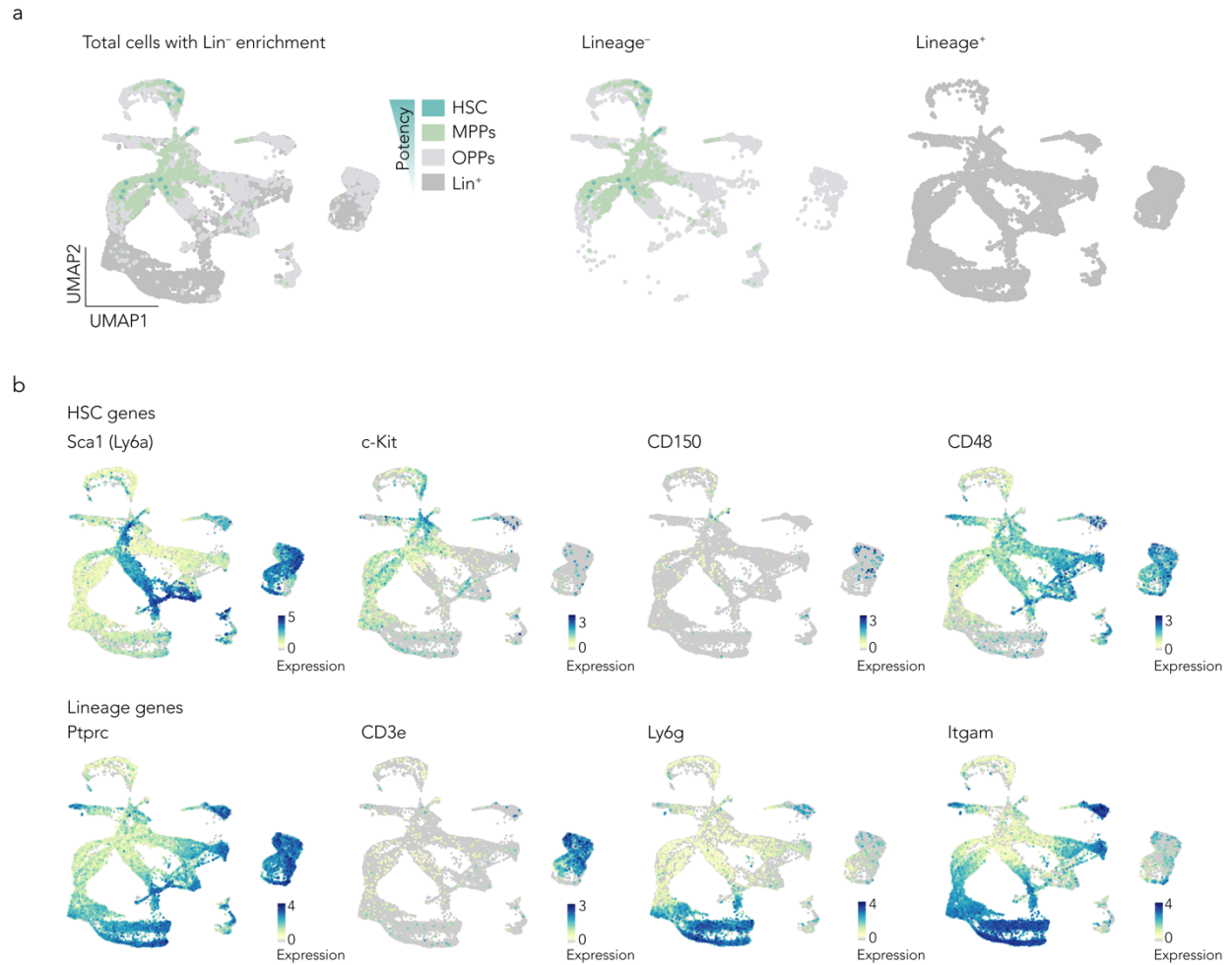
c Formulation parameters

System	Ring micromixer (NanoAssemblr NxGen Mixer; Spark)
Relative flow rate	3:1 (aqueous phase:ethanol phase)
Buffer for aqueous phase	25 mM sodium acetate buffer, pH 5
Mass ratio	10:1 (Lipid:RNA)

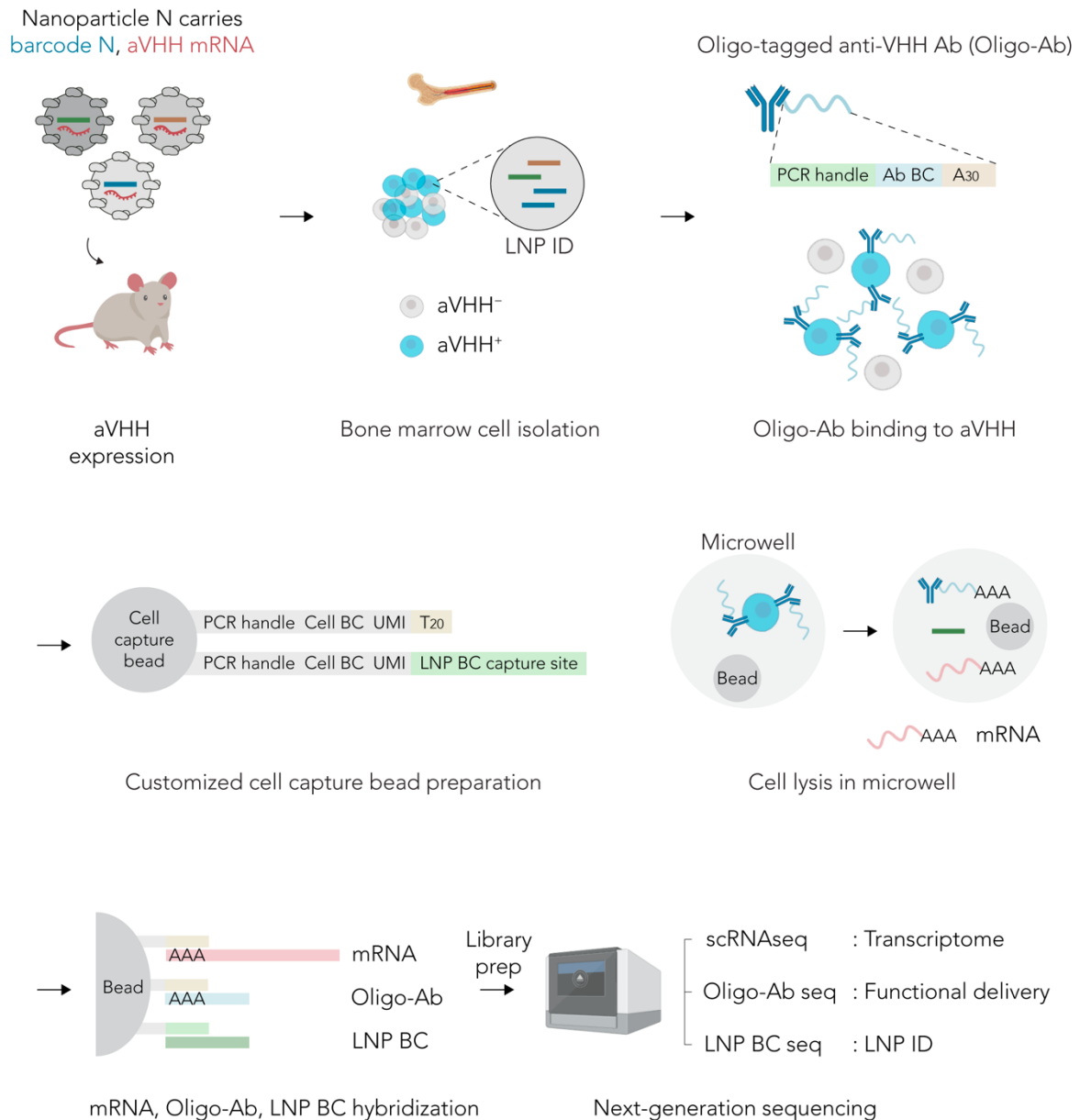
Supplementary Fig. 1 | Compositions for 128 LNPs and formulation parameters. **a**, LNPs were formulated with an ionizable lipid, cholesterol, PEG-lipid, and helper lipid. Formulations were varied by 4 helper lipids, 4 PEG-lipids, and 8 molar ratios. **b**, Chemical structures and molecular weights of lipids used. **c**, Other formulation parameters.



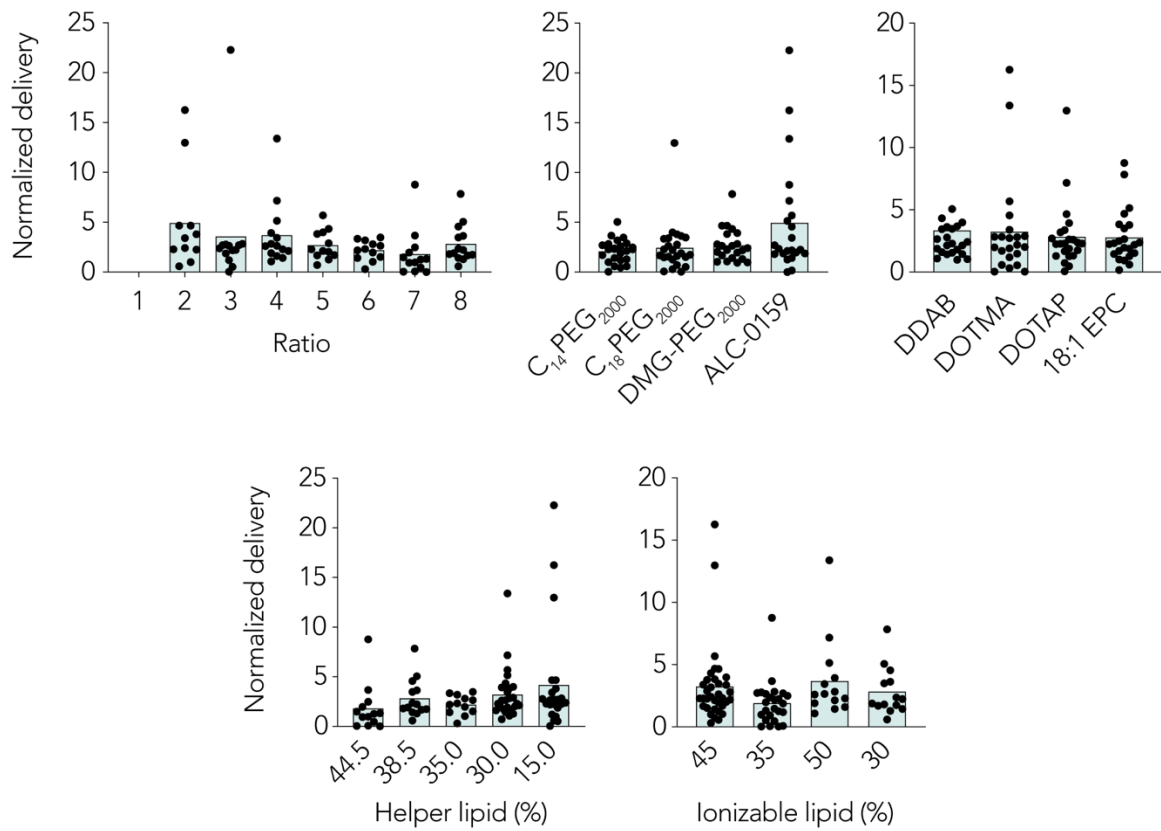
Supplementary Fig. 2 | Enrichment of lineage-negative cells from mouse bone marrow using magnetic-activated cell sorting (MACS). **a**, Mouse bone marrow cells were isolated and labeled with FITC-conjugated anti-mouse lineage cocktail. Then, FITC^{low} population was enriched using MACS. This figure was generated in part using graphics created by [Biorender.com](https://www.biorender.com). **b**, The representative quantification of enrichment. **c**, Lineage-negative cell percentage before and after enrichment (N=3, biological replicates). Data are presented as mean $\pm$ SD. ** $p < 0.01$, analyzed by two-tailed unpaired student's t-test.



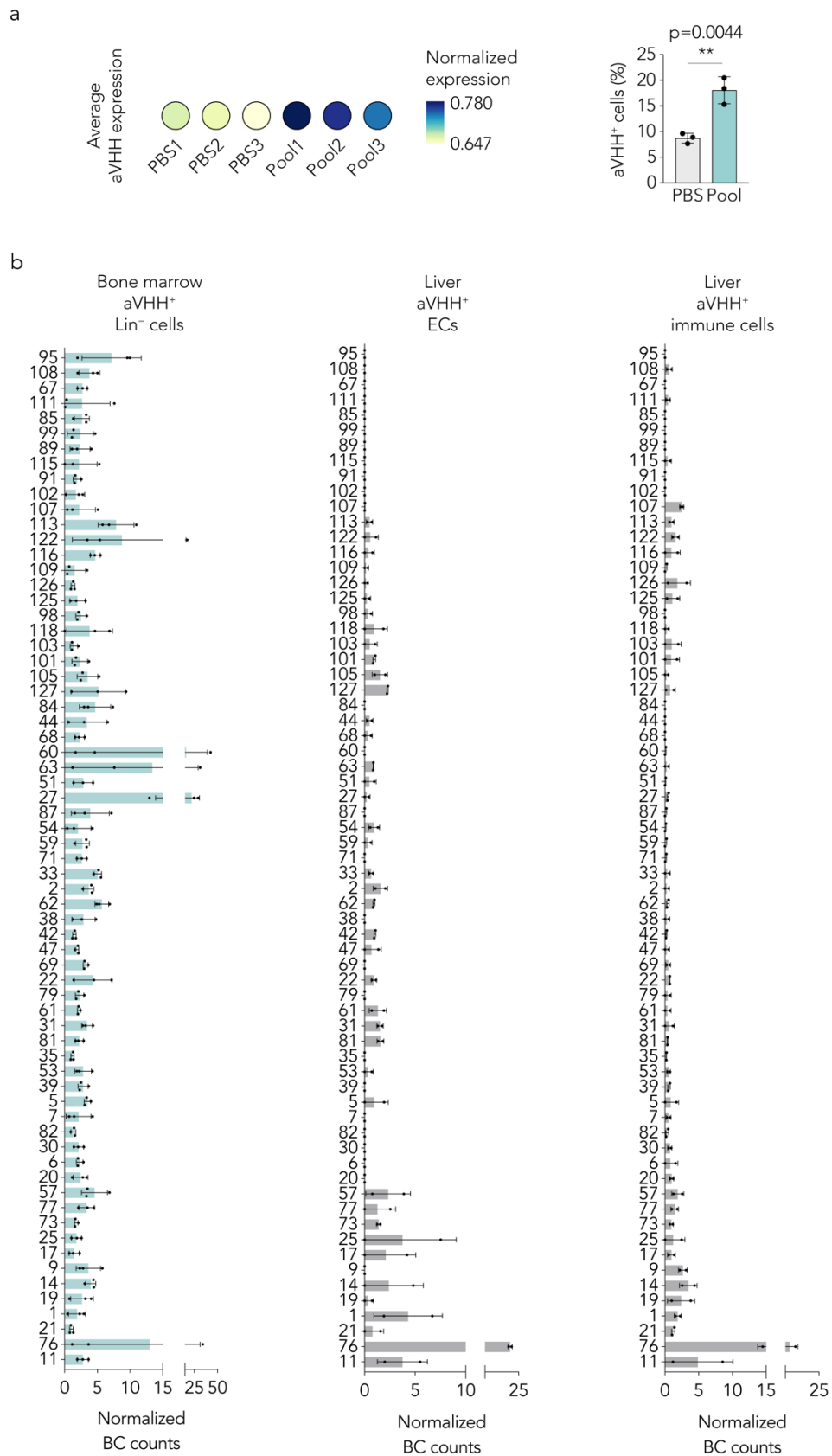
Supplementary Fig. 3 | UMAP representation of 21,618 single cells within the bone marrow. a, Transcriptionally defined lineage-positive and lineage-negative cell populations (Lin⁻). Total mouse bone marrow cells were enriched for Lin⁻ population. **b**, Expression levels of HSC (Sca1 (Ly6a), cKit, CD150, and CD48) and lineage (Ptprc, CD3e, Ly6g, and Itgam) markers used for identifying cell identities are shown.



Supplementary Fig. 4 | Single-cell multiomics-based screening of LNPs. Oligo-tagged anti-VHH antibody (Oligo-Ab) was used to quantify aVHH expression by sequencing reads (methods adapted from CITE-seq¹). Cell capture beads were modified with LNP barcode (BC) capture site to capture barcodes from LNP delivery (methods adapted from SENT-seq²). This figure was generated in part using graphics created by [Biorender.com](https://biorender.com).



Supplementary Fig. 5 | Normalized delivery of LNPs to aVHH⁺Lin⁻ bone marrow cells in mice. Data were represented as a function of molar ratio, PEG content, helper lipid content, helper lipid molar percentage, and ionizable lipid molar percentage.

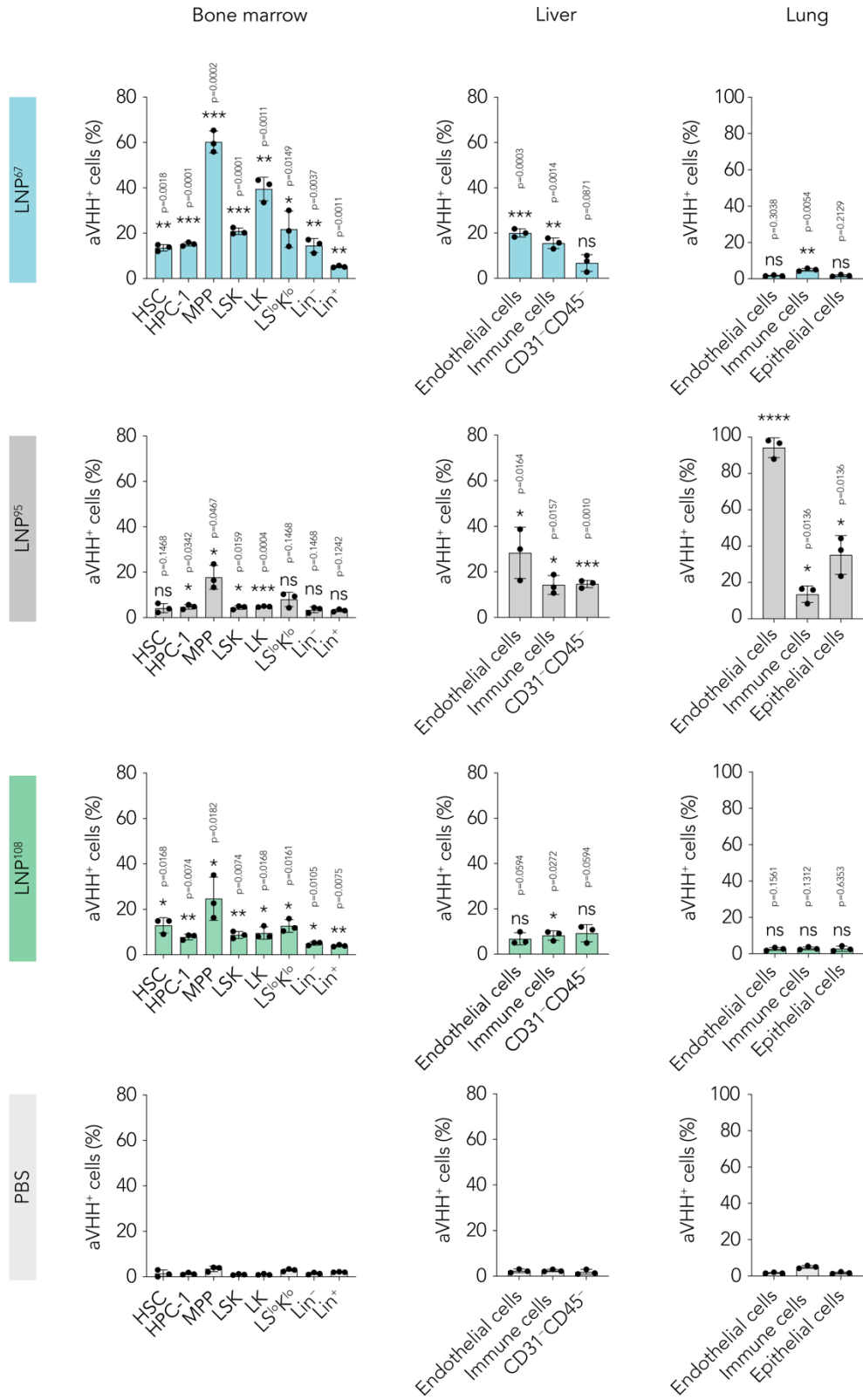


Supplementary Fig. 6 | Lead LNP selection. **a**, Average aVHH expression in 21,618 cells from bone marrow after administration of LNP pool. ** $p < 0.01$, analyzed by unpaired two-tailed student's t-tests. **b**, Normalized barcode counts in aVHH⁺Lin⁻ cells from bone marrow, CD31⁺CD45⁻ cells (endothelial cells, ECs), and CD31⁻CD45⁺ cells (immune cells) from liver. LNPs that have barcode count above 20 are shown. Two naked (unencapsulated) barcodes were included in the pool and their counts were also below 20. Data are presented as mean $\pm$ SD (bone marrow: N=3, liver: N=2, biological replicates).

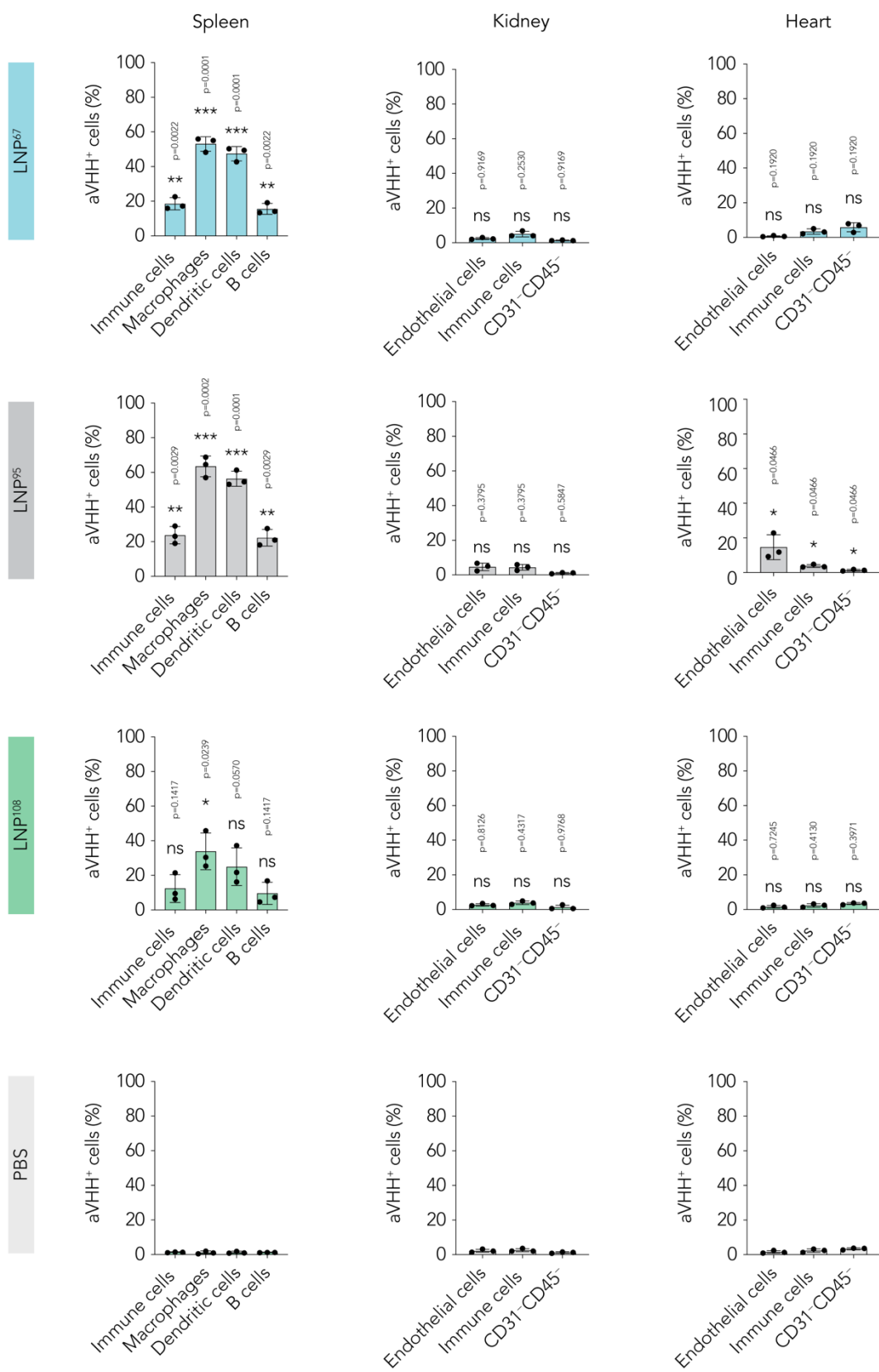
LNP	Ionizable lipid	Cholesterol	PEG-lipid	Helper lipid
LNP ⁶⁷	IL (35)	47.5	C ₁₄ PEG ₂₀₀₀ (2.5)	DOTAP (15)
LNP ⁹⁵	IL (50)	17.5	ALC-0159 (2.5)	DOTAP (30)
LNP ¹⁰⁸	IL (45)	37.5	C ₁₈ PEG ₂₀₀₀ (2.5)	18:1 EPC (15)
LP01 LNP	LP01 (45)	44.0	DMG-PEG (2.0)	DSPC (9)

Supplementary Fig. 7 | Compositions of LNP⁶⁷, LNP⁹⁵, LNP¹⁰⁸, and LP01 LNPs. Mol% is indicated in parentheses.

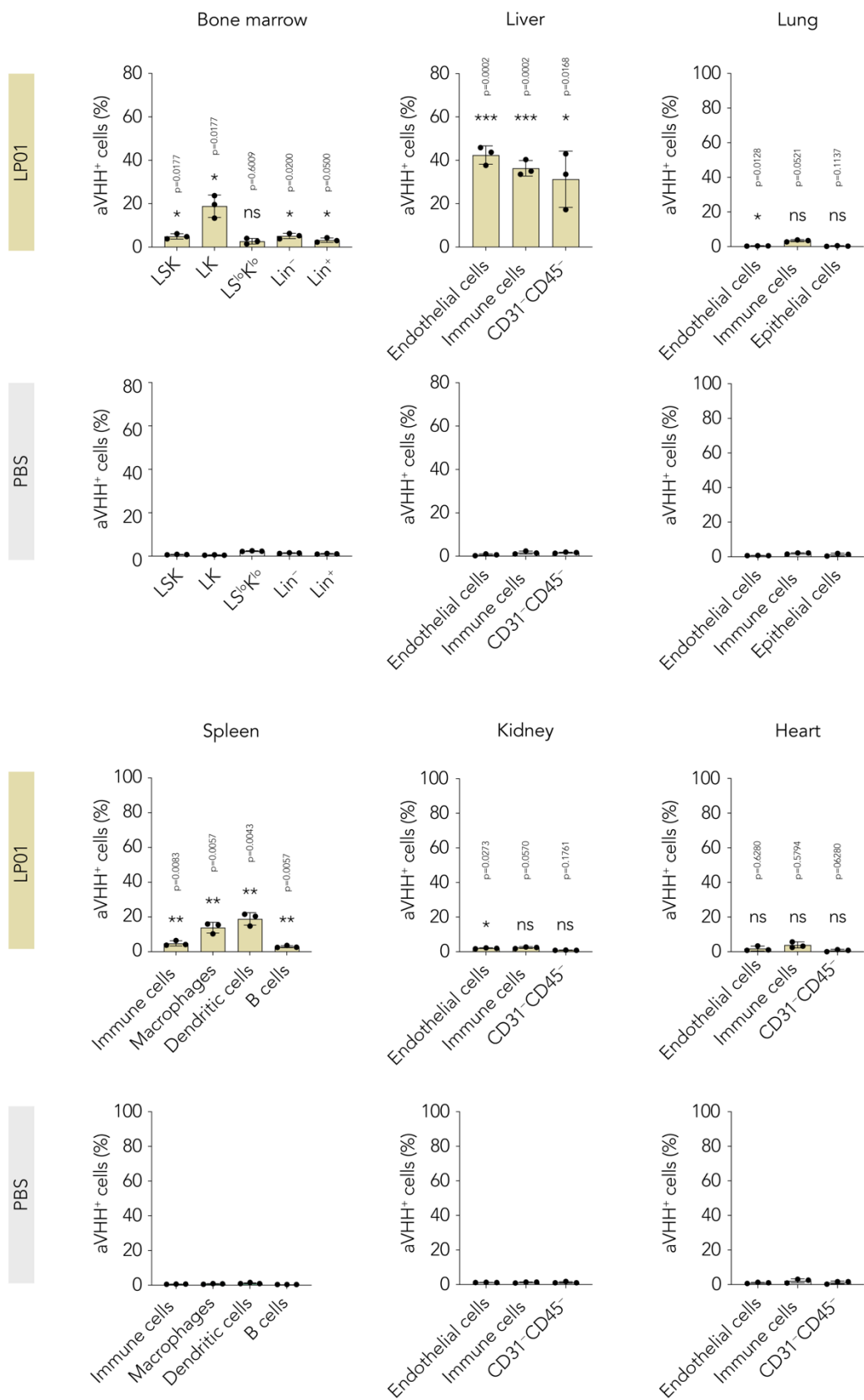
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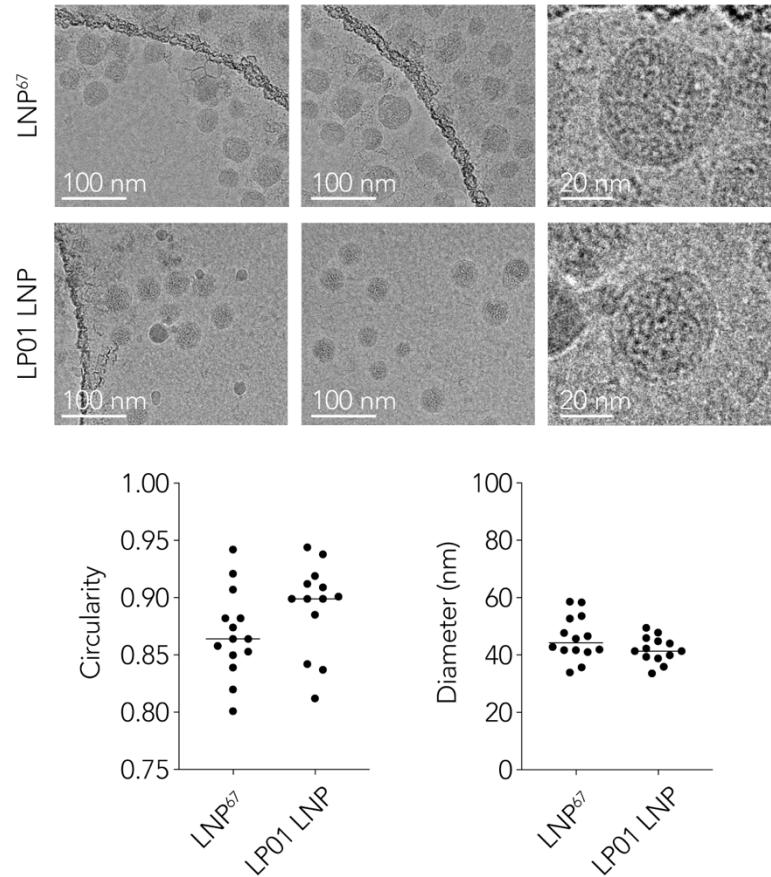
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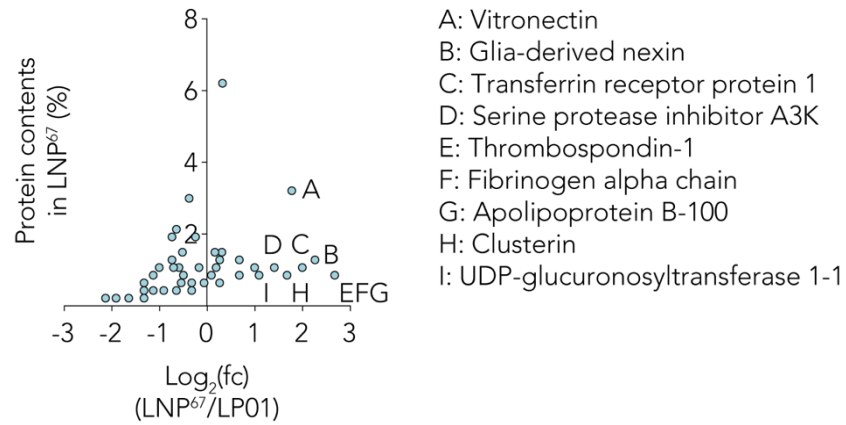
Supplementary Fig. 8 | aVHH expression in 24 cell types from the bone marrow, liver, lung, spleen, kidney, and heart after administration of LNP⁶⁷, LNP⁹⁵, or LNP¹⁰⁸. The percentage of aVHH⁺ cells in each subtype in **a**, bone marrow, liver, and lung, and **b**, spleen, kidney, and heart. Data are presented as mean $\pm$ SD (N = 3, biological replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, and ns = not significant compared to PBS, analyzed by multiple unpaired t-tests (Holm-Sidak's). Individual variance was assumed for each cell type.



Supplementary Fig. 9 | aVHH expression in 21 cell types from the bone marrow, liver, lung, spleen, kidney, and heart after administration of LP01 LNP. Data are presented as mean $\pm$ SD (N = 3, biological replicates). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and ns = not significant compared to PBS, analyzed by multiple unpaired t-tests (Holm-Sidak's). Individual variance was assumed for each cell type.

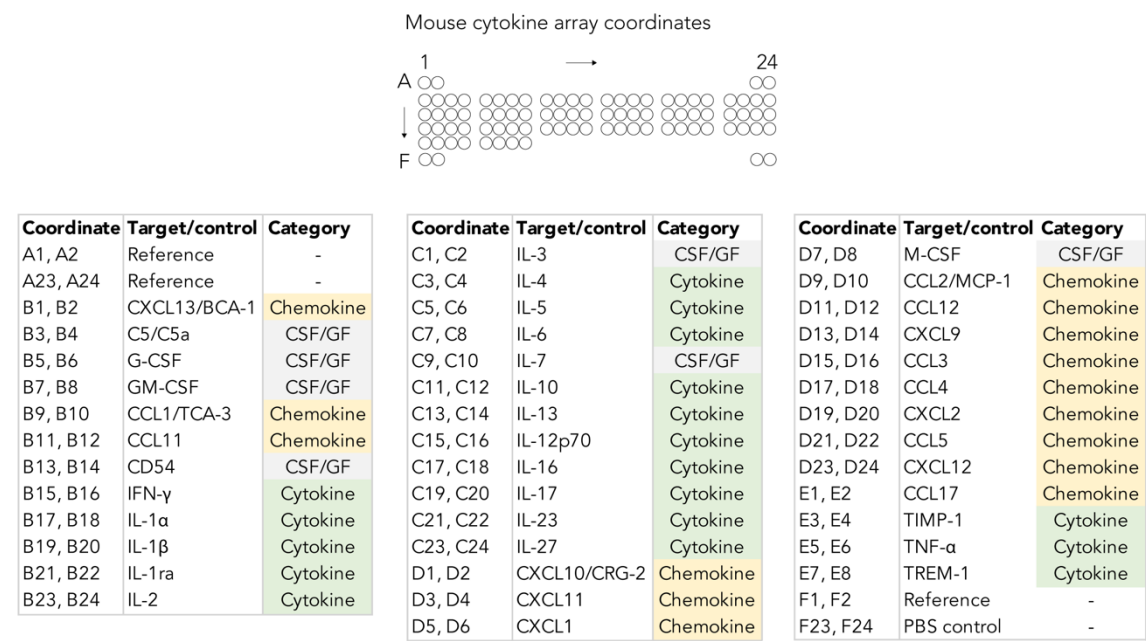


Supplementary Fig. 10 | Cryo-TEM images of LNP⁶⁷ and LP01 LNPs carrying mRNA. Circularity and diameter were measured by ImageJ software. 14 LNP⁶⁷ and 13 LP01 LNPs in the images were analyzed. Lines indicate median value. 4 wide-field (3,000x) and 10 high magnification (40,000x or 80,000x) images were collected, and the representative images are shown.

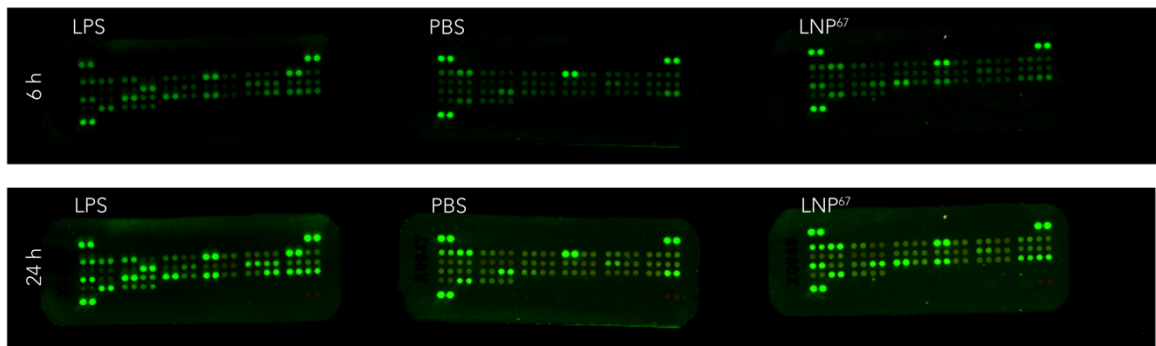


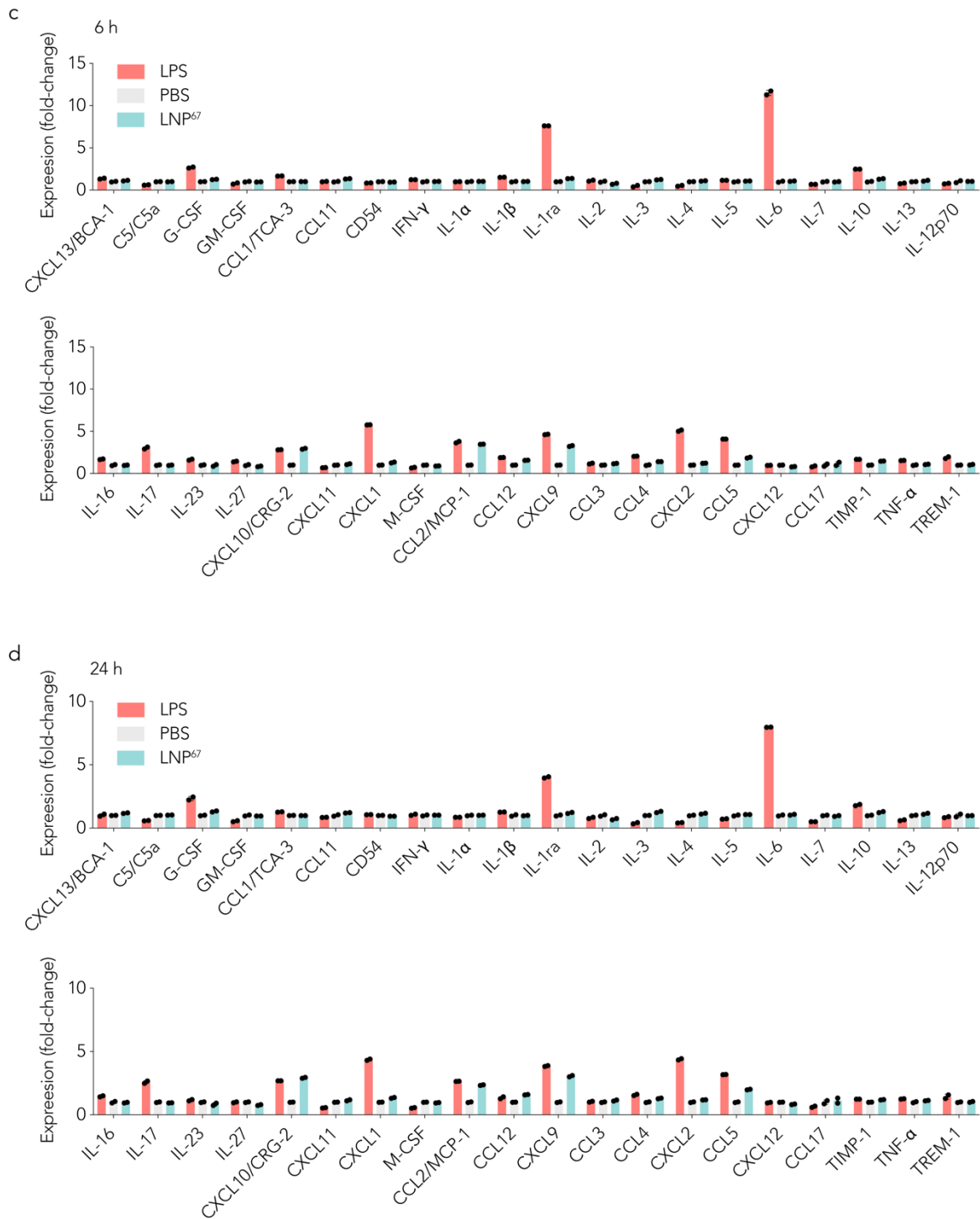
Supplementary Fig. 11 | Sixty-six serum plasma biomolecules adsorbed to the surface of LNP⁶⁷ and LP01 LNPs. The nine molecules with more than a twofold higher abundance in LNP⁶⁷ compared to LP01 LNPs were labeled (A-I, ranked based on abundance). fc: fold-change.

a



b

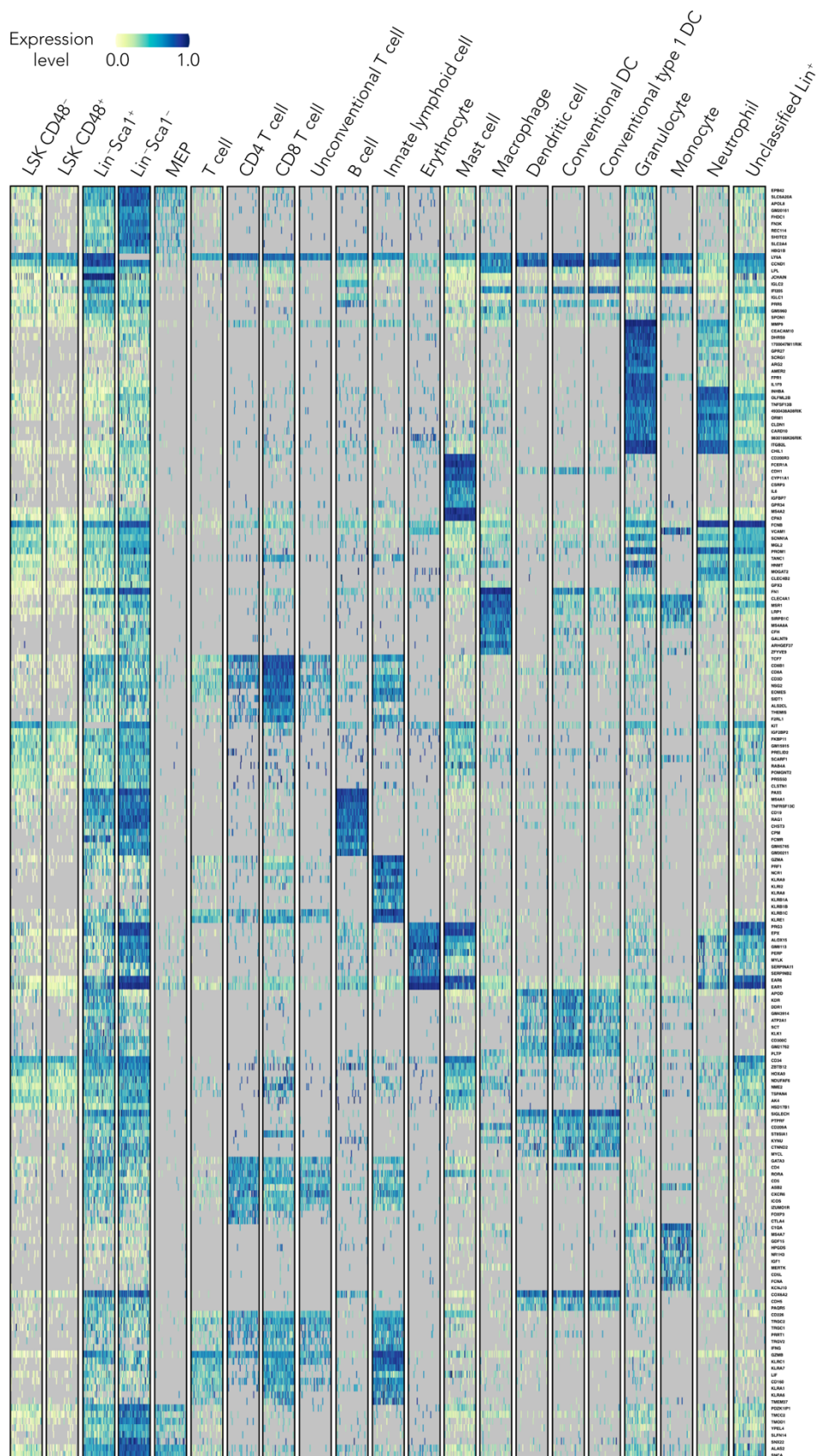




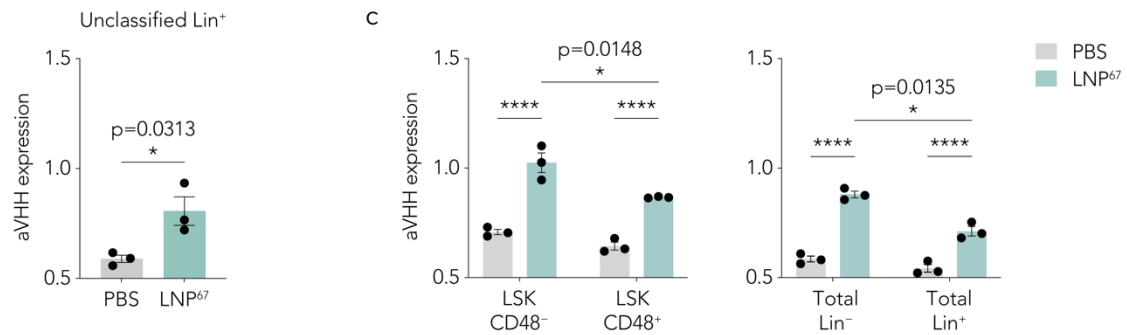
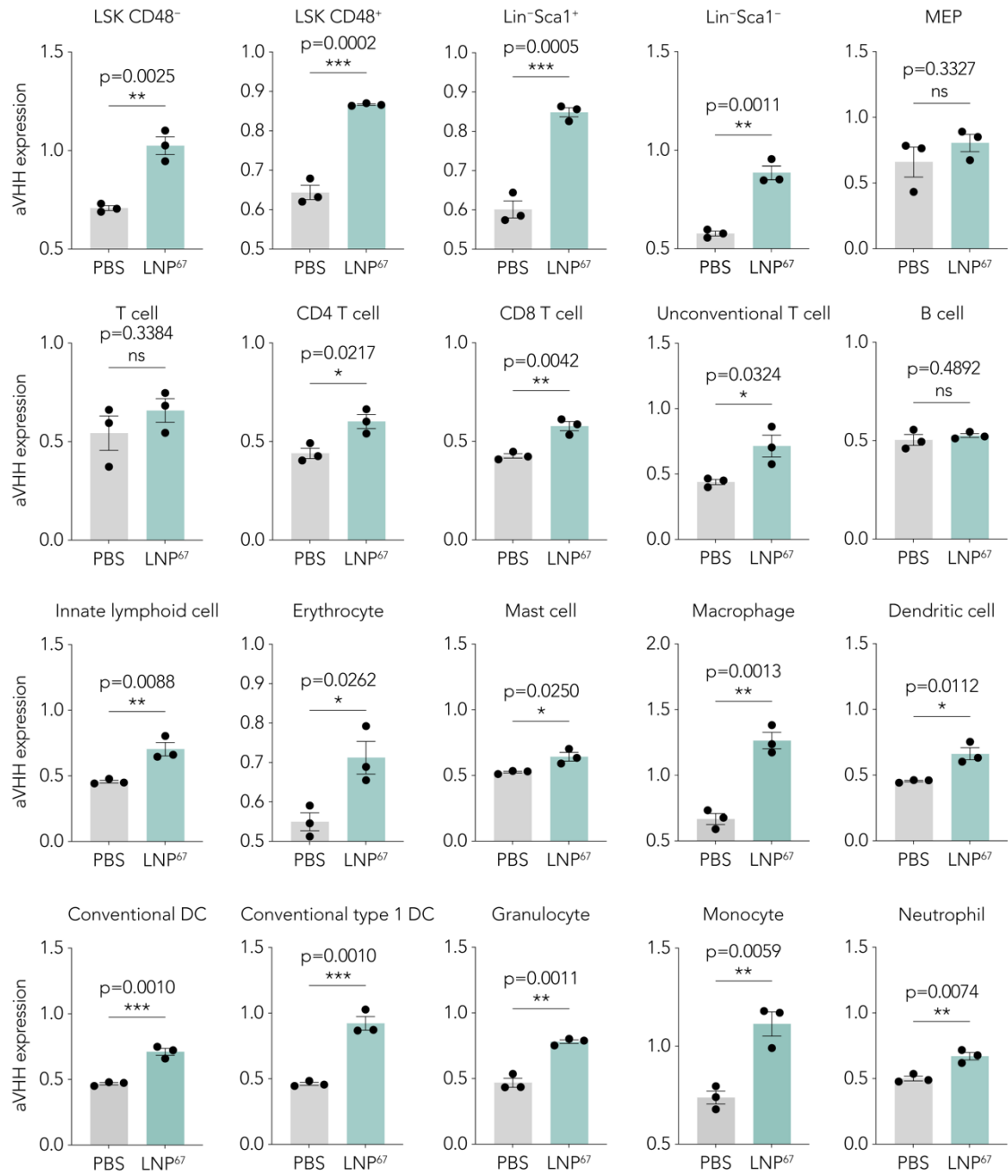
Supplementary Fig. 12 | Serum chemokine, cytokine, and acute-phase protein expression after LNP⁶⁷ administration. **a**, Mouse cytokine array coordinates. **b**, At 6 h and 24 h after administration of LPS (1 mg/kg), PBS, or LNP⁶⁷ (1.5 mg/kg), serum cytokine expression was measured. Raw images of Proteome Profiler Mouse Cytokine Array are shown. **c-d**, Cytokine expression levels in fold change compared to PBS group. Expression levels were quantified by

measuring intensities in **b** with ImageJ software. Data are presented as mean $\pm$ SD. N=3 biological replicates were pooled, and cytokine levels were measured twice.

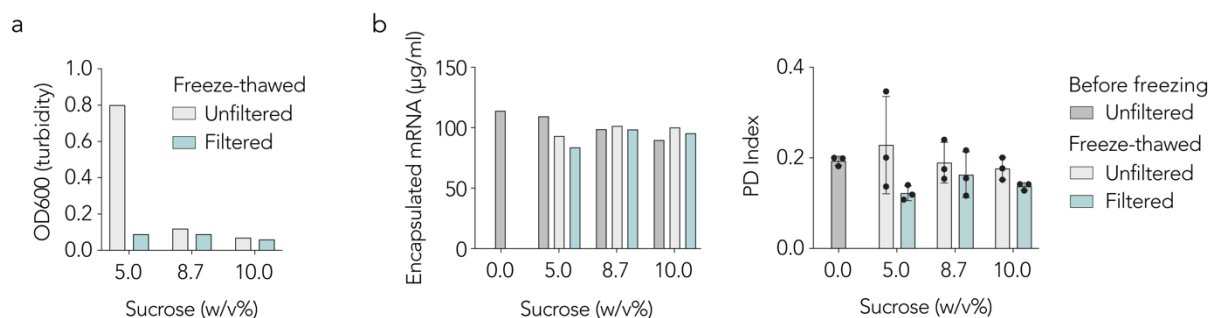
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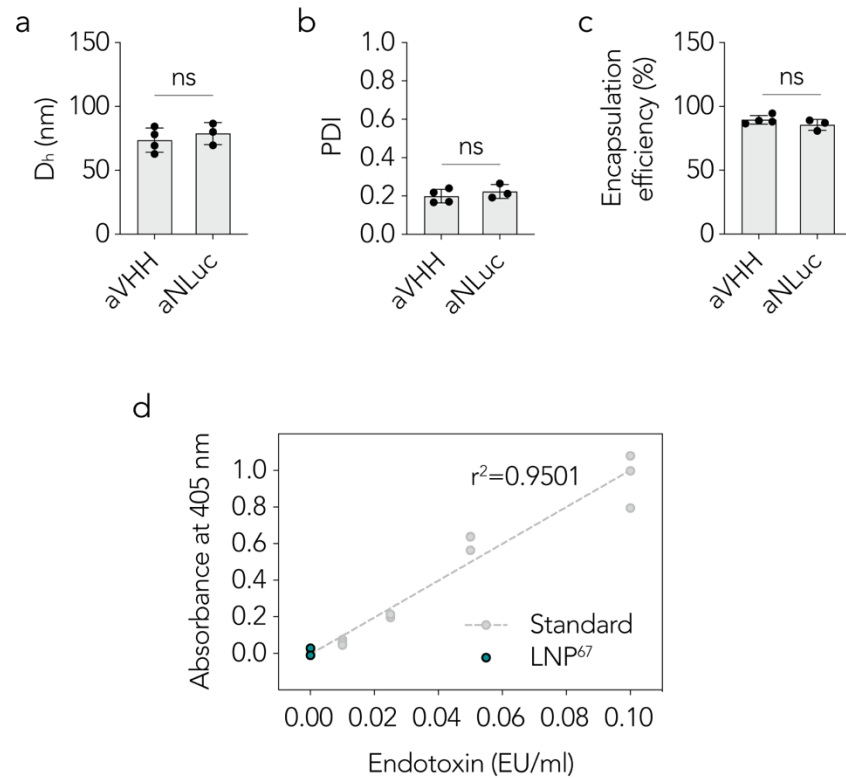
b



Supplementary Fig. 13 | LNP⁶⁷ delivers mRNA to transcriptionally defined HSCs *in vivo*. **a**, Heatmap for visualization of the single-cell expression pattern of cell-type-specific gene markers. **b**, aVHH protein expression in all 21 clusters. **c**, aVHH protein expression levels in LSK CD48⁻, LSK CD48⁺, total lineage⁻, and total lineage⁺ cells. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$ analyzed by two-tailed unpaired student's t-test (LNP⁶⁷ compared to PBS, N = 3, biological replicates). Data are presented as mean $\pm$ SEM for **b** and **c**.



Supplementary Fig. 14 | Optimization of excipients and freeze-thawing conditions. 5.0%, 8.7%, or 10.0% (w/v) sucrose was tested. **a**, Optical density at 600 nm (OD600) after freeze-thawing. 5.0% showed a high turbidity after freeze-thawing, indicating aggregates or precipitates in solution. **b**, Encapsulated mRNA ($\mu\text{g/ml}$) and PD index before and after freezing and filtering with 0.22 μm filters. 5% showed decreased encapsulated mRNA after filtering followed by freeze-thawing. 10% showed the highest stability upon freeze-thawing (minimal changes in turbidity, encapsulated mRNA, and PD index). Data are presented as mean $\pm$ SD (N=3, independent batches).

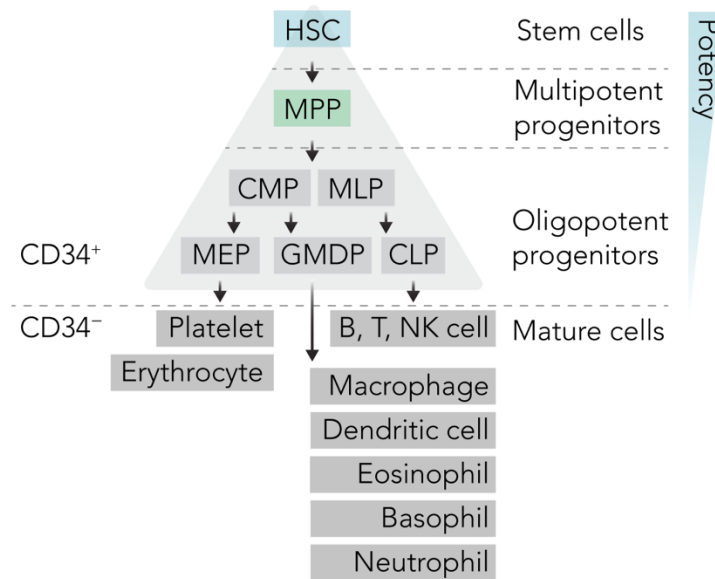


Supplementary Fig. 15 | Physical characteristics of LNP⁶⁷ with different cargo mRNAs and endotoxin testing of LNP⁶⁷. **a-c**, Cargo changes did not affect the physical characteristics of LNP⁶⁷. **a**, Hydrodynamic diameter (D_h , nm), **b**, polydispersity index (PD index), and **c**, encapsulation efficiency (%) of LNP⁶⁷ carrying aVHH (N=4, independent batches) or aNLuc (N=3, independent batches) mRNA. ns: Not significant, analyzed by unpaired two-tailed t-test. Data are presented as mean $\pm$ SD. **d**, Endotoxin testing for LNP⁶⁷. Chromogenic endotoxin quantification method was used with 0.01–0.1 EU/mL range (N=3, independent batches). LNP⁶⁷ endotoxin level was below 0.01 EU/mL.

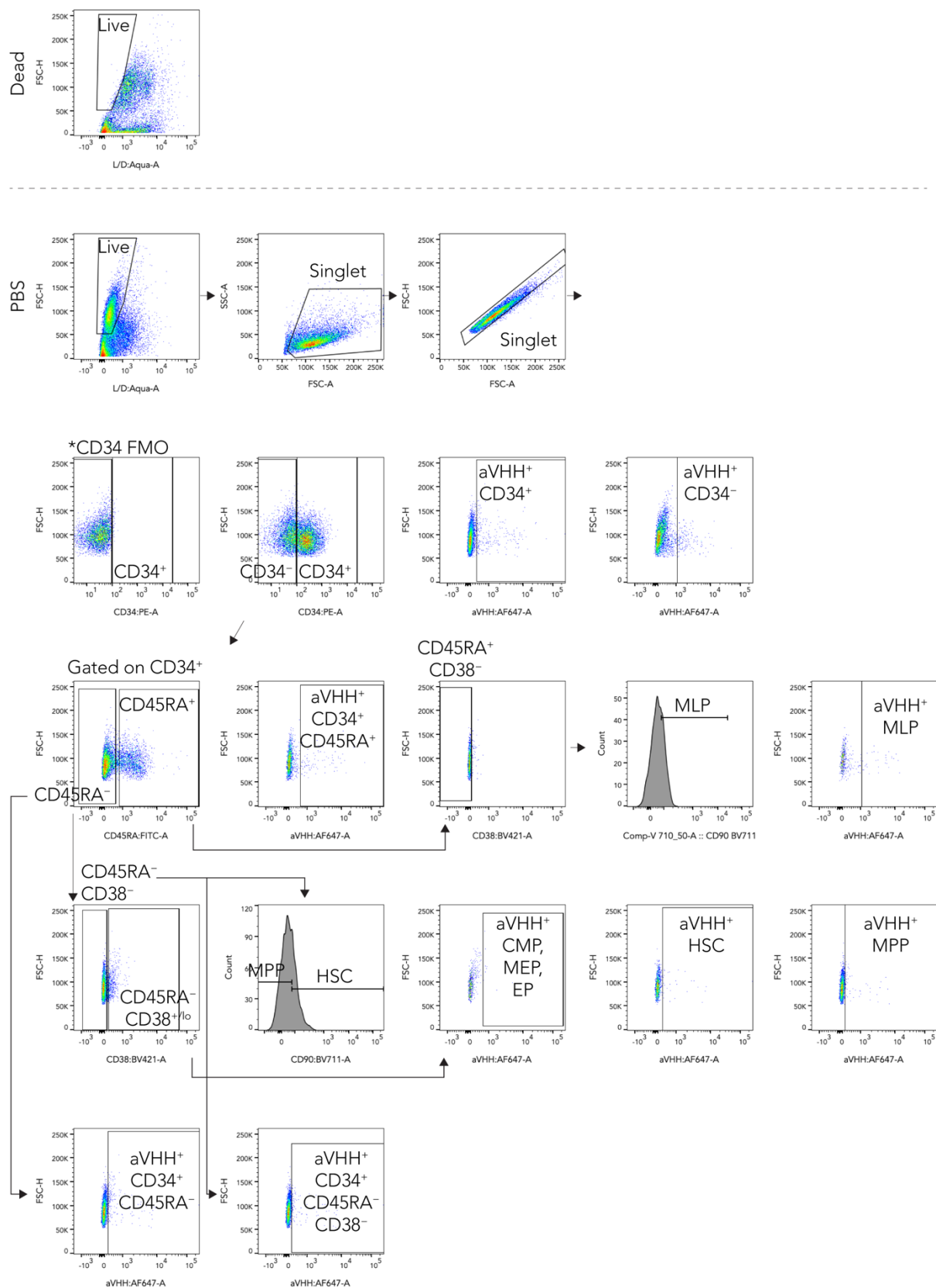
a Cell type definition

Cell type	Markers
Hematopoietic stem cells (HSCs)	CD34 ⁺ CD45RA ⁻ CD38 ⁻ CD90 ⁺
Multipotent progenitor cells (MPPs)	CD34 ⁺ CD45RA ⁻ CD38 ⁻ CD90 ⁻
Multilymphoid progenitor cells (MLPs)	CD34 ⁺ CD45RA ⁺ CD38 ⁻ CD90 ^{lo}
Megakaryocyte-erythroid progenitor and common myeloid progenitor cells (MEP and CMPs)	CD34 ⁺ CD45RA ⁻ CD38 ^{+/lo}
CD34 ⁺ cells (cells with lineage potential)	CD34 ⁺
CD34 ⁻ cells	CD34 ⁻

b Cell type hierarchy

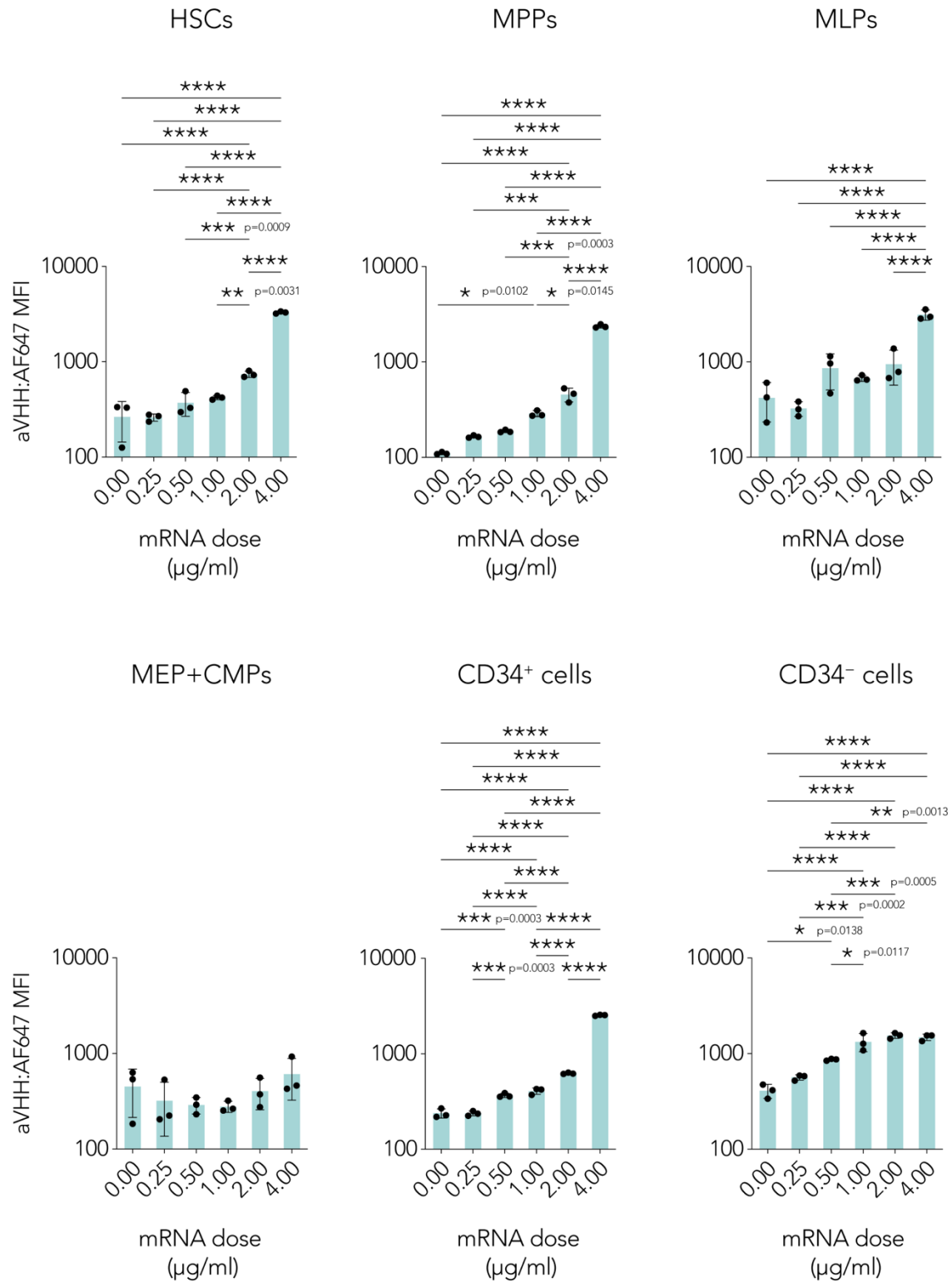


c Human bone marrow CD34⁺ enriched cells



Supplementary Fig. 16 | Cell types in human bone marrow cells and flow cytometry gating strategy for quantifying aVHH expression. **a**, Cell type definitions by surface markers. **b**, Cell type hierarchy. **c**, The representative gating is for the cells from PBS-treated mice and dead cells as control. For a proper gating of CD34, CD34 FMO (fluorescence minus one) sample was included. MLP: multilymphoid progenitor, MPP: multipotent progenitor, HSC: hematopoietic stem cell, CMP: common myeloid progenitor, MEP: megakaryocyte-erythroid progenitor, and EP: erythroid progenitor.

Mean aVHH expression in hematopoietic stem and progenitor cells



Supplementary Fig. 17 | Mean aVHH expression from LNP⁶⁷-treated primary bone marrow cells. AF647: AlexaFluor647. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$, analyzed by one-way ANOVA with Tukey post-hoc test for multiple comparisons. All data are presented as mean $\pm$ SD (n=3, independent wells of cells).

a

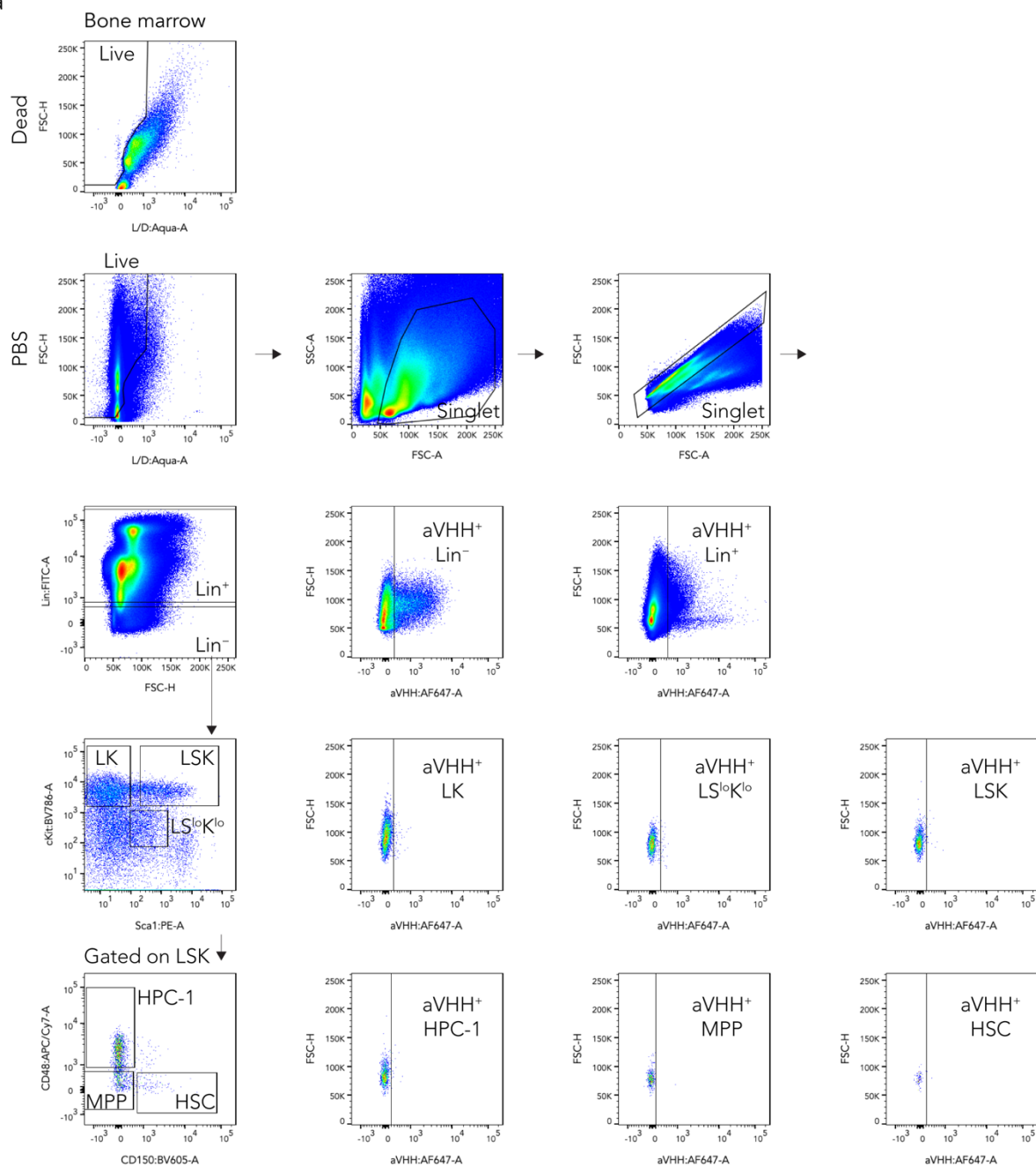
Analyte	Detectable range (pg/ml)
GM-CSF	2.4 – 10000
TGF- α	2.4 – 10000
G-CSF	9.8 – 10000
IFN- γ	9.8 – 10000
IL-2	9.8 – 10000
IL-10	48.8 – 50000
IL-15	2.4 – 10000
sCD40L	2.6 – 2708
IL-17a	2.4 – 10000
IL-1ra	9.8 – 10000
IL-13	39 – 10000
IL-1b	2.4 – 10000
IL-4	19.5 – 10000
IL-5	9.8 – 10000
IL-6	2.4 – 10000
IL-8	9.8 – 10000
MIP-1a	9.8 – 10000
MCP-1	2.4 – 10000
TNF- α	25 – 6400
MIP-1b	2.4 – 10000
IL-12/23 (p40)	39 – 10000
VEGF	39 – 10000
IL-18	48.8 – 50000

b

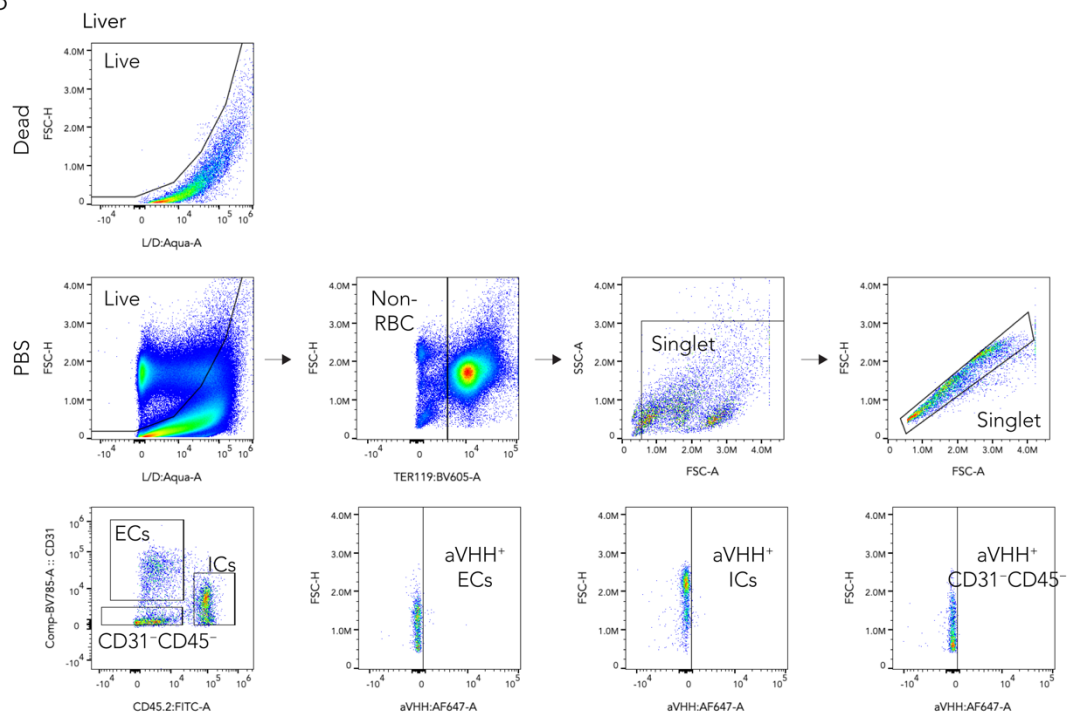
Treatment/Animal ID							
Untreated	0.25 mpk				0.4 mpk		
50566	50670	50714	50749	50750	50611	50761	50762
< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4
< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4
42.75	34.78	38.82	71.69	42.75	< 9.8	30.62	42.75
< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8
10.24	10.24	< 9.8	24.14	< 9.8	< 9.8	< 9.8	10.24
< 48.8	< 48.8	< 48.8	< 48.8	< 48.8	< 48.8	< 48.8	< 48.8
7.89	15.29	11.31	13.12	11.31	9.36	34.22	21.34
146.01	16.76	141.14	176.1	89.95	312.14	62.11	164.13
< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4
25.56	63.69	32.54	72.31	34.05	16.51	943.19	139.74
< 39	< 39	< 39	< 39	< 39	< 39	< 39	< 39
< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4
< 19.5	< 19.5	< 19.5	< 19.5	< 19.5	< 19.5	< 19.5	< 19.5
< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8	< 9.8
< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4	< 2.4
2297.01	591.9	3894.25	2549.94	1732.31	3357.67	1035.03	3838.14
19.06	24.95	10.34	37.94	20.69	< 9.8	17.29	24.95
397.79	191.47	351.42	622.1	246.11	369.64	732.01	344.69
< 25	< 25	< 25	< 25	< 25	< 25	< 25	< 25
30.72	30.03	33	38.37	29.16	39.29	64.8	40.7
< 39	< 39	< 39	< 39	< 39	< 39	< 39	< 39
157.15	225.93	109.88	366.16	185.81	< 39	109.88	212.88
< 48.8	62.41	56.29	64.14	< 48.8	< 48.8	105.73	67.58

Supplementary Fig. 18 | Serum cytokine responses from rhesus macaque after administration of LNP⁶⁷. **a**, Analytes included in the nonhuman primate cytokine multiplex panel and their qualified assay ranges. **b**, Cytokine concentrations (pg/ml) in sera 24 hours after administration of LNP⁶⁷ (0.25 mg/kg or 0.4 mg/kg).

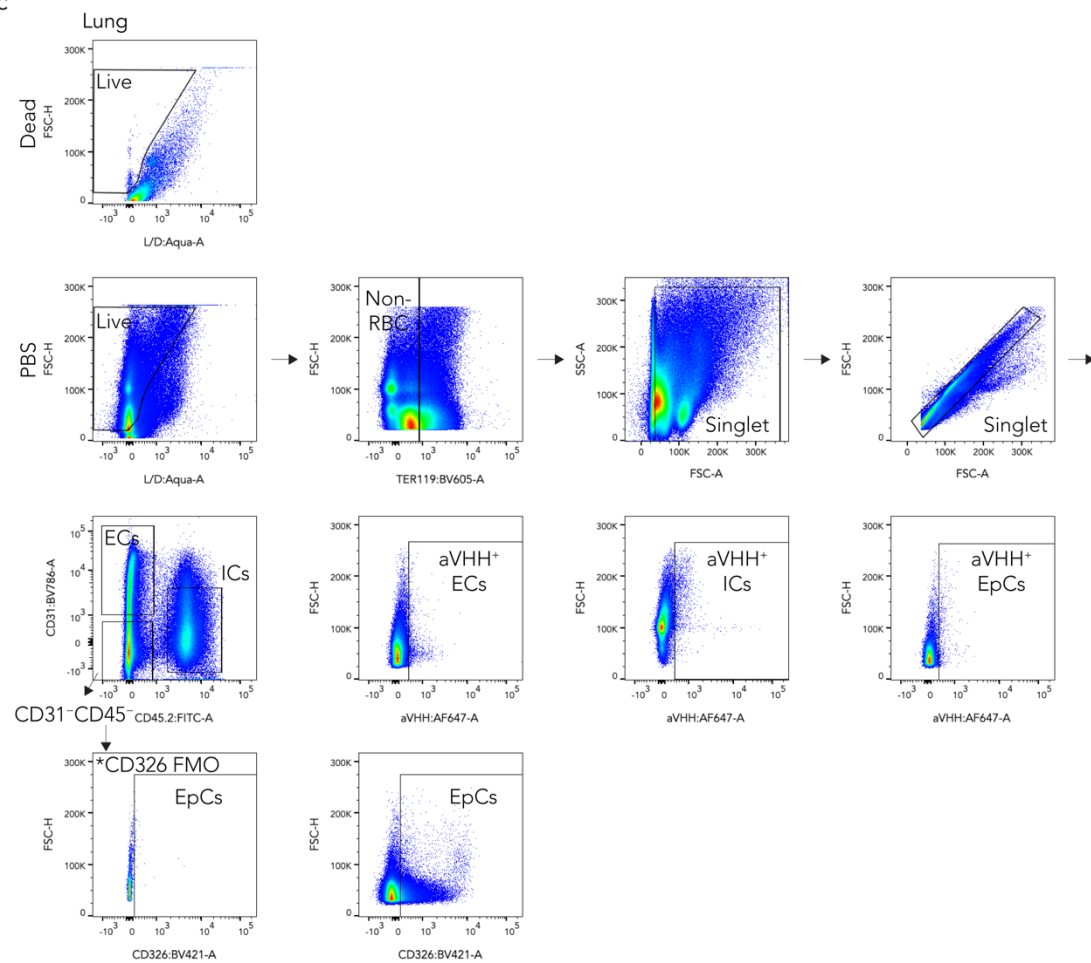
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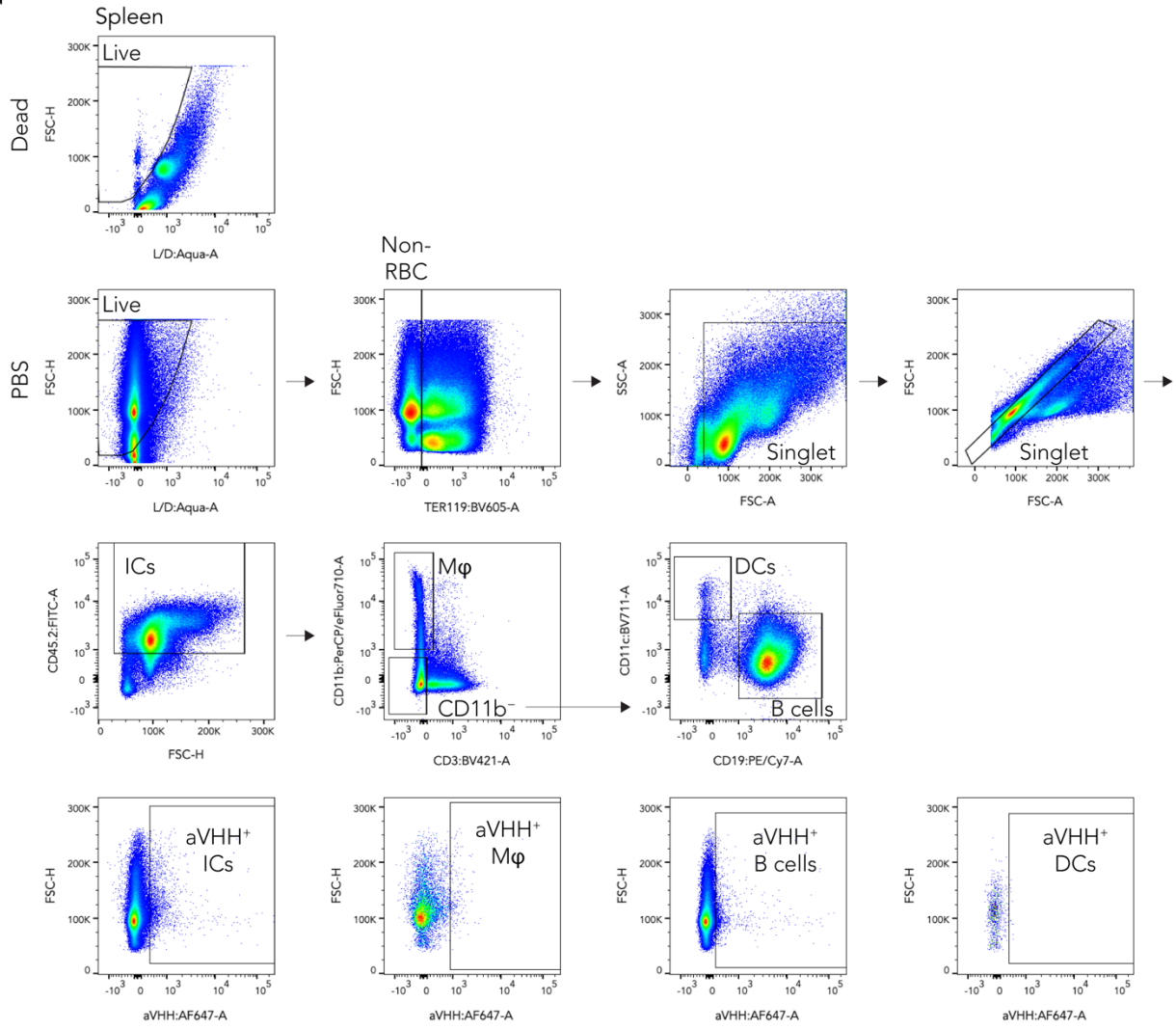
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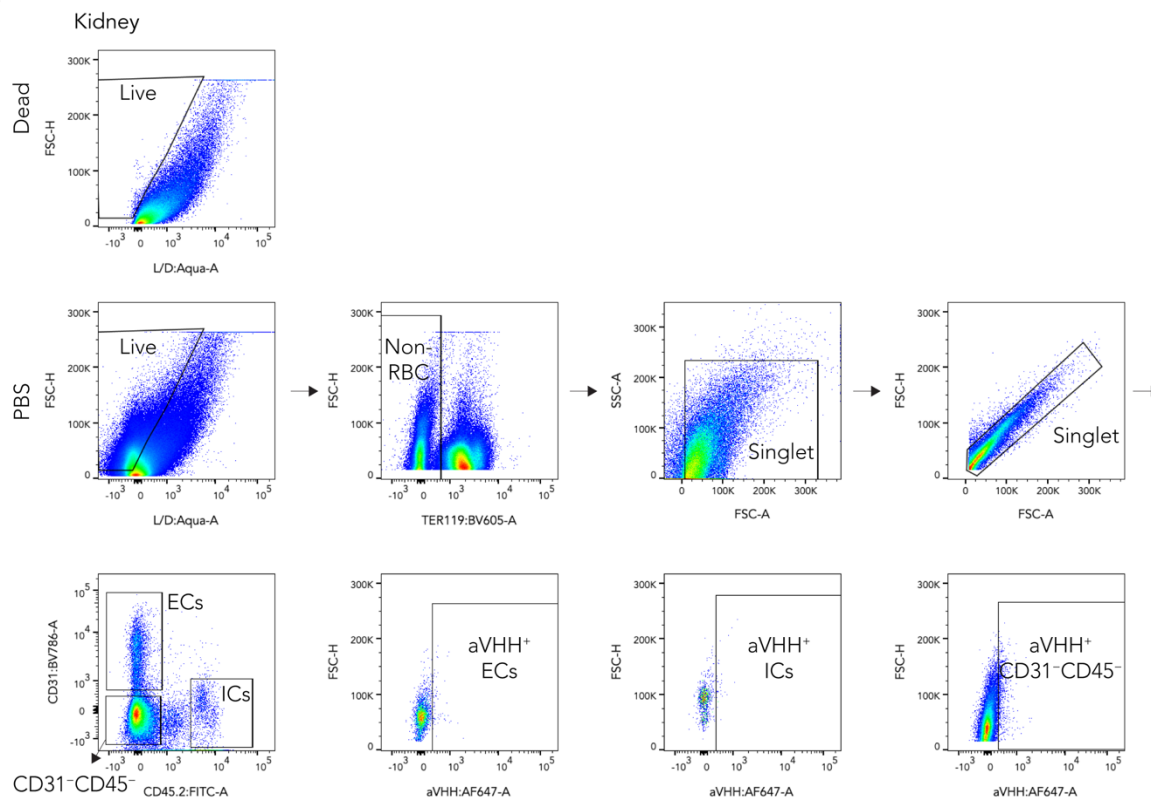
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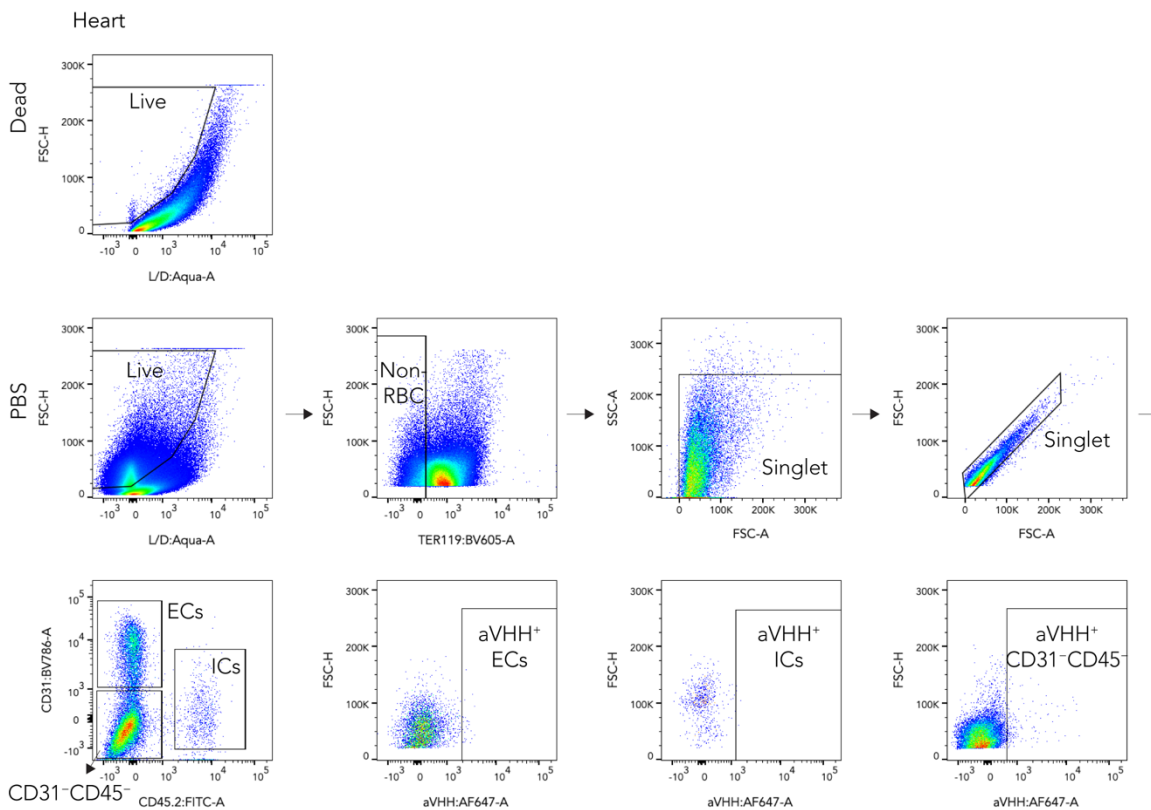
d



e



f



Supplementary Fig. 19 | Flow cytometry gating strategy for quantifying aVHH expression in mouse cells. The representative gating is for the cells from PBS-treated mice and dead cells as control. **a**, Bone marrow, **b**, liver, **c**, lung, **d**, spleen, **e**, kidney, **f**, heart. HSC: hematopoietic stem cell, HPC-1: hematopoietic progenitor cell-1, MPP: multipotent progenitor, LSK: lineage⁻Sca-1⁺cKit⁺, LK: lineage⁻cKit⁺, Lin: lineage, RBC: red blood cell, ECs: endothelial cells, ICs: immune cells, EpCs: epithelial cells, Mφ: macrophage, and DCs: dendritic cells. For a proper gating of CD326 to identify epithelial cells in lung, CD326 FMO (fluorescence minus one) sample was included.

Reference

1. Stoeckius, M. et al., Simultaneous epitope and transcriptome measurement in single cells, *Nat. Methods* **14**, 865–868 (2017).
2. Dobrowolski, C. et al., Nanoparticle single-cell multiomic readouts reveal that cell heterogeneity influences lipid nanoparticle-mediated messenger RNA delivery, *Nat. Nanotechnol.* **17**, 871-879 (2022).