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Title

STING agonist loaded lipid nanoparticles overcome anti-PD-1 resistance in melanoma lung metastasis via NK cell activation

Authors

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Foot note
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Methods

Measurement of IFN-β concentration in the serum

Mice were intravenously administered with the STING-LNP (6 μg of c-di-GMP). After 1.5 or 2 h, the blood samples were collected, and sera were obtained. The concentration of IFN-β in the serum were measured by ELISA kit (R&D systems, Basel, Switzerland) following the manufacturer's instructions.

mRNA expression in the tissues

The B16-F10-luc2 lung metastatic mouse model was prepared. After treatment with the STING-LNPs and the reagents, the lungs, the spleens and the livers were collected. In the case of tumor, the tumor colonies in the lung were collected. The measurement of mRNA expression was carried out as reported previously.[1] The tissues (25 mg) were added to 600 μL of RNAiso Plus (Takara Bio, Shiga, Japan) with zirconia beads (mixture of 1 mm and 2 mm), followed by the homogenization with a Micro Smash homogenizer (MS-100R, TOMY, Tokyo, Japan). RNA was isolated with a Direct-zol RNA MiniPrep (Zymo Research, Irvine, CA) according the manufacturer's instructions. The total RNA was reverse transcribed using a PrimeScript RT reagent Kit with an oligo-dT primer and random 6 mers. Quantitative PCR was carried out on a Light Cycler 480 System in a reaction mixture containing cDNA, with appropriate pairs of primers and the THUNDERBIRD SYBR qPCR Mix. The sequences of primer pairs are shown in the Table S1 in the supplemental material. The mRNA levels were calculated by the comparative CT method using β-glucuronidase (Gusb) as an endogenous gene. The values for the PBS treated mice were set at 1.

Evaluation of killing of B16-F10 cells by NK cells

Mice were intravenously administered with PBS or the STING-LNP (6 μg of c-di-GMP). After 24 h, the spleens were collected and the splenocytes were prepared. NK cells were isolated with MojoSort™ Mouse NK Cell Isolation Kit (Biolegend). The isolated NK cells (1.25×10^4 cells/well, 6.24×10^4 cells/well, or 12.5×10^4 cells/well) were added to B16-F10-luc2 cells (1.25×10^4 cells/well) in 24-well plates, followed by 24 h culture. After washing with PBS, the cell viability was evaluated by a Premix WST-1 Cell proliferation Assay System (Takara Bio Inc., Shiga, Japan). The absorbance of the well without NK cells was set at 100%.

PD-L1 expression in B16-F10-luc2 in vitro

B16-F10-luc2 cells (1×10^5 cells/well) were seeded in 6-well plates and

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cultured for 24 h. Mouse recombinant IFN-β (80 ng/well) or IFN-γ (40 ng/well) were added to the well, followed by a 48 h culture. After collecting the cells, the purified anti-mouse CD16/32 was added to the cells (1×10^6 cells) for blocking. The cells were stained with PE anti-mouse CD274 and the isotype control. The cells were analyzed by flow cytometry (IFN-β: CytoFLEX, IFN-γ: Gallios, Beckman Coulter, Indianapolis, IN). The FlowJo software was used for the data analysis.

Table S1.

Sequences of primer pairs used for the analysis of gene expression at the mRNA level.

| Gene	Forward primer	Reverse primer
Cd3	ATGCGGTGGAACACTTTCTGG	GCACGTCAACTCTACACTGGT
Cd4	CTTCGCAGTTTGATCGTTTTGAT	CCGGACTGAAGGTCACTTTGA
Cd8	AAGAAAATGGACGCCGAACTT	AAGCCATATAGACAACGAAGGTG
Foxp3	TGCAGGGCAGCTAGGTACTTG	TCGGAGATCCCCTTTGTCTTATC
Nk1.1	ACACAGCAAGTATCTACCTCGG	GTAAGACTCGCACTAAGACACTCAT
Pd-1	ACCCTGGTCATTCACTTGGG	CATTTGCTCCCTCTGACACTG
Ifng	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
Pd-l1	GACGCAGGCGTTTACTGCT	GCGGTATGGGGCATTGACTTT
Cd11b	GGGAGGACAAAAACTGCCTCA	ACAACTAGGATCTTCGCAGCAT
Nkg2d	GCACTAACTACCAGTCAACCTG	CTCGAACAACGAACATTGGAGA
Cd69	AAGCGATATTCTGGTGAACTGG	ATTTGCCCATTTCCATGTCTGA
Fasl	TCCGTGAGTTCACCAACCAA	CACTCCAGAGATCAGAGCGG
F4/80	TTGTACGTGCAACTCAGGACT	GATCCCAGAGTGTTGATGCAA
Il-6	CTGCAAGAGACTTCCATCCAG	AGTGGTATAGACAGGTCTGTTGG
Tnfa	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG
Ifnb	CACTGTCTGCTGGTGGAGTTCATC	AAGAGTTACACTGCCTTTGCCATC
Gusb	GTGGTATGAACGGGAAGCAAT	AACTGCATAATAATGGGCACTGT
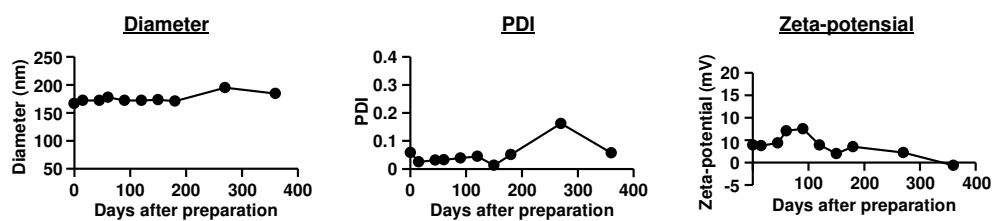
Figure S1. Characteristics of STING-LNPs during storage at 4°C.

STING-LNPs were stored at 4°C for 360 days and their diameters, PDI, zeta-potentials were measured over time.
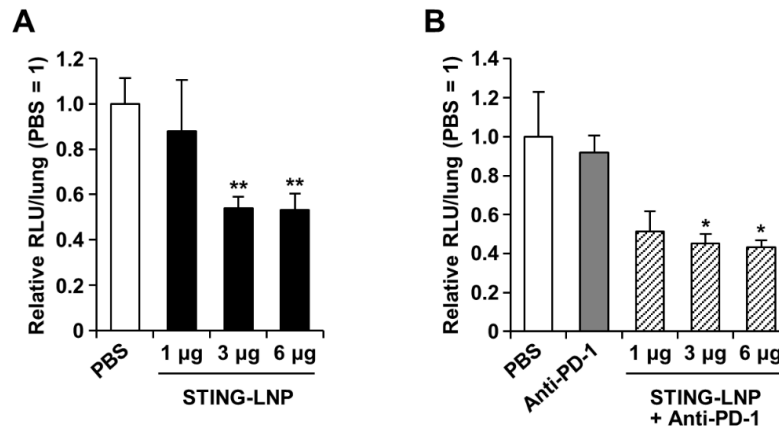
Figure S2. Dose response of antitumor effect against B16-F10 lung metastasis.

(A) Antitumor effect of STING-LNP monotherapy against B16-F10 lung metastasis. B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with the STING-LNP (1, 3 or 6 µg of c-di-GMP) on days 2, 4 and 8. On day 18, the lungs were collected, and the antitumor effect evaluated. The value of RLU per whole lung for the PBS treated mice were set to 1. Data are the mean + SEM (n=4-9, **P<0.01). (B) Antitumor effect of combination therapy against B16-F10 lung metastasis. B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with the STING-LNP (6 µg of c-di-GMP) on days 6, 10 and 14. The mice were intraperitoneally injected with 50 µg of anti-PD-1 on days 8, 12 and 16. On day 18, the lungs were collected, and the antitumor effect evaluated. The value of RLU per whole lung for the PBS treated mice were set to 1. Data are the mean + SEM (n=3-4, *P<0.05).
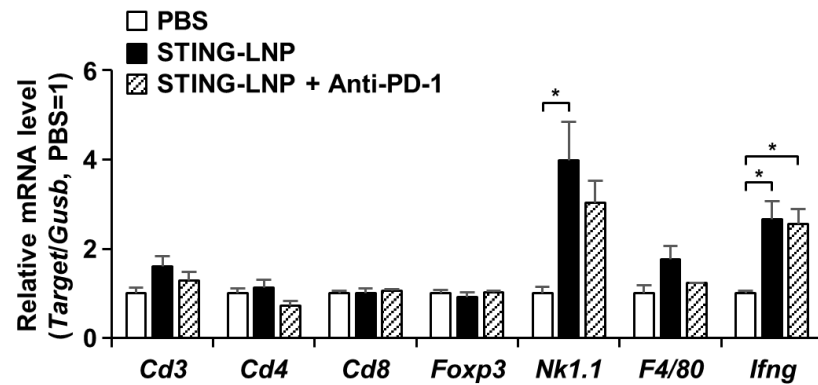
Figure S3. Gene expression at the mRNA level in the lung with B16-F10 metastasis after the combination STING-LNP and anti-PD-1 therapy.

Mice were intravenously injected with B16-F10-luc2 cells (2×10^5 cells). The mice were intravenously injected with the STING-LNP (6 µg of c-di-GMP) on day 6. intraperitoneally injected with 50 µg of anti-PD-1 on day 8. On day 9, the lungs were collected, and the mRNA levels measured by RT-qPCR. The values for the PBS treated mice were set to 1. Data are the mean + SEM (n=3, *P<0.05).
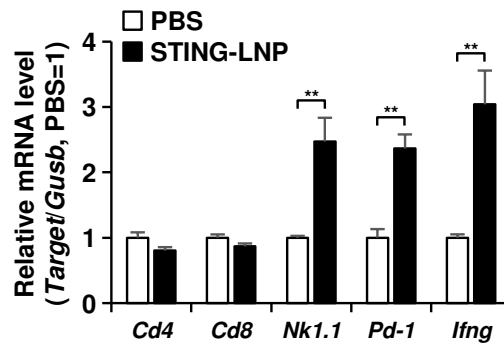
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Figure S4. Gene expression at the mRNA level in the lung with B16-F10 metastasis after the first STING-LNP treatment.

Mice were intravenously injected with B16-F10-luc2 cells (2×10^5 cells). The mice were intravenously injected with the STING-LNP (6 μg of c-di-GMP) on day 2. On day 3, the lungs were collected, and mRNA levels were measured by RT-qPCR. The values for the PBS treated mice were set to 1. Data are the mean + SEM (n=3, **P<0.01).
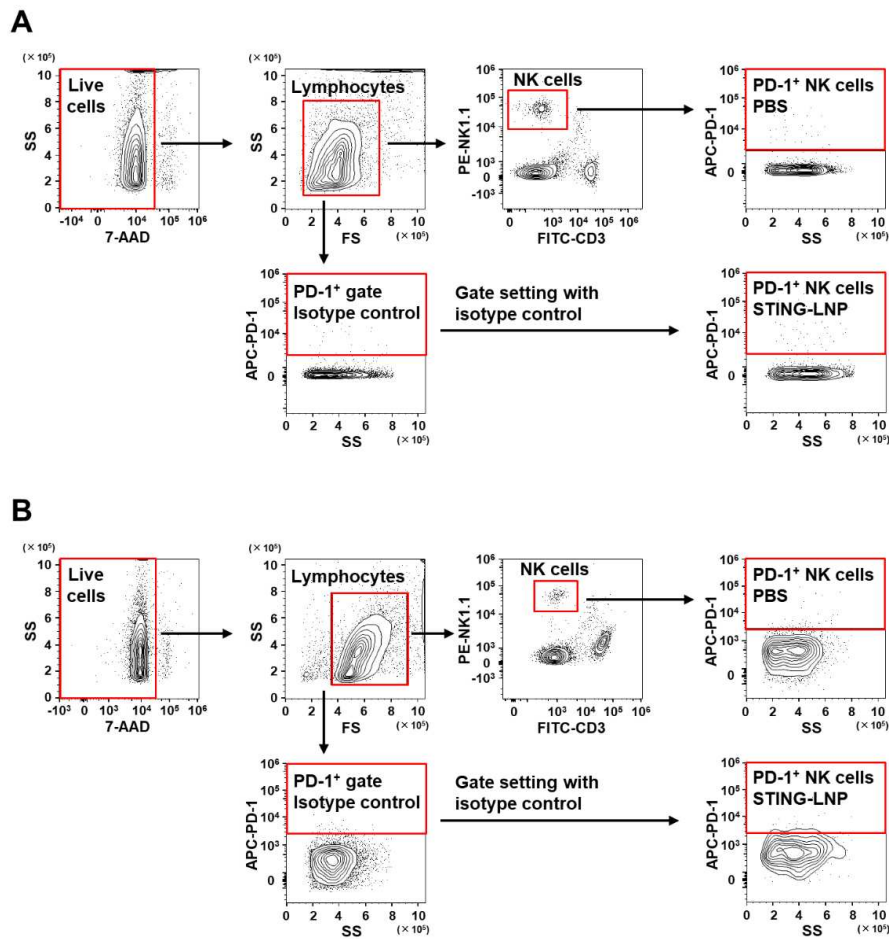
Figure S5. Gating strategy for FCM analysis in the lung and the spleen.

(A) Gating strategy for the FCM analysis of lung NK cells. (B) Gating strategy for the FCM analysis of spleen NK cells. After gating live cells and lymphocytes, NK cells were identified as a CD3$^-$NK1.1$^+$ cells. The PD-1$^+$ gate, the CD69$^+$ gate and the NKG2D$^+$ gate were set by using an isotype control.

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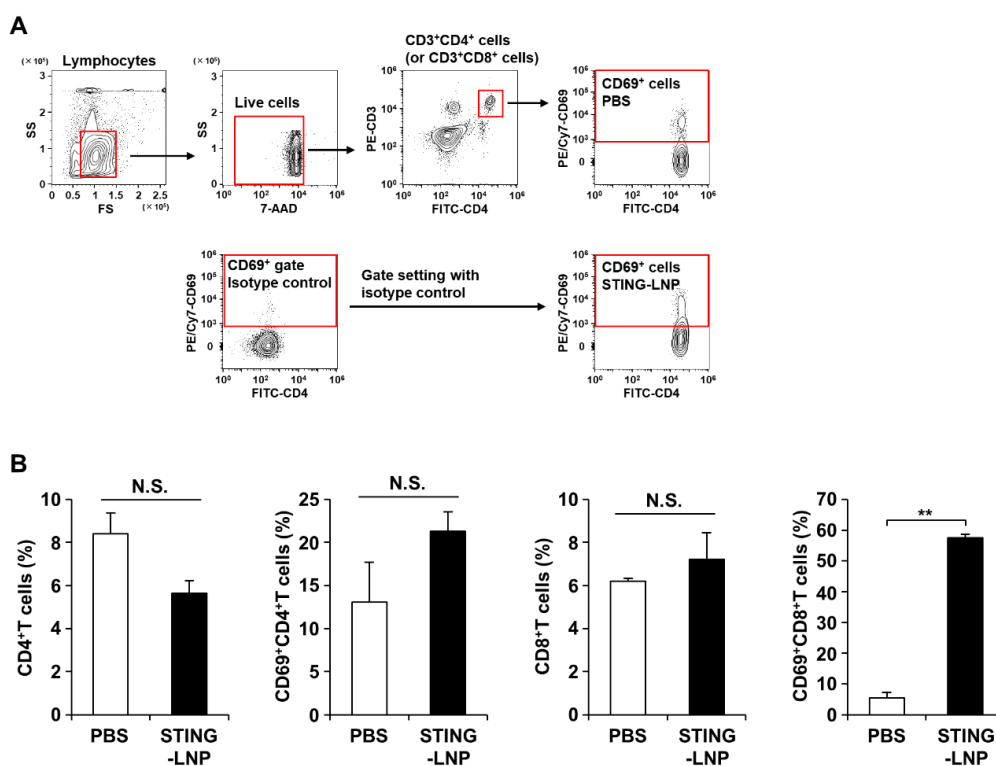
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Figure S6. Analysis of T cell population in the lung after the treatment of STING-LNP.

B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with the STING-LNP (6 µg of c-di-GMP) on days 2, 4 and 8. On day 9, RPMI1640 medium containing 1.25 mg/mL collagenase D was injected into the lung via trachea and the trachea was tied. The lungs were collected and incubated in RPMI1640 medium containing 1.25 mg/mL collagenase D for 30 min at 37°C. The lung cells were then dispersed using scissors and a syringe/18G and 20G needles. After washing the cells, the lymphocyte fraction was collected by Percoll density centrifugation (40% and 80% Percoll). The purified anti-mouse CD16/32 was added to the lymphocytes for blocking. The lymphocytes were then stained with FITC anti-mouse CD4, FITC anti-mouse CD8a, PE anti-mouse CD3, and PE/Cy7 anti-mouse CD69, and the isotype controls (Biolegend). The lymphocytes which were stained with a 7-AAD Viability Staining Solution were analyzed by flow cytometry (Gallios, Beckman Coulter, Indianapolis, IN). The FlowJo software was used for the data analysis. (A) Gating strategy. After gating lymphocyte population and live cell population, CD4+ T cells (CD3+CD4+ cells) and CD8+ T cells (CD3+CD8+ cells) were gated. For gating

the CD69$^+$ cells, the isotype control was used. (B) The percentages of CD4$^+$ T cells, CD69$^+$CD4$^+$ T cells, CD8$^+$ T cells, CD69$^+$CD8$^+$ T cells in the lung. Data are the mean + SEM (n=3, **P<0.01, N.S.: not significant).
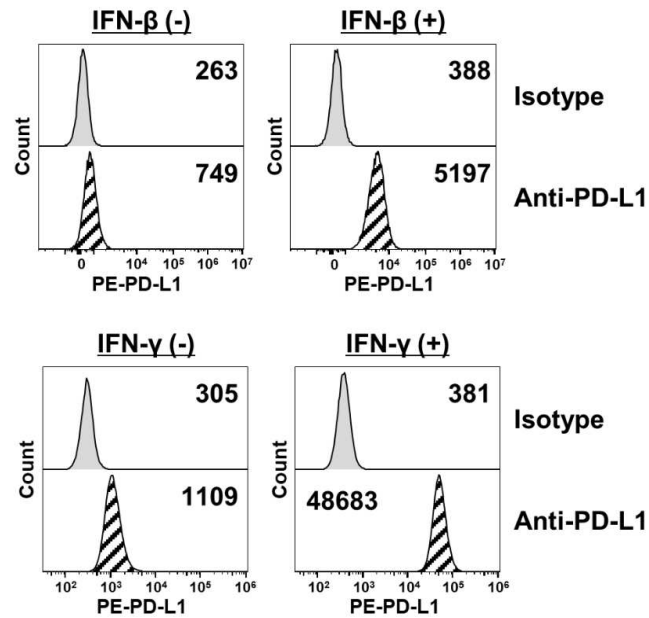
Figure S7. PD-L1 expression in B16-F10-luc2 cells (in vitro).

B16-F10-luc2 cells were treated with IFN-β or IFN-γ for 48 h and then stained with anti-PD-L1. The cells were analyzed by flow cytometry. The filled histogram and the stripped histogram represent the isotype control and the anti-PD-L1 antibody, respectively. The values in the histograms represent the median FI.
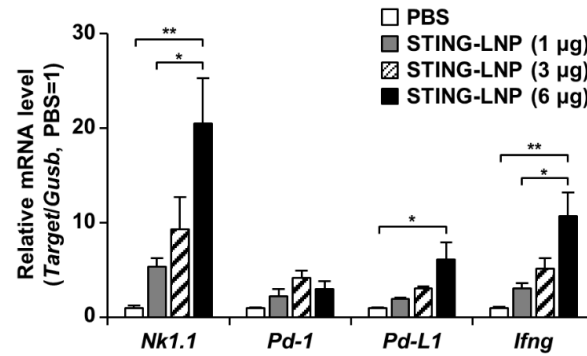
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Figure S8. Dose response of mRNA expression in the B16-F10 colonies.

B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with the STING-LNP (1, 3 or 6 µg of c-di-GMP) on days 12, 14 and 16. On day 18, the B16-F10 colonies were collected, and mRNA levels were measured by RT-qPCR. The values for the PBS treated mice were set to 1. Data are the mean + SEM (n=3, *P<0.05, **P<0.01).
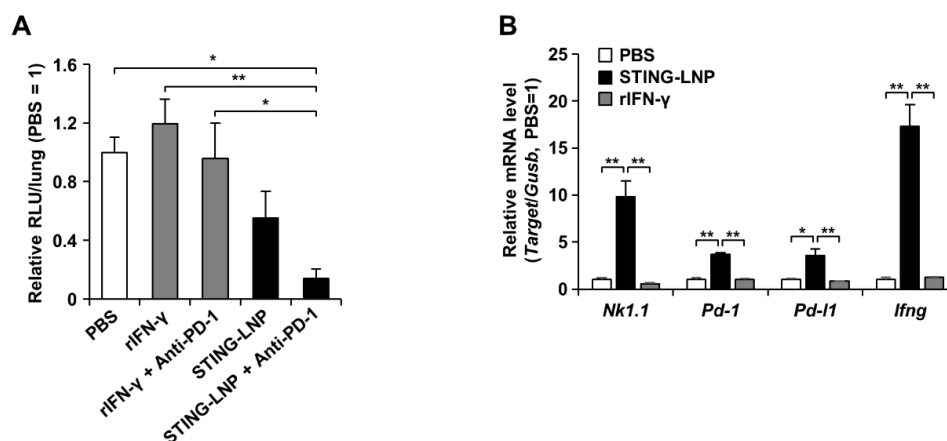
Figure S9. Effect of circulated IFN-γ on the antitumor activity.

(A) Antitumor effect of combination therapy against B16-F10 lung metastasis. B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with recombinant IFN-γ (rIFN-γ) (1.1 μg) or the STING-LNP (6 μg of c-di-GMP) on days 6, 10 and 14. The mice were intraperitoneally injected with 50 μg of anti-PD-1 on days 8, 12 and 16. On day 18, the lungs were collected, and the antitumor effect evaluated. The value of RLU per whole lung for the PBS treated mice were set to 1. Data are the mean + SEM (n=3-4, *P<0.05, **P<0.01). (B) Analysis of mRNA expression in the B16-F10 colonies. B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. The mice were intravenously injected with rIFN-γ (1.1 μg) or the STING-LNP (6 μg of c-di-GMP) on days 12, 14 and 16. On day 18, the B16-F10 colonies were collected, and mRNA levels were measured by RT-qPCR. The values for the PBS treated mice were set to 1. Data are the mean + SEM (n=3, *P<0.05, **P<0.01).

The dose of rIFN-γ was determined based on the IFN-γ concentration in the serum 8 h after the treatment of STING-LNP. The IFN-γ concentration was 776.3 pg/mL. A blood volume of mouse is 1.46 mL.
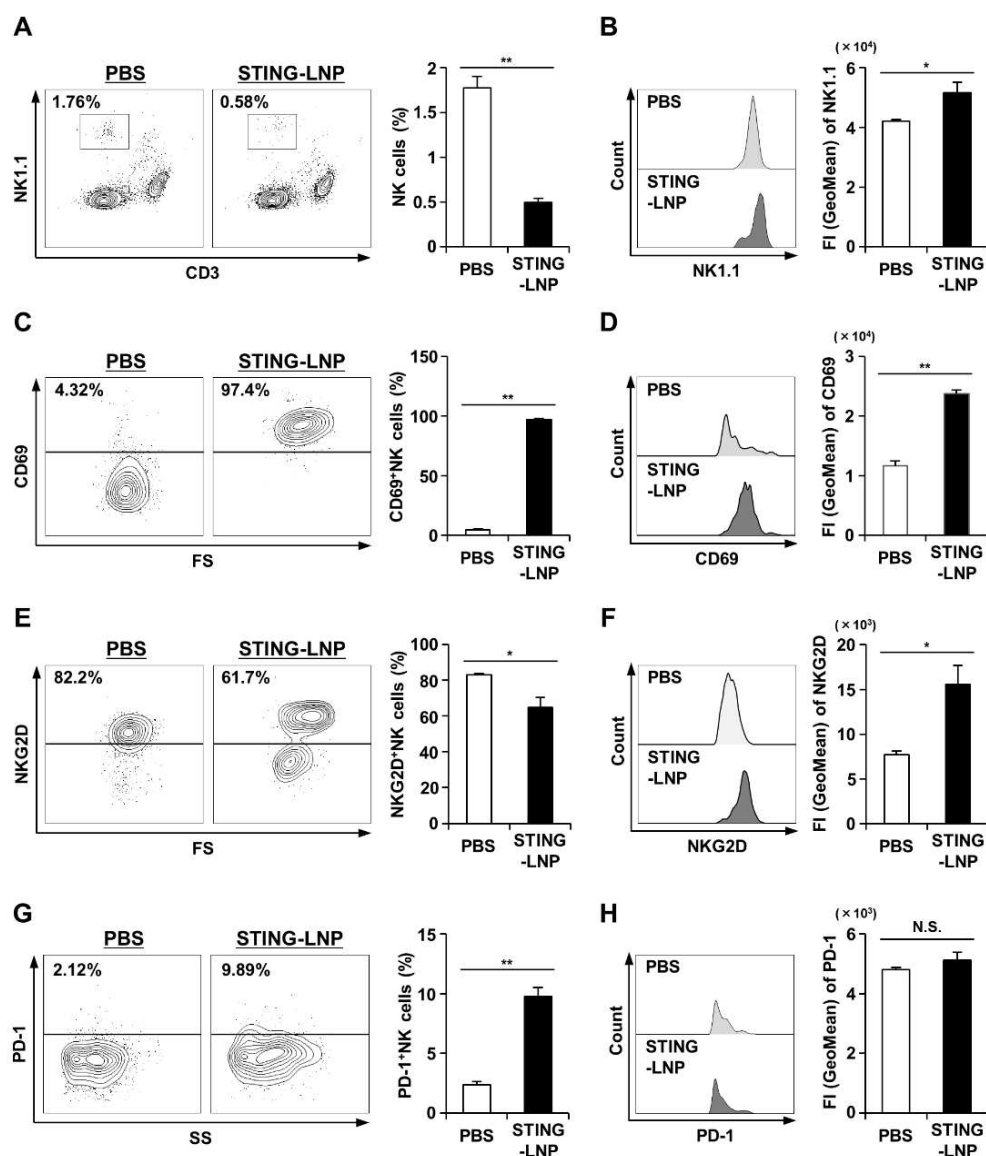
Figure S10. Analysis of NK cells in the spleen after the STING-LNP treatment.

B16-F10-luc2 cells (2×10^5 cells) were intravenously injected to mice. On day 2, the mice were intravenously injected with the STING-LNP (6 μg of c-di-GMP). After 24 h, the spleens were collected and the splenocytes were prepared. The purified anti-mouse CD16/32 was added to the splenocytes (1×10^6 cells) for blocking. The splenocytes were stained with FITC anti-mouse CD3, PE anti-mouse NK1.1, PE/Cy7 anti-mouse CD69, APC anti-mouse CD314, APC anti-mouse CD279 (PD-1) and the

isotype controls (Biolegend). The splenocytes which were stained with a 7-AAD Viability Staining Solution were analyzed by flow cytometry (Gallios). The FlowJo software was used for the data analysis. NK cells were identified as CD3⁻NK1.1⁺ cells. (A) NK cell population. (B) NK1.1 expression of NK cells. (C) CD69⁺ NK cell population. (D) CD69 expression of NK cells. (E) NKG2D⁺ NK cell population. (F) NKG2D expression of NK cells. (G) PD-1⁺ NK cell population. (H) PD-1 expression of NK cells. The FIs (GeoMean) (B, D, F, H) were calculated in whole NK cells. Data are the mean + SEM (n=3, *P<0.05, **P<0.01). N.S.: no significant difference.
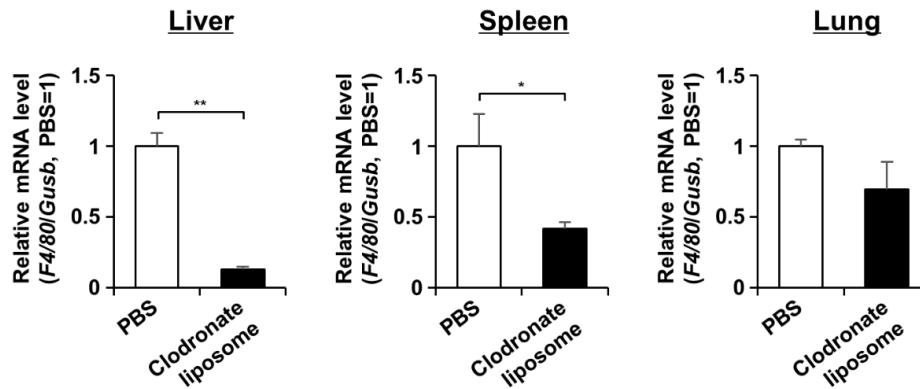
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Figure S11. Macrophage depletion caused by a treatment with clodronate liposomes.

The mice were intravenously injected with 150 µL of clodronate liposomes. After 24 h, the tissues were collected and the mRNA levels of F4/80 were measured by RT-qPCR. The values for the PBS treated mice were set to 1. Data are the mean + SEM (n=3, *P<0.05, **P<0.01).
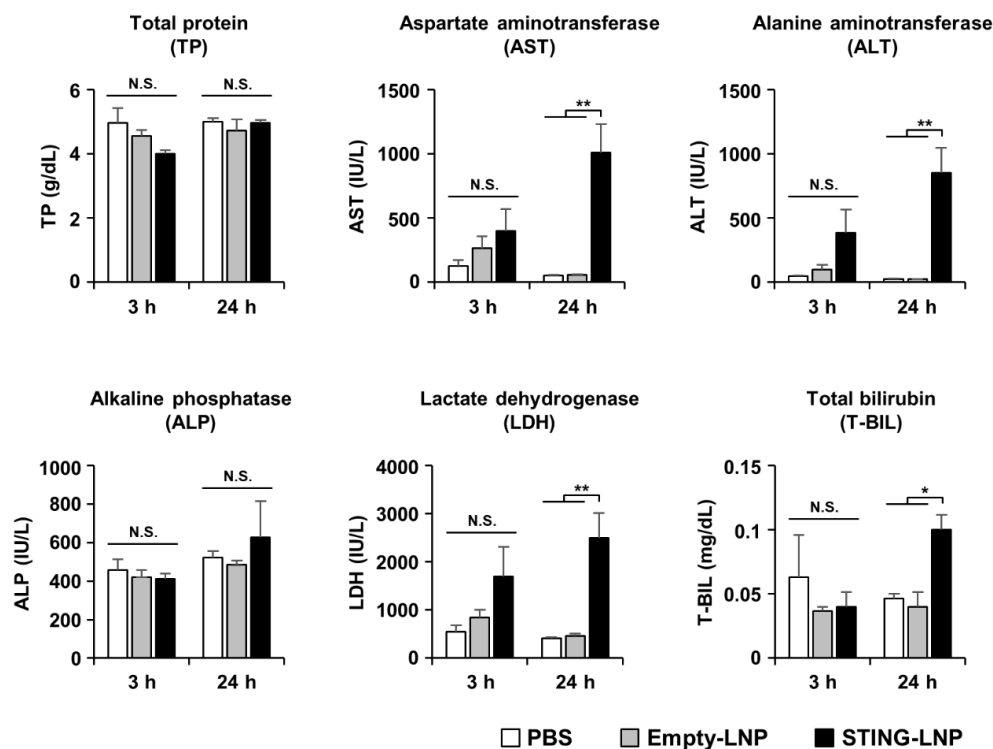
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Figure S12. Biochemical analysis of blood after the administration of the STING-LNP.

Mice were intravenously administered with PBS, empty-LNP (the same amount of lipid as the STING-LNP) and STING-LNP (6 μg of c-di-GMP). Serum was collected after 3 and 24 h and subjected to biochemical analysis (TP: total protein; AST: aspartate aminotransferase; ALT: alanine aminotransferase; ALP: alkaline phosphatase; LDH: lactate dehydrogenase; T-BIL: total bilirubin) by the Oriental Yeast Co., Ltd (Tokyo, Japan). Data are the mean + SEM (n=3, *P<0.05, **P<0.01, N.S.: not significant).

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