Supplementary Information

mRNA lipid nanoparticle-mediated pyroptosis sensitizes immunologically cold tumors to checkpoint immunotherapy

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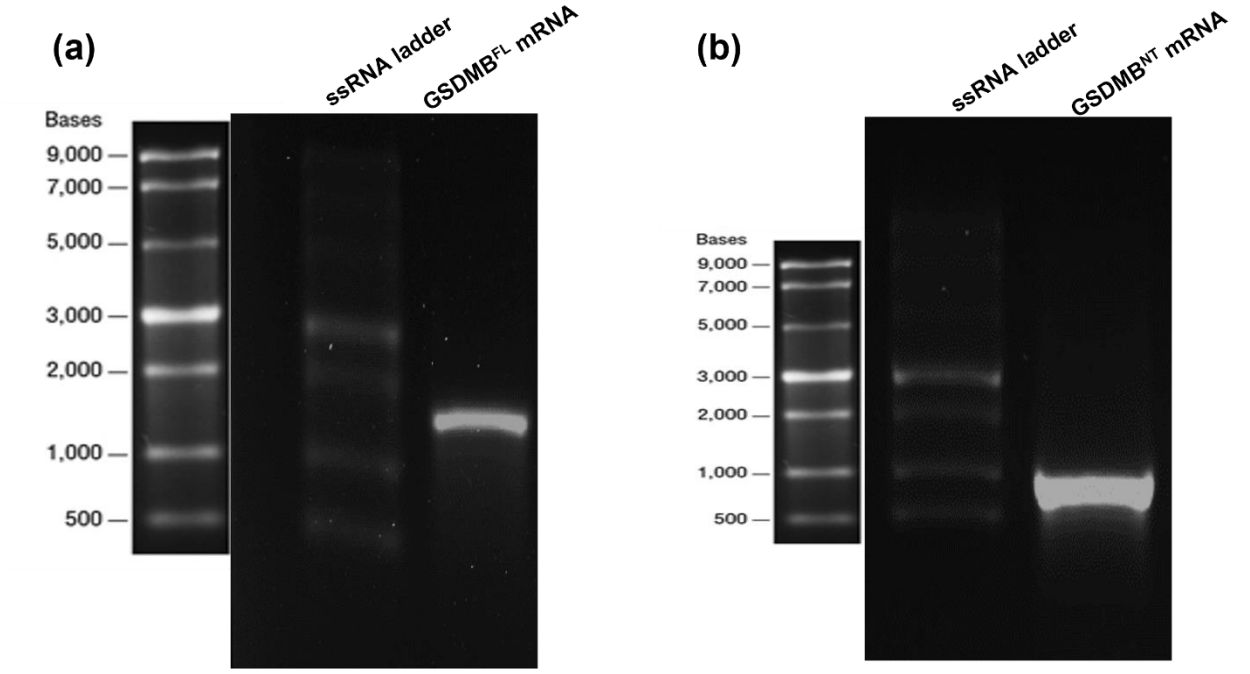
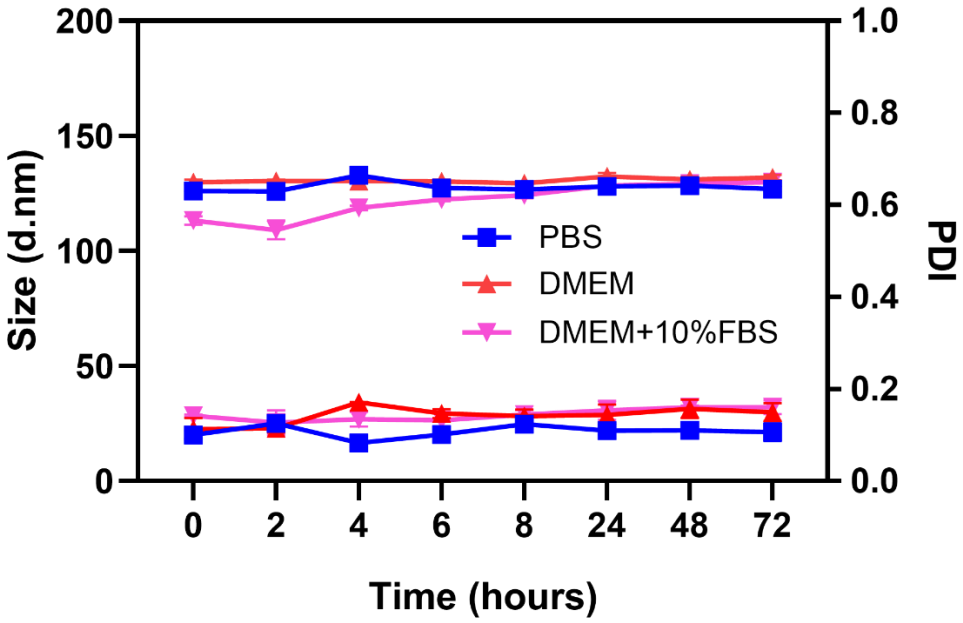
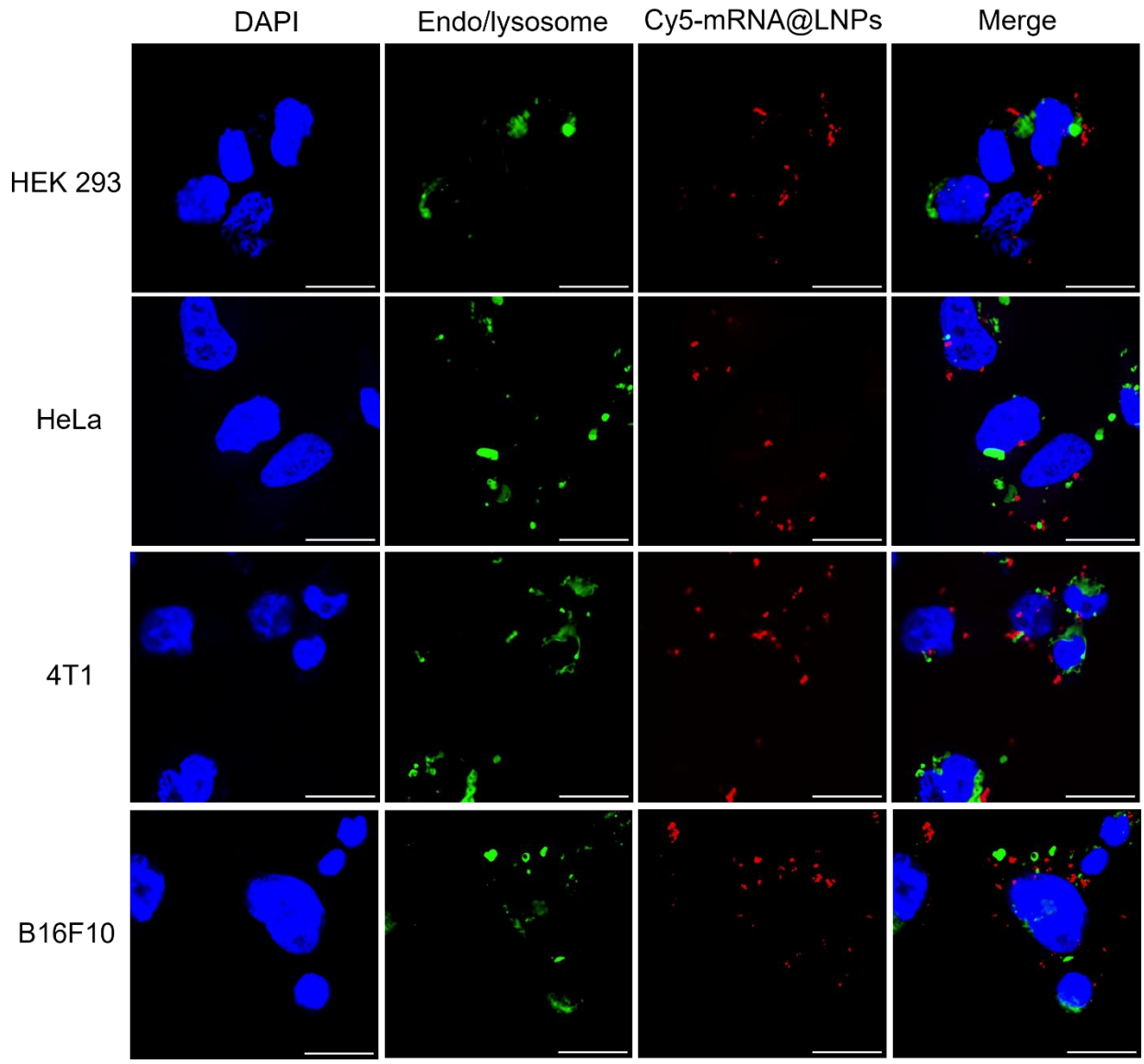


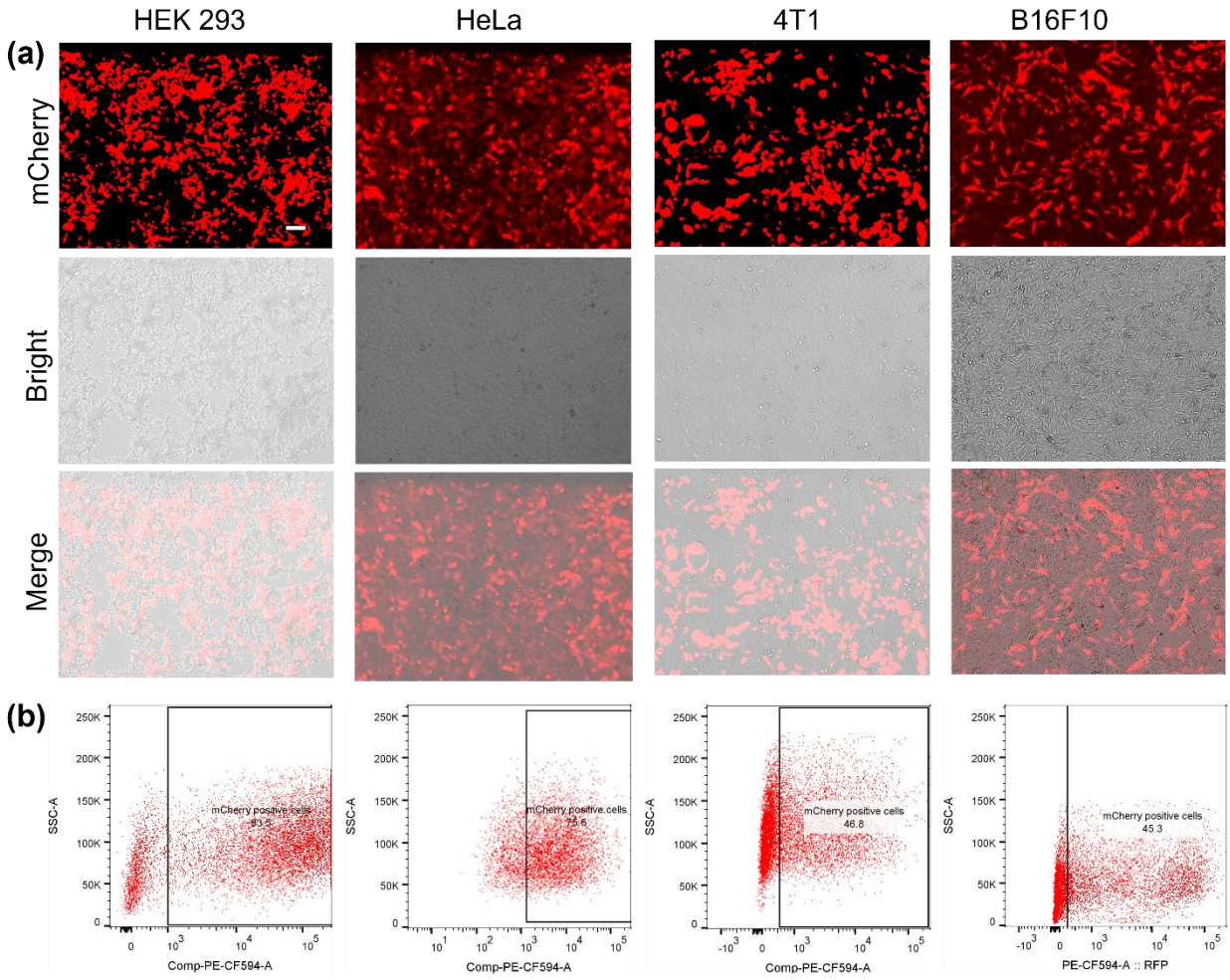
Supplementary Fig. 1 Native agarose gel electrophoresis to identify the size of synthetic mRNAs encoding GSDMB full-length ($GSDMB^{FL}$, **a**), and GSDMB N-terminal ($GSDMB^{NT}$, **b**). Data are representative of three independent experiments.



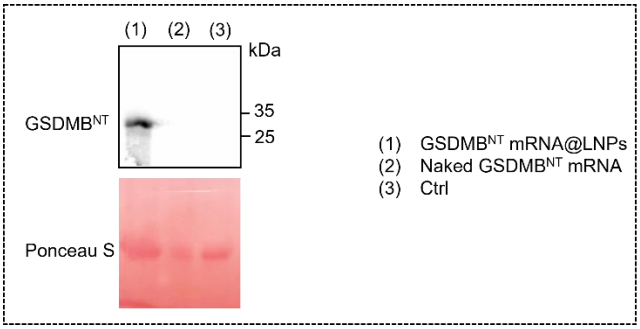
Supplementary Fig. 2 The stability of GSDMB^{NT} mRNA@LNPs in PBS, DMEM, and DMEM + 10% FBS. Data are presented as means ± SD (n = 3). Source data are provided as a Source Data file.



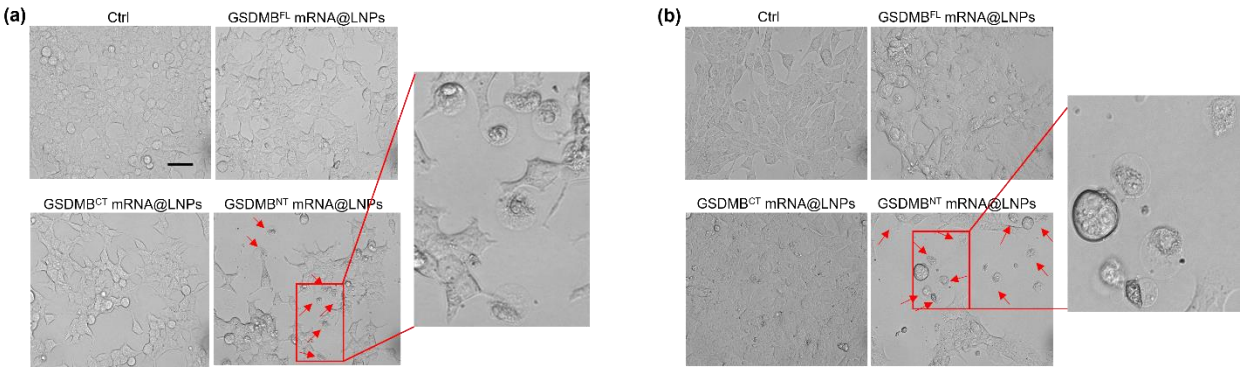
Supplementary Fig. 3 LNP-mediated endosomal/lysosomal escape and cytoplasmic release of Luc ^{Cy5}mRNA in HEK 293, HeLa, 4T1, and B16F10 cells 4 hours after incubation. DAPI (blue), Endo/lysosome (green), Luc ^{Cy5}mRNA@LNPs (red), scale bar = 20 μm. Data are representative of two independent experiments.



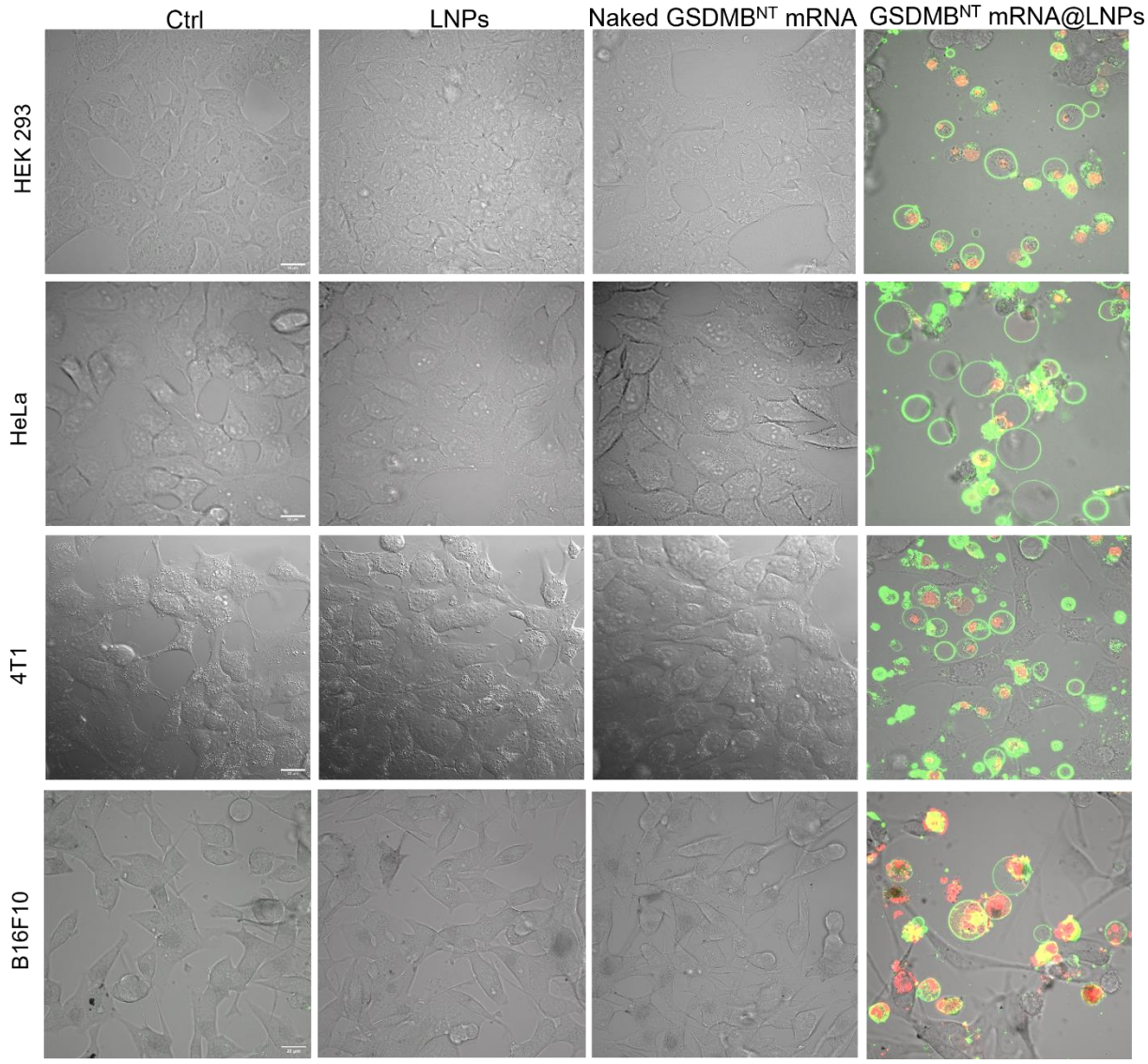
Supplementary Fig. 4 Transfection efficacy of LNPs was determined by fluorescence microscope **(a)** and flow cytometry analysis **(b,** n = 3**)** in four different cell lines. Scale bar = 100 μm. Data in **(a)** is representative of three independent experiments.



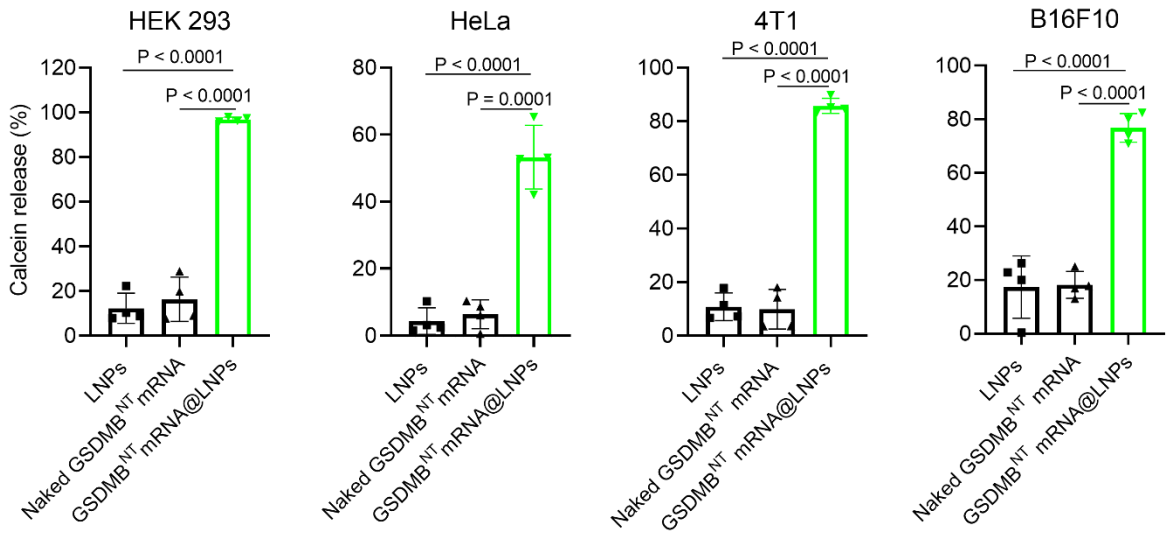
Supplementary Fig. 5 Western blot analysis of GSDMB^{NT} expression.



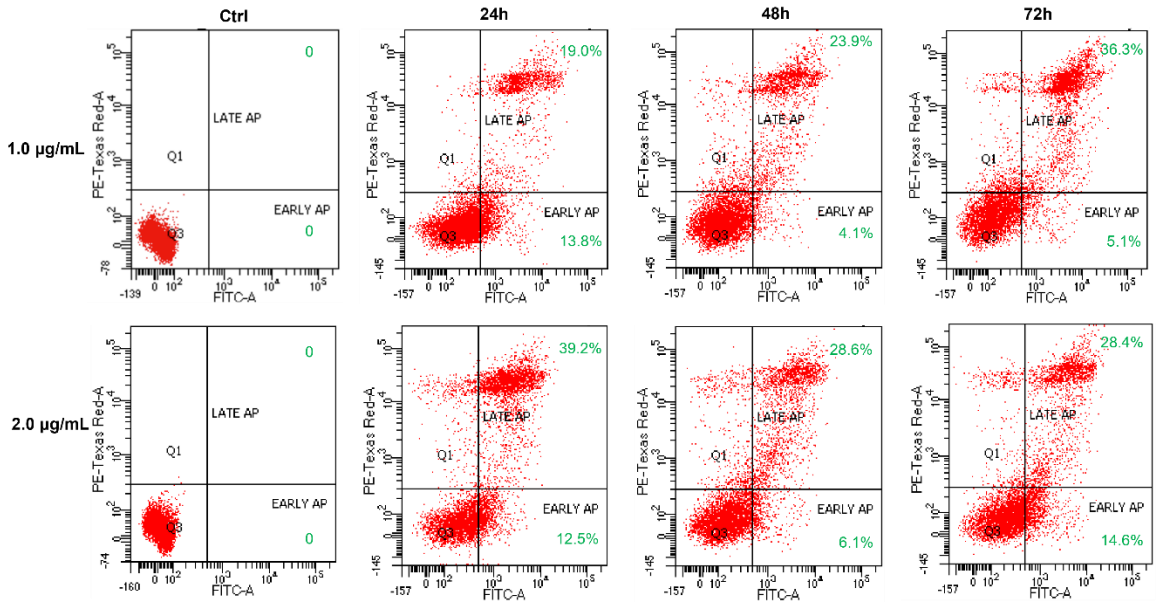
Supplementary Fig. 6 Representative images of HEK 293 **(a)** and 4T1 **(b)** cells transfected by mRNA/LNPs encoding GSDMB full-length (GSDMB^{FL}), GSDMB C-terminal (GSDMB^{CT}), or GSDMB N-terminal (GSDMB^{NT}) after 24 hours of treatment. Scale bar = 50 μm. Data are representative of three independent experiments.



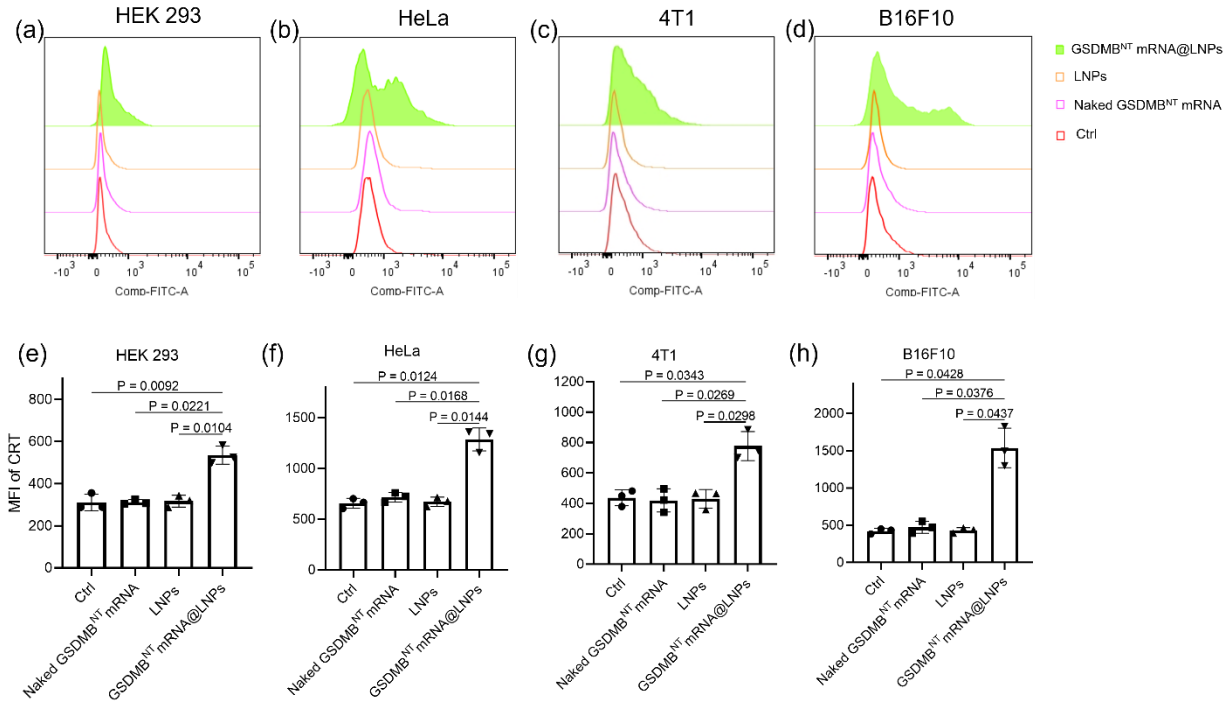
Supplementary Fig. 7 Cell morphologies of the treated HEK293, HeLa, 4T1, and B16F10 cells were detected using a confocal microscope. Before imaging, cells were added with annexin V-FITC and propidium iodide (PI) for 15 mins incubation. Scale bars = 20 μm. All data shown are representative of three independent experiments.



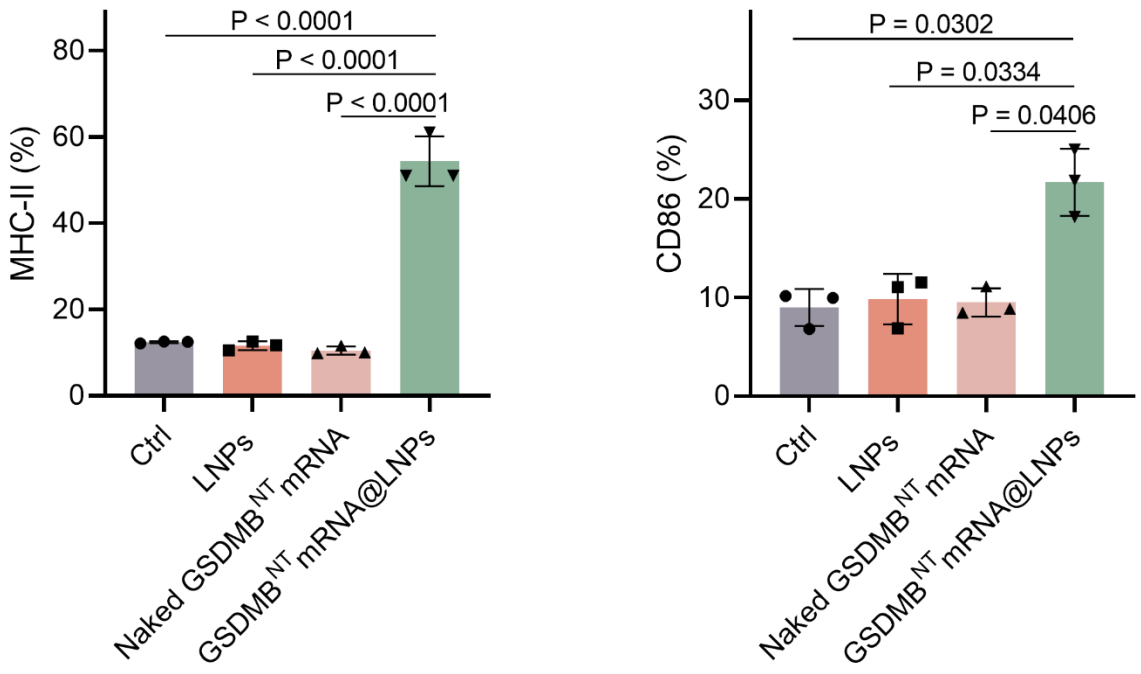
Supplementary Fig. 8 Quantification of calcein AM release. All data are presented as means ± SD (n = 4). Statistical significance was calculated using a two-tailed Student's t test. Source data are provided as a Source Data file.



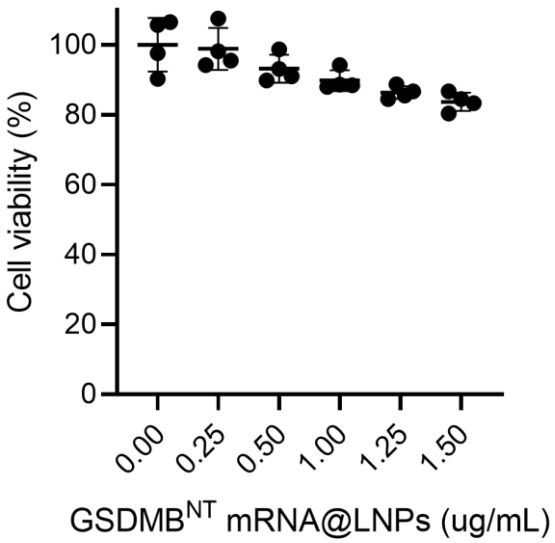
Supplementary Fig. 9 Percentage of apoptotic cells was determined by staining with FITC-Annexin V/PI (n = 3 biological replicates per group). HEK 293 cells were transfected with $GSDMB^{NT}$ mRNA@LNPs at various mRNA concentrations for 24, 48, and 72 hours.



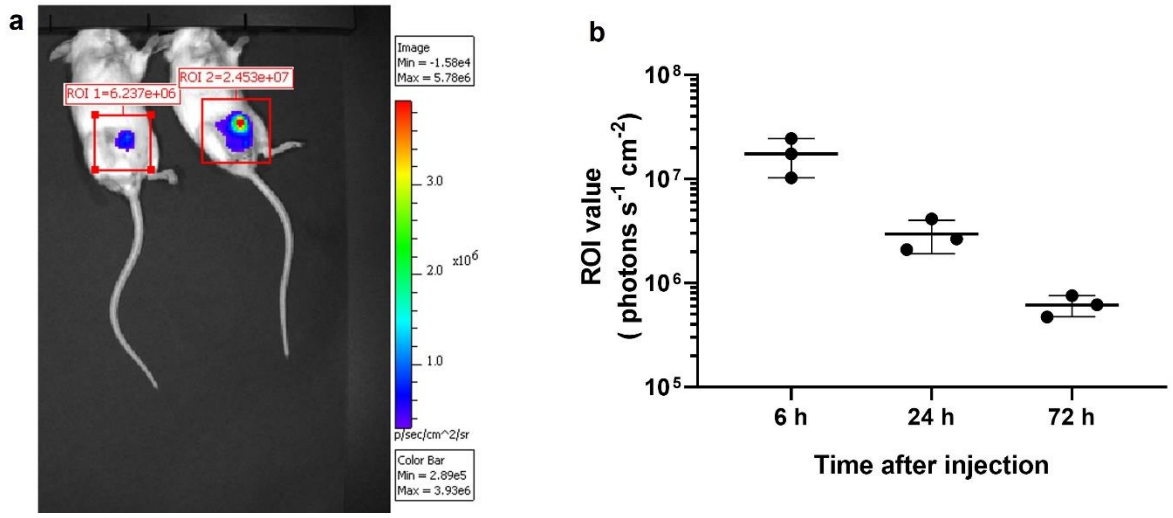
Supplementary Fig. 10 GSDMB^{NT} mRNA@LNP treatment increases calreticulin (CRT) surface exposure of HEK 293 (**a** and **e**), HeLa (**b** and **f**), 4T1 (**c** and **g**), and B16F10 (**d** and **h**) cells. Cells were treated with the indicated treatments for 48 hours, and then collected and stained with an Alexa Fluor 488-labeled CRT antibody for flow cytometry analysis. All data are presented as means ± SD (n = 3). Statistical significance was calculated via one-way ANOVA. Untreated cells served as the control (Ctrl) in all experiments. MFI: mean fluorescence intensity. Source data are provided as a Source Data file.



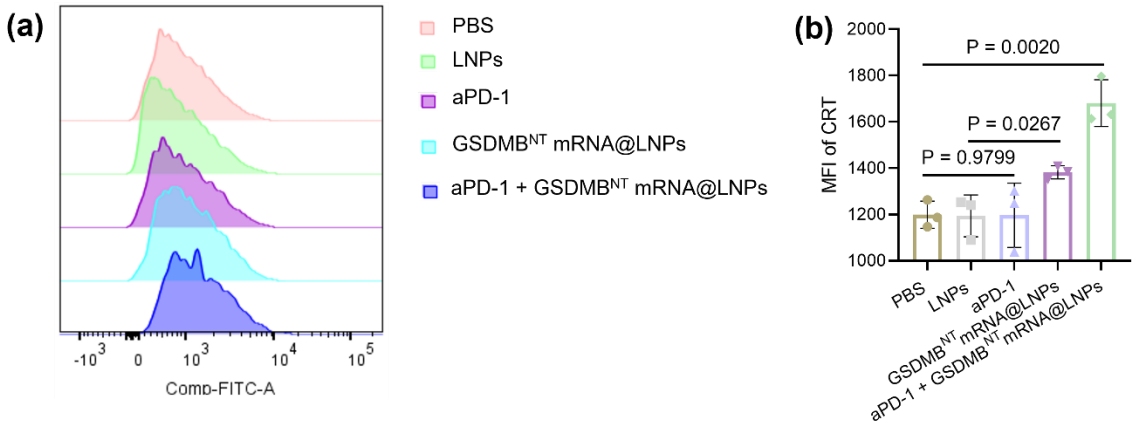
Supplementary Fig. 11 Flow cytometry analysis of DC maturation biomarkers (MHC-II and CD86). Data are presented as means ± SD (n = 3). Statistical significance was calculated via one-way ANOVA. Source data are provided as a Source Data file.



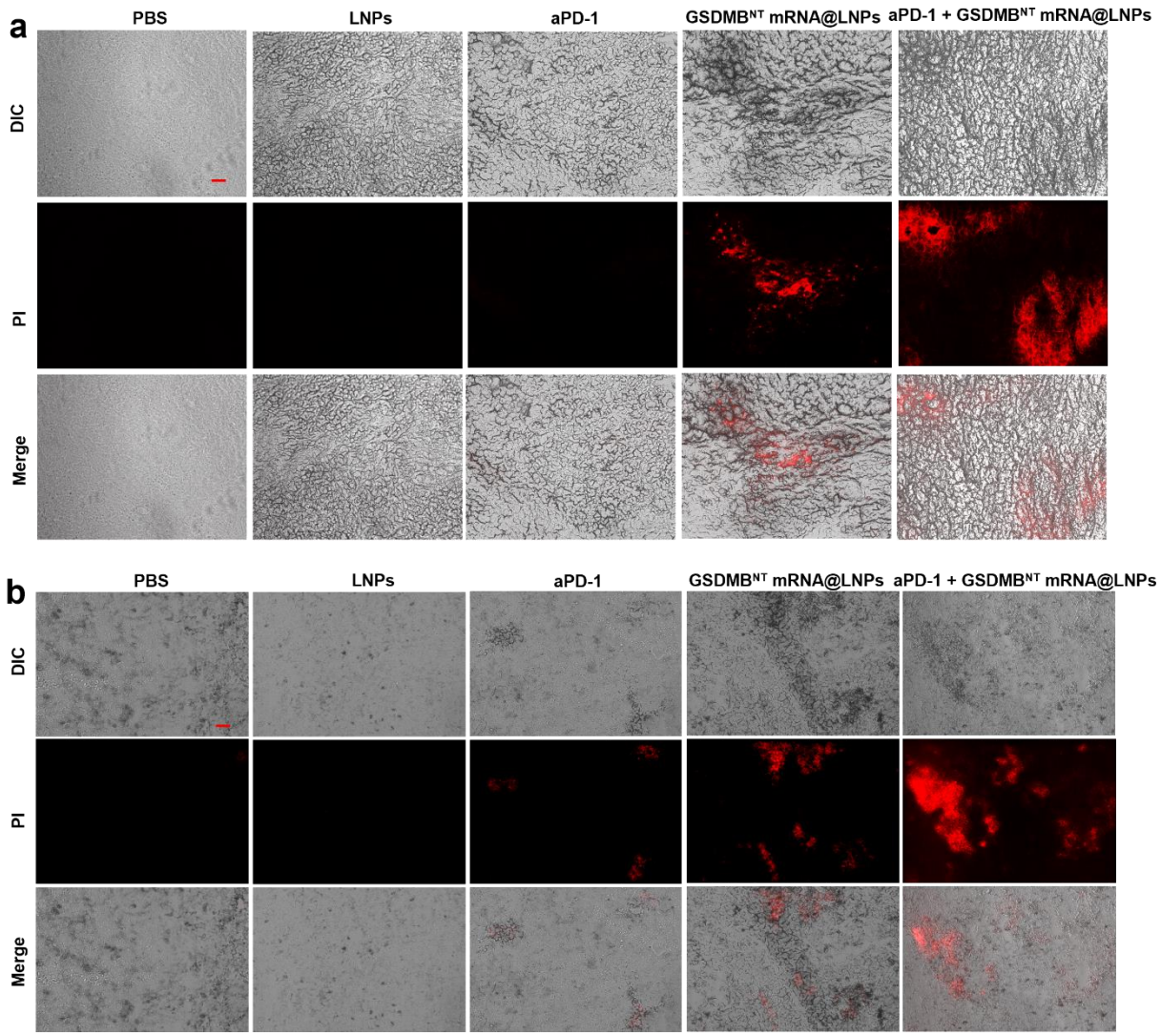
Supplementary Fig. 12 Cell viability of bone marrow-derived macrophages and DCs after treatment with GSDMB^{NT} mRNA@LNPs for 48 hours. Data are presented as means ± SD (n = 4 biological replicates per group). Source data are provided as a Source Data file.



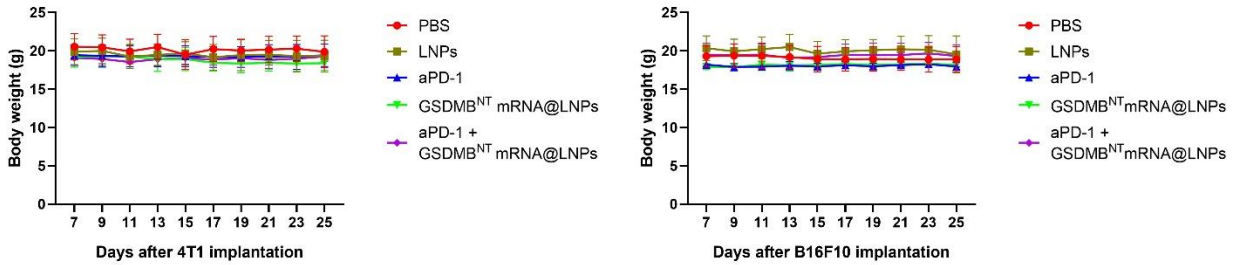
Supplementary Fig. 13 In vivo bioluminescence of luciferase mRNA-encapsulating LNPs of intratumoral injection in orthotopic 4T1 tumors. **a**, Representative images show luciferase activity after 6 hours. Anti-Reverse Cap Analog (ARCA)-capped luciferase mRNA (left) and CleanCap-capped luciferase mRNA (right). **b**, A time course of CleanCap-capped luciferase mRNA bioluminescence activity is shown as photons $s^{-1}cm^{-2}$ as values within regions of interest (ROI). Data are presented as means ± SD (n = 3 mice per group). Source data are provided as a Source Data file.



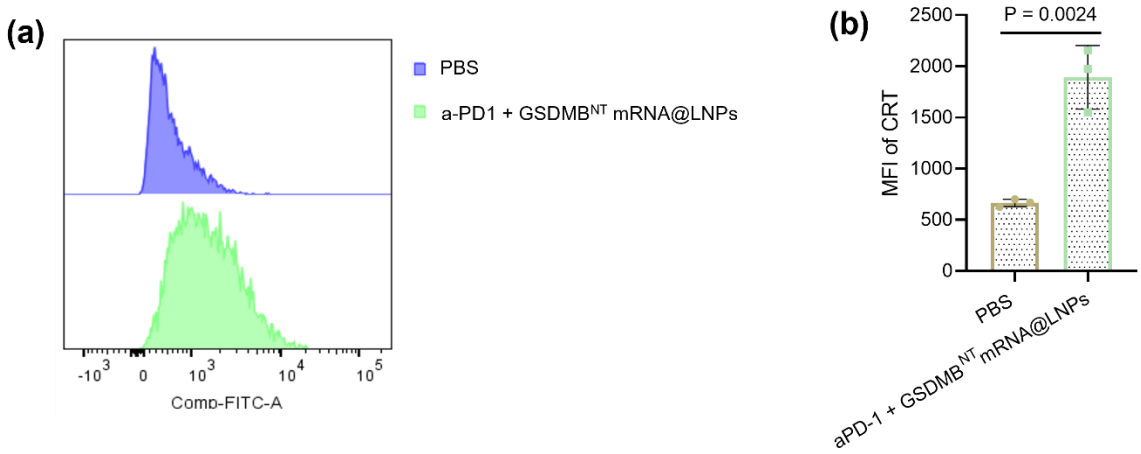
Supplementary Fig. 14 In vivo evaluation of CRT surface exposure in cells isolated from tumor tissues obtained from the 4T1-bearing mouse model. **(a)** Flow cytometry analysis of CRT-positive cells after the indicated treatments. **(b)** Histogram analysis performed by FlowJo software. Data are presented as mean ± SD (n = 3 mice per group). Statistical significance was calculated using a two-tailed Student's t test. MFI: mean fluorescence intensity. Source data are provided as a Source Data file.



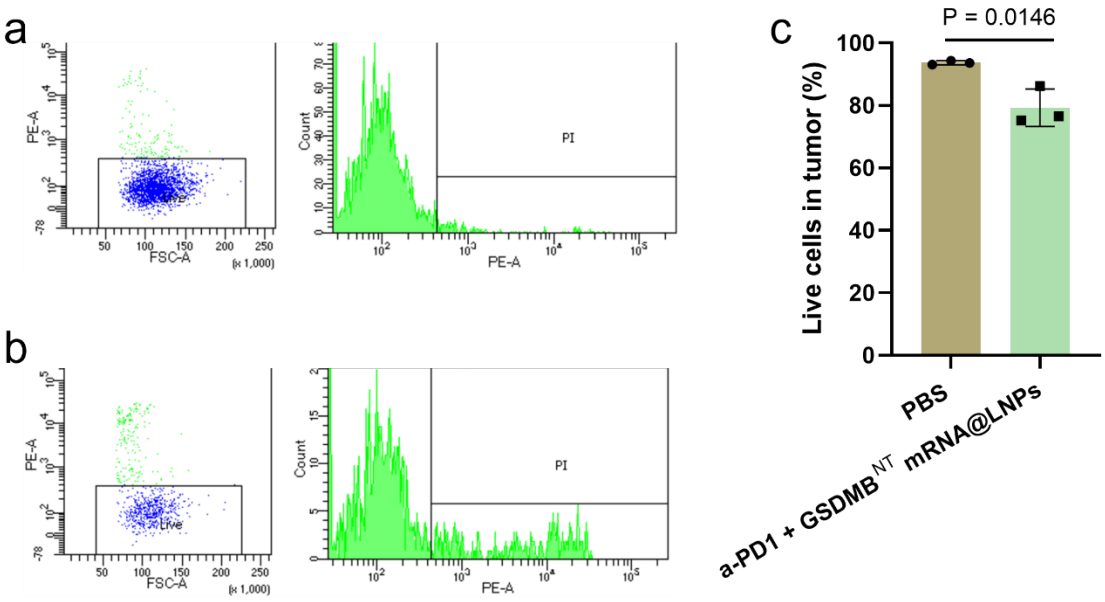
Supplementary Fig. 15 Representative tumor-section images of in vivo pyroptosis cells in the orthotopic 4T1-bearing mouse model (**a**) and B16F10-bearing mouse model (**b**), n = 3 mice per group. Propidium iodide was intravenously injected into the mice before the assay. Scale bar = 50 μm. Data are representative of three independent samples.



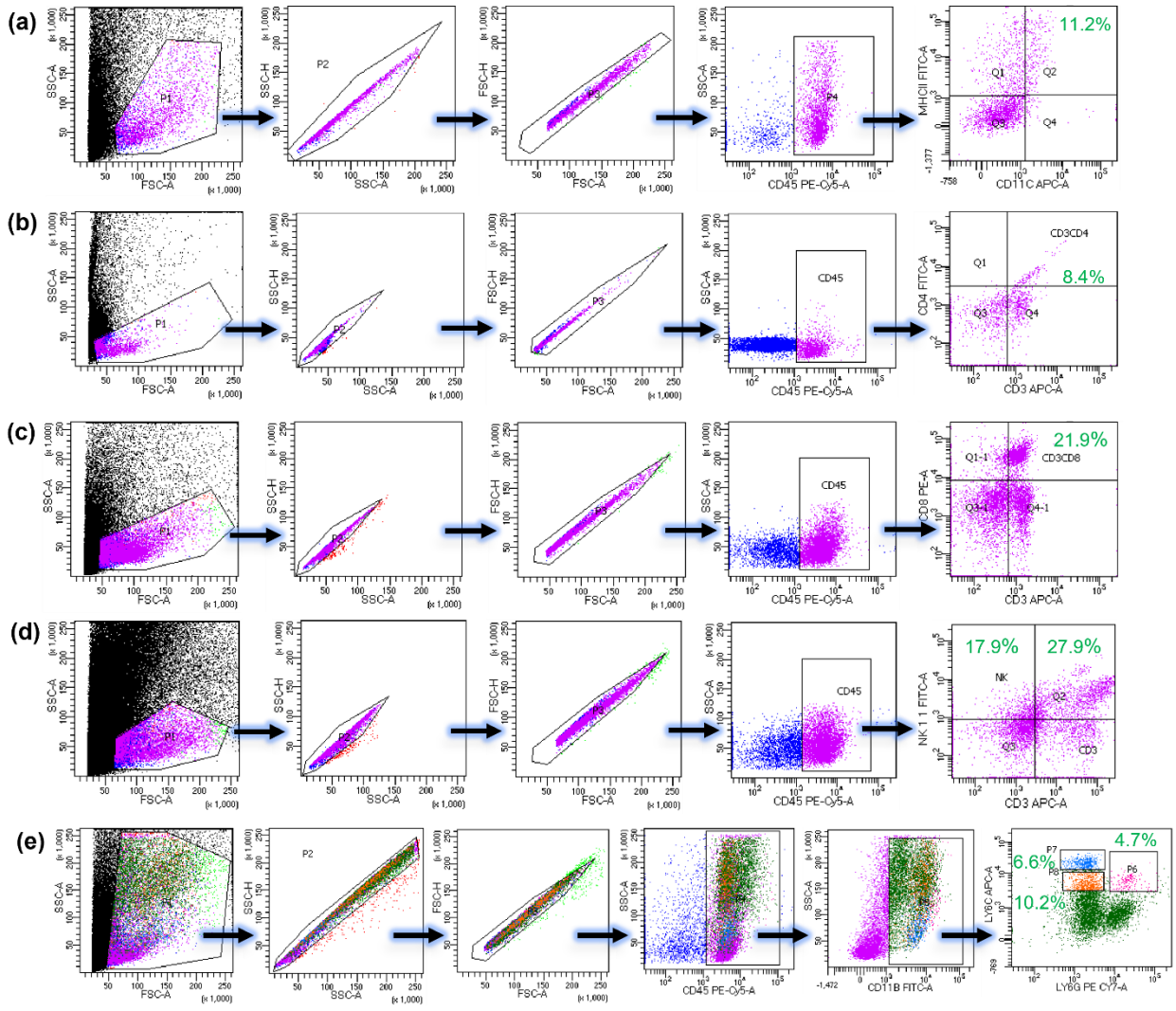
Supplementary Fig. 16 The changes in body weight in 4T1 tumor-bearing mice (left, n = 7 mice per group) and B16F10 tumor-bearing mice (right, n = 8 mice for PBS, aPD-1, or GSDMB^{NT} mRNA@LNP treatment groups, n = 7 mice for LNPs treatment group, and n = 10 mice for aPD-1 + GSDMB^{NT} mRNA@LNP treatment group) with treatments indicated. Data are presented as means ± SD. Source data are provided as a Source Data file.



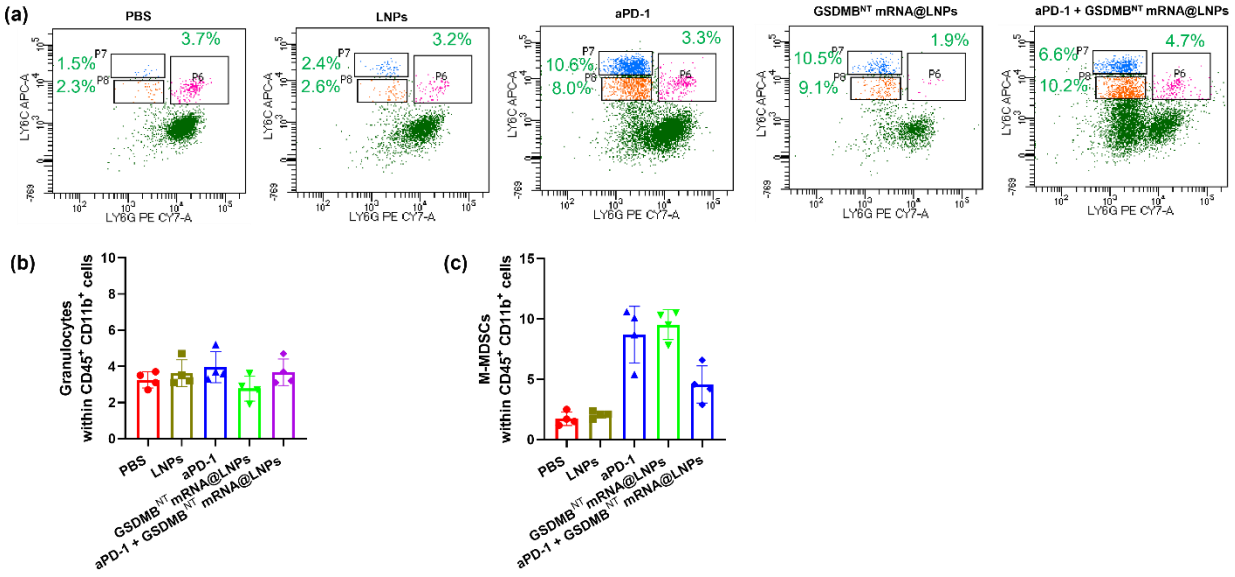
Supplementary Fig. 17 In vivo evaluation of CRT surface exposure in cells isolated from tumor tissues obtained from the B16F10-bearing mouse model. **(a)** Flow cytometry analysis of CRT-positive cells after the indicated treatments. **(b)** Histogram analysis performed by FlowJo software. Data are presented as mean ± SD (n = 3 mice per group). Statistical significance was calculated using a two-tailed Student's t test. MFI: mean fluorescence intensity. Source data are provided as a Source Data file.



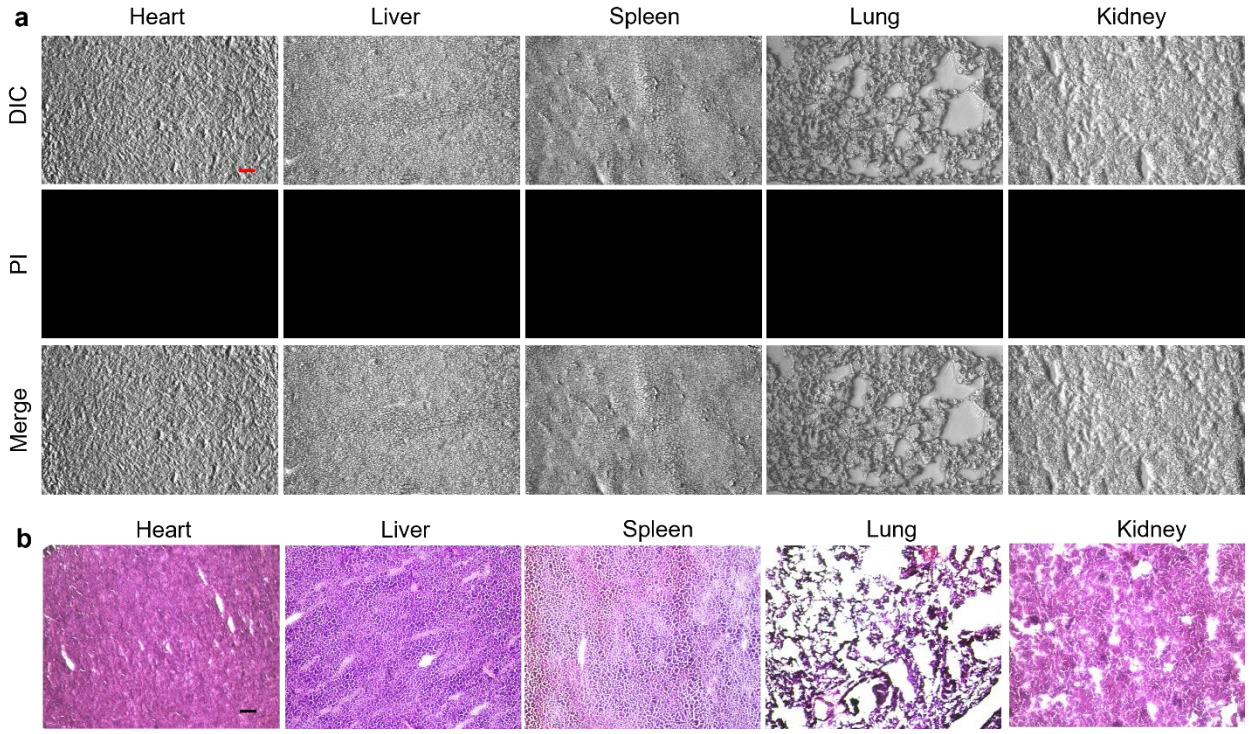
Supplementary Fig. 18 Cell viability assay in B16F10 tumors following combination treatment of aPD1 and GSDMB^{NT} mRNA@LNPs. **a**-**b**, Assessment of the population of PI-positive cells. **c**, Quantitative analysis of live cells in B16F10 tumors. Data are presented as means ± SD (n = 3 mice per group). Statistical significance was calculated using a two-tailed Student's t test. Source data are provided as a Source Data file.



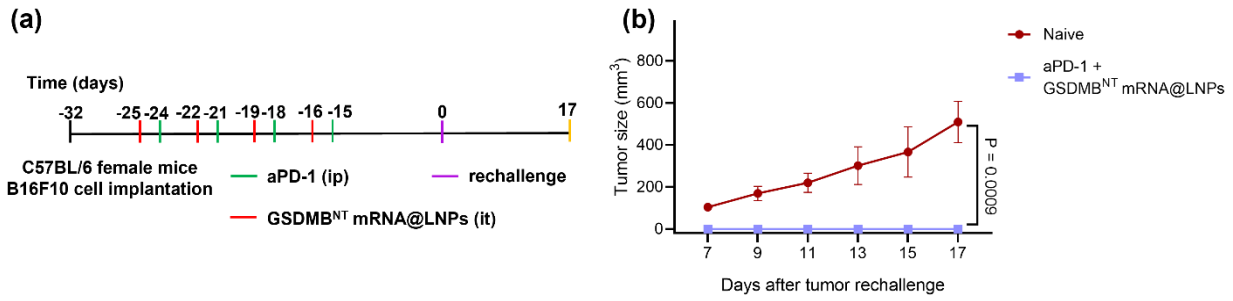
Supplementary Fig. 19 Primary gating strategy for flow cytometric analysis of dendritic cells **(a)**, $CD4^+$ T cells in tumors **(b)**, $CD8^+$ T cells **(c)**, NK and NK T (Q2) cells **(d)**, granulocytes (P6) **(e)**, M-MDSCs (P7), and monocyte cells (P8) in tumors. B16F10 tumor-bearing mice were euthanized, and lymph nodes were isolated on day 18. Singlet cells were selected from the cell population. $CD45^+$ cells were selected from the living cell population. MHC, major histocompatibility complex; FSC-A, forward scatter area; SSC-A, side scatter area; FSC-H, forward scatter height; SSC-H, side scatter height.



Supplementary Fig. 20 Presentative gating strategy **(a)**, quantitative analysis of granulocytes **(b)** and M-MDSCs **(c)** in tumors using flow cytometry. Data are presented as means ± SD (n = 4 mice per group). Source data are provided as a Source Data file.



Supplementary Fig. 21 a, Representative tumor-section images of in vivo pyroptosis cells in major organs in the combinational treatment of aPD-1 and GSDMB^{NT} mRNA@LNPs in B16F10-bearing mice, n = 3 mice per group. Propidium iodide was intravenously injected into the mice before the assay. **b**, H&E staining of major organs in the combination treatment of aPD-1 and GSDMB^{NT} mRNA@LNPs in B16F10 tumor-bearing mice. Scale bar = 50 μm. Data are representative of three independent samples.



Supplementary Fig. 22 Enhanced immunological memory of GSDMB^{NT} mRNA@LNPs in combination with aPD-1 using a B16F10 tumor rechallenge model. **(a)** Experimental timeline for treatment of B16F10 tumor-bearing mice and s.c. rechallenge. B16F10 tumor-bearing mice that had previously received a combination treatment regimen of aPD-1 and GSDMB^{NT} mRNA@LNPs were rechallenged with 5 × 10^5 B16F10 cells on the left flank. Naive mice were subcutaneously implanted with the same number of B16F10 cells on day 0 to serve as a control. The volume of the rechallenged tumors was monitored every two days. **(b)** The tumor growth profile for the naive group and the combination treatment group (GSDMB^{NT} mRNA@LNPs in combination with aPD-1). Data are presented as means ± SD (n = 3 mice per group). Statistical significance was calculated using a two-tailed Student's t test. Source data are provided as a Source Data file.

Supplementary Table 1: mRNA sequences used in this study.

Name	Sequences
5’ UTR	ACUAGUAUUCUUCUGGUCCCCACAGACUCAGAGAGAACCCGCC ACC
3’ UTR	GCUGGAGCCUCGGUGGCCUAGCUUCUUGCCCCUUGGGCCUCC CCCCAGCCCCUCCUCCCCUUCCUGCACCCGUACCCCCGUGGUC UUUGAAUAAAGUCUGAGUGGGCGGC
poly(A)	AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAGCAUAUGACUAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA AAAAAAAAAAAAAAAAAAAA
GSDMB^{NT} mRNA	AUGUUCAGCGUAUUUGAGGAAAUCACAAGAAUUGUAGUUAAGG AGAUGGAUGCUGGAGGGGAUAUGAUUGCCGUUAGAAGCCUUG UUGAUGCUGAUAGAUUCCGCUGCUUCCAUCUGGUGGGGGAGA AGAGAACUUUCUUUGGAUGCCGGCACUACACAACAGGCCUCAC CCUGAUGGACAUUCUGGACACAGAUGGGGACAAGUGGUUAGAU GAACUGGAUUCUGGGCUCCAAGGUCAAAAGGCUGAGUUUCAAA UUCUGGAUAAUGUAGACUCAACGGGAGAGUUGAUAGUGAGAUU ACCCAAAGAAAUAACAAUUUCAGGCAGUUUCCAGGGCUUCCAC CAUCAGAAAAUCAAGAUAUCGGAGAACCGGAUAUCCCAGCAGUA UCUGGCUACCCUUGAAAACAGGAAGCUGAAGAGGGAACUACCC UUUUCAUUCCGAUCAAUUAAUACGAGAGAAAACCUGUAUCUGG UGACAGAAACUCUGGAGACGGUAAAGGAGGAAACCCUGAAAAG CGACCGGCAAUAUAAAUUUUGGAGCCAGAUCUCUCAGGGCCAU CUCAGCUAUAAACACAAGGGCCAAAGGGAAGUGACCAUCCCCC CAAAUCGGGUCCUGAGCUAUCGAGUAAAGCAGCUUGUCUUCCC

	CAACAAGGAGACGAUGAAUAUUCAUUUCAGGGGCAAAACAAAAU CCUUUCCAGAAGAGAAGGAUGGUGCUUCAUCCUGUUUAGGAAA GUAA
GSDMB^{CT} mRNA	AUGUCUUUGGGUUCGGAGGAUUCCAGAAACAUGAAGGAGAAGU UGGAGGACAUGGAGAGUGUCCUCAAGGACCUGACAGAGGAGAA GAGAAAAGAUGUGCUAAACUCCCUCGCUAAGUGCCUCGGCAAG GAGGAUAUUCGGCAGGAUCUAGAGCAAAGAGUAUCUGAGGUCC UGAUUUCCGGGGAGCUACACAUGGAGGACCCAGACAAGCCUCU CCUAAGCAGCCUUUUUAAUGCUGCUGGGGUCUUGGUAGAAGCG CGUGCAAAAGCCAUUCUGGACUUCCUGGAUGCCCUGCUAGAGC UGUCUGAAGAGCAGCAGUUUGUGGCUGAGGCCCUGGAGAAGG GGACCCUUCCUCUGUUGAAGGACCAGGUGAAAUCUGUCAUGGA GCAGAACUGGGAUGAGCUGGCCAGCAGUCCUCCUGACAUGGAC UAUGACCCUGAGGCACGAAUUCUCUGUGCGCUGUAUGUUGUUG UCUCUAUCCUGCUGGAGCUGGCUGAGGGGCCUACCUCUGUCU CUUCCGAUUACAAGGAUGACGACGAUAAGUAA
GSDMB^{FL} mRNA	AUGUUCAGCGUAUUUGAGGAAAUCACAAGAAUUGUAGUUAAGG AGAUGGAUGCUGGAGGGGAUAUGAUUGCCGUUAGAAGCCUUG UUGAUGCUGAUAGAUUCCGCUGCUUCCAUCUGGUGGGGGAGA AGAGAACUUUCUUUGGAUGCCGGCACUACACAACAGGCCUCAC CCUGAUGGACAUUCUGGACACAGAUGGGGACAAGUGGUUAGAU GAACUGGAUUCUGGGCUCCAAGGUCAAAAGGCUGAGUUUCAAA UUCUGGAUAAUGUAGACUCAACGGGAGAGUUGAUAGUGAGAUU ACCCAAAGAAAUAACAAUUUCAGGCAGUUUCCAGGGCUUCCAC CAUCAGAAAAUCAAGAUAUCGGAGAACCGGAUAUCCCAGCAGUA

	UCUGGCUACCCUUGAAAACAGGAAGCUGAAGAGGGAACUACCC UUUUCAUUCCGAUCAAUUAAUACGAGAGAAAACCUGUAUCUGG UGACAGAAACUCUGGAGACGGUAAAGGAGGAAACCCUGAAAAG CGACCGGCAAUAUAAAUUUUGGAGCCAGAUCUCUCAGGGCCAU CUCAGCUAUAAACACAAGGGCCAAAGGGAAGUGACCAUCCCCC CAAAUCGGGUCCUGAGCUAUCGAGUAAAGCAGCUUGUCUUCCC CAACAAGGAGACGAUGAAUAUUCAUUUCAGGGGCAAAACAAAAU CCUUUCCAGAAGAGAAGGAUGGUGCUUCAUCCUGUUUAGGAAA GUCUUUGGGUUCGGAGGAUUCCAGAAACAUGAAGGAGAAGUUG GAGGACAUGGAGAGUGUCCUCAAGGACCUGACAGAGGAGAAGA GAAAAGAUGUGCUAAACUCCCUCGCUAAGUGCCUCGGCAAGGA GGAUAUUCGGCAGGAUCUAGAGCAAAGAGUAUCUGAGGUCCUG AUUUCCGGGGAGCUACACAUGGAGGACCCAGACAAGCCUCUCC UAAGCAGCCUUUUUAAUGCUGCUGGGGUCUUGGUAGAAGCGC GUGCAAAAGCCAUUCUGGACUUCCUGGAUGCCCUGCUAGAGCU GUCUGAAGAGCAGCAGUUUGUGGCUGAGGCCCUGGAGAAGGG GACCCUUCCUCUGUUGAAGGACCAGGUGAAAUCUGUCAUGGAG CAGAACUGGGAUGAGCUGGCCAGCAGUCCUCCUGACAUGGACU AUGACCCUGAGGCACGAAUUCUCUGUGCGCUGUAUGUUGUUGU CUCUAUCCUGCUGGAGCUGGCUGAGGGGCCUACCUCUGUCUC UUCCGAUUACAAGGAUGACGACGAUAAGUAA

Supplementary Table 2: Antibodies used in this study.

Antibody	Clone	Conjugation	Company (Cat#)	Dilution
anti-mouse CD16/32, mAb	93	unconjugated	BioLegend (101302)	1:100
anti-mouse CD45.2, mAb	104	PerCP/Cyanine5.5	BioLegend (109828)	1:100
anti-mouse CD11c, mAb	N418	APC	BioLegend (117310)	1:100
anti-mouse I-A/I-E, mAb	M5/114.15.2	FITC	BioLegend (107605)	1:100
anti-mouse CD3, mAb	17A2	APC	BioLegend (100236)	1:100
anti-mouse CD4, mAb	GK1.5	FITC	BioLegend (100405)	1:100
anti-mouse CD8a, mAb	53-6.7	PE	BioLegend (100708)	1:100
anti-mouse NK-1.1, mAb	PK136	FITC	BioLegend (108706)	1:100
anti-mouse/human CD11b, mAb	M1/70	FITC	BioLegend (101206)	1:100
anti-mouse Ly-6C, mAb	HK1.4	APC	BioLegend (128015)	1:100
anti-mouse Ly-6G, mAb	1A8	PE/Cyanine7	BioLegend (127617)	1:100
anti-mouse CD86, mAb	GL-1	APC	BioLegend (105011)	1:100
rabbit monoclonal [EPR3924] to Calreticulin- ER Marker	EPR3924	Alexa Fluor® 488	Abcam (ab196158)	1:50
rabbit polyclonal to Calreticulin- ER Marker		unconjugated	Abcam (ab2907)	1:500
rabbit monoclonal [EPR21769] to CD8 alpha	EPR21769	unconjugated	Abcam (ab217344)	1:500
rabbit monoclonal [EPR20841] to GSDMB	EPR20841	unconjugated	Abcam (ab215729)	1:1000

mAb, monoclonal antibody.