MANUSCRIPT
Supplementary Information

Delivering cytokine mRNA to secondary lymphoid organs for robust cancer immunotherapy

Benchmark LNP formulation

LNPs were prepared as previously described using a rapid mixing method[1,2]. Briefly, an ethanolic lipid phase containing distearoylphosphatidylcholine (DSPC, 850365; Avanti Polar Lipids), PEG(2K)-DMG (880151; Avanti Polar Lipids), cholesterol (C8667; Sigma-Aldrich), and ALC-0315 (SyMO-Chem B.V.) was prepared to a total volume of 1.17 mL at a molar ratio of 10:50:38:2 (DSPC:ALC-0315:cholesterol:PEG-DMG) with an N/P ratio of 6 (**Supplementary Table 1**). A separate aqueous phase containing 100 μg mRNA in 25 mM sodium acetate (pH 4.0) was prepared in a volume of 3.5 mL organic and aqueous phases were combined at a 1:3 (v/v) organic-to-aqueous flow-rate ratio using rapid T-junction mixing (with an organic flow-rate of 7 mL/min and an aqueous flow-rate of 21 mL/min) to yield mRNA-loaded LNPs, which were immediately transferred to 12–14 kDa MWCO dialysis membranes (Spectra/Por) and dialyzed against 1× PBS (pH 7.4) overnight (18 hours). The next day, the LNPs were passed through a 0.2 µm sterile filter using aseptic technique, concentrated by centrifugal filtration at 1100× g using 100 kDa MWCO Vivaspin filters (Amicon), and then adjusted to the desired final mRNA concentration. Unless otherwise specified, formulations were stored at 4 °C until use.

	Phospholipid			Ionizable cationic material			Cholesterol		Tricaprylin		ApoA1	mRNA
Formulation	**type**	**mg**	**mol%**	**type**	**mg**	**mol%**	**mg**	**mol%**	**mg**	**mol%**	**mg**	**mg**
aNP_{HMJ1}	DMPC	1.2	20	HMJ1	0.6	3	0.7	20	2.4	57	0.5	0.1
aNP_{HMJ2}	DMPC	1.2	20	HMJ2	0.6	3	0.7	20	2.4	57	0.5	0.1
aNP_{ALC}	DMPC	1.2	17	ALC-0315	1.3	17	0.7	17	2.4	49	0.5	0.1

mRNA sequences

IL-2:

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GA-3'

IFN-y:

5'AUGAACGCCACCCACUGCAUCCUGGCCCUGCAGCUGUUCCUGAUGGCCGUGAGCGGCUGCUACUGCCACGGCAC
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mCherry:

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Supplementary Table 1. Formulation compositions and mRNA sequences

Target	Fluorochrome	Type	Isotype species	Isotype Ig	Isotype fragment	Clone	Immunogen	Company	Catalog number
CD115 (CSF-1R)	BV421	Monoclonal	Rat	IgG2a	κ	AFS98	-	Biolegend	135513
Ly6C	FITC	Monoclonal	Rat	IgG2c	κ	HK1.4	L3 cloned CTL cells	Biolegend	128006
F4/80	APC	Monoclonal	Mouse	IgG1	κ	QA17A29	Murine macrophages	Biolegend	157306
Ly6G	APC-Cy7	Monoclonal	Rat	IgG2a	κ	1A8	Ly-6G transfected EL-4J cell line	Biolegend	127624
CD11b	BV785	Monoclonal	Rat	IgG2b	κ	M1/70	C57BL/10 splenocytes	Biolegend	101243
CD45	PerCP	Monoclonal	Rat	IgG2b	κ	30-F11	Mouse thymus or spleen	Biolegend	103130
CD11c	PE-Cy7	Monoclonal	Armenian Hamster	IgG		N418	Mouse spleen dendritic cells	Biolegend	117318

Supplementary Table 2. Myeloid cell populations flow cytometry panel

Target	Fluorochrome	Type	Isotype species	Isotype Ig	Isotype fragment	Clone	Immunogen	Company	Catalog number
CD31 (PECAM-1)	BV711	Monoclonal	Rat	IgG2b	κ	W18222B	Mouse CD31 transfected cell line	Biolegend	102449
CD68	PerCP-Cy5.5	Monoclonal	Rat	IgG2a	κ	FA-11	Purified Con A receptor glycoproteins from the P815 cell line	Biolegend	137010
ASGPR1	CoraLite Plus 488	Polyclonal	Rabbit	IgG	-	-	ASGR1 fusion protein (asialoglycoprotein receptor 1)	Proteintech	CL488-11739
CD45	PE-Cy7	Monoclonal	Rat	IgG2b	κ	30-F11	Mouse thymus or spleen	Biolegend	103113

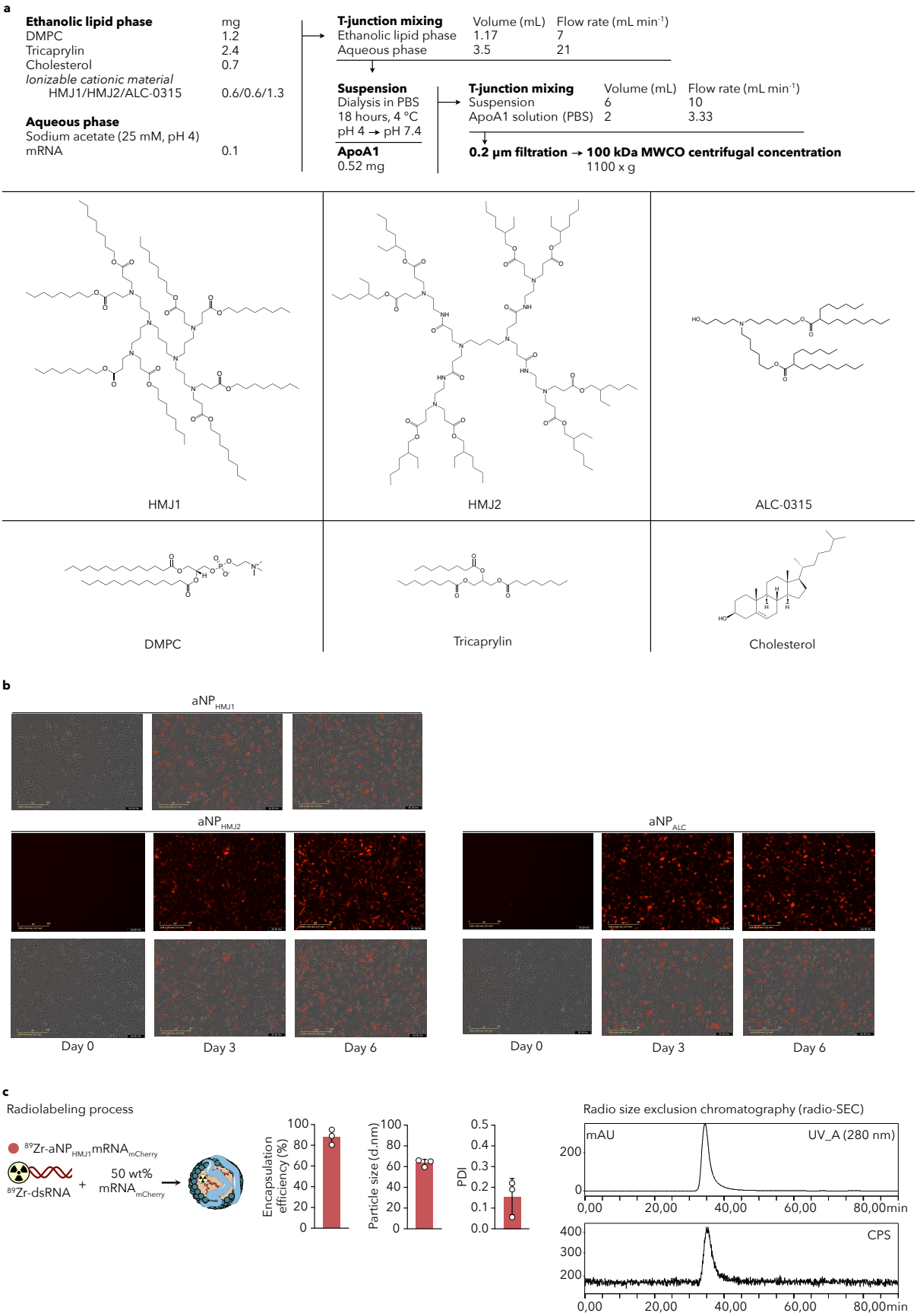
Supplementary Table 3. Liver cell populations flow cytometry panel

Target	Fluorochrome	Type	Isotype species	Isotype Ig	Isotype fragment	Clone	Immunogen	Company	Catalog number
CD3	APC	Monoclonal	Rat	IgG2b	κ	17A2	γδTCR-positive T-T hybridoma D1	Biolegend	100236
CD4	FITC	Monoclonal	Rat	IgG2b	κ	GK1.5		Biolegend	100406
CD8a	PE	Monoclonal	Rat	IgG2a	κ	53-6.7	Mouse thymus or spleen	Biolegend	100708
CD19	BV650	Monoclonal	Rat	IgG2a	κ	6D5	Mouse CD19-expressing K562 human erythroleukemia cells	Biolegend	115541
NK-1.1	Spark Red 718	Monoclonal	Mouse	IgG2a	κ	S17016D	Mouse NK1.1-transfectants	Biolegend	156534
CD11b	eFluor 506	Monoclonal	Rat	IgG2b	κ	M1/70		ThermoFisher	69-0112-82
CD45	PerCP	Monoclonal	Rat	IgG2b	κ	30-F11	Mouse thymus or spleen	Biolegend	103130

Supplementary Table 4. Lymphoid cell populations flow cytometry panel

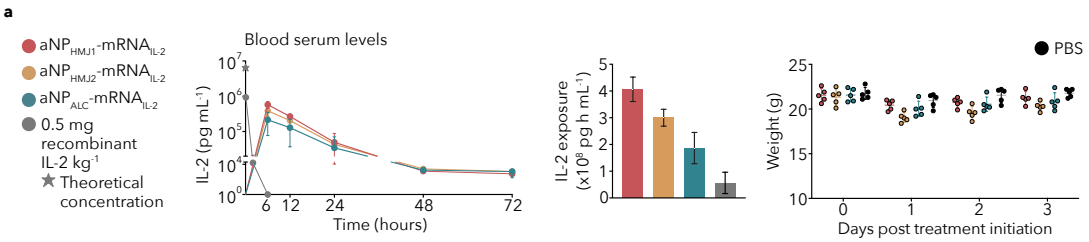
Target	Fluorochrome	Type	Isotype species	Isotype Ig	Isotype fragment	Clone	Company	Catalog number
CD3	Alexa Fluor 700	Monoclonal	Mouse	IgG1	λ	SP34-2	BD Biosciences	557917
CD4	Pacific blue	Monoclonal	Mouse	IgG2b	κ	OKT4	Biolegend	317428
CD8	APC-Fire750	Monoclonal	Mouse	IgG1	κ	SK1	Biolegend	344746
CD11c	PE	Monoclonal	Mouse	IgG1	κ	3.9	Biolegend	301606
CD14	APC	Monoclonal	Mouse	IgG2a	κ	M5E2	Biolegend	301808
CD16	BV785	Monoclonal	Mouse	IgG1	κ	3G8	Biolegend	302046
CD20	FITC	Monoclonal	Mouse	IgG2b	κ	2H7	Biolegend	285006
CD45	V500	Monoclonal	Mouse	IgG1	κ	D058-1283	BD Biosciences	558411
HLA-DR	BUV395	Monoclonal	Mouse	IgG2a	κ	G46-6	BD Biosciences	564040

Supplementary Table 5. Non-human primate immune cell populations flow cytometry panel

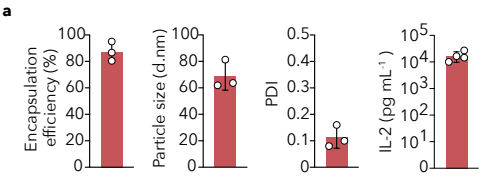


Supplementary Figure 1. Intravenous aNP administration enables mRNA translation in splenic monocytes **a**, Formulation process of the aNP-mRNA with the chemical structures of the components; DMPC, tricaprylin, cholesterol, and ionizable cationic molecules HMJ1, HMJ2, and ALC-0315 **b,** Incucyte images acquired at 0, 3, and 6 days after transfection. Bone marrow–derived macrophages were transfected for 1 h with 5 μg/mL of

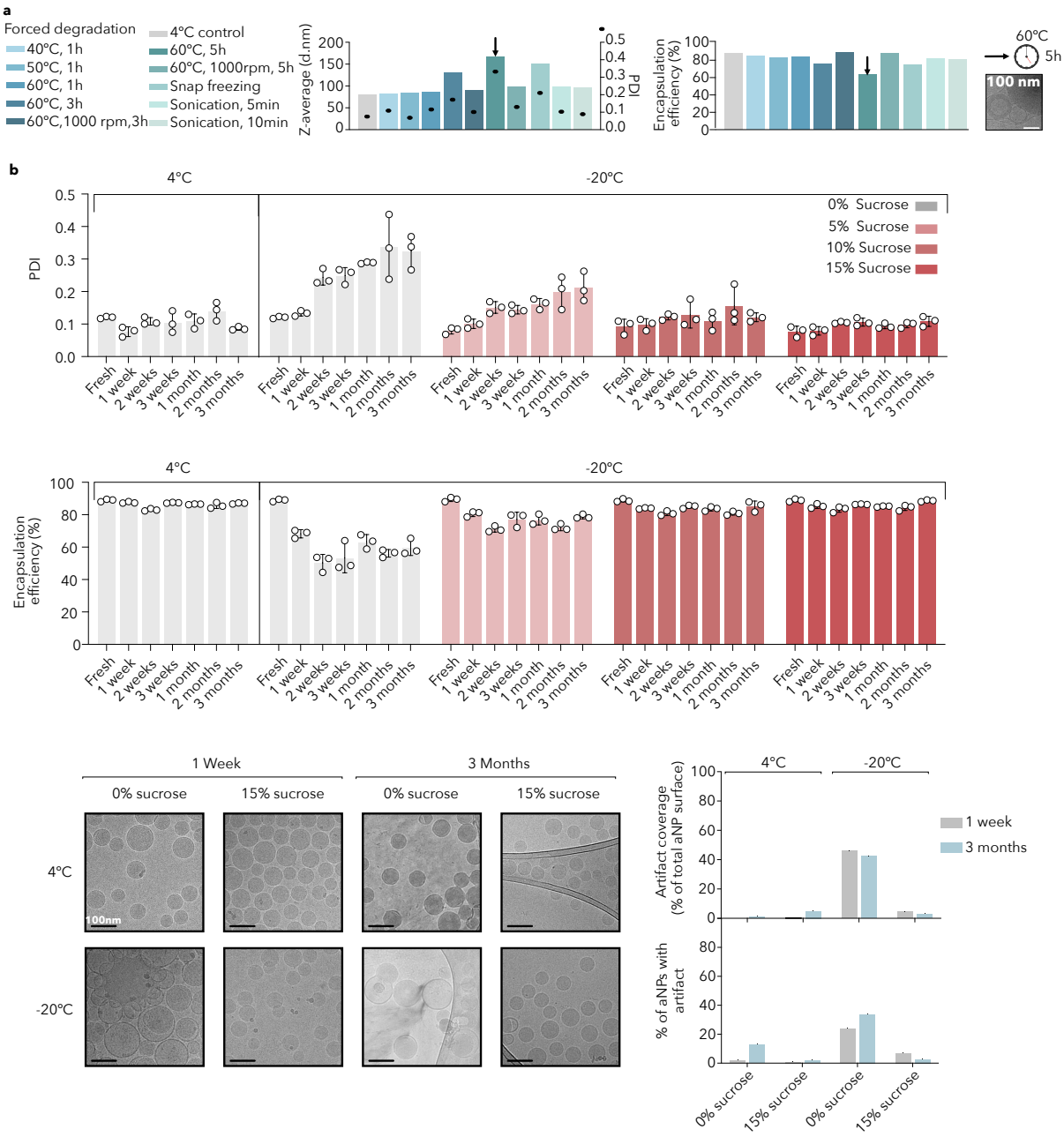
aNP_{HMJ1}-$mRNA_{mCherry}$, aNP_{HMJ2}-$mRNA_{mCherry}$, and $aNP_{ALC-0315}$-$mRNA_{mCherry}$, after which the medium was refreshed. **c**, (left) Radiolabeling scheme for ^{89}Zr-aNP_{HMJ1}-$mRNA_{mCherry}$. Equal weight fractions (50 wt%) of ^{89}Zr-siRNA and $mRNA_{mCherry}$ were combined to formulate ^{89}Zr-aNP_{HMJ1}-$mRNA_{mCherry}$. (middle) mRNA encapsulation efficiency, particle size measured as hydrodynamic diameter and expressed as the number mean average, and associated poly dispersity index (PDI) of ^{89}Zr-aNP_{HMJ1}-$mRNA_{mCherry}$ (n = 3 formulation batches). (right) Radio size exclusion chromatography (radio-SEC) analysis of ^{89}Zr-aNP_{HMJ1}-$mRNA_{mCherry}$. Elution profiles were acquired on a Superose® 6 Increase 10/300 GL column at 0.25 mL/min in PBS.



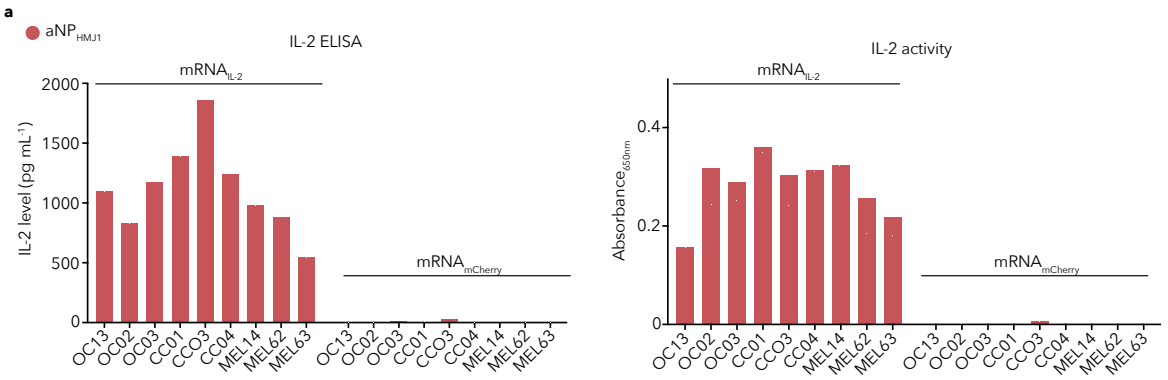
Supplementary Figure 2. Interleukin-2 production in secondary lymphoid organs drives effective and safe tumor growth inhibition
a, (left) Blood IL-2 serum levels determined 6, 12, 24, 48, and 72 hours following intravenous administration of 0.5 mg/kg aNP_{HMJ1}-$mRNA_{IL-2}$, aNP_{HMJ2}-$mRNA_{IL-2}$, $aNP_{ALC-0315}$-$mRNA_{IL-2}$, and recombinant IL-2, presented as measured value and theoretical recombinant IL-2 concentration value of 6.5 x 10^6 pg/mL, calculated for a 22 g mouse administered a dose of 0.5 mg rIL-2 / kg body weight, assuming a total blood volume of 1.7 mL. (middle) Area under the curve of blood IL-2 serum concentrations (pgxh/mL) was calculated for each group. Data are presented as mean ± s.e.m. (n = 4–9 mice; total data points: aNP_{HMJ1}-$mRNA_{IL-2}$, 52; aNP_{HMJ2}-$mRNA_{IL-2}$, 30; $aNP_{ALC-0315}$-$mRNA_{IL-2}$, 44; recombinant IL-2, 10). (right) Mice weight determined 0, 1, 2, and 3 days after treatment initiation. Data represent mean ± s.d. of one experiment (n = 5 mice).



Supplementary Figure 3. Intravenous aNP treatment induces CD8+ T cell expansion in tumor-bearing mice
a, mRNA encapsulation efficiency, particle size measured as hydrodynamic diameter and expressed as the number mean average, and associated poly dispersity index (PDI) of ^{89}Zr-aNP_{HMJ1}-$mRNA_{IL-2}$ (n = 3 formulation batches). IL-2 blood serum level 24 hours after injecting ^{89}Zr-aNP_{HMJ1}-$mRNA_{IL-2}$ (n = 4 mice, 0.16 mg $mRNA_{IL-2}$/kg, 8 μCi per mouse).

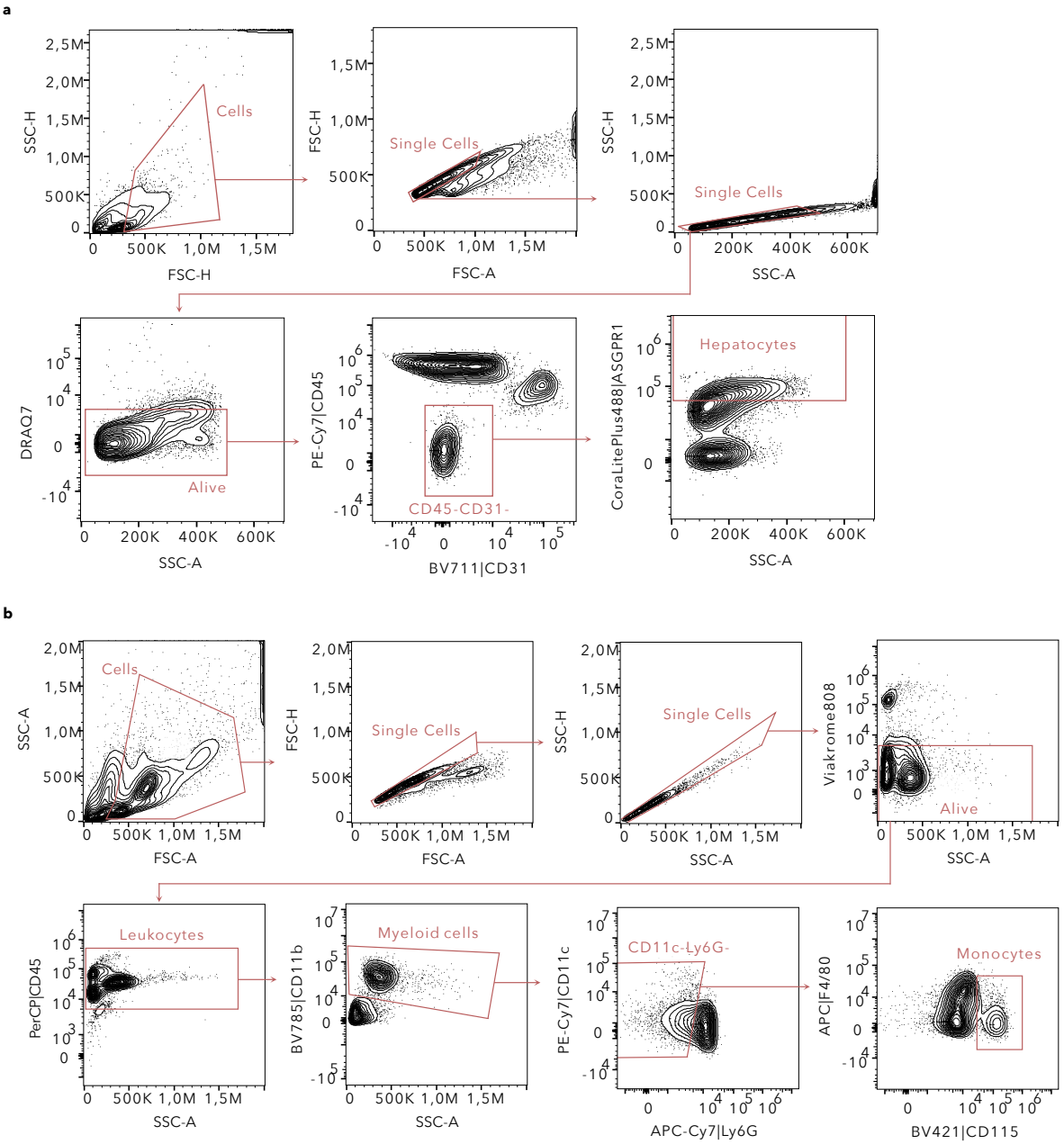


Supplementary Figure 4. Reproducible aNP manufacturing and long-term storing strategy
a, Forced degradation studies of aNP$_{HMJ1}$-mRNA$_{fLuc}$ under various stress conditions. Physicochemical analysis is represented by Z-average (bars, left Y-axis), PDI (black dots, right Y-axis), and encapsulation efficiency (bars, right panel). The most destructive condition was further analyzed by cryo-EM. All cryo-EM scale bars represent 100 nm. **b**, aNP$_{HMJ1}$-mRNA$_{fLuc}$ nanoparticles were formulated with increasing sucrose concentrations as cryoprotectants and assessed over the course of 3 months at -20 °C. Polydispersity index (PDI) and encapsulation efficiency were measured at the indicated timepoints. Cryo-EM images depict nanoparticle morphology after 1 week or 3 months at 4ºC or -20ºC, with or without 15 w/v% sucrose cryoprotection. For quantification of artifacts resulting from these different storing conditions, 100 representative nanoparticles were randomly selected from each Cryo-TEM image across all storage conditions. Structural artifacts, including blebbing and empty particles, were annotated independently. These data were used to determine the percentage of nanoparticles exhibiting artifacts and the percentage of the total nanoparticle surface area occupied by artifacts. Scale bars indicate 100nm.



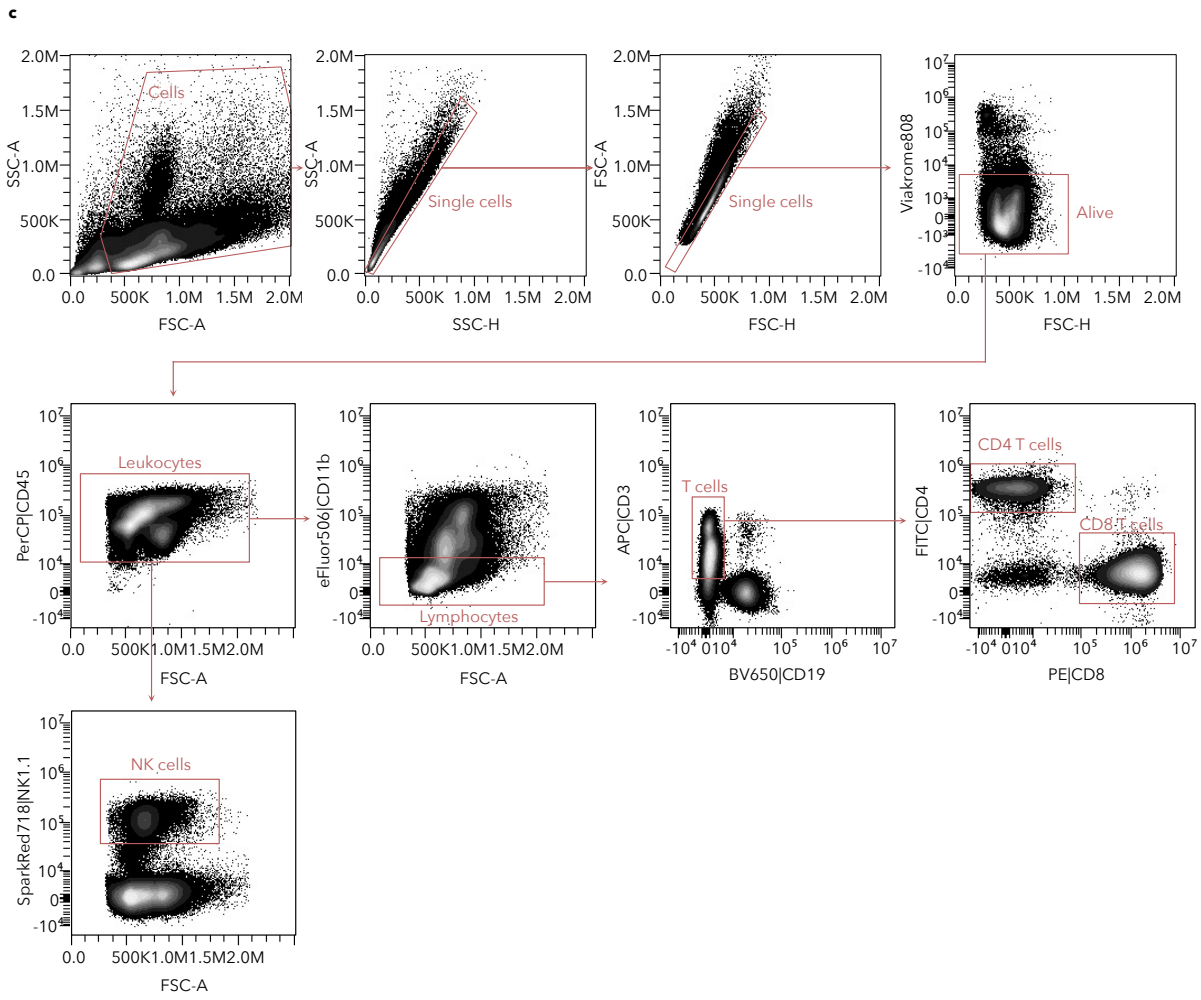
Supplementary Figure 5. Patient studies

a, Primary monocytes from cancer patients are transfected with $mRNA_{IL\text{-}2}$ or $mRNA_{mCherry}$ (2.5 μg mRNA/mL) with aNP_{HMJ1}. IL-2 secretion was quantified by ELISA (pg/mL, left) and IL-2 activity was measured by HEK-Blue IL-2 reporter cells, quantified via SEAP colorimetric readout (absorbance at 650 nm, right).



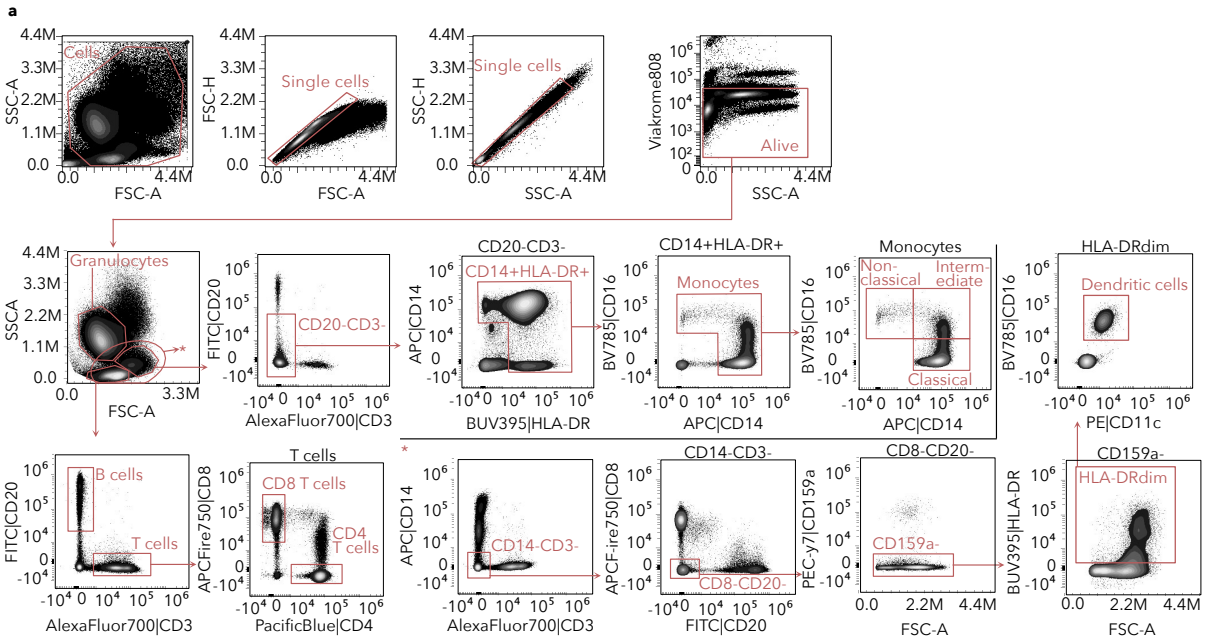
Supplementary Figure 6. Gating strategies to identify mouse hepatocytes and immune cells with flow cytometry

a, Liver cell populations flow cytometry gating. **b,** Myeloid cell populations flow cytometry gating.



Supplementary Figure 6. Gating strategies to identify mouse hepatocytes and immune cells with flow cytometry

c, Lymphoid cell populations flow cytometry gating



Supplementary Figure 7. Gating strategies to identify non-human primate immune cells with flow cytometry

a, Non-human primate immune cell populations flow cytometry gating

References

1. Kulkarni, J. A. *et al.* On the Formation and Morphology of Lipid Nanoparticles Containing Ionizable Cationic Lipids and siRNA. *ACS Nano* **12**, 4787–4795 (2018).
2. Jeffs, L. B. *et al.* A scalable, extrusion-free method for efficient liposomal encapsulation of plasmid DNA. *Pharm Res* **22**, 362–372 (2005).