
1 *Supplementary Information for*

2 **α PD-1-conjugated acid-cleavable nanodrugs overcomes cellular immunotherapy**
3 **barriers in pancreatic tumors**

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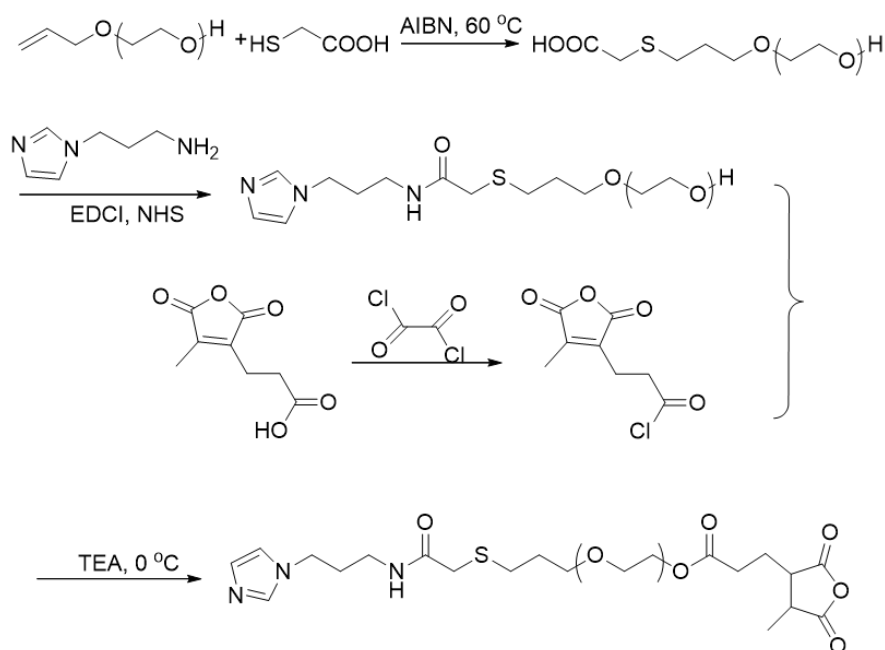
18 Ziqi Gan, E-mail: zgan@houstonmethodist.org

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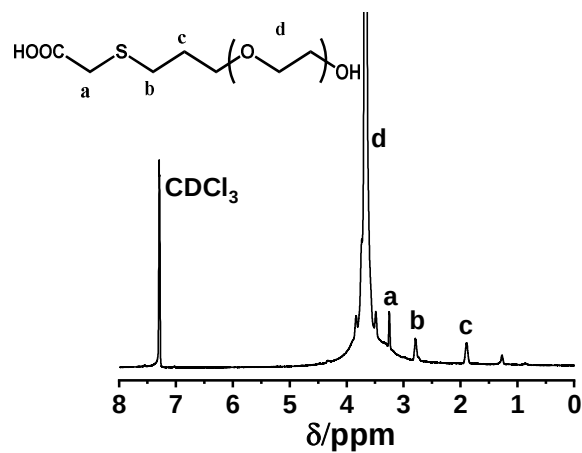
20 Zecong Xiao, E-mail: xiaozc5@mail.sysu.edu.cn

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Results



Supplementary Figure 1 Synthesis of NAID-PEG-CDM.

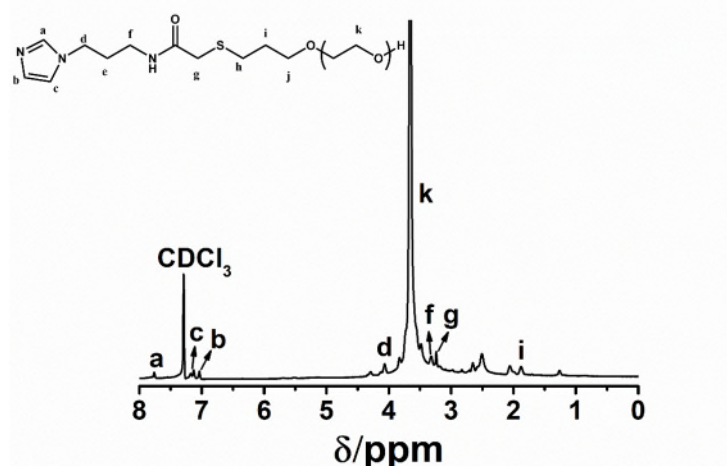


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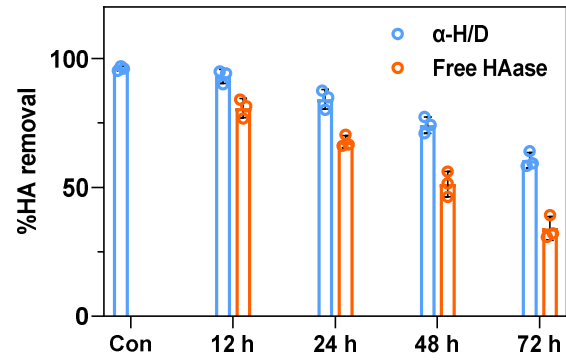
29 **Supplementary Figure 2** ¹H nuclear magnetic resonance (¹H NMR) spectrum of
30 COOH-PEG-OH. ¹H NMR (400 MHz, chloroform-d₁, 298 K): δ (ppm)= 3.46-3.87
31 ppm (-CH₂CH₂O-) of PEG, d), 3.25 ppm (COOH-CH₂-S-, a), 2.79 ppm (-SCH₂CH₂-,
32 b), 1.89 ppm (-SCH₂CH₂CH₂-, c). Data are representative of three independent
33 experiments with similar results. Source data are provided as a Source Data file.

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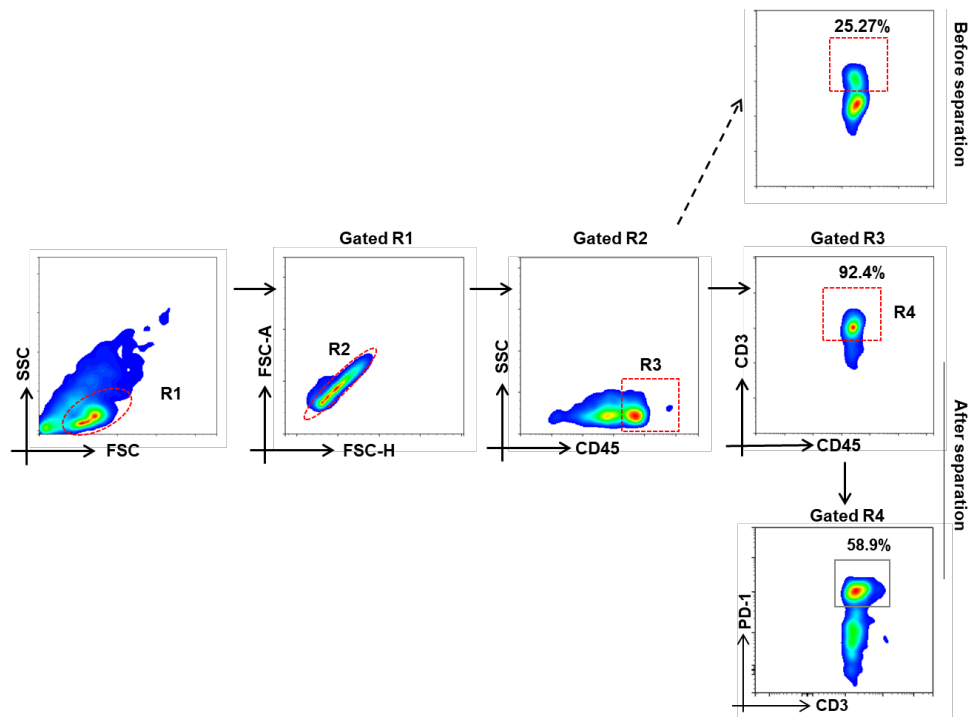
Supplementary Figure 3 ^1H nuclear magnetic resonance (^1H NMR) spectrum of NAID-PEG-OH. ^1H NMR (400 MHz, chloroform- d_1 , 298 K): δ (ppm)= 7.68 ppm (- $\text{CH}_2\text{NCHNCHCH-}$ of NAID, a), 6.97-7.23 ppm (- $\text{CH}_2\text{NCHNCHCH-}$ of NAID, b, c), 4.07 ppm (- $\text{CH}_2\text{NCHNCHCH-}$ of NAID, d), 3.46-3.87 ppm (-($\text{CH}_2\text{CH}_2\text{O}$)- of PEG, k), 3.33 ppm (- $\text{CH}_2\text{CH}_2\text{NHCO-}$, f), 2.51 ppm (- $\text{NHCOCH}_2\text{S-}$, g), 1.89 ppm (- $\text{SCH}_2\text{CH}_2\text{CH}_2\text{-}$, i). Data are representative of three independent experiments with similar results. Source data are provided as a Source Data file.



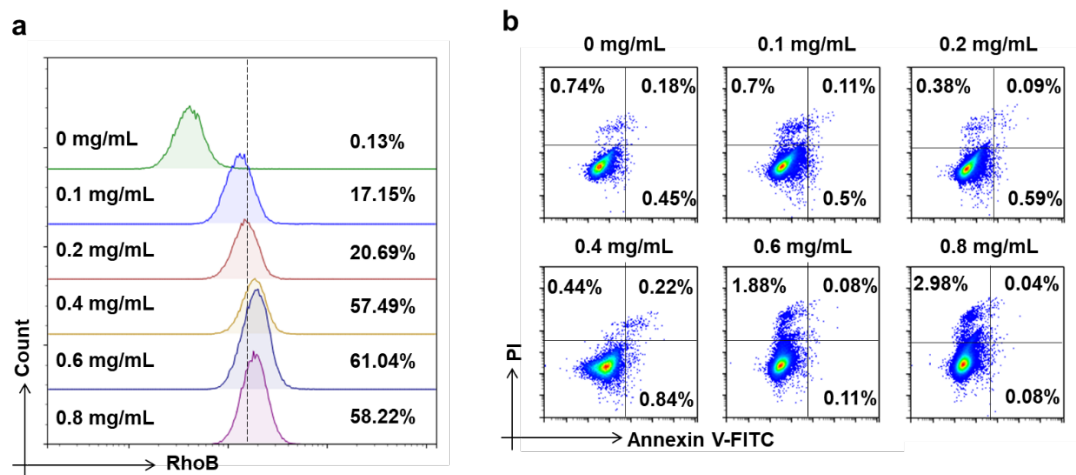
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47 **Supplementary Figure 4** Hyaluronidase (HAase) enzymatic activity at different time
48 points was detected by enzyme-linked immunosorbent assay (ELISA) at pH~6.5), data
49 points represent mean $\pm$ SD ($n = 3$ independent experiments). Source data are provided
50 as a Source Data file.

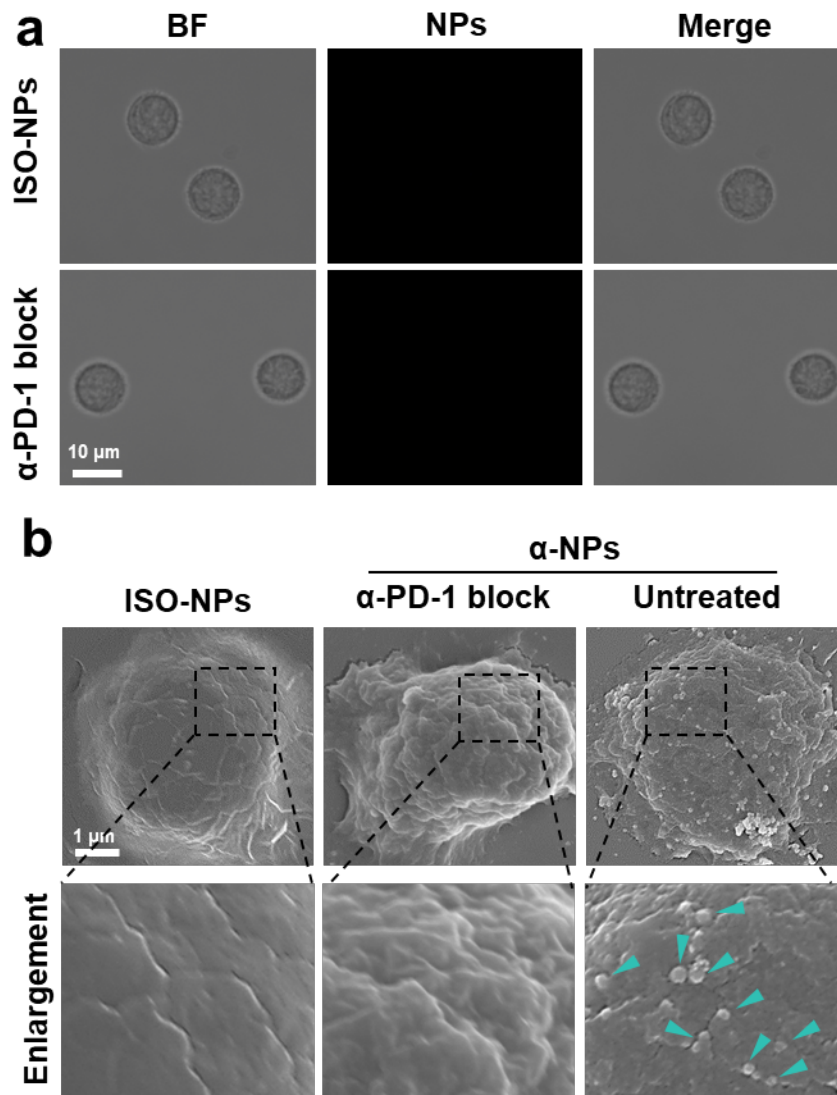
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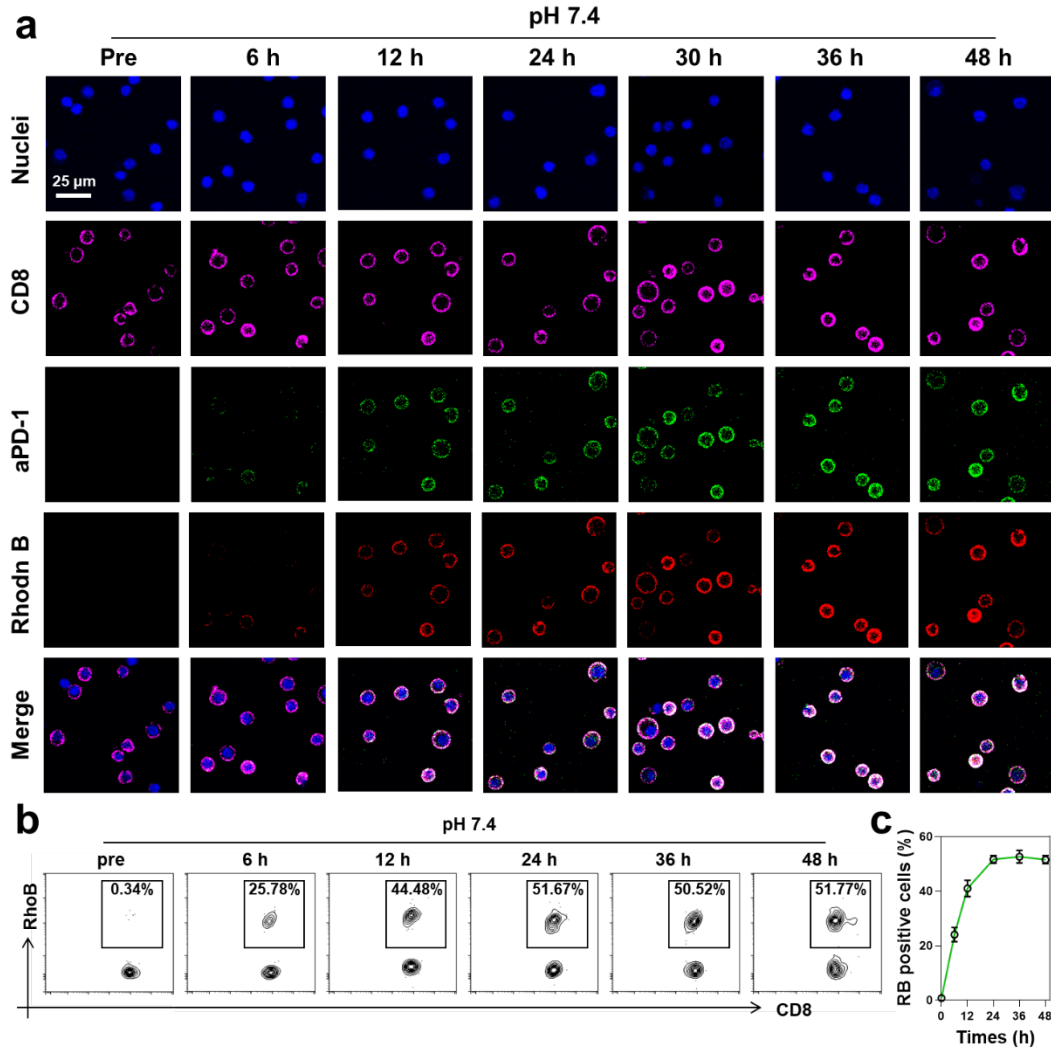
Supplementary Figure 5 Characteristics of tumor-infiltrating lymphocyte (TILs). Representative flow cytometry gating strategies for T lymphocytes panel of magnet isolation CD3⁺ T cell (TILs) from Panc02 tumor using Miltenyi mouse CD3 microbeads.



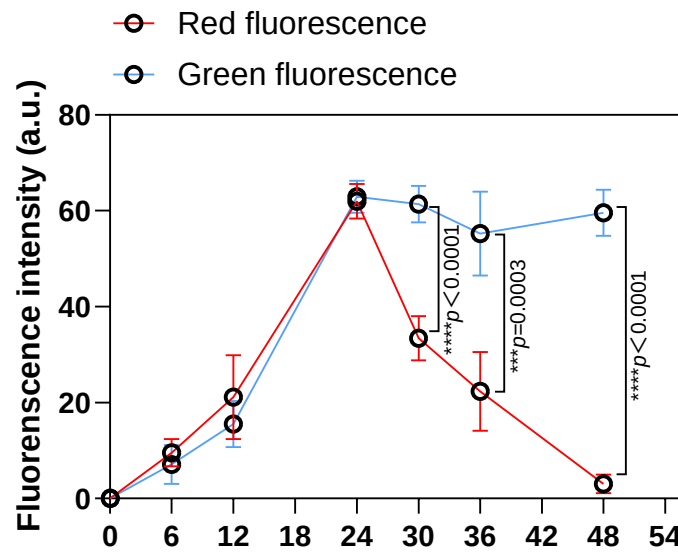
Supplementary Figure 6 Tumor-infiltrating lymphocytes (TILs) incubated with α -rhodamine B (RhoB) nanoparticles (NPs) at varying concentrations for 24 h. (a) Relative RhoB level of TILs carrying nanodrug backpacks analyzed with flow cytometry. (b) Representative images of TILs apoptosis analyzed by flow cytometry.



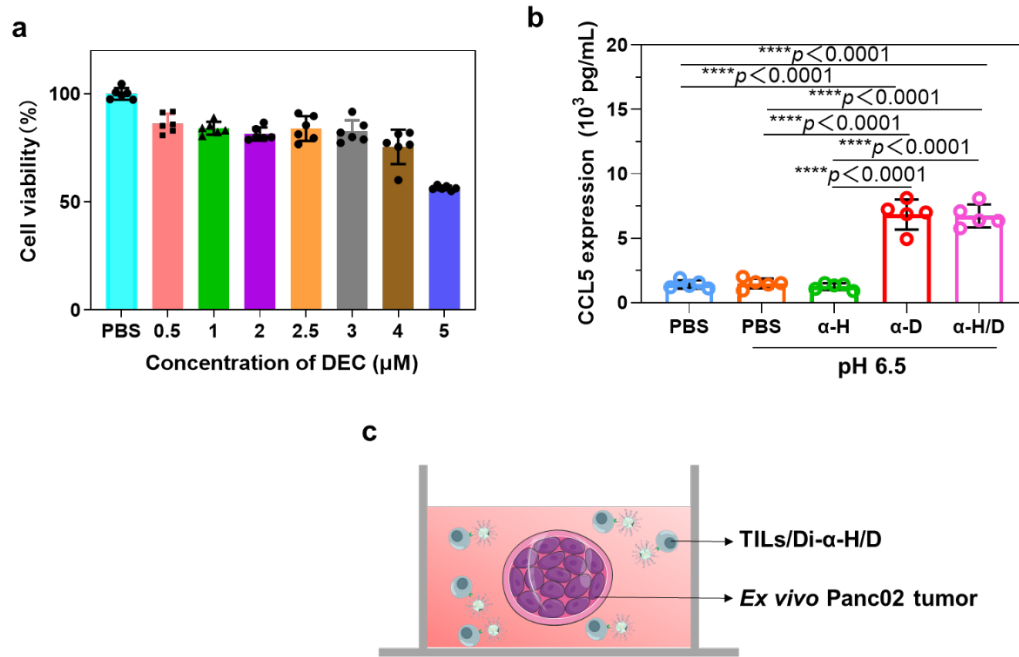
Supplementary Figure 7 Characterization of nanoparticle conjugation with tumor-infiltrating lymphocytes (TILs). (a) Confocal laser scanning microscopy (CLSM) images showing nanoparticles (NPs) bound to TILs. (b) Scanning electron microscopy (SEM) images demonstrating conjugation of NPs (indicated by green arrows) to the membrane surfaces of TILs. Data are representative of three independent experiments with similar results.



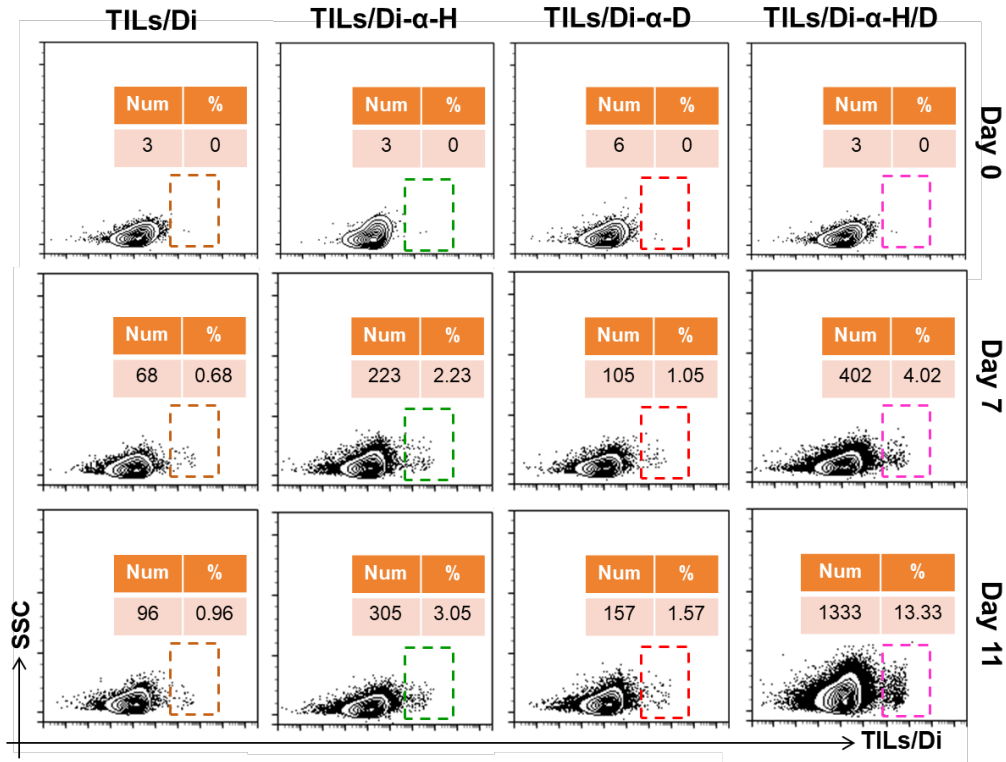
Supplementary Figure 8 (a) Confocal laser scanning microscopy (CLSM) images showing the binding of α - rhodamine B (RhoB) nanoparticles to TILs at pH 7.4. T cell marker CD8 was labeled with Alexa Fluor[®] 647 (purple fluorescence), α PD-1 was labeled with Alexa Fluor[®] 488 (green fluorescence), and the nanoparticle was labeled with Rhodamine B (RhoB, red fluorescence) (concentration of α -RhoB nanoparticles: 0.4 mg mL⁻¹). (b)Typical and (c) quantitative flow cytometric results showing the binding of α -RhoB nanoparticles to T cells at pH 7.4, data points represent mean $\pm$ SD (n = 3 independent samples). Source data are provided as a Source Data file.



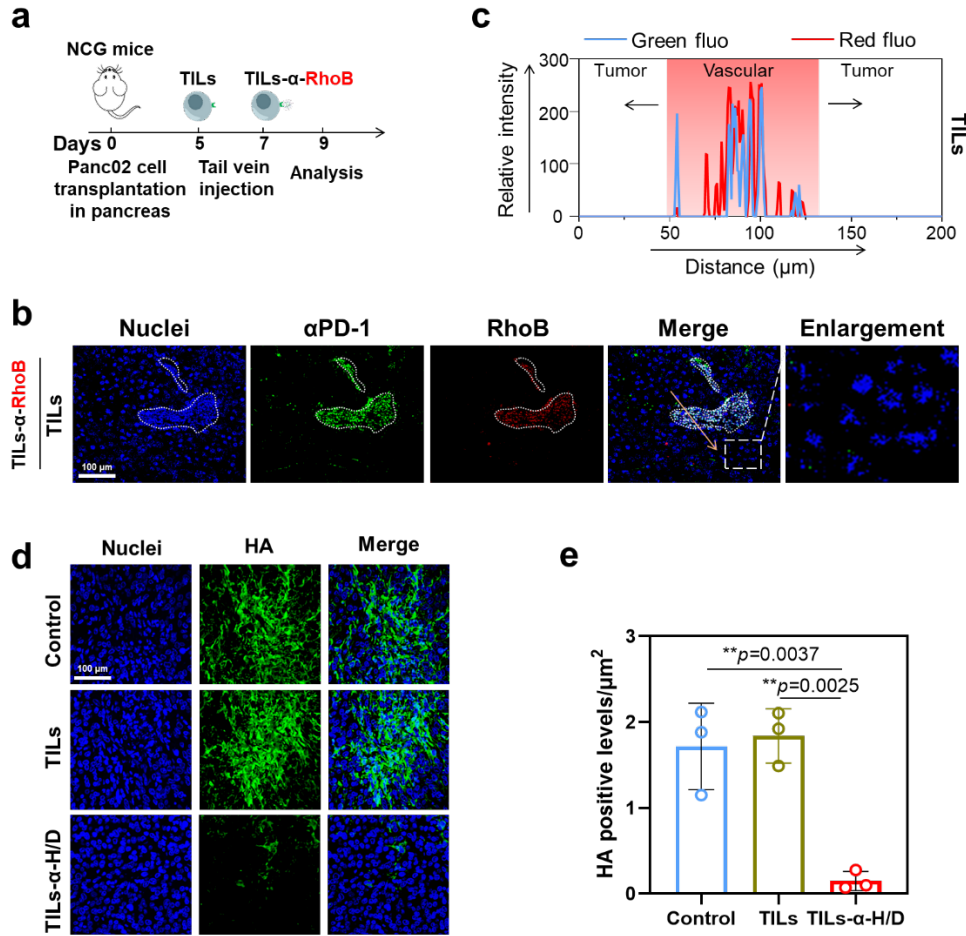
Supplementary Figure 9 Quantification of fluorescence intensity from Fig. 3e, data points represent mean $\pm$ SD (n = 5 independent samples). Statistical significance was calculated using two-sided unpaired t-test, $***p < 0.001$ and $****p < 0.0001$. Source data are provided as a Source Data file.



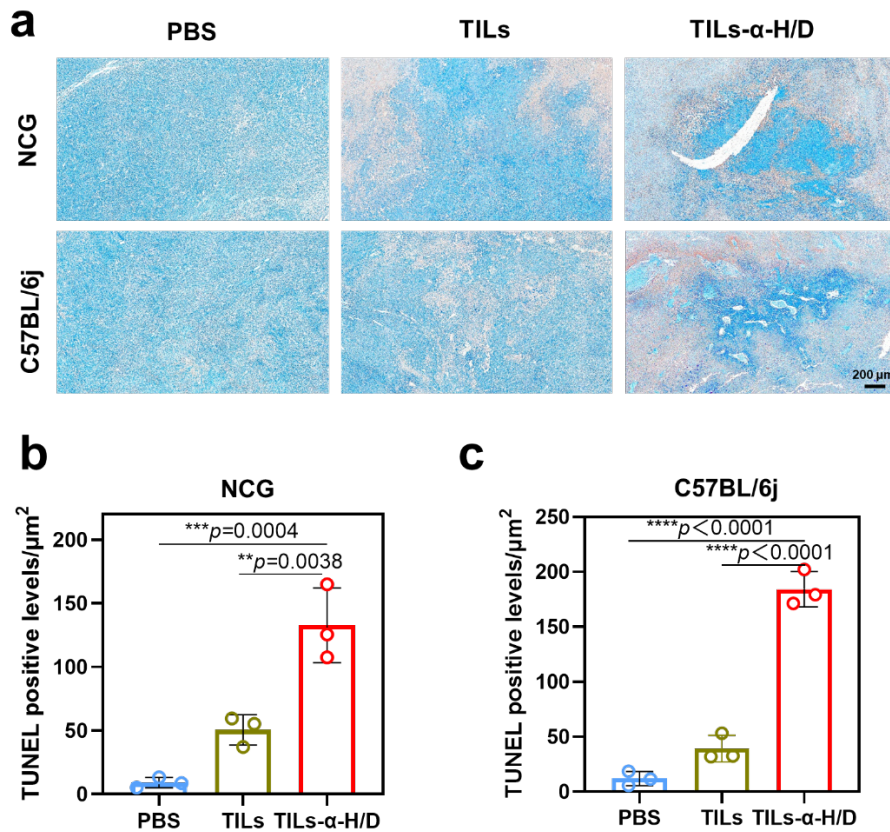
Supplementary Figure 10 (a) Cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, data points represent mean $\pm$ SD ($n = 6$ independent samples). (b) Quantification of CCL5 in the supernatant 48 h after the treatment, data points represent mean $\pm$ SD ($n = 5$ independent samples). Statistical significance was calculated using unpaired one-way ANOVA test, **** $p < 0.0001$. (c) Illustration of an ex vivo tumor-coculture model to evaluate the infiltration capabilities of TILs/Di- α -H/D. Source data are provided as a Source Data file.



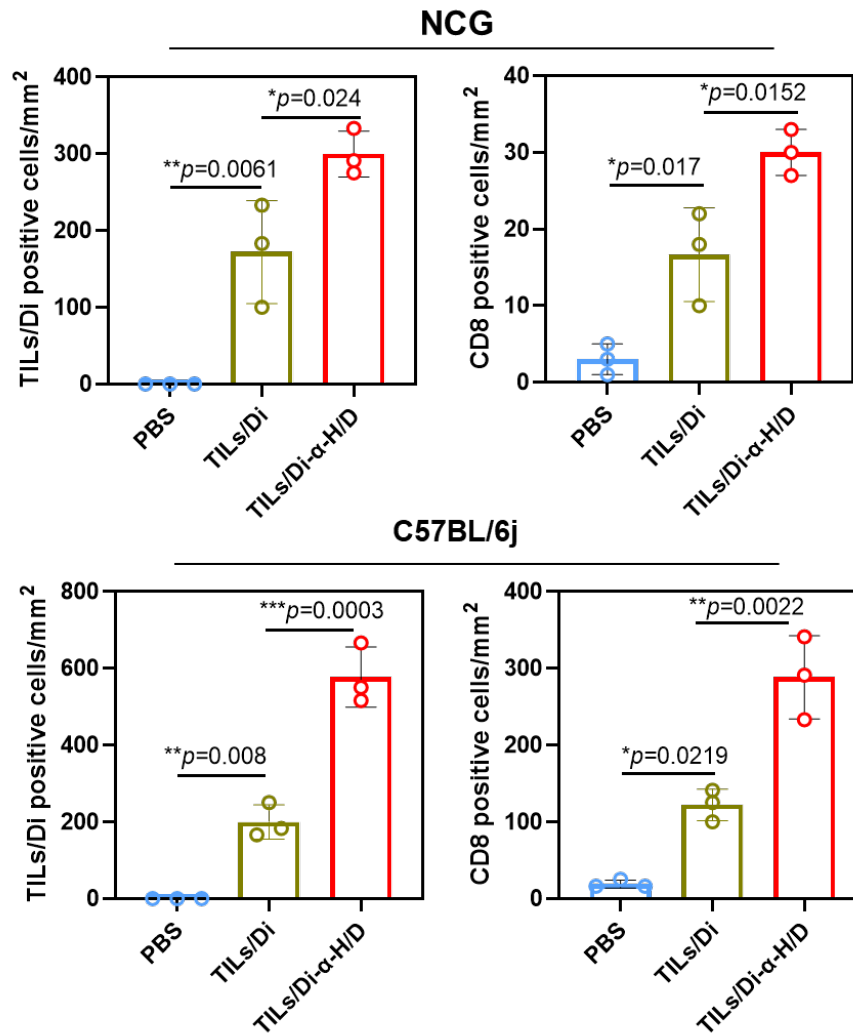
Supplementary Figure 11 Detection of the numbers of intratumoral tumor-infiltrating lymphocytes (TILs) in NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) mice by flow cytometry.



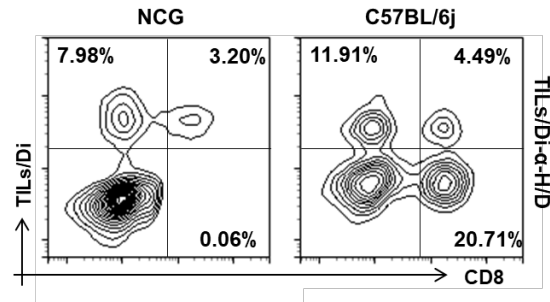
Supplementary Figure 12 (a) Illustration of experimental designs for figure (b and c). (b) Confocal laser scanning microscopy (CLSM) images of the tumor section exhibiting the colocalization of nanodrug and anti-PD-1 antibody (α PD-1) on T cells after vein injection of nanodrug-carrying TILs. α PD-1 was labeled with Alexa Fluor[®] 488 (green), the nanodrug was labeled with RhoB (red), and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) (blue), scale bar: 100 μ m. (c) Fluorescence intensity profiles across the cell along the selected line (indicated by an orange arrow in Figure (b)). (d) The immunofluorescence staining of hyaluronic acid (HA), HA was labeled with Alexa Fluor[®] 488 (green), and cell nuclei were labeled with DAPI (blue), scale bar: 100 μ m. (e) Quantification of HA expression in Supplementary Figure 12d, data points represent mean $\pm$ SD (n = 3 mice). Statistical significance was calculated using unpaired one-way ANOVA test, $**p < 0.01$. Data in (b and d) are representative of three independent experiments with similar results. Source data are provided as a Source Data file.



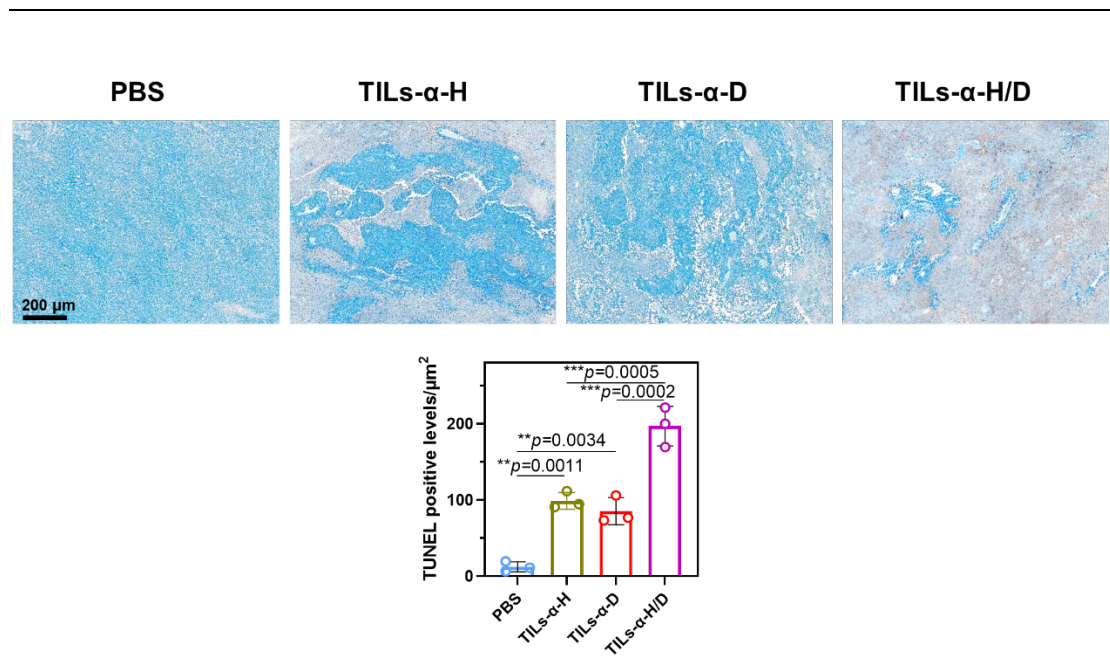
Supplementary Figure 13 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining (a) and quantification of apoptotic cells in tumor sections from NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) (b) and C57BL/6j (c) mice treated with various therapies, data points represent mean $\pm$ SD (n = 3 mice), scale bar: 200 μm . Statistical significance was calculated using unpaired one-way ANOVA test, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$. Source data are provided as a Source Data file.



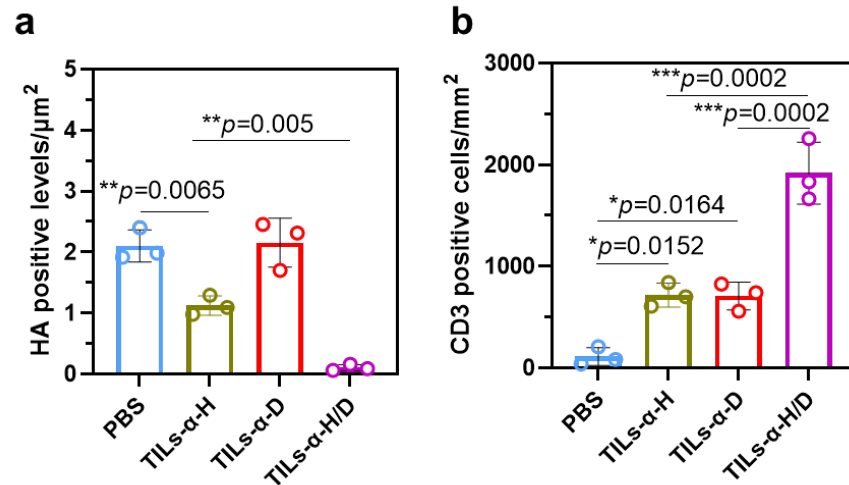
Supplementary Figure 14 Quantification of TIL/Di⁺ and CD8⁺ cells in tumor sections (including NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) and C57BL/6j mice) based on immunofluorescence results from Fig. 5m, data points represent mean ± SD (n = 3 mice). Statistical significance was calculated using unpaired one-way ANOVA test, *p < 0.05, **p < 0.01, and ***p < 0.001. Source data are provided as a Source Data file.



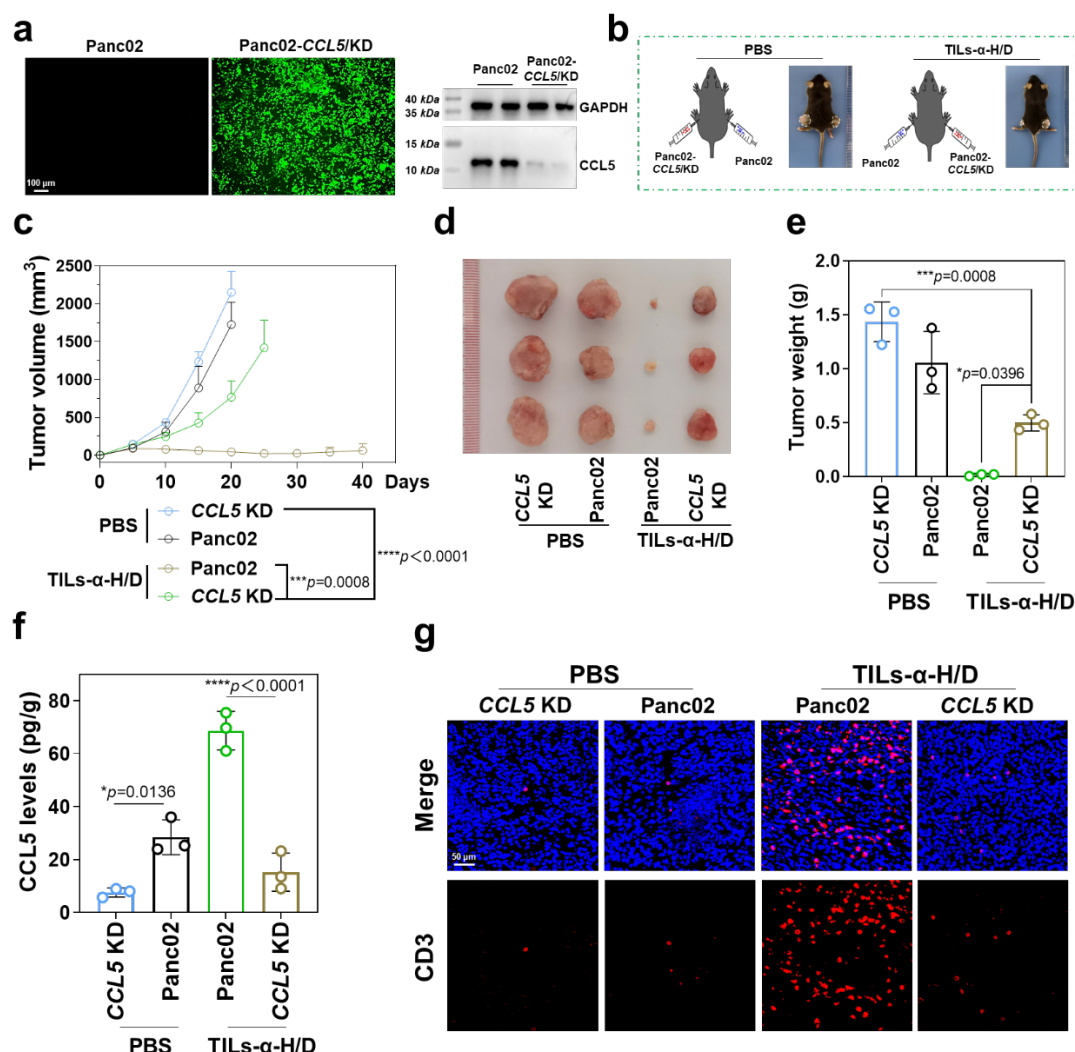
Supplementary Figure 15 Detection of the percentages of injected tumor-infiltrating lymphocytes (TILs) and the CD8⁺ T cells of tumor tissue in NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) and C57BL/6j mice receiving nanodrug-carrying TILs treatment by flow cytometry.



Supplementary Figure 16 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining and quantification of apoptotic cells in orthotopic tumor sections from C57BL/6j mice after different treatments, data points represent mean $\pm$ SD (n = 3 mice), scale bar: 200 μm. Statistical significance was calculated using unpaired one-way ANOVA test, $**p < 0.01$, $***p < 0.001$. Source data are provided as a Source Data file.

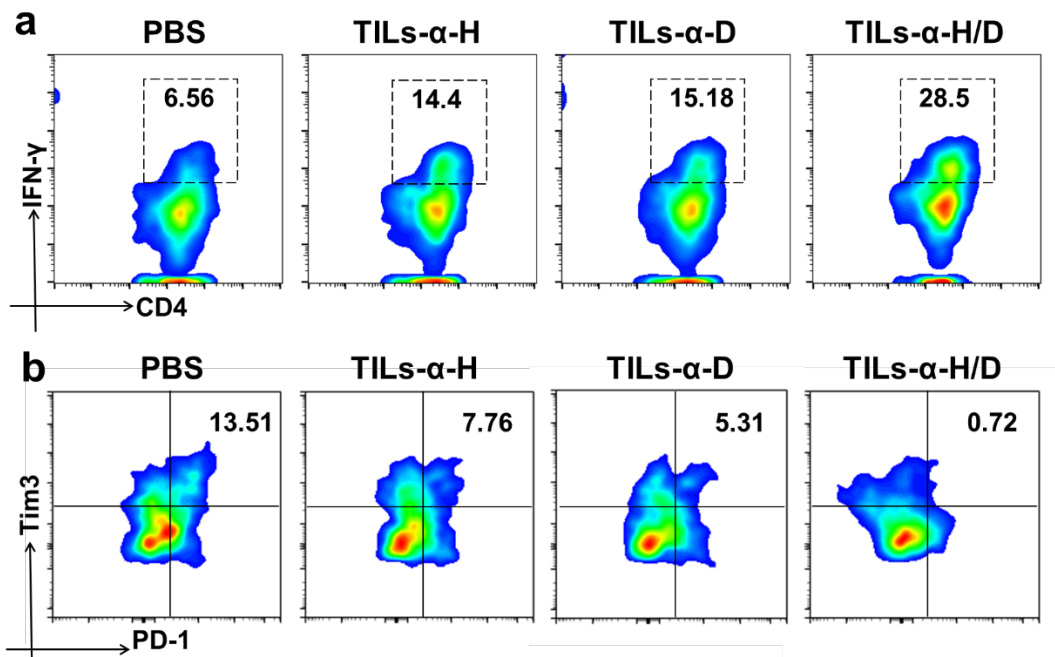


Supplementary Figure 17 (a) Quantification of hyaluronic acid (HA) expression in orthotopic tumor sections from C57BL/6j mice based on immunofluorescence results from Fig. 6g, data points represent mean $\pm$ SD ($n = 3$ mice). (b) Quantification of CD3⁺ cells in orthotopic tumor sections from C57BL/6j mice based on immunofluorescence results from Fig. 6g, data points represent mean $\pm$ SD ($n = 3$ mice). Statistical significance was calculated using unpaired one-way ANOVA test, * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$. Source data are provided as a Source Data file.

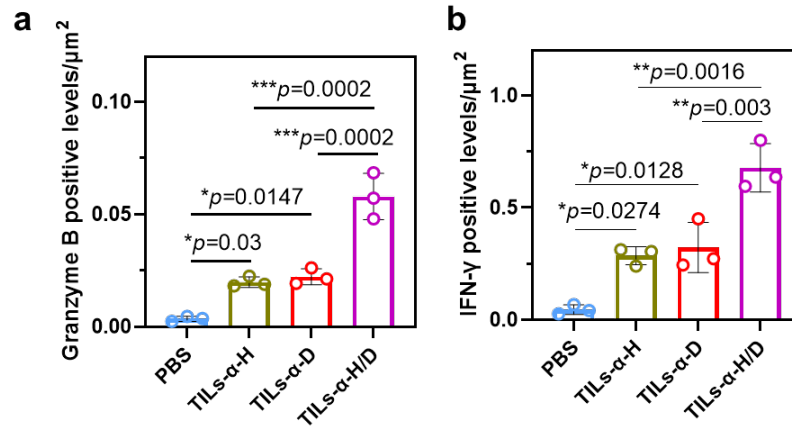


Supplementary Figure 18 (a) Panc02 cells were transfected with lentiviral recombinant vectors encoding C–C motif chemokine ligand 5 (*CCL5*) shRNA and green fluorescent protein (GFP) to establish stable *CCL5* knockdown cell lines. Green fluorescence indicates GFP expression, scale bar: 100 μ m. (b) Schematic illustration of the establishment of a subcutaneous Panc02 tumor model. The left and right hind limbs of the same C57BL/6j mouse were inoculated with Panc02 cells and Panc02 *CCL5*/KD cells, respectively. (c) Tumor sizes were measured after inoculation with Panc02 or Panc02 *CCL5*/KD cells, data points represent mean $\pm$ SD (n = 5 mice). (d) Representative images of subcutaneous tumors from Panc02 and Panc02 *CCL5*/KD tumor-bearing mice treated with different formulations on day 20. (e) Tumor weights from Panc02 and Panc02 *CCL5*/KD tumor-bearing mice after treatment with different formulations on day 20, data points represent mean $\pm$ SD (n = 3 mice). (f) Quantification of intratumoral CCL5 levels after different treatments, determined using an enzyme-

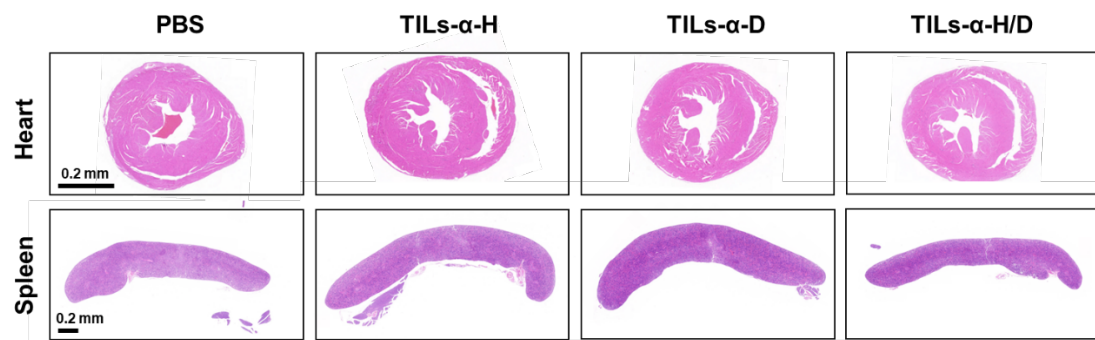
linked immunosorbent assay (ELISA) kit, data points represent mean $\pm$ SD (n = 3 mice).
(g) Immunofluorescence analysis of CD3⁺ T cell infiltration in Panc02 and Panc02
CCL5/KD tumor tissues on day 20 following various treatments. CD3⁺ T cells were
labeled with Alexa Fluor 555 (red), and cell nuclei were stained with 4',6-diamidino-2-
phenylindole (DAPI) (blue), scale bar: 50 μ m. Statistical significance was calculated
using unpaired one-way ANOVA test, * p < 0.05, *** p < 0.001, and **** p < 0.0001.
Data in (a, b and g) are representative of three independent experiments with similar
results. Source data are provided as a Source Data file.



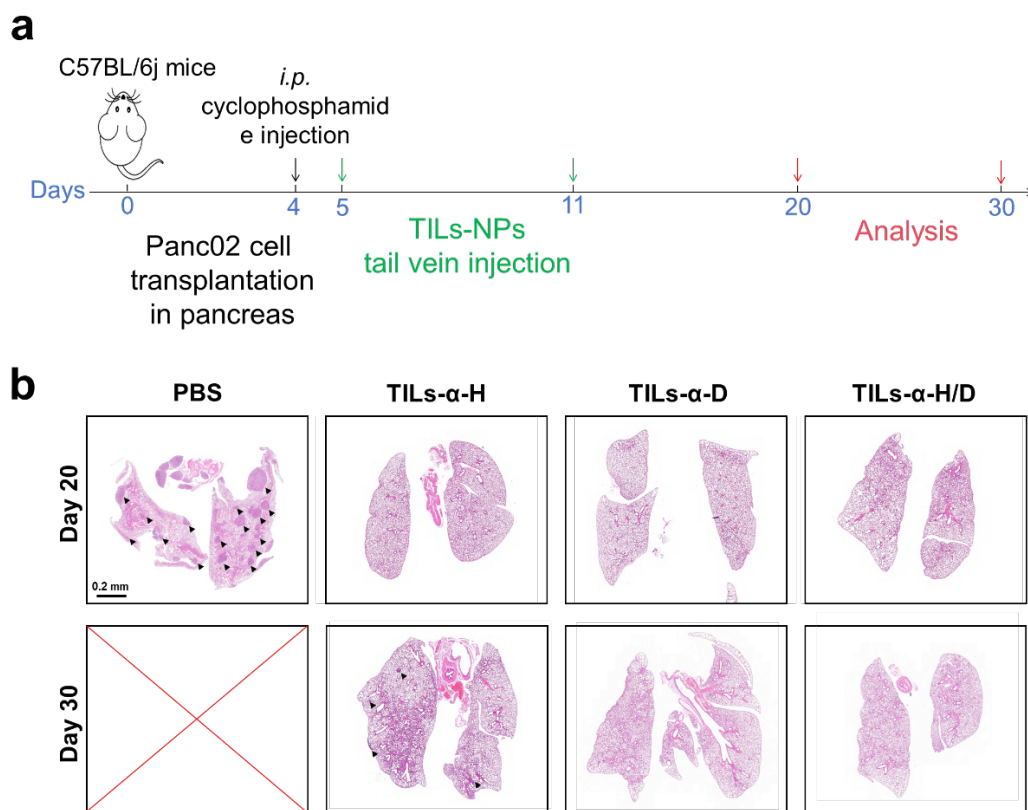
Supplementary Figure 19 (a) Flow cytometry analysis of CD4⁺IFN-γ⁺ T cells in orthotopic pancreatic tumors after various treatments (gate on CD3⁺CD4⁺CD8⁻ T cells). (b) Flow cytometry analysis of exhausted CD4⁺ TILs (PD-1⁺Tim3⁺CD4⁺ T cells; gated on CD3⁺CD4⁺CD8⁻ T cells) after various treatments.



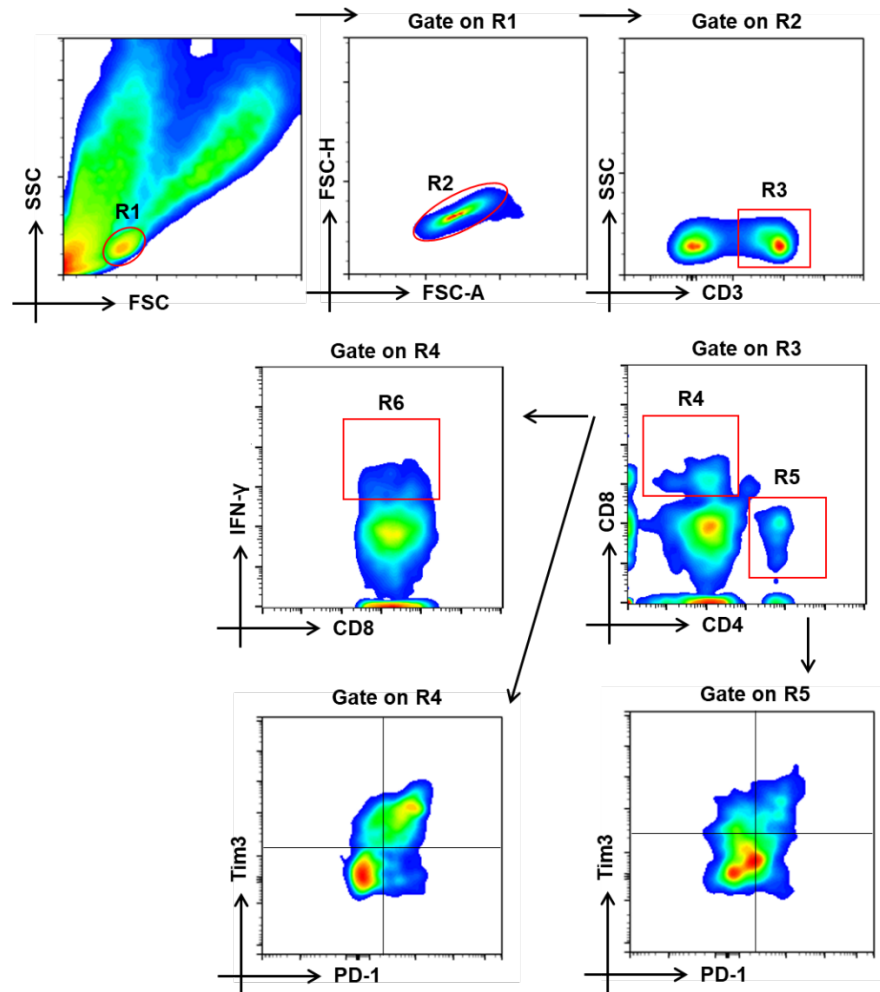
Supplementary Figure 20 (a) Quantification of granzyme B expression in tumor tissues based on immunohistochemical analysis from Fig. 6n, data points represent mean $\pm$ SD ($n = 3$ mice). (b) Quantification of interferon- γ (IFN- γ) expression in tumor tissues based on immunohistochemical analysis from Fig. 6n, data points represent mean $\pm$ SD ($n = 3$ mice). Statistical significance was calculated using unpaired one-way ANOVA test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Source data are provided as a Source Data file.



Supplementary Figure 21 Hematoxylin and eosin (H&E) staining of heart and spleen, scale bar: 0.2 mm. Data are representative of three independent experiments with similar results.



Supplementary Figure 22 (a) Schematic illustration of the experimental design for the anti-spontaneous metastasis in the orthotopic pancreatic cancer model. (b) Hematoxylin and eosin (H&E) staining of lung tissues from mice bearing orthotopic pancreatic tumors after receiving different treatments, scale bar: 0.2 mm. Data are representative of three independent experiments with similar results.



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202 **Supplementary Figure 23** Gating strategy of Fig. 6h-m.