

Supplementary Materials

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Supplementary Methods

Cell lines and media

The KPC(*LSL-Kras*^{G12D/+};*LSL-Trp53*^{R172H/+};*Pdx-1-Cre*) tumor cell line is a PDAC cell line that was previously established from a C57Bl/6 background mouse model as previously described¹⁷. The KPC cells were cultured at a temperature of 37°C in a 5% CO₂ incubator using RPMI 1640 media(Life Technologies). The media was supplemented with 10% heat-inactivated fetal bovine serum(HI-FBS, Benchmark), 1% penicillin/streptomycin(pen/strep, Life Technologies), 1% MEM Non-Essential Amino Acids Solution(MEM-NEAA, Life Technologies), 1% L-glutamine(Life Technologies), and 1% sodium pyruvate(Sigma). Harvested tumor-infiltrating immune cells were processed using T cell culture media consisting of RPMI 1640 media(Life Technologies) supplemented with 10% heat-inactivated fetal bovine serum(HI-FBS, Benchmark), 1% penicillin/streptomycin(pen/strep, Life Technologies), 1% MEM Non-Essential Amino Acids Solution(MEM-NEAA, Life Technologies), 1% HEPES(Life Technologies), 1% L-glutamine(Life Technologies), and 0.05% 2-mercaptoethanol(Sigma).

Mice and in vivo experiments

The hemi-spleen preclinical pancreatic cancer metastatic model using KPC cells was performed as described previously¹⁸. Briefly, on day 0, the spleen was eviscerated from the anesthetized mouse, clipped and divided in half. One-half of the spleen was injected with 7.5×10^5 KPC cells resuspended in 100 μ L of PBS with the anti-clumping agent (Life Technologies) at a dilution of 1:1000 and flushed with 150 μ L of PBS in the same syringe. The injected hemi-spleen segment was then surgically removed to eliminate residual tumor cells. The KPC cells were passaged and cultured until day 7, on which 5×10^5 cells were subcutaneously injected into the bilateral flanks

of the postoperative mice. Small-animal ultrasound (Vevo770, VisualSonics) was employed to screen for liver metastatic lesion on day 12. Mice met the specified inclusion criteria were randomly assigned to the treatment groups: 1) a liver metastatic lesion length between 3 to 5 mm as measured by ultrasonography, 2) absence of in situ tumors within the abdominal cavity, 3) presence of palpable bilateral subcutaneous tumors, and 4) overall good health.

The mice were monitored twice a week until death and euthanized humanely by CO₂ inhalation if any of the following survival endpoints were met: hunched posture, lethargy, dehydration, and rough hair coat. Liver metastatic lesion size was measured using small-animal ultrasound, while SubQ tumors were measured with calipers. TGI of the liver metastatic lesion and SubQ tumors was calculated during the dosing period using the formula: $\%TGI = (1 - [Tt/T0/Ct/C0] / 1 - [C0/Ct]) \times 100$, where Tt is the median tumor volume of the treated group at time t, T0 is the median tumor volume of the treated group at time 0, Ct is the median tumor volume of the control group at time t and C0 is the median tumor volume of the control group at time 0. A TGI of greater than 50% is considered meaningful.

Cell staining and flow cytometry

On day 21, various samples were collected from mice that received intratumoral injections of STING agonist, including the specific liver metastatic lesion received dosing injection, the whole left liver (including other metastatic lesions), and one of the SubQ tumors. Tumor-infiltrating immune cells were analyzed from these samples. First, the samples were mechanically minced using the gentleMACS Dissociator with Tumor Dissociation Kit for mouse tissues (Miltenyi Biotec, San Diego, CA, USA) and processed as previously described to collect the lymphocytes¹⁹.

Then, leukocytes were isolated from the liver and tumor samples and stained with the Live Dead Aqua Dead Cell Kit (Invitrogen). The leukocytes were washed and blocked using mouse Fc antibody (BD Pharmingen) for 10 minutes on ice. Afterwards, cell-surface antibodies were used to stain the cells, including CD45-PerCP-Cy5.5 (Biolegend), CD4-APC-A750 (Biolegend), CD8a-PE/Cy7 (Biolegend), PD-1-FITC (Biolegend), CD45-APC-Cy7 (Biolegend), CD11b-PE TexasRed (Invitrogen), MHC II (I-A, I-E)- FITC (Biolegend), CD11c-APC (Biolegend), F4/80-PE/Cy7 (Invitrogen), CD8a-V450 (Biolegend), and CD103-PE (Biolegend), for a 30-minute incubation on ice. The cells were then washed twice and resuspended in FACS buffer, and flow cytometry was performed using CytoFLEX (Beckman Coulter). Flow data were analyzed using the CytExpert software (Beckman Coulter).

Luminex Based Cytokine Profiling

For serum cytokine detection analyses, peripheral blood was collected from submandibular facial vein on day 14 six hours after the first treatment. After collection, the blood was immediately centrifuged at 10 000 g and the resulting serum was collected. A total of 44 individual analytes were included in this study, which were divided into three panels (cat. MCYTMAG-70K-PX32, MHSTCMAG-70K, and MTH17MAG-47K). Mouse-specific Luminex reagents were used for the analysis, sourced from Millipore Sigma. The cytokine measurements were performed using protocols provided by the manufacturer, which were miniaturized to fit the 384-well format (REF)²⁰. The lyophilized cytokine standard cocktails (Millipore Sigma) were prepared according to the manufacturer's instructions. 30 μ L of mouse serum was submitted for profiling in the 96-well format at -70°C . The samples were then thawed on ice and diluted 1:1 in the Luminex kit assay buffer. Next, 10 μ L of the diluted serum, standard, and kit-provided control samples were

transferred to 384-well assay plates (Greiner, cat. 781096) and mixed with 10 μ L Luminex bead reagents. The plates were shaken overnight at 4°C in the dark and washed twice with 90 μ L of 1X wash buffer using a magnetic plate washer (Biotek). Then, 10 μ L of the detection antibody solution was added to each well and mixed. The plates were shaken for 1 hour at room temperature in the dark. After adding 10 μ L of Streptavidin labeled Phycoerythrin to each well, the assay plates were incubated for 30 min at room temperature in the dark. Following the incubation period, the plates were washed with 90 μ L of 1X wash buffer, and 80 μ L of sheath fluid were added to each well. The plates were then measured using the Bio-Plex 3D system (Bio-Rad), and the data were analyzed using the Bio-Plex Software 3.0. A total of 70 μ L and a minimum of 50 bead events per analyte were acquired. The mean fluorescence intensity data were converted into pg/mL using a 4-parametric logistic fit model based on the standard curve.

Statistical analyses

Statistical analyses and graphing were conducted using GraphPad Prism software (GraphPad Software). The tumor inhibition rates were analyzed using the unpaired t-test while Kaplan-Meier curves and log-rank tests were utilized to evaluate the survival outcomes across the groups. The unpaired t-test was used to determine the mean values for cell number, cytokine expression, and NanoString data. A p-value <0.05 was considered statistically significant.

Supplemental Figures

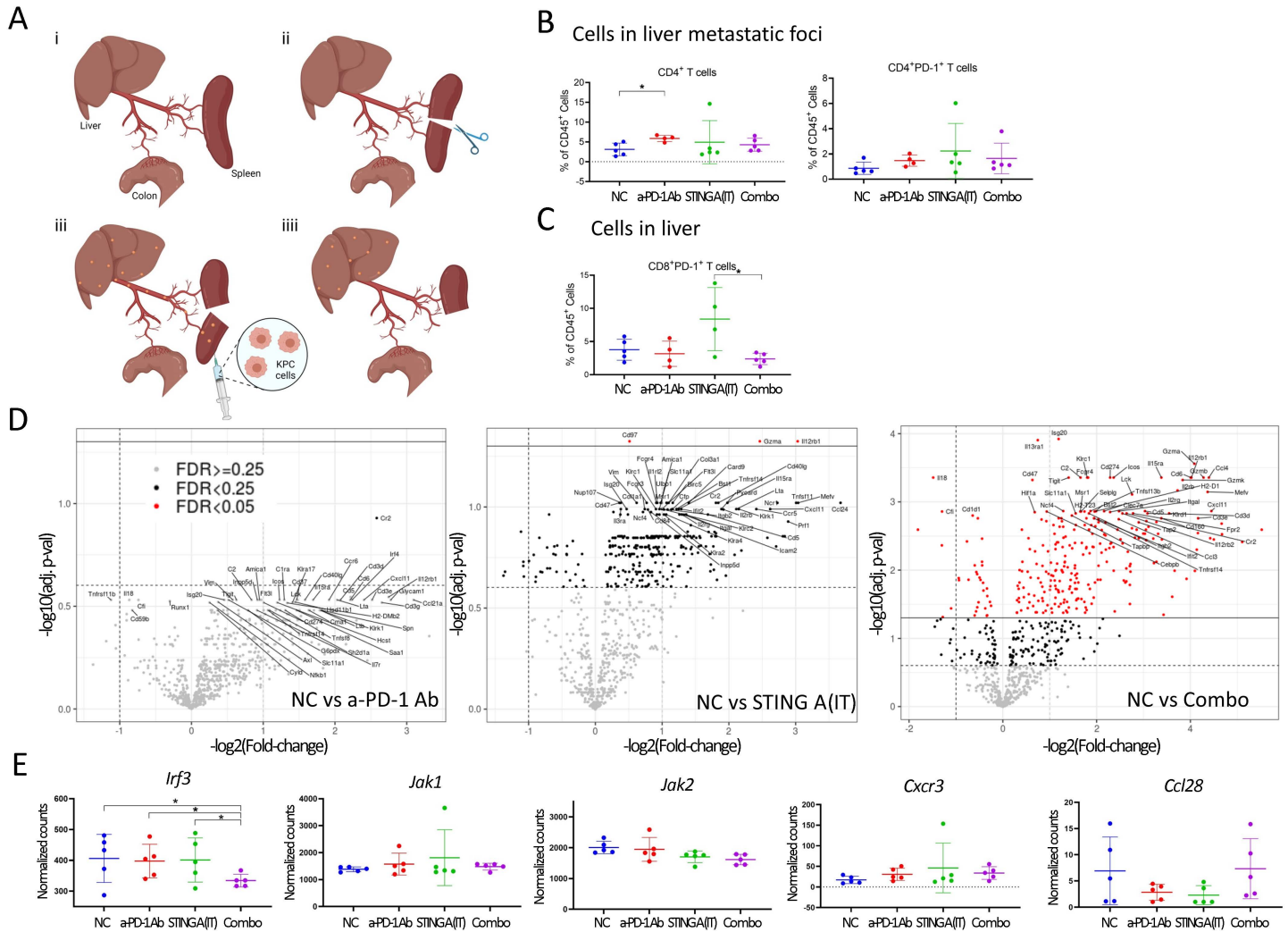


Fig. S1. Metastatic model building and gene expression in the liver metastatic lesions: (A) The schema of the hemisplenectomy procedure and splenic vessel injection to form liver metastases. **(B)** Percentages of the CD4⁺ and CD4⁺ PD-1⁺ T cells among CD45⁺ leucocytes in the target liver metastatic lesion. **(C)** Percentages of the CD8⁺ PD-1⁺ T cells among CD45⁺ leucocytes in the non-target liver metastases. **(D)** Volcano map of differentially expressed genes in target liver metastatic lesion between the vehicle control and three treatment groups, respectively. Dashed horizontal line indicates the false discovery rate at 0.25 while the solid horizontal line is at 0.05. Dashed vertical line at -1 indicates the $-\log_2(\text{Fold-change})$. **(E)** Expression of *Irf3*, *Jak1*, *Jak2*, *Cxcr3*, and *Ccl28* in the target liver metastatic lesions from different treatment groups. NC, vehicle/isotype antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by unpaired t test; * $p < 0.05$. Remaining comparisons are non-significant.

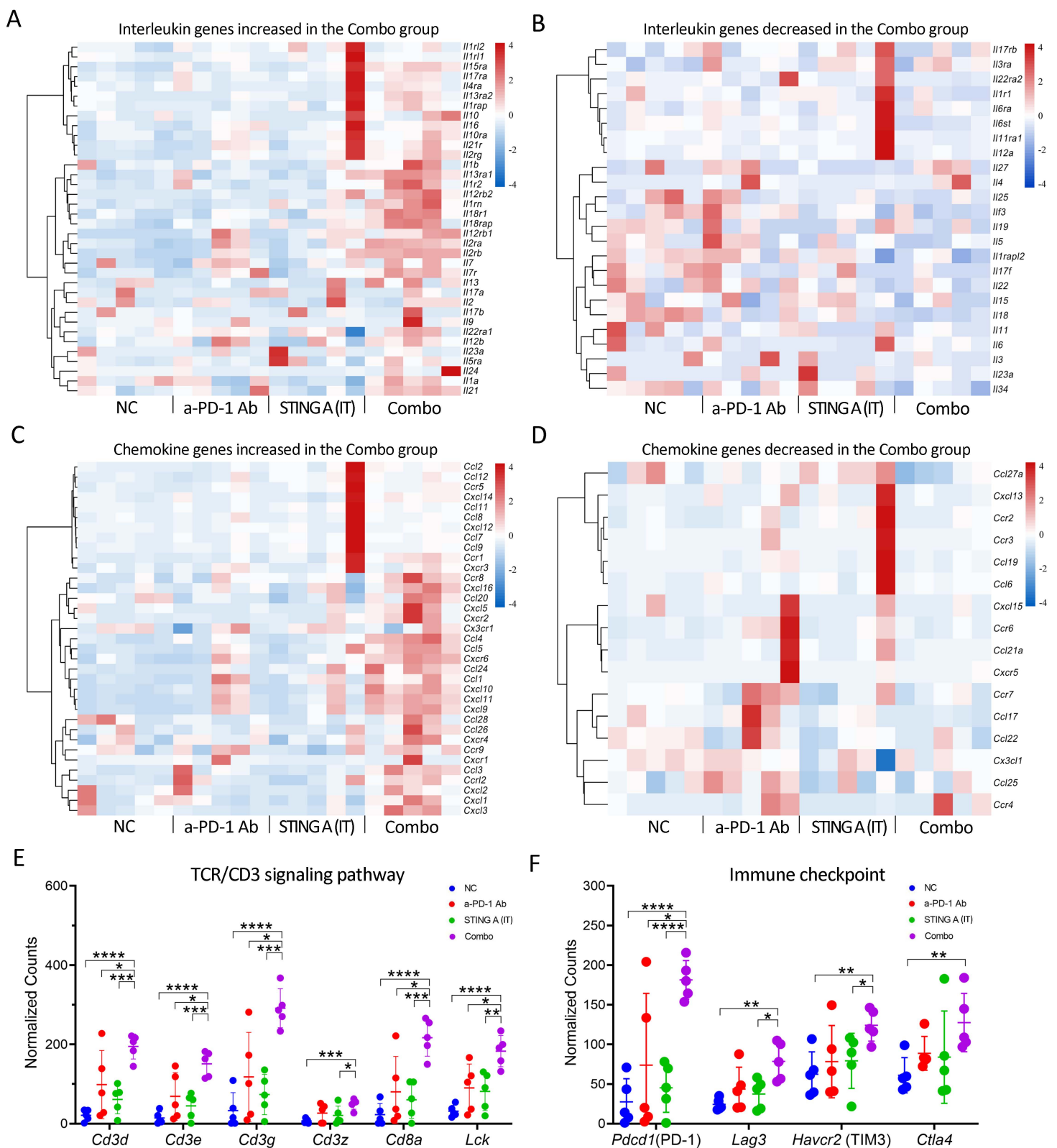


Fig. S2. Expression of genes in the liver target liver metastatic lesions between treatment groups: Heatmap of increased and decreased genes in the families of interleukin (A-B) and chemokine (C-D) in the Combo group comparing to the vehicle control treatment group. (E) Expression of genes in the TCR/CD3 signaling pathways in the target liver metastatic lesions from different treatment groups. (F) Expression of genes in the families of immune checkpoint activators in the target liver metastatic lesions from different treatment groups. NC, vehicle/isotype

antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by unpaired t test; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

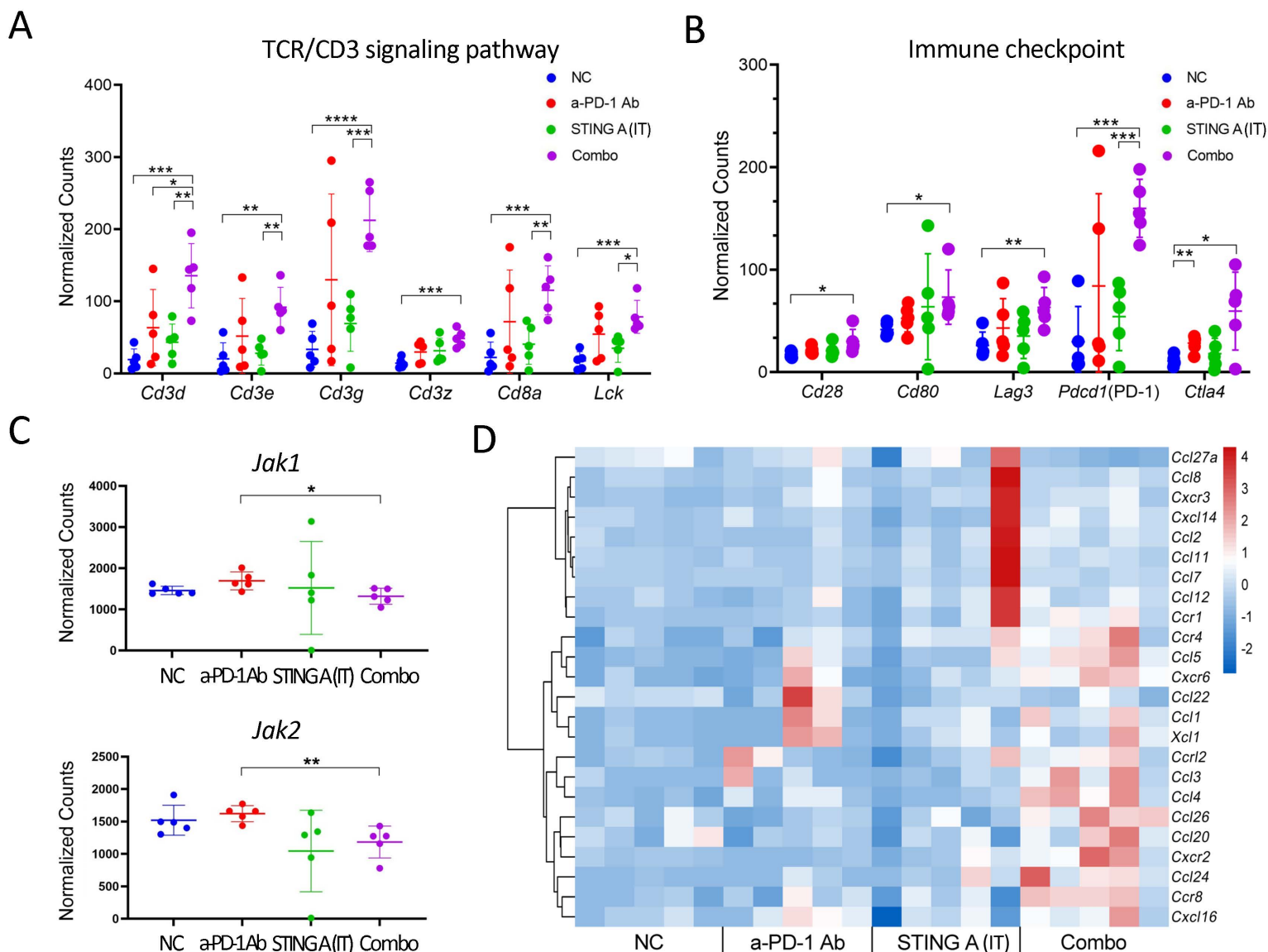


Fig. S3. Gene expression in SubQ tumors: (A) Expression of genes in the TCR/CD3 signaling pathway in SubQ tumors from different treatment groups. (B) Expression of genes in the families of immune checkpoint activators in SubQ tumors from different treatment groups. (C) Expression of *Jak1* and *Jak2* in the SubQ tumors from different treatment groups. (D) Heatmap of the chemokine family genes that showed increasing trend in the Combo group comparing to the vehicle control treatment group. Data are shown as the mean \pm SD; comparison by unpaired t test; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

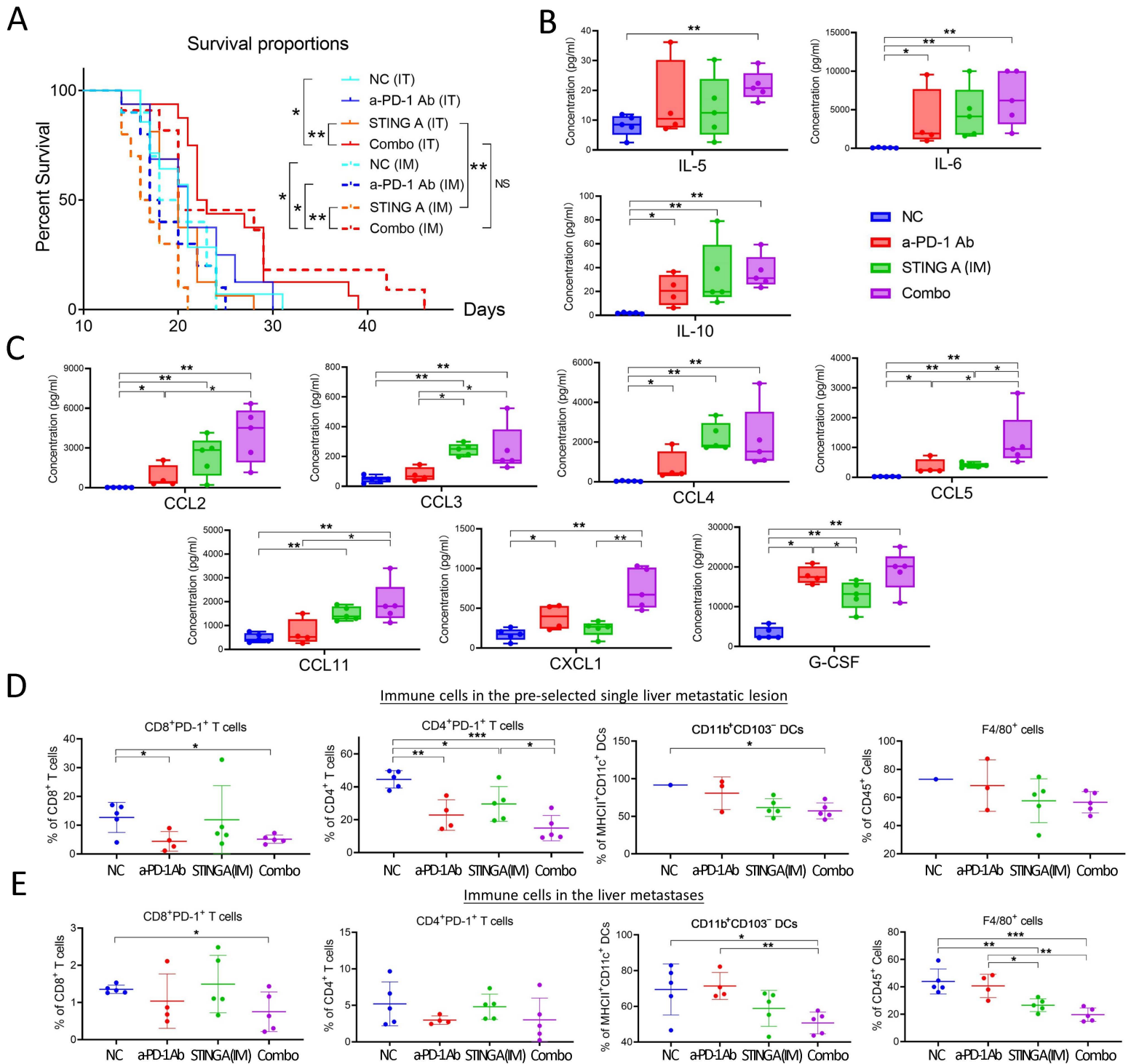


Fig. S4. Survival curves for IT and IM groups; serologic concentration of cytokines from IM group and infiltrating immune cells in the IM group: (A) Combined Kaplan-Meier's survival curves compare the survival in different intratumoral (IT) and intratumoral (IM) treatment groups. (B) The comparison of the serologic concentration of IL-5, IL-6 and IL-10 that collected 6 hours after the first IM injection among all treatment groups. (C) Comparison of serum concentrations of CCL2, CCL3, CCL4, CCL5, CCL11, CXCL1, and G-CSF collected 6 hours after the first IM injection between treatment groups. Percentages of the CD8⁺ PD-1⁺ among CD8⁺ T cells, the CD4⁺ PD-1⁺ T cells among CD4⁺ T cells, the CD11b⁺ CD103⁻ subtype DC among MHCII⁺ CD11c⁺ DCs,

and F4/80⁺ cells among CD45⁺ leucocytes in the pre-selected single liver metastatic lesion (**D**) and non-target liver metastases (**E**). NC, vehicle/isotype antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by Log-rank test for A and by unpaired t test for others; *p < 0.05; **p < 0.01; ***p < 0.001.

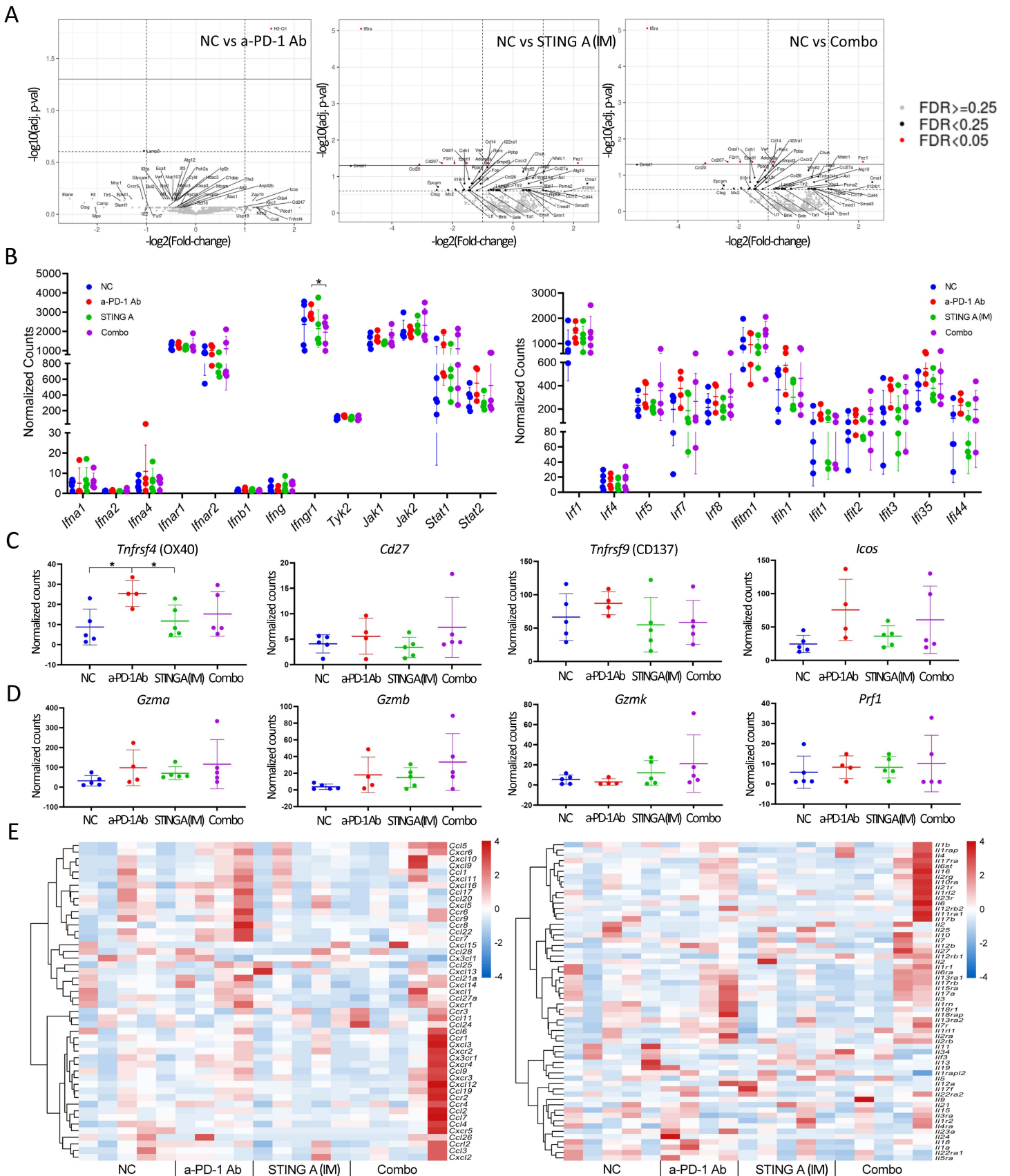


Fig. S5. Gene expression of the metastatic lesions in IM group: (A) Volcano map of differentially expressed genes in the pre-selected, target single liver metastatic lesions between the vehicle control and three treatment groups, respectively. Dashed horizontal line indicates the false discovery rate at 0.25 while the solid horizontal line is at 0.05. Dashed vertical line at -1

indicates the $-\log_2(\text{Fold-change})$. **(B)** Expression of genes in the IFN-response pathways in the pre-selected, target single liver metastatic lesions from different treatment groups. Expression of genes as indicated, in the gene families of the T cell co-stimulatory factors **(C)** and the activation of effector T cells **(D)** in the pre-selected single liver metastatic lesion. **(E)** Heatmap of genes in the families of interleukin and chemokine in the pre-selected single liver metastatic lesion in the Combo group comparing to the vehicle control treatment groups. NC, vehicle/isotype antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by unpaired t test; * $p < 0.05$.

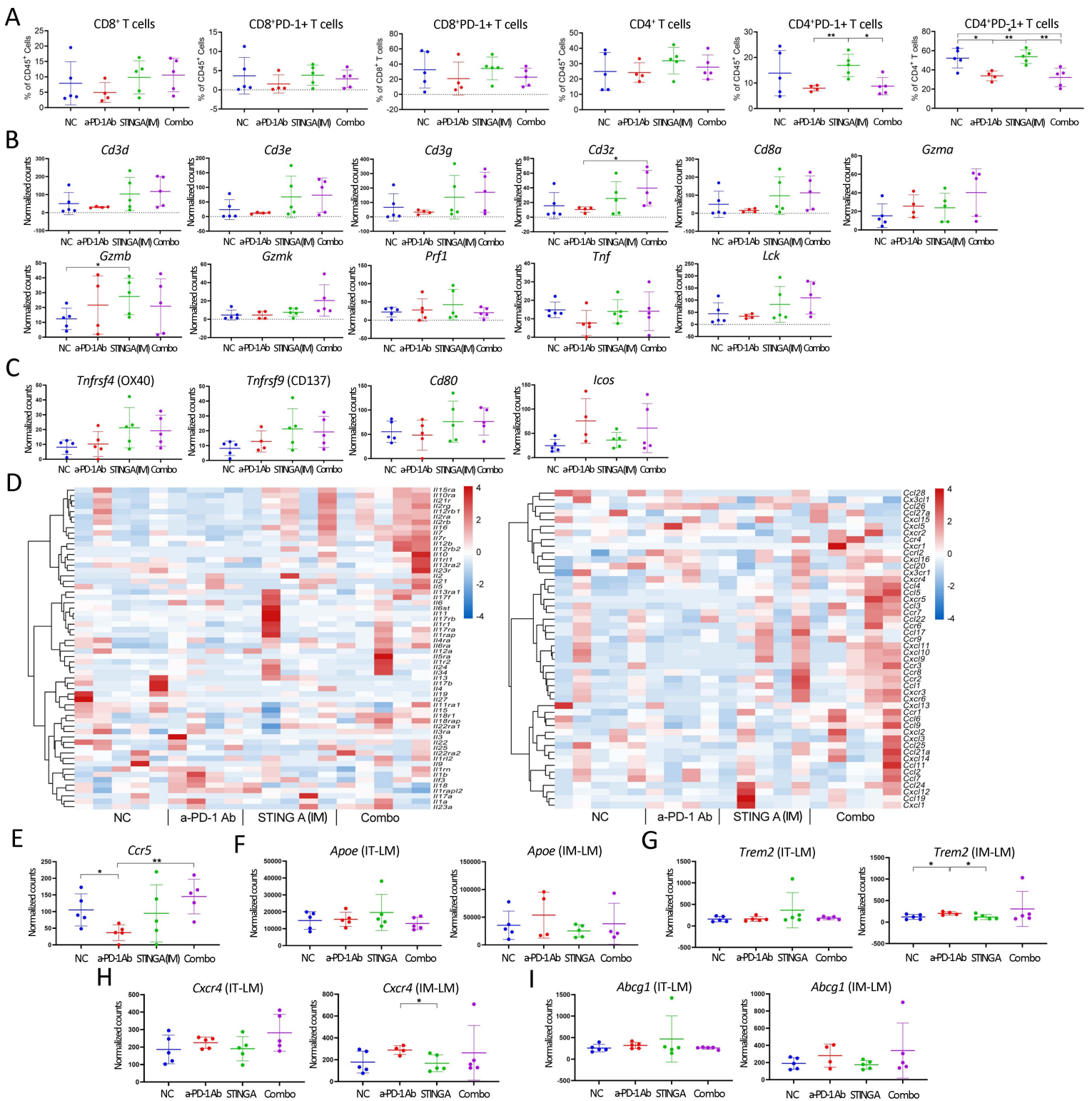


Fig. S6. Immune cells infiltrating status and gene expression of the SubQ tumors in IM group: (A) Percentages of immune cells in the remote SubQ tumors of IM experiment, such as the CD8⁺, CD8⁺PD-1⁺, CD4⁺, and CD4⁺PD-1⁺ T cells among CD45⁺ leucocytes, the CD8⁺PD-1⁺ among CD8⁺ T cells, and the CD4⁺PD-1⁺ T cells among CD4⁺ T cells, respectively. Differentially expressed genes in the distant SubQ tumors of IM experiment from different treatment groups, including those in the gene families of T cell functional status (B) and T cell co-stimulatory factors (C). (D) Heatmap of genes in the families of interleukin and chemokine in the remote SubQ tumors of IM experiment in the Combo group comparing to the vehicle control treatment groups.

Expression of *Ccr5* (**E**) in the remote SubQ tumors of IM experiment and *ApoE* (**F**), *Trem2* (**G**), *Cxcr4* (**H**), and *Abcg1* (**I**) in the liver metastatic lesions from both intratumoral and intramuscularly treated mice. NC, vehicle/isotype antibody control; STING A, STING agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, STING A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by unpaired t test; *p < 0.05; **p < 0.01.

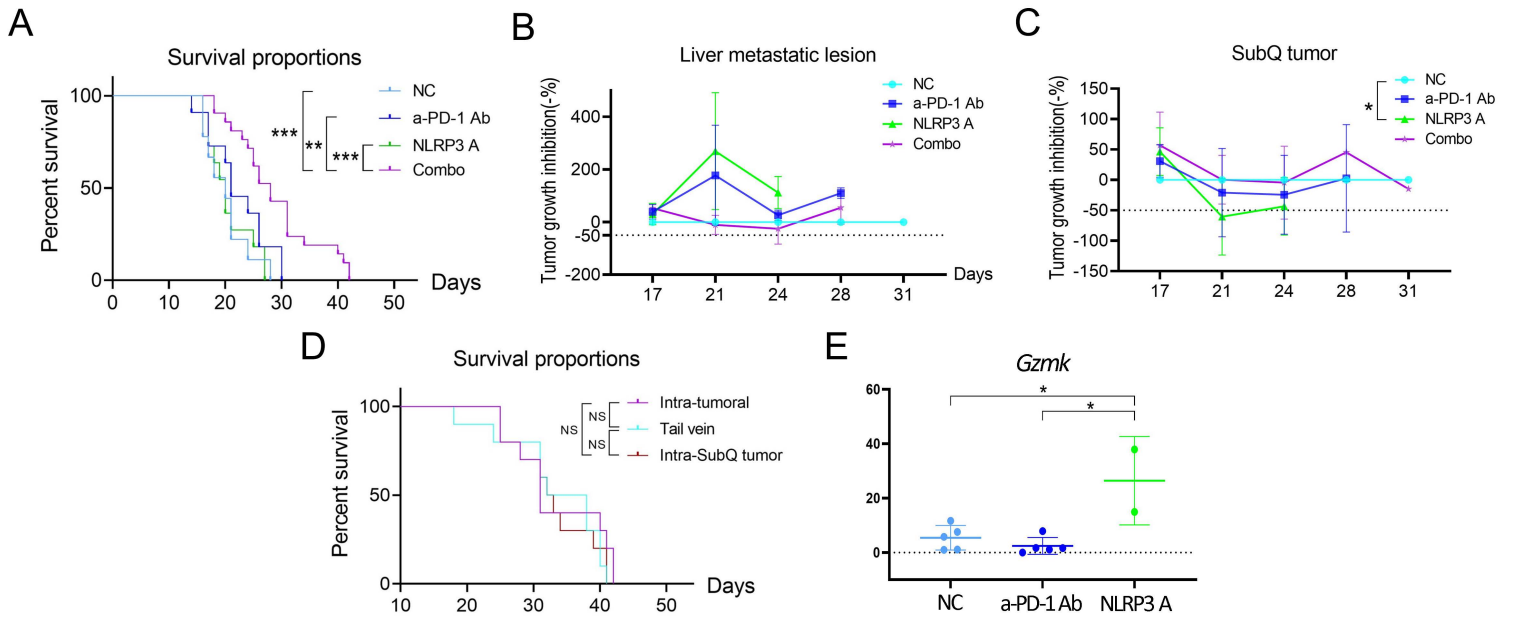


Fig. S7. Systemic administration and intratumoral administration of NLRP3 agonist in combination with anti-PD-1 antibody induced antitumor efficacy: (A) Kaplan-Meier's survival curves compare different treatment groups. TGI of the injected liver metastatic lesion (B) and remote SubQ tumor (C) during the treatment period. Dashed line at -50% indicates statistically significant TGI. (D) Kaplan-Meier's survival curves comparing different routes of administration of NLRP3 agonist including intratumoral, tail-vein injection, and intra-subcutaneous tumor injection. (E) Expression of *Gzmk* in the target liver metastatic lesions from different treatment groups. NC, vehicle/isotype antibody control; NLRP3 A, NLRP3 agonist; a-PD-1 Ab, anti-PD-1 antibody; Combo, NLRP3 A+a-PD-1 Ab. Data are shown as the mean \pm SD; comparison by Log-rank test for A and D, and by unpaired t test for others; * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS, not significant.