

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

Corresponding Author: Dr Ziqi Gan

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

In this study, Zecong Xiao et al. present an innovative strategy to enhance TIL therapy by engineering TILs with α PD-1-conjugated, acid-sensitive ZIF-8 nanoparticles. The authors designed these nanoparticles to encapsulate hyaluronidase and decitabine, while tethering α PD-1 antibodies to the surface to enable stable binding to TILs. This system allows the nanoparticles to remain associated with TILs during circulation and to selectively release their payloads in the acidic tumor microenvironment. Upon release, hyaluronidase facilitates stromal degradation, while decitabine promotes CCL5-mediated TIL recruitment, establishing a self-amplifying loop that enhances TIL infiltration and antitumor efficacy. Using the pancreatic cancer model, the authors demonstrate that this approach effectively overcomes key barriers encountered by conventional ACT strategies in immune-resistant solid tumors. Overall, this work offers a promising direction for the development of TIL therapy.

Below I list my concerns.

1. The rationale for selecting ZIF-8 over other nanocarriers (e.g., lipid-based or polymeric nanoparticles) is not fully justified. What are the advantages of ZIF-8 in terms of pH sensitivity and protein encapsulation efficiency?
2. How are the nanodrugs loaded onto TILs exactly? The manuscript mentions the binding through PD-1/anti-PD-1 antibody interactions. Is this achieved simply by co-culturing TILs with nanodrugs in cytokine-containing TIL medium? On Line 590, various nanodrug concentrations (e.g., 0.1, 0.2, 0.4, 0.6, 0.8 mg/mL) are listed for coculture with TILs. How were these concentrations determined, considering the multiple payloads carried by the nanodrugs?
3. Is Di labeling, similar to CFSE, heritable during TIL proliferation, thereby allowing dye dilution? Could a portion of the Di⁺ immune cells represent proliferative progeny of the infused TILs?
4. For a more rigorous evaluation of in vivo antitumor effects, it would be important to include an additional control group treated with TILs plus drugs (without nanocarriers), to better highlight the superiority of the nanocarrier system.
5. In Fig.3b, an in vitro assay was performed to assess TIL proliferation. What was the TIL-to-tumor cell coculture ratio? The methodology section does not describe this experiment in details.
6. In Fig.S6, the legend describes the optimal nanodrug concentration for TIL loading. However, the figure legend instead discusses the "relative ROS level of MSCs" (Line 117) and "MSC apoptosis" (Line 118). Could this be a typographical error?
7. In Figure 6h and 6i, the authors could show the proportion of CD4⁺ and IFN- γ ⁺CD4⁺ T cells, as well as the exhaustion status of CD8⁺ and CD4⁺ TILs across different groups.
8. All the staining results (Figure 3e, 4i, 5l, 5m, 6f, 6g, 6j, 7e and so on) should include statistical analysis. Same for the charts.
9. A minor suggestion: in the schematic diagrams, the nanodrugs are depicted as being almost the same size as the TILs. Given that the nanodrugs are intended to coat the TIL membrane, it would be more accurate and less misleading to depict the nanoparticles smaller relative to the TILs. Additionally, the arrangement and labeling of small panels within the large figures could be improved for clarity and logical flow.

Reviewer #2

(Remarks to the Author)

The manuscript by Ziqi Gan and collaborators describes the creation of specific nanoparticles which, when combined to tumor infiltrating lymphocytes (TILs) greatly enhanced their antitumor activity. This work is of good quality, complete, nicely

organized and written, with most data shown supporting conclusions by authors. However, I have few main concerns that should be addressed by authors.

Main concerns:

- I do not understand why/how these anti-PD1 conjugated nanoparticles are not internalized by TILs? This is very strange, especially considering the specific engagement of the antibody with the receptor PD1. This raises the question of whether the membrane binding is truly specific and mediated by PD1 recognition by the antibody, or simply a non-specific coating of the cells via adsorptive binding. Are the nanoparticles non conjugated to anti-PD1 antibody still able to bind TILs' membranes? I couldn't find such control in figures, especially in figure 3 in which authors shows the coating of TILs with alpha-H/D nanoparticles. Authors have to show this control and explain the absence of internalization of the nanoparticles.
- How can these findings be applied in a clinical context? Most PDAC patients (80-85%) are not treated surgically, and only undergo biopsy. How can we isolate enough TILs under these conditions? Moreover, in their experimental setting, the isolated TILs were obtained from tumors one week old, which is too short to achieve exhaustion compared with TILs isolated from patients whose tumors are several years old. These limitations should be mentioned in the discussion section.
- Effect of hyaluronidase action and promotion of metastasis. It is known that targeting the extracellular matrix to make it less dense is a double-edge sword. Authors showed the efficacy of their nanodrug-carrying TILs in eliminating metastases in an artificial metastases model, injecting tumor cells in the tail vein. Hence, even if these TILs could favor the production of metastases, they are expected to eradicate them. However, in the longer term, the hyaluronidase action of these TILs may favor metastatic occurrence. Authors should look longer times (much longer than 7 days) after last TILs injection and in a spontaneous metastatic model, that their treatment do not favor metastasis. At least, this limitation should be mentioned in the discussion section.
- CCL5 may not be the only Decitabine-induced protein involved in recruitment of CD3+ T cells. The KO or KD of CCL5 is required to demonstrate its real and unique implication in this recruitment.
- Fig. 3 e,f,g, a control with continuous pH 7.4 in place of 6.5 is missing. The fluorescence could be lost without the pH switch from 7.4 to 6.5.
- Fig. S8a, Day 0 control is missing (not so important but...). More important, when observing more Di+ cells it seems that the total number of cells studied by flow cytometry was also much more important. Values should be expressed as percentage or, the total number of studied cells should be the same between all conditions.
- Results section, Fig. 4l, "extracellular" should be replace by "extravascular" I guess. Regarding this experiment shown in Fig. 4K-l, in control, mice should have been injected at day 5 with TILs to serve as control for TILs- α -H/D injected mice. Also, is there any evidence that hyaluronic acid has been degraded in TILs- α -H/D injected mice compared to control mice? The probable disintegration or cleavage of CDM bonds within the tumor microenvironment should be demonstrated. At least, acidic intratumoral pH should be confirmed.
- Fig. 5l and Fig. 6f need of a specific marker of apoptosis to confirm a difference in apoptosis since H&E staining cannot discriminate between the different forms of cell death.
- In the results section of Fig 5m, contrary to the authors' assertion, there was no overlap of green and red signals in NCG mice treated with TILs-a-H/D, indicating that most CD8 T cells were endogenous and not exogenous TILs. In C57BL/6j mice, it seems that there is an increased number of exogenous TILs so, consequently, also an increased number of CD8+ exogenous TILs in addition the endogenous CD8+ TILs. Fig. 5o, in the t-SNE analysis, the red dots indicate endogenous TILs since they are Di-&CD8+, contrary to what is indicated in the text. In fact, this whole paragraph should be carefully checked and rewritten in a clearer manner.

Minor points :

- In Fig. 4d "Tumor" should be written in white since it is not so visible in red. I missed it at first observation...
- In legend of Figure 4, (m) shows the fluorescence intensity profiles from line selected in "l" not "i".
- Result section, fig. 7b, I think the authors wanted to write "TILs-a-H" and "TILs-a-D" instead of two times "TILs-a-H/D".
- Fig. 5k and not 5i in discussion.

Reviewer #3

(Remarks to the Author)

In this manuscript, the authors use nanoengineered tumour-infiltrating lymphocyte (TIL) therapy using aPD-1-conjugated acid-cleavable ZIF-8 nanoparticles in pancreatic cancer models. They show that using TILs, enhances antitumor efficacy by releasing hyaluronidase and decitabine in acidic tumor microenvironments promotes stroma degradation and CCL5 secretion.

This topic is clinically relevant since available treatment options is severely lacking for pancreatic cancer and TIL therapy has now progressed into clinical trials in other cancer types. The authors used various methods and took the time to validate their constructs.

General comments

Most subcutaneous, metastatic and orthotopic model with panc02 cells have a treatment start on day 5. Most likely, the tumours and metastasis are barely growing at that time. It would be good to show if you have tumour/metastasis established on day 5 or do an experiment when you start the treatment on later days when the tumours/metastasis are established. The in vivo results do not determent if there are cytotoxic or cytostatic treatment effect and if this treatment is effective in already established tumours. Additionally, apoptosis assays or stainings would be beneficial. Are the cancer cells really dying?

Grammar and proof-reading are needed in few places. i.e. line 61, 317, 465, 594, 610, 618, 654, 655

A lot of abbreviations missing both in main text and figure texts. i.e. ICB, PVP, Cys, MOF, NSAID-PEG-CEM while some are abbreviated multiple times i.e. DEC, aPD-1, TIL

The authors declare competing interests. This should be explained.

Specific comments

Results

- Fig. 2l wrongly marked as i (line 133)
- Fig. 5d missing from results text.
- Fig 7c missing from results text.

Methods

Missing from the method section:

o No mention of how cyclophosphamide was given and in what dose. No manufacturer or how it was dissolved. (line 285 and 306 and figure text 5)

o No mention of how MRI was done. Manufacture? How were the mice anaesthetised? For what cancer model and what days was it done?

o 2-MIN: what is it and where does it come from?

The preparation of a-H/D nanoparticle/TILs and nanodrug-carrying TILs preparation/In vitro release:

- Information missing where all the incorporated materials come from: FITC, DEC, MOF, aPD-1, CDM
- At specific time intervals = state what time intervals
- Fresh buffer: what does this buffer contain?
- Fluorospectrophotometer and chromatography info missing.

Tils and nanodrug-carrying TILS preparation/in vitro cell binding:

- Were the cells injected? In what volume? In what solution?
- Were the mice anesthetized? If so, how?
- No information about collagenase IV and DNase
- Hoechst manufacture?

ELISA and biochemistry index and histology analysis:

- Info missing about RIPA buffer, protease inhibitors, TNF and IL6

TILs killing assay in vitro:

- Info missing of following: Confocal dishes, Calcein-AM, CLSM, D-luciferin, Microplate reader
- Various treatments: state what was done
- Panc02 luc: How were they transfected/transduced and with what plasmid?

Animal model/in vivo and ex vivo fluorescence imaging:

- Did the mice receive pain management?
- How were the mice anesthetised?
- How were the cells diluted? PBS? Matrigel?
- How much liquid was injected?
- How were the mice euthanized?
- Missing MRI info, how was that data obtained?
- Manufacture of in vivo fluorescence imaging system missing.

Histological analysis

- "Section of tumor and organ tissue": where were the tissues cut? Only in one place or in several places throughout the tissues to give more 3D analysis? What liver lobe, kidney and lung were chosen?

Staining:

- Manufacturer of H&E staining kit missing
- No information about secondary antibodies
- DAPI manufacturer missing
- Confocal microscopy type missing
- DAB kit manufacturer missing
- Hematoxylin manufacturer missing
- What kind of Pathology Imaging System?

Flow cytometry:

- CFSE manufacturer missing
- Red cell lysis solution manufacturer missing
- Abbreviation for flow cytometry is claimed but then never used: FCM

Figure/figure legends:

All figures and text are very small and difficult to read and understand.

In figure 3 you have "(*** p < 0.001)" in the figure text but the p value explanation is missing in all the other figures.

Figure 1.

- o Good schematic figure but would need more detailed figure text and all the abbreviations needs to be explained.
- o "Death tumor cells", should probably say "dead"
- o Increase the size of the letters above the arrows
- o 2-MIN never explained. What is it and where does it come from?

Figure 2:

- o Missing figure legend for b and f.
- o b and c are wrongly named. Should be c and d.
- o h. "Zata" should be "Zeta"
- o k, l, m: look very similar. Would be clearer to have a title in each graph.

Figure 3

- o Abbreviations needed for CFSE and CLSM
- o i. scalebar not visible
- o k. x-axis = "TILs distribution", not very clear. What is the difference between the dots on the left side of the graph to the once on the right?

Figure 4

- o b, n. You show the mouse on its back, and it has high expression from the right side. However, I would expect the high signal should be coming from the pancreas on the left side?
- o m. very unclear figure. What is x-axis? What do you mean by Gray value? Also, no white lines in Figure i as stated in the figure text.
- o Are there no statistical analysis done for any of the figures? If so, the info is missing in the figure text.

Figure 5

- o d. No mention of cyclophosphamide. How was it given and in what dose?
- o k and n are missing statistics
- o m. missing scalebar in the enlargement images
- o g. make sure all the text is visible.

Figure 6

- o c. would be good to have also a figure of the size of the tumour when the treatment started as a baseline.

Figure 7

- o k. More info about the markers in the figure text, what is each measuring?
- o e. images of liver don't seem to be from the same liver lobe. What liver lobes were collected? Where all of them sectioned? Where the sections throughout to make a 3D analysis?

Supplementary information:

Fig. S6

- o Abbreviation of ROS and MSCs are missing

Fig. S7

- o Missing statistics information
- o Why two PBS groups? What's the difference between them?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Based on the authors' comprehensive revisions, I am pleased to confirm that the manuscript has achieved significant improvements in scientific rigor and clarity. The inclusion of critical validation experiments has satisfactorily addressed all substantive concerns. Specifically, the conclusive demonstration of nanoparticle-TIL binding specificity through isotype controls and PD-1 blockade studies (Supplementary Figure 7), along with the addition of a control group treated with TILs plus free drugs (without nanocarriers), robustly validates the superior in vivo antitumor efficacy of the nanocarrier system (Figure 5). The author has optimized and adjusted the layout and details of the article, significantly enhancing both the quality of the manuscript and its readability. I accept the revised manuscript without further revision. I commend the authors for their exemplary responsiveness and wish them success with the publication of this impactful work.

Reviewer #2

(Remarks to the Author)

In their revised version of their manuscript, authors have successfully replied to most of my concerns.

There are only two remaining minor points:

- First, in supplementary figure 11, graphics still display high differences between TILs/Di cells and TILs/Di-a-H/D whereas they should display similar amounts.
- Second, in the figure R1 for reviewers, on one out of three tumors has a real acidic pH of 6.47. The two others are pretty close to 7 which is not so acidic!

Reviewer #3

(Remarks to the Author)

This manuscript has been revised to my satisfaction. My concerns have been addressed and detailed corrections have been made.

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Point-by-point response to reviewer comments

Response to Reviewer #1(TIL therapy, nanotherapy):

In this study, Zecong Xiao et al. present an innovative strategy to enhance TIL therapy by engineering TILs with α PD-1-conjugated, acid-sensitive ZIF-8 nanoparticles. The authors designed these nanoparticles to encapsulate hyaluronidase and decitabine, while tethering α PD-1 antibodies to the surface to enable stable binding to TILs. This system allows the nanoparticles to remain associated with TILs during circulation and to selectively release their payloads in the acidic tumor microenvironment. Upon release, hyaluronidase facilitates stromal degradation, while decitabine promotes CCL5-mediated TIL recruitment, establishing a self-amplifying loop that enhances TIL infiltration and antitumor efficacy. Using the pancreatic cancer model, the authors demonstrate that this approach effectively overcomes key barriers encountered by conventional ACT strategies in immune-resistant solid tumors. Overall, this work offers a promising direction for the development of TIL therapy. Below I list my concerns.

Response: We sincerely appreciate your thoughtful and positive evaluation of our manuscript. Your comments and subsequent concerns are highly valuable to us, and we believe addressing them will substantially strengthen our manuscript. We have carefully and thoroughly responded to each concern raised, ensuring clarity and rigor in our revised submission.

1. The rationale for selecting ZIF-8 over other nanocarriers (e.g., lipid-based or polymeric nanoparticles) is not fully justified. What are the advantages of ZIF-8 in terms of pH sensitivity and protein encapsulation efficiency?

Response: We thank the reviewer for the suggestion. Various platforms, such as ionic lipids, polymeric nanoparticles, mesoporous silica, and sol-gel matrices, have been developed to deliver proteins, which are susceptible to conformational changes during formulation and storage due to their delicate structures. These platforms aim to stabilize proteins; however, they face substantial challenges, including low loading efficiency, poor confinement, and restricted pore sizes of the supporting materials (*Enzyme Microb. Technol.*, 2007, 40, 1451–1463).

ZIF-8, which consists of metal nodes (Zn^{2+}) linked by organic ligands (2-methylimidazole), is a classic porous material with tailorable ultrahigh porosity (up to 90% free volume) and a high surface area (beyond $600 \text{ m}^2\text{g}^{-1}$) (*Science* 2013, 341, 1230444). This ultrahigh porosity provides ample space for protein encapsulation, and the tightly surrounding ZIF-8 layer significantly stabilizes proteins through conformational confinement, enhancing both loading efficiency and protection of bioactivity (*Angew. Chem. Int. Ed.*, 2020, 59, 8786-8798). Moreover, the pH-triggered protonation of 2-methylimidazole in the tumor microenvironment can lead to ZIF-8 decomposition, promoting the controlled release of encapsulated proteins. Therefore, to optimize loading efficiency, ensure bioactivity protection during circulation, and facilitate on-demand release in the tumor microenvironment, ZIF-8 was selected as the carrier for this study. We believe this choice provides significant advantages in terms of both stability and release control, addressing the challenges faced by other nanocarriers.

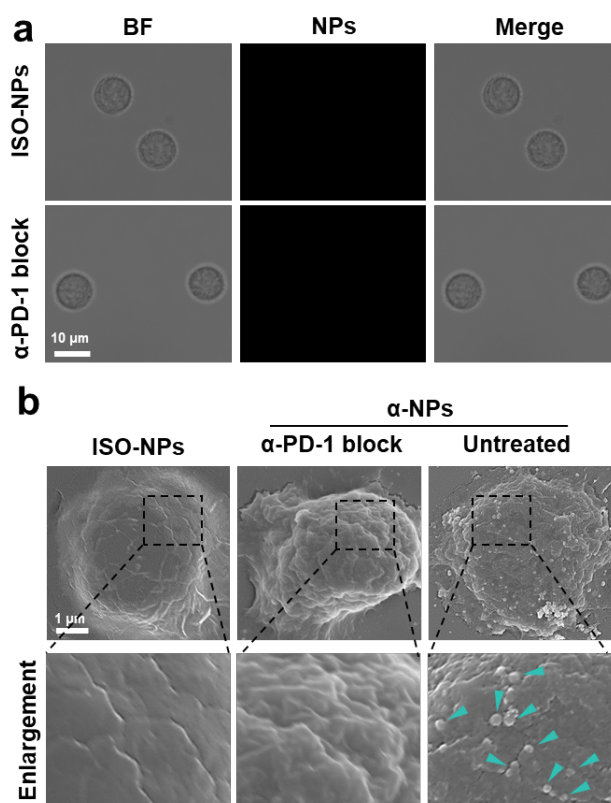
2. How are the nanodrugs loaded onto TILs exactly? The manuscript mentions the binding through PD-1/anti-PD-1 antibody interactions. Is this achieved simply by co-culturing TILs with nanodrugs in cytokine-containing TIL medium? On Line 590, various nanodrug concentrations (e.g., 0.1, 0.2, 0.4, 0.6, 0.8 mg/mL) are listed for coculture with TILs. How were these concentrations determined, considering the multiple payloads carried by the nanodrugs?

Response: We thank the reviewer for this valuable comment. As described in our manuscript, the nanodrugs were loaded onto tumor infiltrating lymphocytes (TILs) through specific interactions between anti-PD-1 antibodies (α -PD-1) on the nanoparticle surface and PD-1 receptors expressed on TILs. This was indeed achieved through a simple co-incubation of the nanodrugs with TILs. Similar antibody–receptor-mediated strategies for constructing cell-nanoparticle conjugates have also been documented in previous studies (*Nat. Biomed. Eng.*, 2024, 8, 1347-1365).

To further confirm that the binding was specifically mediated by PD-1/ α -PD-1 interactions, we conducted additional validation experiments (**Supplementary Figure 7**). First, we prepared nanoparticles surface-modified with an isotype control antibody

(ISO-NPs). Confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM) imaging revealed that ISO-NPs did not bind to TILs. Subsequently, TILs were pretreated with free α -PD-1 to block PD-1 receptors prior to incubation with α -NPs, which also resulted in no detectable binding. In contrast, direct incubation of α -NPs with untreated TILs led to clear nanoparticle attachment to the TIL surface, as confirmed by SEM (indicated by green arrows). These results collectively confirm that nanoparticle loading onto TILs is specifically mediated through PD-1/anti-PD-1 interactions.

Regarding the nanodrug concentrations (0.1, 0.2, 0.4, 0.6, and 0.8 mg mL⁻¹) used for co-culture with TILs, these values were based on the mass of lyophilized ZIF-8 nanoparticles encapsulating rhodamine B and surface-functionalized with α -PD-1 (α -RhoB NPs). These concentrations reflect the nanoparticle dosage used during incubation and were selected to optimize the nanoparticle-to-cell ratio for efficient nanodrug loading onto TILs.



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.

3. Is Di labeling, similar to CFSE, heritable during TIL proliferation, thereby allowing dye dilution? Could a portion of the Di⁻ immune cells represent proliferative progeny of the infused TILs?

Response: We thank the reviewer's comments. We understand the concern regarding the potential dilution of Di during TIL proliferation and whether Di⁻ immune cells might represent proliferative progeny of the infused TILs. In this study, we used the lipophilic membrane dye Di to label TILs for tracking their tumor infiltration. Unlike CFSE, which covalently binds intracellular proteins and is equally inherited by daughter cells during each cell division, resulting in a well-known dilution effect suitable for proliferation studies, Di integrates into the lipid bilayer of the cell membrane and exhibits strong membrane stability. It is not evenly distributed to daughter cells during each cell division, and thus its signal does not dilute as rapidly or consistently as CFSE.

Indeed, as the reviewer rightly noted, since Di is generally not evenly partitioned between daughter cells during division, it is theoretically possible that a subset of Di⁻ immune cells may originate from proliferating infused TILs. We fully acknowledge this possibility. However, our results argue against this being a significant contributor to the Di⁻ population in our study. To specifically address this point, we would like to highlight a critical observation from our experiments (Fig. 5n, o): In NCG immunodeficient mice (which lack endogenous T cells), we did not detect a notable population of Di⁻CD8⁺ T cells following TIL infusion. In contrast, in immunocompetent C57BL/6j mice, a clear population of Di⁻CD8⁺ T cells was present. This difference strongly suggests that the Di⁻CD8⁺ T cells we observed in our C57BL/6j models are primarily host-derived endogenous T cells, rather than the proliferative progeny of infused Di-labeled TILs that have lost the dye.

Additionally, previous studies have validated the stability and applicability of Di for *in vivo* tracking of cell distribution. For example, Ping *et al.* (*Nat. Nanotechnol.* 2023, 18, 933–944 (Fig. 3) used Di to label TILs and successfully monitored their tumor infiltration following adoptive transfer *via* fluorescence imaging and flow cytometry.

In summary, we believe Di is a reliable membrane-labeling tool for assessing TIL infiltration under our experimental conditions.

4. For a more rigorous evaluation of *in vivo* antitumor effects, it would be important to include an additional control group treated with TILs plus drugs (without nanocarriers), to better highlight the superiority of the nanocarrier system.

Response: We thank the reviewer for this constructive suggestion. In response, we have included an additional control group (TILs + α /H/D, *i.e.*, TILs combined with free drugs without nanocarriers) in the subcutaneous tumor model to more rigorously evaluate the *in vivo* antitumor effects. To ensure consistency and comparability across all groups, we repeated the therapeutic experiments for all treatment groups—including PBS, TILs, TILs + α /H/D, TILs- α -H, TILs- α -D, and TILs- α -H/D—in both NCG and C57BL/6j mouse models.

As shown in **Fig. 5a-k** in the revised manuscript, the TILs- α -H/D group exhibited significantly greater tumor suppression compared to the TILs + α /H/D group. Moreover, TILs- α -H/D treatment markedly prolonged mouse survival in both the NCG and C57BL/6j subcutaneous tumor models, suggesting the superiority of the nanocarrier system over the administration of free drugs.

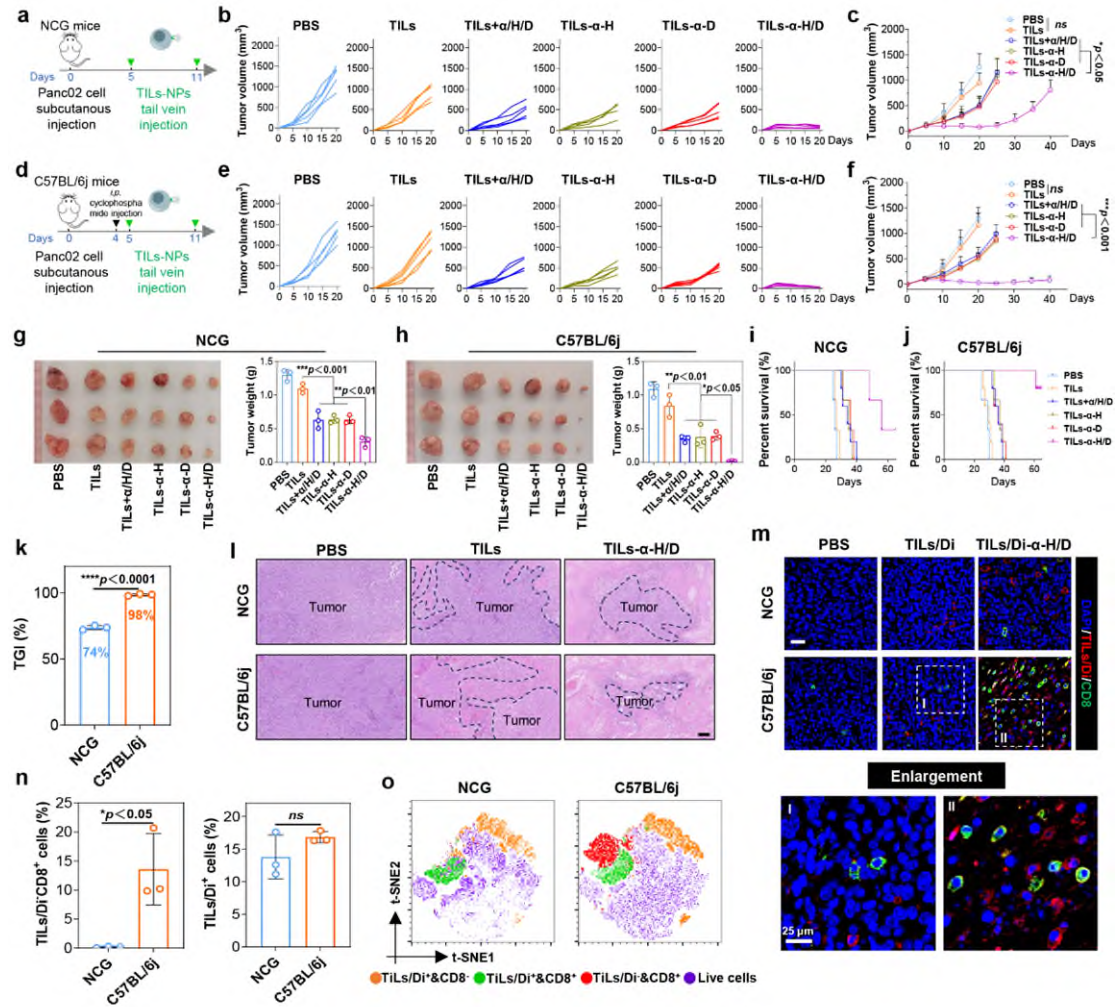


Fig. 5 Effect of antitumor and immune regulation *in vivo*. (a) Schematic of the experimental design for the antitumor study in NOD-Prkdc^{em26}Cd52^{Il2rg^{em26}Cd22}/Nju (NCG) mice. (b) Individual tumor growth curves for Panc02 tumor-bearing NCG mice receiving different treatments. (c) Tumor growth in Panc02 tumor-bearing NCG mice, mean ± SD, *n* = 5 mice per group. Statistical significance: one-way analysis of variance (ANOVA), *ns* = no significance, **p* < 0.05. (d) Experimental design for the antitumor study in C57BL/6j mice, with cyclophosphamide intraperitoneal injection (200 mg kg⁻¹). (e) Individual tumor growth curves for Panc02 tumor-bearing C57BL/6j mice receiving different treatments. (f) Tumor growth in Panc02 tumor-bearing C57BL/6j mice, mean ± SD, *n* = 5 mice per group. Statistical significance: one-way ANOVA, *ns* = no significance, ****p* < 0.001. (g) Images of subcutaneous tumors and tumor weights from Panc02 tumor-bearing NCG mice after 20 days, mean ± SD, *n* = 3 mice per group. Statistical significance: one-way ANOVA, ***p* < 0.01, ****p* < 0.001. (h) Images of

subcutaneous tumors and tumor weights from Panc02 tumor-bearing C57BL/6j mice after 20 days, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, $*p < 0.05$, $**p < 0.01$. (i) Cumulative survival of NCG mice, $n = 5$ mice per group. (j) Cumulative survival of C57BL/6j mice, $n = 5$ mice per group. (k) Tumor growth inhibition (%) in NCG and C57BL/6j mice treated with nanodrug-carrying TILs, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: Students t-test, $****p < 0.0001$. (l) Hematoxylin and eosin (H&E) staining of tumor tissues at 20 days, tumor margins marked with a dotted line (scale bar: 200 μ m). (m) Confocal laser scanning microscope (CLSM) images of tumor sections showing TILs and CD8⁺ T cells. Red fluorescence: TILs, green fluorescence: CD8⁺ T cells, blue fluorescence: nuclei (scale bar: 50 μ m). (n) Quantification of TILs and CD8⁺ T cells in tumor tissue from NCG and C57BL/6j mice, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: Student's t-test, $ns =$ no significance, $*p < 0.05$. (o) t-distributed stochastic neighbor embedding (t-SNE) plots of flow cytometry data from tumor samples in NCG and C57BL/6j mice treated with TILs- α -H/D. Red dots: endogenous CD8⁺ T cells, green/orange dots: exogenous TILs.

5. In Fig.3b, an *in vitro* assay was performed to assess TIL proliferation. What was the TIL-to-tumor cell coculture ratio? The methodology section does not describe this experiment in details.

Response: We thank the reviewer for pointing out the missing detail regarding the TIL-to-tumor cell coculture ratio used in the *in vitro* proliferation assay (Fig. 3b). In this experiment, TILs and Panc02 tumor cells were cocultured using a transwell system at a 1:1 ratio (5×10^4 cells each per well). We have now added a detailed description of this protocol to the revised Methods section under "TILs and nanodrug-carrying TILs preparation" in the revised manuscript.

6. In Fig.S6, the legend describes the optimal nanodrug concentration for TIL loading. However, the figure legend instead discusses the "relative ROS level of MSCs" (Line 117) and "MSC apoptosis" (Line 118). Could this be a typographical error?

Response: We thank the reviewer for their careful attention to detail. This was a

typographical error in the original legend of Fig. S6 (now Supplementary Figure 6 in the revised version). The incorrect references to "relative ROS level of MSCs" and "MSC apoptosis" have been removed, and the figure legend has been revised to accurately reflect the experimental content.

7. In Figure 6h and 6i, the authors could show the proportion of CD4⁺ and IFN- γ ⁺CD4⁺ T cells, as well as the exhaustion status of CD8⁺ and CD4⁺ TILs across different groups.

Response: We thank the reviewer for this helpful suggestion. The proportions of CD4⁺ and IFN- γ ⁺CD4⁺ T cells have been added in the revised manuscript. Specifically, the percentage of CD4⁺ T cells is shown in **Fig. 6h, i** in the revised manuscript, and the proportion of IFN- γ ⁺CD4⁺ T cells is presented in **Supplementary Figure 19a** and **Fig. 6k**. Notably, both populations were significantly increased in the TIL- α -H/D treatment group compared to the other three groups.

In addition, we added the *in vivo* experiments and analyzed the exhaustion status of CD4⁺ and CD8⁺ TILs by flow cytometry across different treatment groups. As shown in **Fig. 6l, m** and **Supplementary Figure 19b**, the TIL- α -H/D group exhibited the lowest proportion of exhausted TILs—including PD-1⁺Tim3⁺CD4⁺ T cells and PD-1⁺Tim3⁺CD8⁺ T cells—among all four groups. The corresponding descriptions of these results have also been included in the revised manuscript.

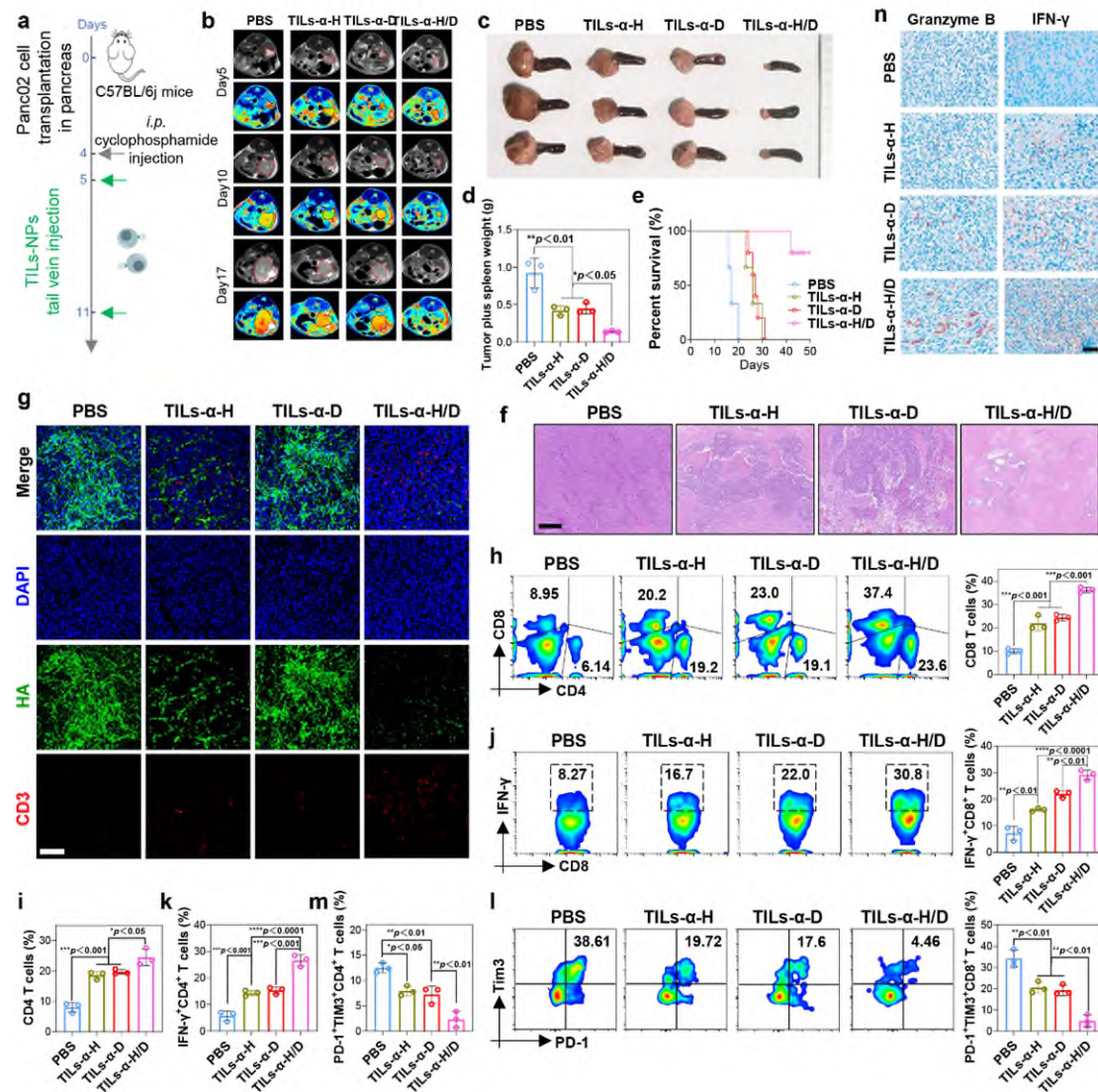
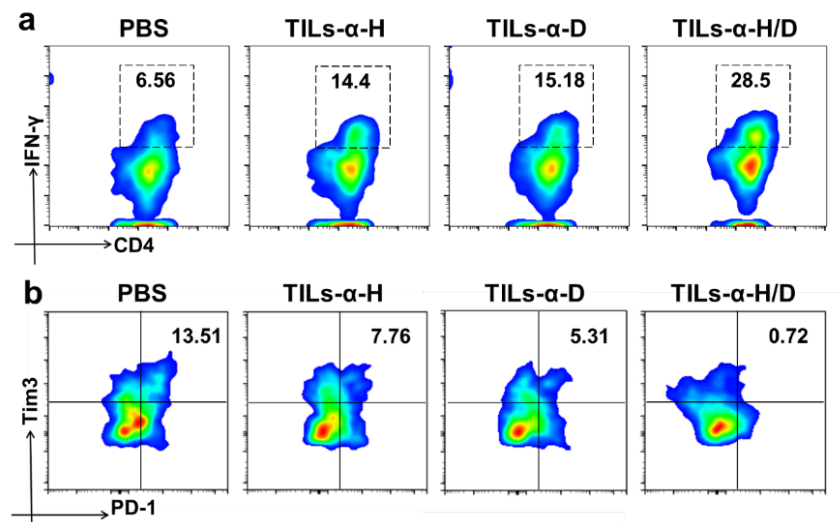


Fig. 6 Antitumor efficacy of treatments in an orthotopic Panc02 pancreatic tumor model in C57BL/6j mice. (a) Schematic of the experimental design for the antitumor study in C57BL/6j mice. (b) Tumor growth was monitored by magnetic resonance imaging (MRI) at 5, 10, and 17 days post-treatment initiation. (c) Representative photographs of excised orthotopic pancreatic tumors and spleens from each treatment group. (d) Weights of tumors and spleens at the study endpoint, mean $\pm$ standard deviation (SD), $n = 3$ mice per group. Statistical significance: one-way analysis of variance (ANOVA), $*p < 0.05$, $**p < 0.01$. (e) Cumulative survival of mice bearing orthotopic tumors after different treatments, $n = 5$ mice per group. (f) Hematoxylin and eosin (H&E) staining of tumor tissues, scale bar: 200 μ m. (g) Double immunofluorescence for hyaluronic acid (HA) and CD3⁺ T cells in tumor sections, HA (labeled with Alexa Fluor[®]488, green), CD3⁺ T cells (labeled with Alexa Fluor[®]555,

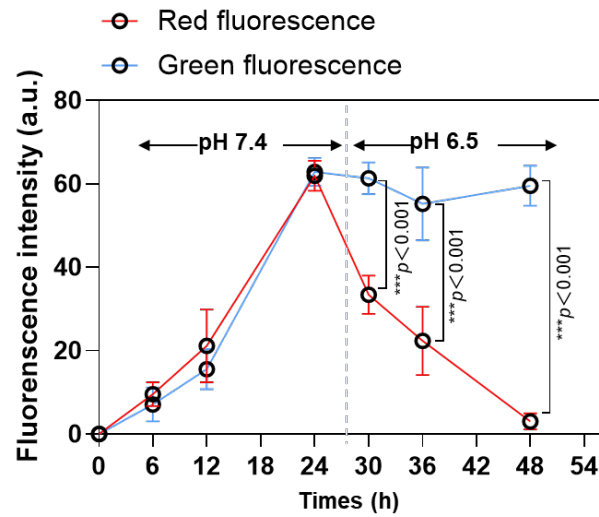
red), nuclei (stained with 4',6-diamidino-2-phenylindole (DAPI), blue), scale bar: 100 μm . (h) Flow cytometry quantification of CD8⁺ T cells (gated on CD3⁺ T cells) in orthotopic tumors, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, *** $p < 0.001$. (i) Quantification of CD4⁺ (gated on CD3⁺ T cells) T cells in tumors, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, * $p < 0.05$, *** $p < 0.001$. (j) Flow cytometry analysis of CD8⁺IFN- γ ⁺ T cells (gated on CD3⁺CD4⁻CD8⁺ T cells) in tumors, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, ** $p < 0.01$, **** $p < 0.0001$. (k) Quantitative analysis of CD4⁺IFN- γ ⁺ T cells (gated on CD3⁺CD4⁺CD8⁻ T cells) in tumors, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, *** $p < 0.001$, **** $p < 0.0001$. (l) Flow cytometry analysis of exhausted CD8⁺ TILs (PD-1⁺Tim3⁺CD8⁺; gated on CD3⁺CD4⁻CD8⁺ T cells), mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, ** $p < 0.01$. (m) Quantification of exhausted CD4⁺ TILs (PD-1⁺Tim3⁺CD4⁺; gated on CD3⁺CD4⁺CD8⁻ T cells), mean $\pm$ SD, $n = 3$ mice per group. Statistical significance: one-way ANOVA, * $p < 0.05$, ** $p < 0.01$. (n) Immunohistochemical staining of granzyme B and interferon- γ (IFN- γ) in tumor tissues, scale bar: 50 μm .



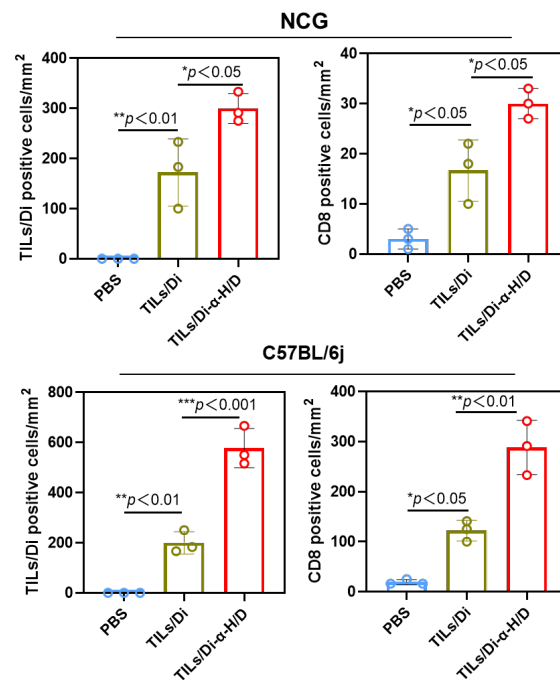
Supplementary Figure 19 (a) Flow cytometry analysis of CD4⁺IFN- γ ⁺ T cells in orthotopic pancreatic tumors after various treatments (gate on CD3⁺CD4⁺CD8⁻ T cells). (b) Flow cytometry analysis of exhausted CD4⁺ TILs (PD-1⁺Tim3⁺CD4⁺ T cells; gated on CD3⁺CD4⁺CD8⁻ T cells) after various treatments.

8. All the staining results (Figure 3e, 4i, 5l, 5m, 6f, 6g, 6j, 7e and so on) should include statistical analysis. Same for the charts.

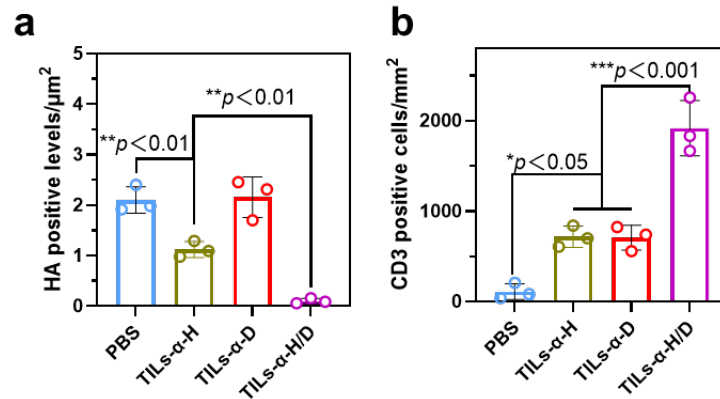
Response: As suggested, statistical analysis for the quantification of CCL5 levels in tumors after different treatments (Fig. 4i) has been added to the revised manuscript. Additionally, statistical analyses for the staining results shown in Figs. 3e, 5m, 6g, and 6j (corresponding to Fig. 6n in the revised manuscript) have also been included. These results are now presented as **Supplementary Figure 9**, **Supplementary Figure 14**, **Supplementary Figure 17** and **Supplementary Figure 20**, respectively. Moreover, regarding the hematoxylin and eosin (H&E) staining results in Figs. 5l and 6f, and in order to enable more accurate quantification of apoptotic cells in tumor tissues—as also suggested by Reviewer #2 and Reviewer #3—we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on adjacent tumor sections taken from the same tumors and anatomical regions as those used for H&E staining. This approach allowed for precise identification and quantification of apoptosis in anatomically matched regions across treatment groups. As shown in **Supplementary Figure 13** and **Supplementary Figure 16**, TUNEL staining and its quantification revealed that TIL- α -H/D treatment significantly promoted tumor cell apoptosis in both the subcutaneous tumor models (NCG and C57BL/6j mice) and the orthotopic tumor model (C57BL/6j mice), compared to the other treatment groups. These quantitative results are consistent with the representative staining images provided in the manuscript. In addition, the statistical analysis corresponding to Fig. 7e has already been included in the original version of the manuscript (see Fig. 7f).



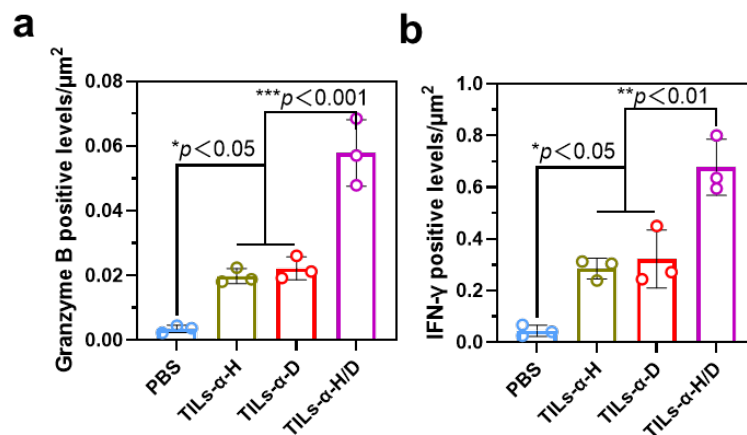
Supplementary Figure 9 Quantification of fluorescence intensity from Fig. 3e, mean $\pm$ SD, $n = 5$ independent cell samples per group. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, *** $p < 0.001$.



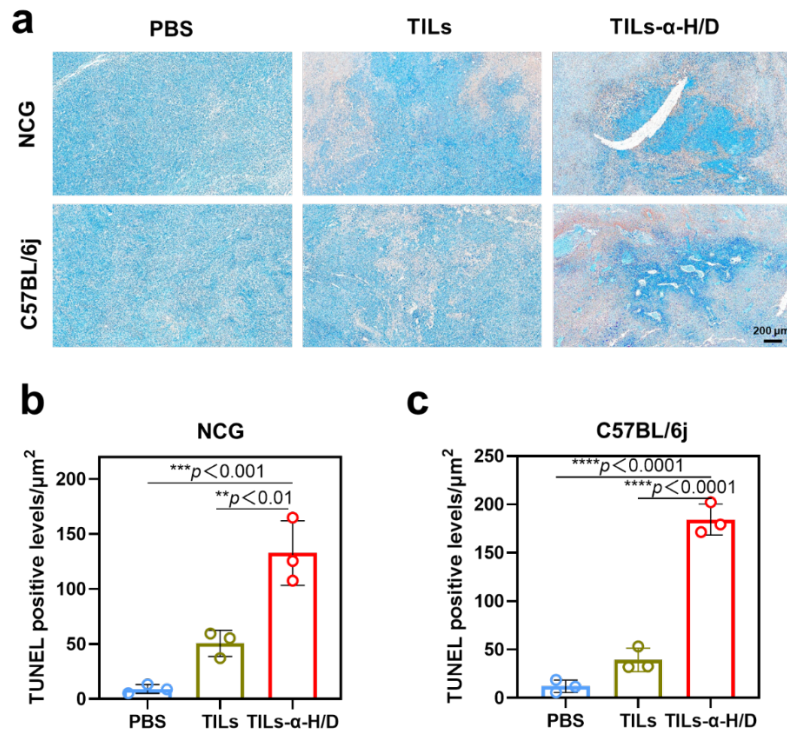
Supplementary Figure 14 Quantification of TIL/DI⁺ and CD8⁺ cells in tumor sections (including NOD-Prkdc^{em26Cd52}Il2rg^{em26Cd22}/Nju (NCG) and C57BL/6j mice) based on immunofluorescence results from Fig. 5m, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



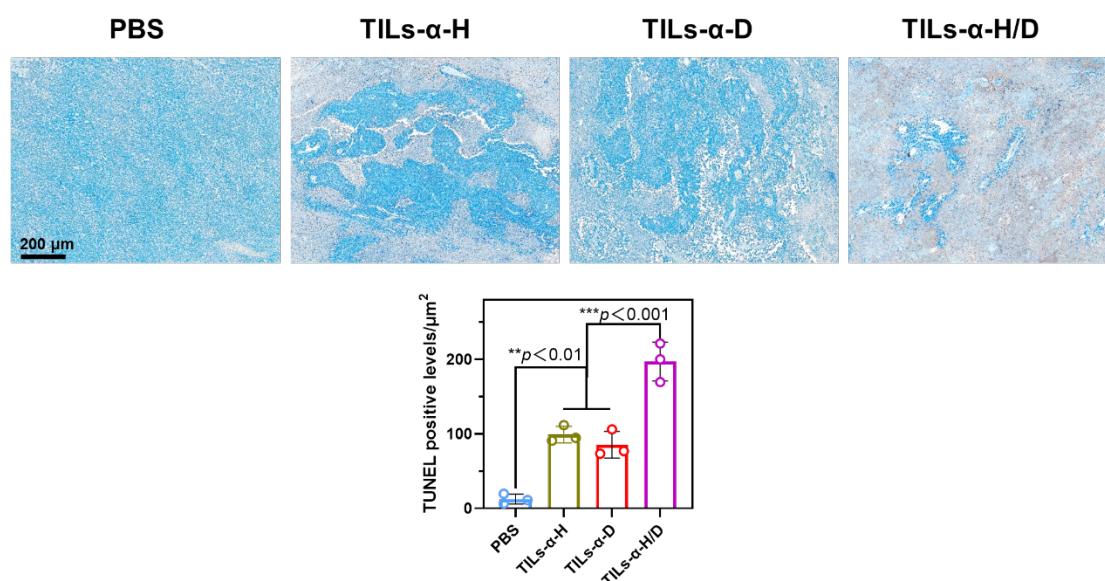
Supplementary Figure 17 (a) Quantification of hyaluronic acid (HA) expression in orthotopic tumor sections from C57BL/6j mice based on immunofluorescence results from Fig. 6g, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, ** $p < 0.01$. (b) Quantification of CD3⁺ cells in orthotopic tumor sections from C57BL/6j mice based on immunofluorescence results from Fig. 6g, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way ANOVA test, * $p < 0.05$, *** $p < 0.001$.



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



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μ m. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μm . Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, $**p < 0.01$, $***p < 0.001$.

9. A minor suggestion: in the schematic diagrams, the nanodrugs are depicted as being almost the same size as the TILs. Given that the nanodrugs are intended to coat the TIL membrane, it would be more accurate and less misleading to depict the nanoparticles smaller relative to the TILs. Additionally, the arrangement and labeling of small panels within the large figures could be improved for clarity and logical flow.

Response: We thank the reviewer for this helpful suggestion. The relative size of the nanodrugs in all schematic diagrams has been adjusted to appear smaller compared to the TILs, better reflecting their membrane-coating role. Additionally, we have revised the arrangement and labeling of the small panels within the composite figures as much as possible to improve clarity and logical flow.

Response to Reviewer #2 (pancreatic cancer therapy/biology):

The manuscript by Ziqi Gan and collaborators describes the creation of specific nanoparticles which, when combined to tumor infiltrating lymphocytes (TILs) greatly enhanced their antitumor activity. This work is of good quality, complete, nicely organized and written, with most data shown supporting conclusions by authors. However, I have few main concerns that should be addressed by authors.

Response: We thank the reviewer for the positive evaluation of our work and for recognizing the quality, completeness, and organization of our study. We truly appreciate your thoughtful comments and constructive suggestions, which have been highly valuable in helping us further improve the manuscript.

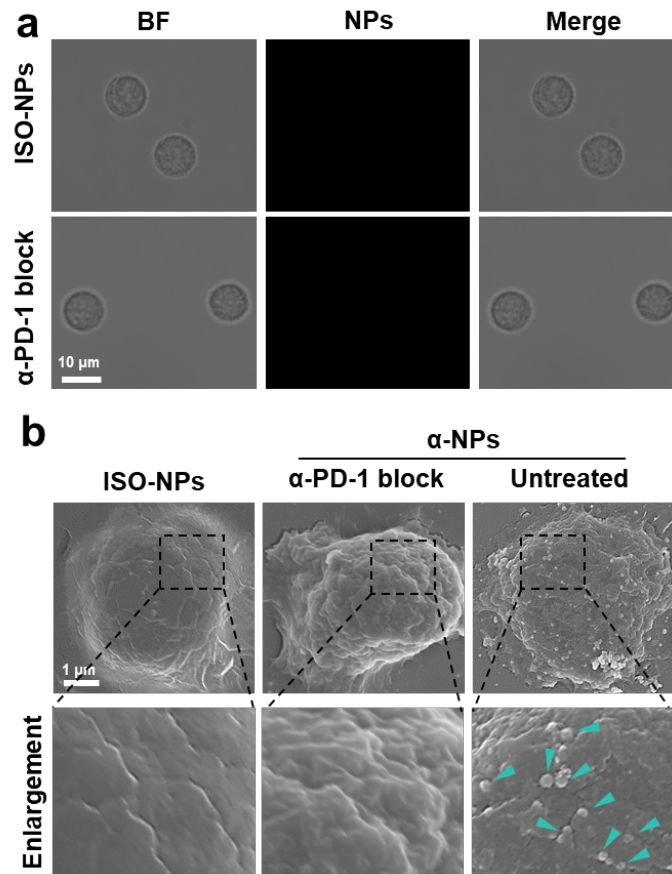
Main concerns:

- I do not understand why/how these anti-PD1 conjugated nanoparticles are not internalized by TILs? This is very strange, especially considering the specific engagement of the antibody with the receptor PD1. This raises the question of whether the membrane binding is truly specific and mediated by PD1 recognition by the antibody, or simply a non-specific coating of the cells via adsorptive binding. Are the nanoparticles non conjugated to anti-PD1 antibody still able to bind TILs' membranes? I couldn't find such control in figures, especially in figure 3 in which authors shows the coating of TILs with alpha-H/D nanoparticles. Authors have to show this control and explain the absence of internalization of the nanoparticles.

Response: Thank you for your valuable comments. As suggested, we conducted additional validation experiments to confirm that membrane binding is indeed specific and mediated by PD-1 recognition *via* the anti-PD-1 antibody (**Supplementary Figure 7**). Specifically, we first prepared nanoparticles surface-modified with an isotype control antibody (ISO-NPs). Confocal laser scanning microscope (CLSM) and scanning electron microscope (SEM) imaging demonstrated that ISO-NPs failed to bind to tumor infiltrating lymphocytes (TILs). Next, TILs were pretreated with free anti-PD-1 antibodies to block the PD-1 receptors before incubation with α -NPs, which also showed no detectable binding. In contrast, direct incubation of α -NPs with untreated TILs resulted in clear nanoparticle attachment to the TIL membrane, as evidenced by

SEM (Indicated by green arrows). These results collectively demonstrate that nanoparticle loading onto TILs is specifically mediated by PD-1/anti-PD-1 interactions, rather than by nonspecific adsorption onto the cell surface.

Regarding your question about why the anti-PD-1-conjugated nanoparticles are not internalized by TILs, we believe several factors may account for this observation: First, T lymphocytes generally exhibit limited capacity to internalize nanoparticle-sized materials compared to professional phagocytic cells such as macrophages or dendritic cells. Due to their non-phagocytic nature and inherently low endocytic activity, nanoparticles bound to their surface typically remain membrane-associated rather than being internalized (*ACS Nano*, 2021, 15(3): 3736–3753). Second, after PD-1 antibody conjugation, our nanoparticles exhibit a negative surface charge (-16.7 ± 1.4 mV). It is well established that negatively charged nanoparticles have reduced internalization efficiency due to electrostatic repulsion from the negatively charged cell membrane, which further reduces cellular uptake (*Int. J. Pharm.*, 2024, 666:124799). Furthermore, our SEM results (**Supplementary Figure 7b**) clearly show α -NPs retained on the TIL membrane. This observation is consistent with our previous findings (*Sci. Adv.*, 2020, 6: eaay7785) and other studies (*ACS Nano*, 2019, 13(2): 1469–1478), which reported that anti-PD-1-modified nanoparticles remain membrane-bound after *in vivo* administration and can be transported into tumors *via* T cell-mediated hitchhiking. We hope this explanation satisfactorily addresses your concerns.



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.

- How can these findings be applied in a clinical context? Most PDAC patients (80-85%) are not treated surgically, and only undergo biopsy. How can we isolate enough TILs under these conditions? Moreover, in their experimental setting, the isolated TILs were obtained from tumors one week old, which is too short to achieve exhaustion compared with TILs isolated from patients whose tumors are several years old. These limitations should be mentioned in the discussion section.

Response: Thank you for these insightful comments. We have now added a dedicated paragraph in the *Discussion* section to underscore these important limitations. These limitations have been mentioned in the discussion section as follows: “Despite these encouraging results, two limitations of our study should be clarified here. First, while

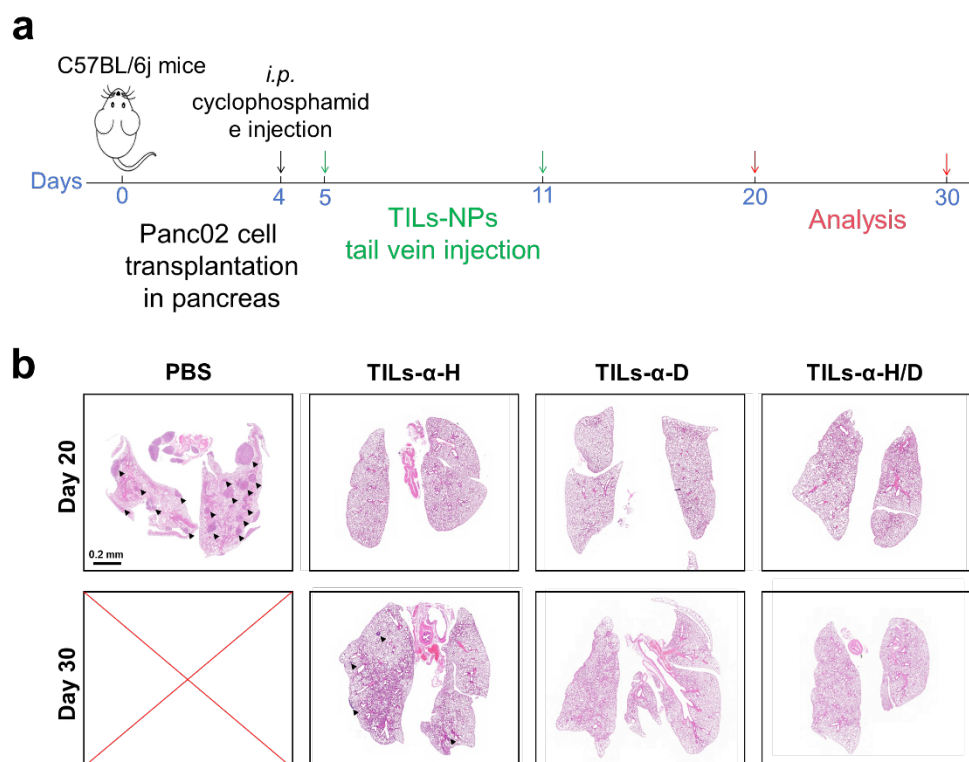
approximately 15-20% of PDAC patients undergo surgical resection, the majority only receive diagnostic biopsies^{48, 49}. Ensuring sufficient TIL yield from limited biopsy samples remains a potential constraint. Encouragingly, emerging biopsy-derived TIL (bTIL) protocols have demonstrated recovery of $> 5 \times 10^6$ functional TILs from core-needle biopsies in melanoma, with comparable phenotypic and functional profiles to those obtained from surgical resections⁵⁰. Some of these approaches are already being evaluated in clinical trials (e.g., NCT05470283). Extending and optimizing these protocols for PDAC may expand patient eligibility. Second, the TILs used here were derived from a rapidly established murine tumor model, which may not fully reflect the exhaustion states of TILs in long-standing human PDAC. While T-cell dysfunction programs often initiate within the first 1-2 weeks of tumorigenesis⁵¹⁻⁵³, human TILs experience more chronic and multifactorial exhaustion. Future studies should therefore validate this strategy in longer-established murine models or patient-derived xenografts to strengthen clinical translatability.”

- Effect of hyaluronidase action and promotion of metastasis. It is known that targeting the extracellular matrix to make it less dense is a double-edge sword. Authors showed the efficacy of their nanodrug-carrying TILs in eliminating metastases in an artificial metastases model, injecting tumor cells in the tail vein. Hence, even if these TILs could favor the production of metastases, they are expected to eradicate them. However, in the longer term, the hyaluronidase action of these TILs may favor metastatic occurrence. Authors should look longer times (much longer than 7 days) after last TILs injection and in a spontaneous metastatic model, that their treatment do not favor metastasis. At least, this limitation should be mentioned in the discussion section.

Response: Thank you for this insightful comment. As you mentioned, targeting the extracellular matrix (ECM) to reduce its density can indeed be a double-edged sword, as ECM degradation has been reported to potentially facilitate tumor invasion and metastasis by weakening structural barriers and promoting tumor cell migration (*Sci Transl Med.* 2024;16(728):eadg3840). However, PDAC features a highly desmoplastic microenvironment (~70% stromal content), which is recognized as an independent negative prognostic factor and a major contributor to immune evasion (*J Clin Oncol.*

2022;40(24):2789-2805). Within this stroma, hyaluronic acid (HA) is a key determinant of the physical barriers and degrading it is considered a rational approach to improving immune access. Indeed, preclinical studies combining PEGylated hyaluronidase (PEGPH20) with gemcitabine showed significantly reduced metastatic burden in PDAC mouse models, including metastases to liver, lungs, diaphragm, and mesenteric lymph nodes in mice model (*Cancer cell.* 2012;21(3):418-429.). Moreover, early-phase clinical data demonstrated improved response rates and progression-free survival in stage IV metastatic PDAC patients with HA-degrading strategies (*Clin Cancer Res.* 2016;22(12):2848–2854).

In light of your concern and prior reports regarding the potential pro-metastatic effect of HAase, we appreciate your suggestion to evaluate the long-term impact of TILs carrying HAase nanodrugs in a spontaneous metastasis model. To address this, we conducted additional *in vivo* experiments using an orthotopic pancreatic tumor model. As shown in **Supplementary Figure 22**, spontaneous lung metastases (indicated by black triangles) were observed in the PBS control group by day 20, whereas no metastases were detected in the TILs- α -H and TILs- α -H/D treatment groups, suggesting that HAase-loaded TILs did not promote metastasis at this time point. To further assess the long-term risk of metastasis, we extended the observation period to day 30 and performed histological analysis of lung tissues. Small metastatic lesions were observed in the TILs- α -H group, indicating that HAase-loaded TILs alone may not fully suppress metastatic progression. However, this does not necessarily imply a metastasis-promoting effect of TILs- α -H. Importantly, in the TILs- α -H/D group, no metastatic lesions were observed even at day 30, demonstrating the superior efficacy of our combinatorial nanodrug delivery system—comprising TILs, anti-PD-1 antibody, HAase, and decitabine (DEC) —in preventing spontaneous metastasis. These results are consistent with our findings from the artificial metastasis model presented in the original version (Fig. 7e). The new experimental data have been added to the revised manuscript to support these conclusions.



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.

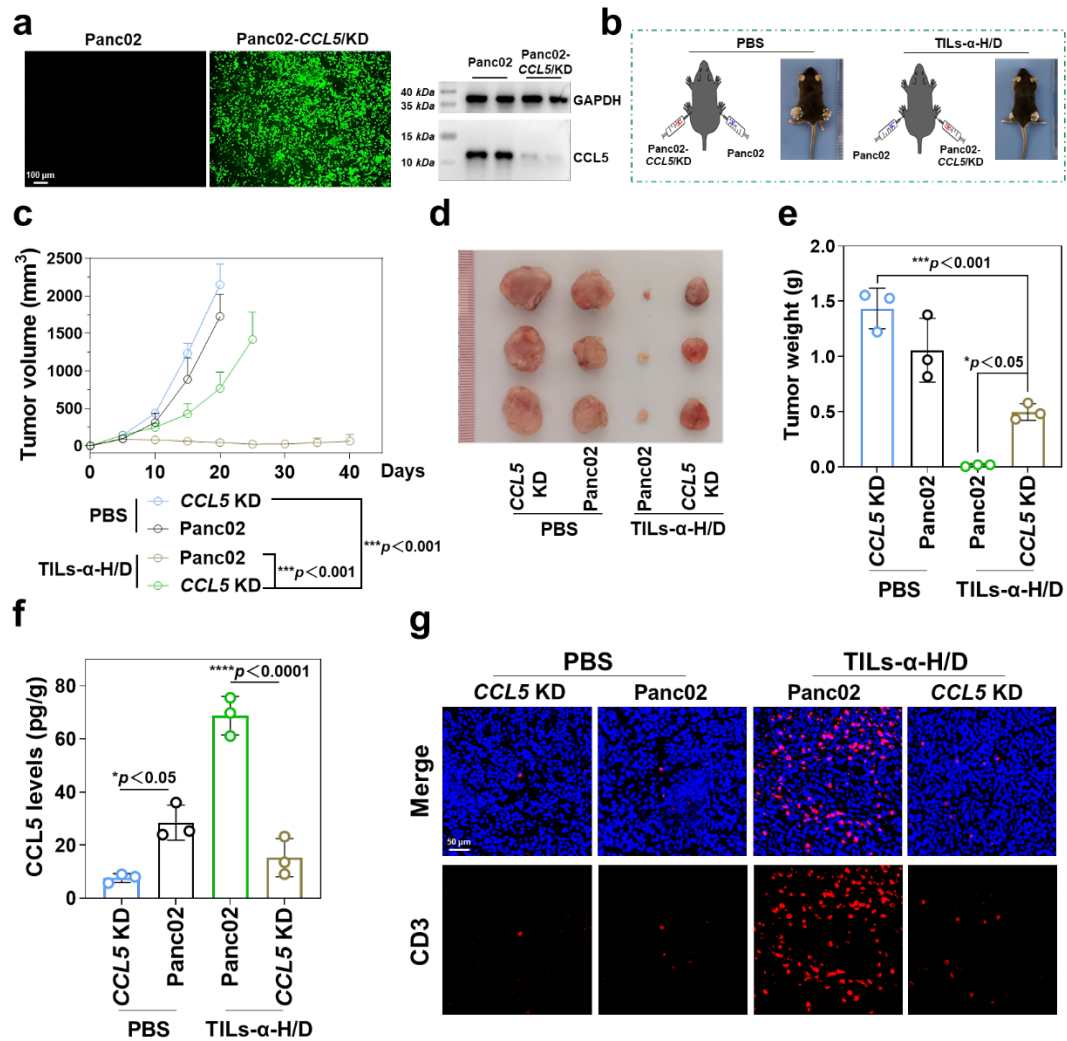
- CCL5 may not be the only Decitabine-induced protein involved in recruitment of CD3⁺ T cells. The KO or KD of CCL5 is required to demonstrate its real and unique implication in this recruitment.

Response: Thank you for this valuable suggestion. Indeed, CCL5 may not be the only decitabine-induced protein involved in CD3⁺ T cell recruitment, as we did not evaluate the effect of decitabine on the expression of other T cell chemoattractants. In our study, we focused on CCL5 based on prior reports highlighting its role in promoting CD3⁺ T cell infiltration and its upregulation by decitabine in tumors (*Cancer Cell*, 2019,35(6):885–900.e10). Therefore, we selected decitabine-induced CCL5 expression as a representative mechanism for mediating T cell recruitment into the tumor.

Meanwhile, as per your suggestion, to directly investigate the specific role of CCL5 in this process, we established a stable *CCL5* knockdown Panc02 cell line by

transducing cells with lentiviral vectors encoding *CCL5*-targeting short hairpin RNA (shRNA) and green fluorescent protein (GFP). As shown in **Supplementary Figure 18a**, GFP fluorescence was clearly observed in Panc02/*CCL5* KD cells, and western blot analysis confirmed a significant reduction in *CCL5* expression, indicating the successful construction of the *CCL5* knockdown cell line. Next, we subcutaneously inoculated Panc02 and Panc02/*CCL5* KD cells into the left and right hindlimbs of the same C57BL/6j mouse, respectively, followed by treatment with PBS or TILs- α -H/D (**Supplementary Figure 18b**). As shown in **Supplementary Figure 18c–e**, tumors derived from *CCL5* knockdown cells grew significantly faster than those from Panc02 cells after TILs- α -H/D treatment. ELISA further confirmed a significant decrease in intratumoral *CCL5* levels in the knockdown tumors (**Supplementary Figure 18f**). To assess T cell infiltration, we further performed immunofluorescence staining of CD3⁺ T cells within the tumors. As shown in **Supplementary Figure 18g**, tumors derived from Panc02/*CCL5* KD cells exhibited significantly fewer CD3⁺ T cells compared to the Panc02 group when treated with TILs- α -H/D.

Combined with our original findings (Fig. 4i), which showed that treatment with TILs combined anti-PD-1 antibody and HAase (TILs- α -H group) did not significantly induce *CCL5* expression, these results suggest that *CCL5* upregulation in the TILs- α -H/D group is primarily attributed to decitabine. And this *CCL5* upregulation contributes to enhanced CD3⁺ T cell infiltration. When *CCL5* was knocked down in tumor cells, decitabine-carrying TILs failed to elevate intratumoral *CCL5* levels, and T cell infiltration was markedly reduced. These results collectively demonstrate that, within the context of this study, *CCL5* is a critical decitabine-induced protein responsible for recruiting CD3⁺ T cells. The relevant results have been included in the revised version.



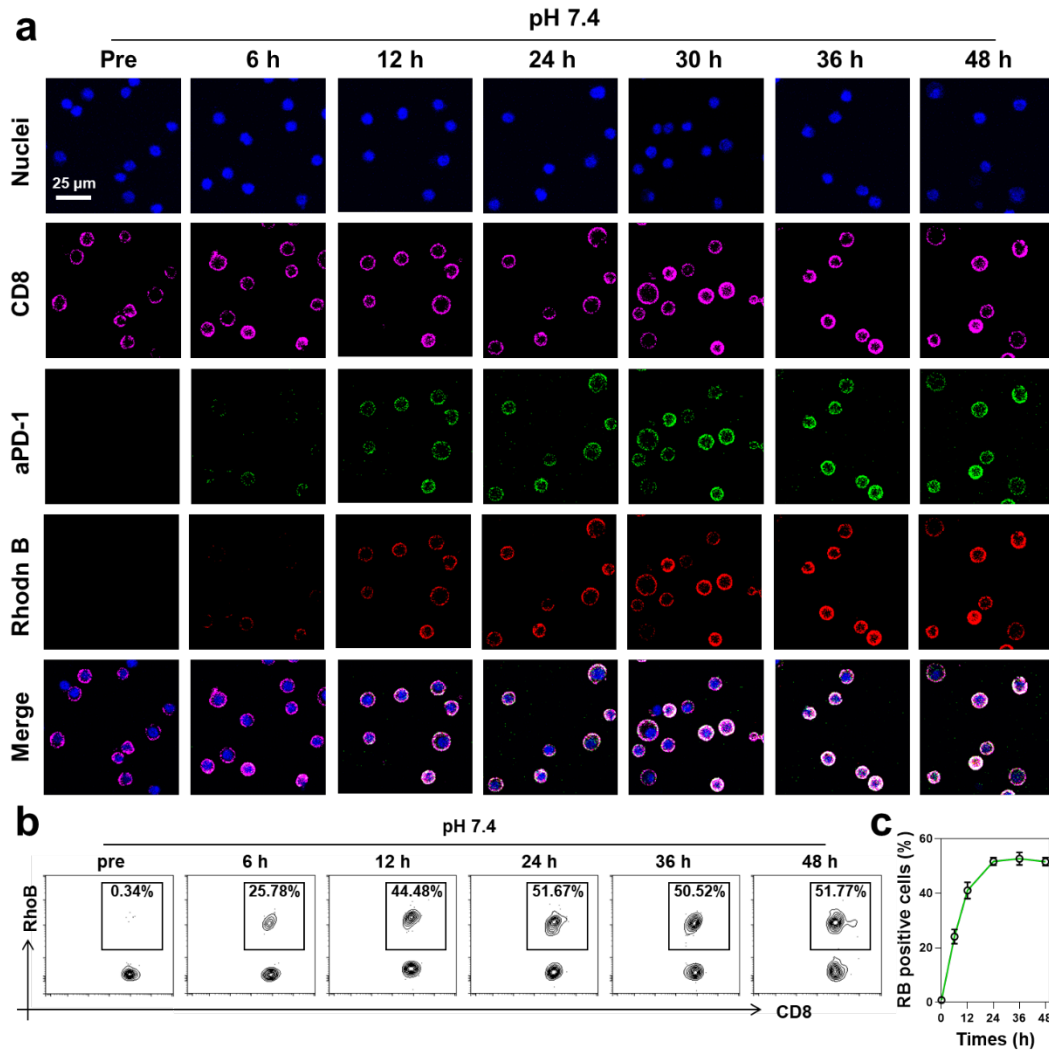
Supplementary Figure 18 (a) Panc02 cells were transfected with lentiviral recombinant vectors encoding C–C motif chemokine ligand 5 (*CCL5*) shRNA and green fluorescent protein (GFP) to establish stable *CCL5* knockdown cell lines. Green fluorescence indicates GFP expression, scale bar: 100 μ m. (b) 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, mean $\pm$ SD, $n = 5$ mice per group. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, $***p < 0.001$. (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, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way ANOVA test, $*p < 0.05$, $***p < 0.001$. (f) Quantification of intratumoral CCL5 levels after different treatments, determined using an enzyme-linked immunosorbent assay (ELISA) kit, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way ANOVA test, $*p < 0.05$, $****p < 0.0001$. (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.

- Fig. 3 e,f,g, a control with continuous pH 7.4 in place of 6.5 is missing. The fluorescence could be lost without the pH switch from 7.4 to 6.5.

Response: Thank you for your suggestion. As you pointed out, the original manuscript did not provide sufficient evidence to distinguish whether the observed decrease in red fluorescence was specifically caused by the pH switch from 7.4 to 6.5, or by other factors such as spontaneous fluorescence quenching over time. In response to your comment, we conducted additional experiments to monitor fluorescence changes under a continuous pH~7.4 condition. As shown in **Supplementary Figure 8**, both green fluorescence (labeling anti-PD-1 antibody) and red fluorescence (labeling the nanoparticle) progressively increased during the 24 h incubation at pH~7.4, consistent with the trend observed in Fig. 3e. Notably, after an additional 24 h incubation under the same pH~7.4 condition, the red fluorescence on the cell membrane showed no appreciable decline.

Moreover, flow cytometry results further demonstrated a gradual increase in the percentage of RhoB-positive CD8⁺ TILs at pH 7.4. When incubation was extended for 24 h under pH~7.4, there was no significant decrease in the proportion of RhoB-positive CD8⁺ T cells. These results confirm that the loss of red fluorescence observed under the pH-switch condition is indeed attributable to the change from pH~7.4 to ~6.5. The corresponding data and descriptions have been added to the revised manuscript.

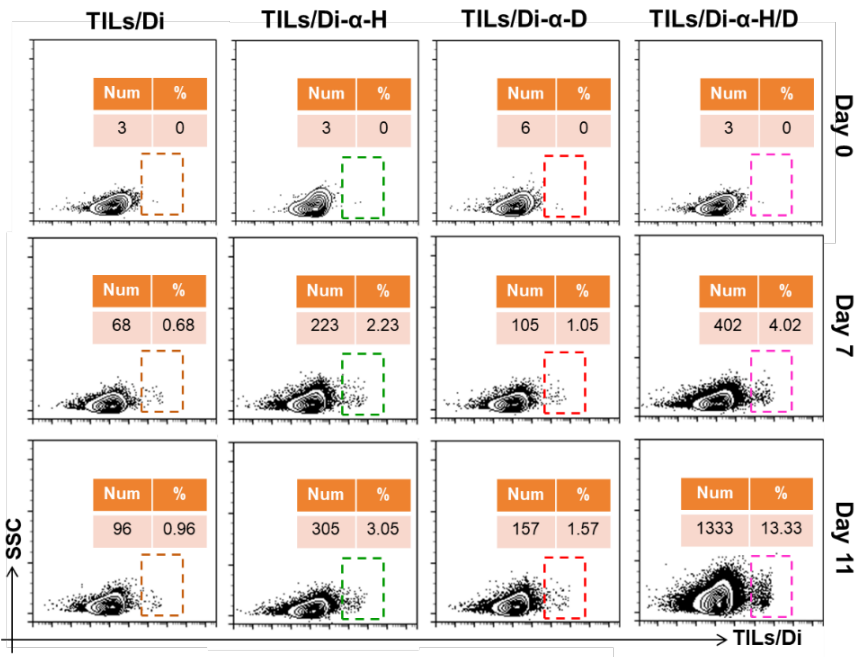


Supplementary Figure 8 (a) Confocal laser scanning microscopy (CLSM) images showing the binding of α -rhodamine B (RhoB) nanoparticles to TILs at pH 7.4. T cell marker CD8 was labeled with Alexa Fluor[®] 647 (purple fluorescence), α PD-1 was labeled with Alexa Fluor[®] 488 (green fluorescence), and the 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, mean $\pm$ SD, $n = 3$ independent cell samples per group.

-Fig. S8a, Day 0 control is missing (not so important but...). More important, when observing more Di⁺ cells it seems that the total number of cells studied by flow cytometry was also much more important. Values should be expressed as percentage or,

the total number of studied cells should be the same between all conditions.

Response: Thank you for your suggestion, we have included the day 0 control data in the revised version (**Supplementary Figure 11**). Meanwhile, we agree that normalizing the data is important for accurate comparison and it is more meaningful to use the number of injected cells (1×10^4) as the reference total. By calculating the ratio of intratumoral Di⁺ cells to the number of injected cells, we can more accurately reflect the proportion of cells that successfully infiltrated into the tumor. In the original version, the percentage quantification related to Fig. S8a was already provided in Fig. 4h. To improve clarity and ensure direct correspondence between figures, we have now added both the absolute number (Num) of infiltrated cells and their percentage (%) relative to the injected cell population in the revised manuscript.



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

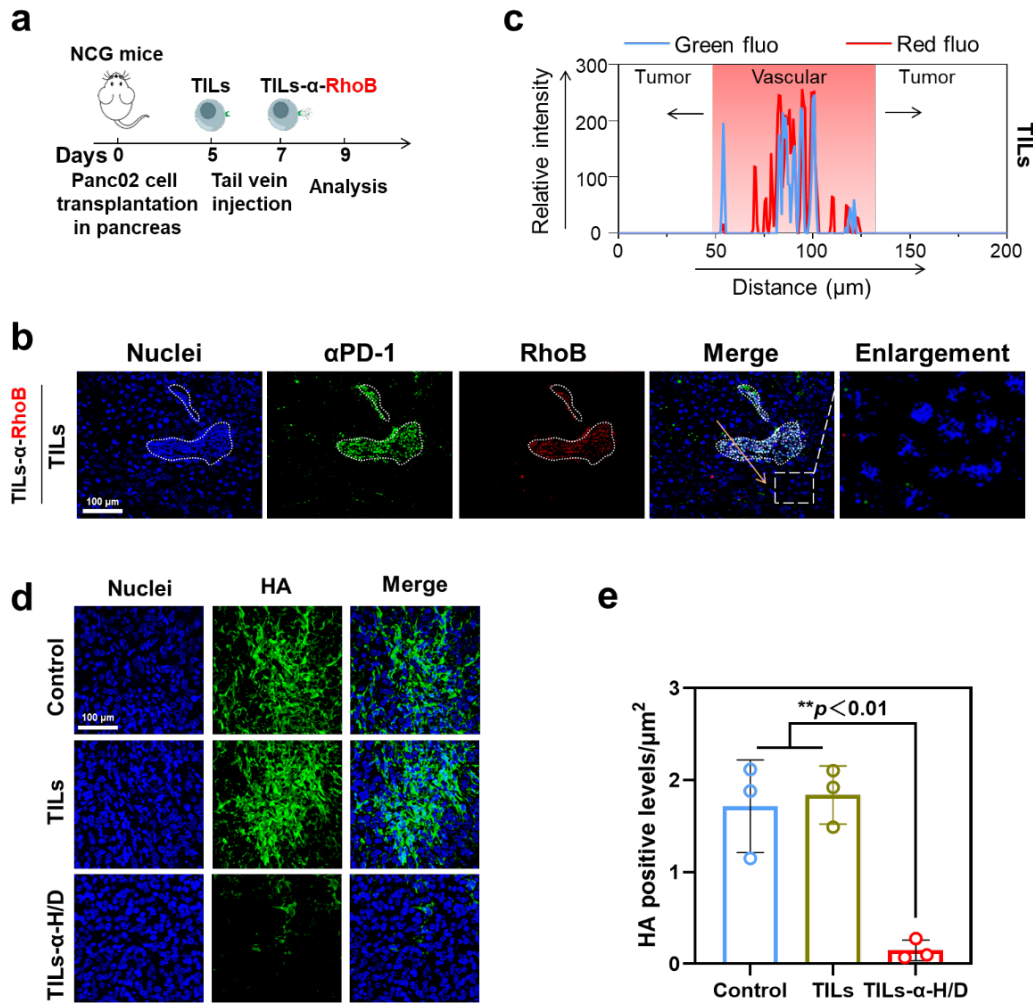
- Results section, Fig. 4l, "extracellular" should be replace by "extravascular" I guess. Regarding this experiment shown in Fig. 4K-l, in control, mice should have been injected at day 5 with TILs to serve as control for TILS-α-H/D injected mice. Also, is

there any evidence that hyaluronic acid has been degraded in TILs- α -H/D injected mice compared to control mice? The probable disintegration or cleavage of CDM bonds within the tumor microenvironment should be demonstrated. At least, acidic intratumoral pH should be confirmed.

Response: Thank you for your valuable comments. As you pointed, the term "extracellular" in Fig. 4l has been corrected to "extravascular" in the revised manuscript. Regarding the experiment shown in Fig. 4k–l, we agree that a proper control group is essential. Therefore, we have included an additional group in which mice received TILs (TILs group) *via* intravenous injection on day 5 to serve as a control for TILs- α -H/D treatment (**Supplementary Figure 12a**). As shown in **Supplementary Figure 12b, c**, similar to the PBS control group (Fig. 4l, m), strong colocalization of green and red fluorescence was observed within the vasculature immediately following TILs- α -RhoB administration. However, minimal fluorescence was detected outside the vessels, likely due to the dense extracellular matrix (ECM) in pancreatic tumors forming a physical barrier that hinders the extravasation of nanodrug-carrying TILs.

To confirm hyaluronic acid (HA) degradation following TILs- α -H/D treatment, we performed immunofluorescence staining to assess HA levels within tumor tissues. As shown in **Supplementary Figure 12d**, HA expression was reduced in the TILs- α -H/D group compared to both the PBS and TILs groups, demonstrating that intratumoral HA was effectively degraded in TILs- α -H/D–treated mice. These additional data and the corresponding descriptions have been incorporated into the revised manuscript.

In response to your concern regarding cleavage of CDM linkers in the tumor microenvironment, we have previously demonstrated the pH-responsive behavior of CDM linkages under acidic conditions (see Figs. 2 and 3). As you suggested, we further validated the acidic nature of the tumor microenvironment. Specifically, we adopted a method described in *Advanced Materials* (*Adv Mater.* 2023;35(21): e2300216) and used the pH-sensitive fluorescent probe (2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester, BCECF-AM) to measure intratumoral pH. As shown in **Fig. R1**, the results confirmed that the tumor tissues in our model exhibit an acidic pH environment, thereby supporting the likelihood of CDM bond cleavage *in vivo*.



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, mean $\pm$ SD, $n = 3$ mice per group. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, $**p < 0.01$.

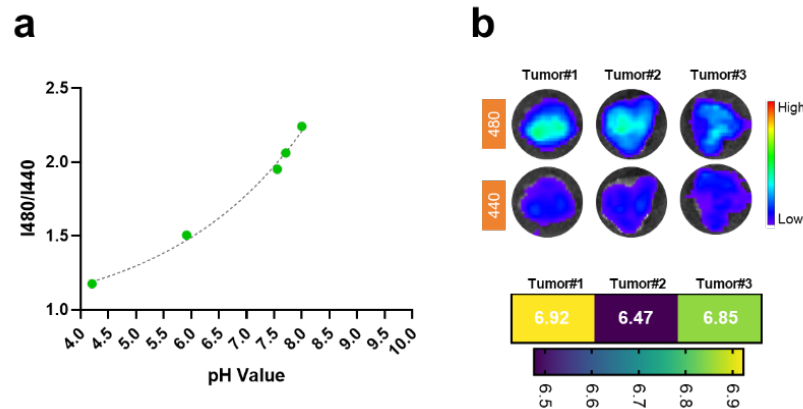
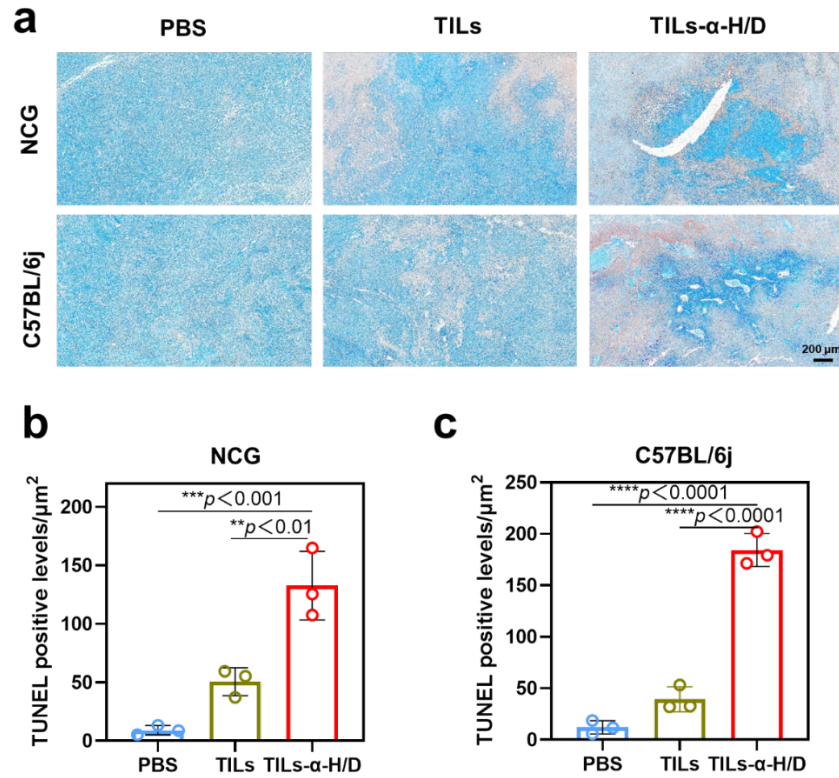


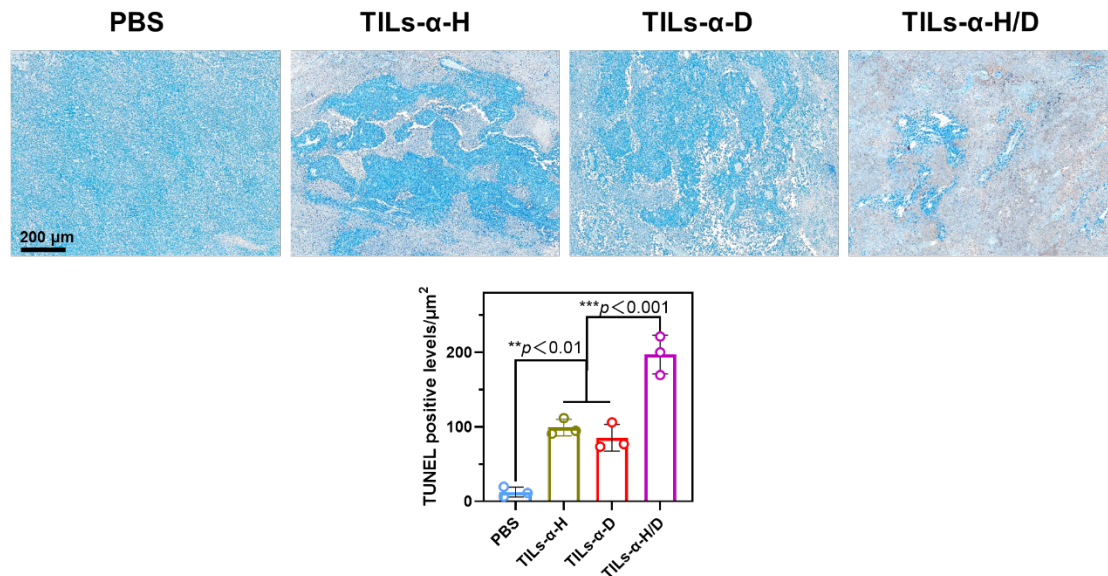
Fig.R1 (a) Fluorescence spectra of BCECF-AM at different pH values. (b) Multispectral fluorescence images of intratumor pH and the corresponding calculated pH value.

- Fig. 5l and Fig. 6f need of a specific marker of apoptosis to confirm a difference in apoptosis since H&E staining cannot discriminate between the different forms of cell death.

Response: As suggested, we performed Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on adjacent tumor sections taken from the same tumors and anatomical regions as those used for hematoxylin and eosin (H&E) staining (Fig. 5l and 6f). This approach allowed for precise identification and quantification of apoptosis in anatomically matched regions across treatment groups. As shown in **Supplementary Figure 13 and 16**, TUNEL staining and its quantification revealed that TIL- α -H/D treatment significantly promoted tumor cell apoptosis in both the subcutaneous tumor models (NCG and C57BL/6j mice) and the orthotopic tumor model (C57BL/6j mice), compared to the other treatment groups. These quantitative results are consistent with the representative staining images provided in the manuscript. These additional data and the corresponding descriptions have been incorporated into the revised manuscript.



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μm . Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μ m. Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, ** $p < 0.01$, *** $p < 0.001$.

- In the results section of Fig 5m, contrary to the authors' assertion, there was no overlap of green and red signals in NCG mice treated with TILs-a-H/D, indicating that most CD8 T cells were endogenous and not exogenous TILs. In C57BL/6j mice, it seems that there is an increased number of exogenous TILs so, consequently, also an increased number of CD8⁺ exogenous TILs in addition the endogenous CD8⁺ TILs. Fig. 5o, in the t-SNE analysis, the red dots indicate endogenous TILs since they are Di-&CD8⁺, contrary to what is indicated in the text. In fact, this whole paragraph should be carefully checked and rewritten in a clearer manner.

Response: Thank you for your careful review and for pointing out the inconsistency in our interpretation. We agree that the original description was unclear and may have led to confusion regarding the identification of exogenous versus endogenous CD8⁺ T cells, particularly in Fig. 5m and Fig. 5o. We have carefully checked and rewritten the corresponding paragraph in the revised manuscript to more accurately reflect the observations.

The revised text now reads as follows: “As shown in **Fig. 5m** and Supplementary **Fig. 14**, a small number of CD8⁺ T cells (identified by green fluorescence only, representing endogenous T cells) were detected in PBS-treated C57BL/6j mice, suggesting partial immune recovery from cyclophosphamide-induced immunosuppression. Both NCG and C57BL/6j mice treated with TILs- α -H/D showed substantial infiltration of Di-labeled TILs (red fluorescence), which was greater than that observed in the TILs group. Notably, in NCG mice, most CD8⁺ T cells were Di⁺, indicating that they were primarily infused TILs rather than endogenous T cells. In contrast, C57BL/6j mice exhibited both a large number of Di⁺ TILs and a substantial population of CD8⁺ T cells labeled solely by green fluorescence, indicating successful recruitment of endogenous CD8⁺ T cells following immunosuppression recovery. These findings were further supported by flow cytometry analysis (Supplementary **Fig. 15** and **Fig. 5n**). In the C57BL/6j mice receiving TILs/Di- α -H/D treatment, approximately 15% of the CD45⁺ cells were exogenous Di⁺ TILs, and 20% were Di⁻CD8⁺ endogenous T cells (Supplementary **Fig. 15**). Quantitative flow cytometry revealed similar proportions of Di⁺CD45⁺ cells in both NCG and C57BL/6j tumors, but Di⁻CD8⁺ T cells reached 10% only in C57BL/6j mice (**Fig. 5n**). t-SNE analysis further revealed extensive infiltration of infused TILs (orange and green dots) in tumor tissues from both mouse strains, whereas a notable population of endogenous CD8⁺ T cells (red dots) was only observed in C57BL/6j mice (**Fig. 5o**). These results indicate that nanodrug-loaded TILs not only deliver infused TILs effectively but also potentiate endogenous T-cell responses following immune recovery.”

Minor points:

- In Fig. 4d "Tumor" should be written in white since it is not so visible in red. I missed it at first observation...

Response: Thank you for your suggestion. We have modified the label color to white in the revised manuscript to improve visibility.

- In legend of Figure 4, (m) shows the fluorescence intensity profiles from line selected in "l" not "i".

Response: Thank you for your careful observation. We have corrected the figure legend accordingly in the revised version.

- Result section, fig. 7b, I think the authors wanted to write "TILs-a-H" and "TILs-a-D" instead of two times "TILs-a-H/D".

Response: Thank you for pointing this out. We have revised the text to correctly reflect "TILs- α -H" and "TILs- α -D" as intended.

- Fig. 5k and not 5i in discussion.

Response: Thank you for your attention to detail. We have corrected the figure reference from 5i to 5k in the revised manuscript.

Response to Reviewer #3 (PDAC, CAFs):

In this manuscript, the authors use nanoengineered tumour-infiltrating lymphocyte (TIL) therapy using aPD-1-conjugated acid-cleavable ZIF-8 nanoparticles in pancreatic cancer models. They show that using TILs, enhances antitumor efficacy by releasing hyaluronidase and decitabine in acidic tumor microenvironments promotes stroma degradation and CCL5 secretion.

This topic is clinically relevant since available treatment options is severely lacking for pancreatic cancer and TIL therapy has now progressed into clinical trials in other cancer types. The authors used various methods and took the time to validate their constructs.

Response: We sincerely appreciate your thoughtful and supportive comments regarding our manuscript. It is encouraging that you find the topic clinically relevant, especially in the context of the limited treatment options currently available for pancreatic cancer. As you pointed out, TIL therapy is indeed an emerging and promising strategy that has shown encouraging results in clinical trials for other cancer types, and we are hopeful that our work can contribute to expanding its potential applications to pancreatic cancer.

General comments

Most subcutaneous, metastatic and orthotopic model with panc02 cells have a treatment start on day 5. Most likely, the tumours and metastasis are barely growing at that time. It would be good to show if you have tumour/metastasis established on day 5 or do an experiment when you start the treatment on later days when the tumours/metastasis are established. The in vivo results do not determent if there are cytotoxic or cytostatic treatment effect and if this treatment is effective in already established tumours. Additionally, apoptosis assays or stainings would be beneficial. Are the cancer cells really dying?

Response: Thank you for your insightful comments. We fully agree that the timing of treatment initiation and confirmation of tumor establishment are crucial for evaluating therapeutic efficacy. To address this, we conducted additional experiments to verify that tumors or metastases were indeed established by day 5 in all models. In the subcutaneous model, tumors reached approximately 100 mm³ by day 5, as shown in

Figs. 5c and 5f. In the orthotopic model, MRI presented in our original manuscript (Fig.6b) already showed tumor formation on day 5, marked by dashed lines. However, due to the small image size, the tumor boundaries may have been unclear. We have now enlarged and re-displayed this data in **Fig. R2a** for better visualization. Furthermore, in the metastatic model, we performed hematoxylin and eosin (H&E) staining on lung tissues collected five days after tail vein injection of Panc02 cells. As shown in **Fig. R2b**, metastatic nodules were clearly observed in all three mice (indicated by black arrowheads), confirming that lung metastases had formed by day 5. These results demonstrate that tumor burden was present across all models at the time of treatment initiation.

To determine whether our treatment exerts a cytotoxic effect, we further conducted terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on tumor tissues collected from each group. As presented in **Supplementary Figures 13** and **16**, the TILs- α -H/D treatment group exhibited a significant increase in TUNEL-positive cells compared to the other groups, indicating that the nanodrug-loaded TILs effectively induced apoptosis in tumor cells *in vivo*. These results provide direct evidence that the therapeutic effect observed is not merely cytostatic but involves active induction of cancer cell death. The apoptosis analysis results and corresponding descriptions have been incorporated into the revised manuscript. We hope this addresses your concerns regarding tumor establishment and treatment mechanism.

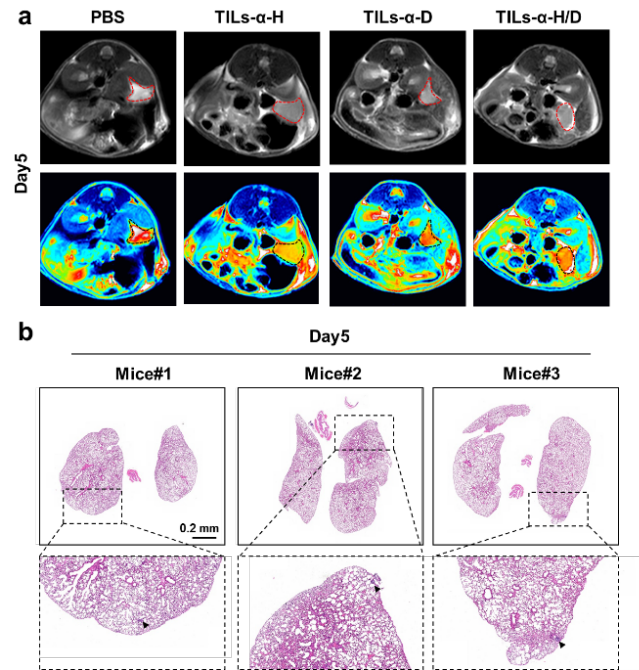
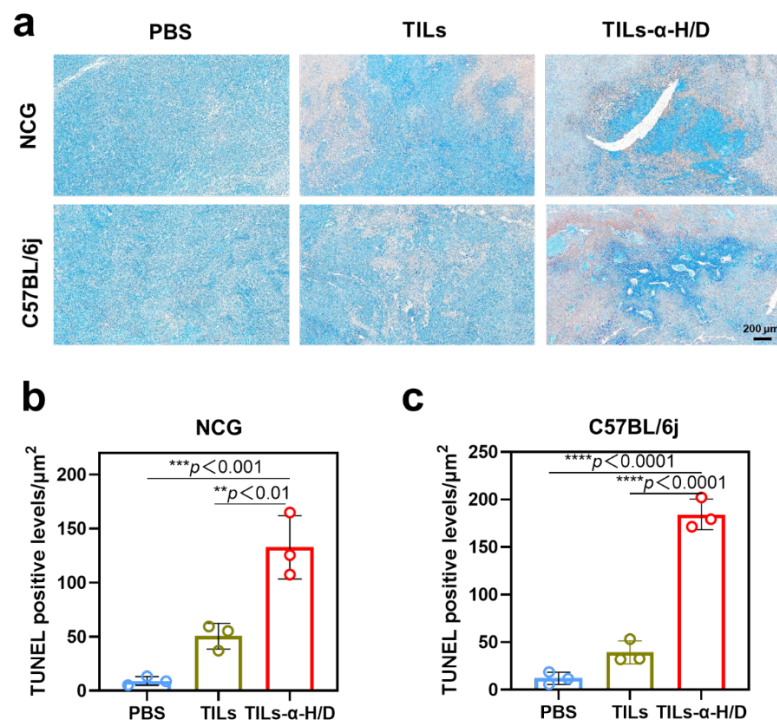
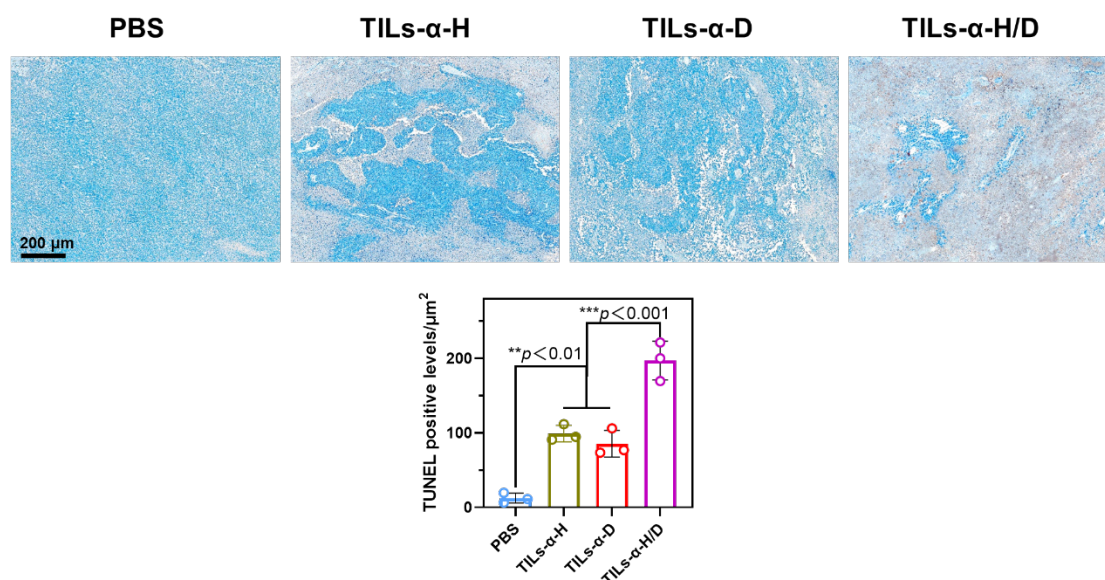


Fig. R2 (a) Orthotopic pancreatic tumor growth was monitored by magnetic resonance imaging (MRI) on day 5 in different groups. (b) Hematoxylin and eosin (H&E) staining.



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μm . Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.



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, mean $\pm$ SD, $n = 3$ mice per group, scale bar: 200 μm . Statistical significance was calculated using a one-way analysis of variance (ANOVA) test, $**p < 0.01$, $***p < 0.001$.

Grammar and proof-reading are needed in few places. *i.e.* line 61, 317, 465, 594, 610, 618, 654, 655

Response: As suggested, we have carefully revised the manuscript for grammar and style. All corrections are incorporated in the revised version and highlighted for your convenience (note: line numbers may have shifted slightly due to edits).

A lot of abbreviations missing both in main text and figure texts. *i.e.* ICB, PVP, Cys, MOF, NSAID-PEG-CEM while some are abbreviated multiple times *i.e.* DEC, aPD-1, TIL

Response: Thank you for pointing this out. We have thoroughly audited the main text, figure panels, and figure legends to standardize abbreviation usage. Specifically, we

now spell out all terms at first mention and use the abbreviation thereafter (per journal style), including ICB, PVP, Cys, MOF, and NAID–PEG–CDM. We also removed redundant, repeated definitions of DEC, α PD-1, and TIL, ensuring each is defined only at first occurrence.

The authors declare competing interests. This should be explained.

Response: This was a typographical error in the original submission. It has been corrected to: “The authors declare no competing interests.” in the revised manuscript.

Specific comments

Results

- Fig. 2l wrongly marked as i (line 133)
- Fig. 5d missing from results text.
- Fig 7c missing from results text.

Response: Thank you for your suggestions. The incorrect reference to Fig. 2l has been corrected, and the descriptions for Figs. 5d and 7c have been incorporated into the revised manuscript.

Methods

Missing from the method section:

- No mention of how cyclophosphamide was given and in what dose. No manufacturer or how it was dissolved. (line 285 and 306 and figure text 5)

Response: Thank you for pointing this out. In the revised manuscript, we have added the missing details regarding cyclophosphamide administration in the *Animal Model* section of the *Methods*. Specifically, cyclophosphamide (Selleck, S1217) was dissolved in 0.9% physiological saline and administered intraperitoneally at a dose of 200 mg kg⁻¹ one day prior to TIL treatment.

- No mention of how MRI was done. Manufacture? How were the mice anaesthetised? For what cancer model and what days was it done?

Response: Thank you for highlighting this. While MRI was briefly described in the

original submission, we have now expanded the *Methods* section (see the *In vivo antitumor activity* section) and the relevant figure legend in the revised manuscript to clearly report the manufacturer, anesthesia protocol, model, and time points. Specifically, the revised text reads: “To measure tumor growth in the orthotopic pancreatic tumor model (Panc02 in C57BL/6j mice), tumor-bearing mice were anesthetized with sodium pentobarbital (75 mg kg⁻¹) and scanned on days 5, 10, and 17 using a 3-T clinical MR scanner (Achieva, Philips Medical Systems, Best, The Netherlands) equipped with a 50-mm small animal coil (Zhongzhi Medical Technologies, Suzhou, China). T₂-weighted images were then acquired.”

- 2-MIN: what is it and where does it come from?

Response: Thank you for your comment. We believe you were referring to 2-MIM, which stands for 2-methylimidazole, the organic linker used for ZIF-8 synthesis. It was purchased from J&K Chemical Ltd. This information is already provided in our original Supplementary Information (see the *Reagents and materials* section).

The preparation of α -H/D nanoparticle/TILS and nanodrug-carrying TILs preparation/*In vitro* release:

- Information missing where all the incorporated materials come from: FITC, DEC, MOF, α PD-1, CDM

Response: Thank you for the suggestion. We have added the missing sourcing information for FITC, DEC, α PD-1, and CDM in the revised Supplementary Information, with changes highlighted. The MOF was synthesized in our laboratory, and the complete synthetic procedures are now provided in detail in the *Methods* section (see the *preparation of α -H/D nanoparticle* section) of the revised manuscript.

- At specific time intervals = state what time intervals

Response: Thank you for your comment. In the revised manuscript, we have specified the exact time intervals for sampling. The release measurements were performed at 1, 2, 4, 6, 8, 12, and 24 h, as now indicated in the *Methods* section (see the *In vitro release* section).

- Fresh buffer: what does this buffer contain?

Response: Thank you for pointing this out. In the revised manuscript, we have clarified that the “fresh buffer” refers to phosphate-buffered saline (PBS).

- Fluorospectrophotometer and chromatography info missing.

Response: Thank you for your comment. In the revised manuscript (see the *Methods*, *In vitro release* section), we have added full details of the instruments used. Specifically, fluorescence spectra were collected using a fluorospectrophotometer (F-7100, Hitachi High-Tech Co., Ltd., Tokyo, Japan) with an excitation wavelength of 495 nm and an emission wavelength of 595 nm. Decitabine (DEC) was quantified by high-performance liquid chromatography (HPLC) using an Agilent Technologies 1260 Infinity II (California, USA).

Tils and nanodrug-carrying TILS preparation/in vitro cell binding:

- Were the cells injected? In what volume? In what solution?

Response: Yes. C57BL/6j mice were subcutaneously inoculated with 1×10^6 Panc02 cells suspended in 100 μ L of sterile PBS. This information has been added to the revised manuscript in the *Methods* section (see the *Animal Model* section).

- Were the mice anesthetized? If so, how?

Response: Yes. Mice were anesthetized with sodium pentobarbital at a dose of 75 mg kg^{-1} administered intraperitoneally prior to tumor cell inoculation. This information has been added to the revised manuscript (see the *Methods*, *Animal Model* section).

- No information about collagenase IV and DNase

Response: The sources and details of collagenase IV and DNase I used for tumor digestion have now been added to the revised manuscript. Specifically, collagenase IV (Sigma-Aldrich, USA) and DNase I (Sigma-Aldrich, USA) were employed during enzymatic digestion of tumor tissue, as described in the *Methods* section (see the *TILs and nanodrug-carrying TILs preparation* section).

- Hoechst manufacture?

Response: The manufacturer information for Hoechst 33342 has been added to the revised manuscript. Specifically, Hoechst 33342 (Beyotime, China) was used for nuclear staining as described in the *Methods* section (see the *In vitro T cell binding* section).

ELISA and biochemistry index and histology analysis:

- Info missing about RIPA buffer, protease inhibitors, TNF and IL6

Response: We have added the missing sourcing details in the revised version. Specifically, the RIPA buffer and protease inhibitor cocktail were purchased from Beyotime (China) (see the *Methods, ELISA and biochemistry index* section). The vendors for all ELISA kits had already been provided in the original Supplementary Information (See the *Reagents and Materials* section); in particular, the TNF- α and IL-6 ELISA kits were obtained from Westang Biotechnology (Shanghai, China).

TILs killing assay in vitro:

- Info missing of following: Confocal dishes, Calcein-AM, CLSM, D-luciferin, Microplate reader

Response: we have added the missing sourcing and equipment details in the revised manuscript (see the *Methods, TILs killing assay in vitro* section). Specifically, confocal dishes were purchased from Nest (China); Calcein-AM was obtained from Beyotime (China); the confocal laser scanning microscope used LSM710 (Germany); D-luciferin substrate was purchased from Aladdin (China); and bioluminescence was measured using a microplate reader (TECAN, Switzerland).

- Various treatments: state what was done

Response: Thank you for the comment. In the revised manuscript, we have specified the details of the “various treatments” in the *Methods* section (see the *TILs killing assay in vitro* section). Specifically, Panc02 cells were treated with one of the following: PBS (Control), TILs at pH 7.4, TILs- α -H/D at pH 7.4, TILs at pH 6.5, or TILs- α -H/D at pH 6.5.

- Panc02 luc: How were they transfected/transduced and with what plasmid?

Response: Thank you for the comment. The Panc02-Luc cells were purchased directly from Wuhan Pricella Biotechnology Co., Ltd (Wuhang, China). According to the supplier's information, these cells are stably transduced with a lentiviral vector encoding the firefly luciferase gene.

Animal model/in vivo and ex vivo fluorescence imaging:

- Did the mice receive pain management?

Response: Yes. All mice received pain management as required by the SYSU Animal Ethics Committee, in compliance with institutional and national animal welfare guidelines. This information has been added to the revised *Methods* section (see the *Animal model* section).

- How were the mice anesthetised?

Response: Mice were anesthetized with sodium pentobarbital (75 mg kg⁻¹, intraperitoneally) before all surgical procedures. This detail is now included in the revised *Methods* section (see the *Animal model* section).

- How were the cells diluted? PBS? Matrigel?

Response: Panc02 or Panc02-Luc cells were suspended in sterile PBS for injection. This has been specified in the revised manuscript (see the *Animal model* section).

- How much liquid was injected?

Response: For the orthotopic pancreatic tumor model, 1×10^6 Panc02 cells were injected in a total volume of 10 μ L PBS. For the subcutaneous tumor model and the systemic metastasis model, 1×10^6 cells were injected in a total volume of 100 μ L PBS. This information has been added to the revised *Methods* section (see the *Animal model* section).

- How were the mice euthanized?

Response: At the experimental endpoint or for ex vivo analysis, mice were euthanized

by carbon dioxide (CO₂) inhalation followed by cervical dislocation, in compliance with the SYSU Animal Ethics Committee guidelines. This detail has been added to the revised *Methods* section (see the *Animal model* section).

- Missing MRI info, how was that data obtained?

Response: Tumor growth in the orthotopic pancreatic tumor model (Panc02 in C57BL/6j mice) was measured by MRI on days 5, 10, and 17 after tumor implantation. Mice were anesthetized with sodium pentobarbital (75 mg kg⁻¹, *i.p.*) and scanned using a 3-T clinical MR scanner (Achieva, Philips Medical Systems, Best, The Netherlands) equipped with a 50-mm small-animal coil (Zhongzhi Medical Technologies, Suzhou, China). T₂-weighted images were then acquired. These details have now been incorporated into the revised *Methods* section (see the *In vivo antitumor activity* section).

- Manufacture of in vivo fluorescence imaging system missing.

Response: The *in vivo* fluorescence imaging system used was the FX PRO (Carestream, USA). This information has been added to the revised *Methods* section (see the *In vivo and ex vivo fluorescence imaging* section).

Histological analysis

- “Section of tumor and organ tissue”: where were the tissues cut? Only in one place or in several places throughout the tissues to give more 3D analysis? What liver lobe, kidney and lung were chosen?

Response: In this study, representative sections were collected from each organ for histological analysis. For the liver, sections were obtained from the left lateral lobe; for the kidney, longitudinal sections were prepared from the left kidney; and for the lung, the largest lobe, *i.e.*, the left lung was selected. Sections were obtained at a single standardized site for each organ to maintain consistency across all samples, and each organ was sectioned at one representative site for 2D histological comparison between treatment groups instead of multiple sites for full 3D reconstruction. This information has been added to the revised manuscript (see the *Methods, Histological analysis* section).

Staining:

- Manufacturer of H&E staining kit missing

Response: The missing manufacturer information has been added. The H&E staining kit was purchased from Solarbio (China). This information is now included in the revised manuscript (see *Methods*, the *Histological analysis* section).

- No information about secondary antibodies

Response: The information regarding all antibodies has already been provided in the *Antibody* section of the original Supplementary Information. Specifically, fluorescently labeled secondary antibodies for immunofluorescence were purchased from Abcam (UK), and HRP-conjugated secondary antibodies for immunohistochemistry were purchased from Arigo Biolaboratory (China).

- DAPI manufacturer missing

Response: The DAPI used for nuclear staining was purchased from Beyotime (China). This information is now included in the revised manuscript (see the *Methods*, *Immunofluorescence staining* section).

- Confocal microscopy type missing

Response: The revised manuscript (see the *Methods*, *Immunofluorescence staining* section) specifies that confocal imaging was performed using an LSM710 confocal laser scanning microscope (Germany).

- DAB kit manufacturer missing

Response: The revised manuscript (see the *Methods*, *Immunohistochemical staining* section) now states that the DAB kit was purchased from ZSGB-BIO (China).

- Hematoxylin manufacturer missing

Response: The revised manuscript (see the *Methods*, *Immunohistochemical staining* section) now specifies that hematoxylin was purchased from Solarbio (China).

- What kind of Pathology Imaging System?

Response: The revised manuscript (see the *Methods, Immunohistochemical staining* section) specifies that tissue section images were acquired and analyzed using the Perkin-Elmer Vectra Pathology Imaging System (USA).

Flow cytometry:

- CFSE manufacturer missing

Response: The CFSE used for TIL proliferation studies was purchased from Thermo Scientific (USA). This information has been added to the revised manuscript (see the *Methods, Flow cytometric analysis* section).

- Red cell lysis solution manufacturer missing

Response: The red cell lysis solution was purchased from Solarbio (China). This information has been added to the revised manuscript (see the *Methods, Flow cytometric analysis* section).

- Abbreviation for flow cytometry is claimed but then never used: FCM

Response: The abbreviation “FCM” has been removed from the revised manuscript to avoid inconsistency.

Figure/figure legends:

All figures and text are very small and difficult to read and understand.

Response: Thank you for your valuable feedback. We apologize for the issue with the readability of the figures and text. In response to your comment, we have made the following revisions to improve clarity: We have increased the text size in the figures where possible to enhance readability. Additionally, all the figure legends have been revised to improve their clarity.

In figure 3 you have “(*** $p < 0.001$)” in the figure text but the p value explanation is missing in all the other figures.

Response: Thank you for your comment. We have now added the p-value explanations

in the figure legends of the revised manuscript. Specifically, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ are presented in Fig. 3h, while $****p < 0.0001$ and *ns* are presented in Figs. 3j and 3l.

Figure 1.

- Good schematic figure but would need more detailed figure text and all the abbreviations needs to be explained.

Response: Thank you for your recognition of the schematic figure. In response to your comment, we have added more detailed figure text and included explanations for all abbreviations in the figure legend.

- “Death tumor cells”, should probably say “dead”

Response: Thank you for your suggestion. The term "death" has been corrected to "dead" in the revised manuscript (**Fig.1**).

- Increase the size of the letters above the arrows

Response: As suggested, we have increased the size of the letters above the arrows in the revised manuscript (**Fig.1**).

- 2-MIN never explained. What is it and where does it come from?

Response: Thank you for pointing this out. Apologies for the oversight. "2-MIN" refers to 2-methylimidazole (2-MIM), a chemical compound commonly used in various chemical synthesis processes. In the revised manuscript, we have provided a detailed explanation of this compound. Specifically, 2-MIM was purchased from J&K Chemical Ltd. (Beijing, China). This information has now been included in the revised supplementary materials for further clarity.

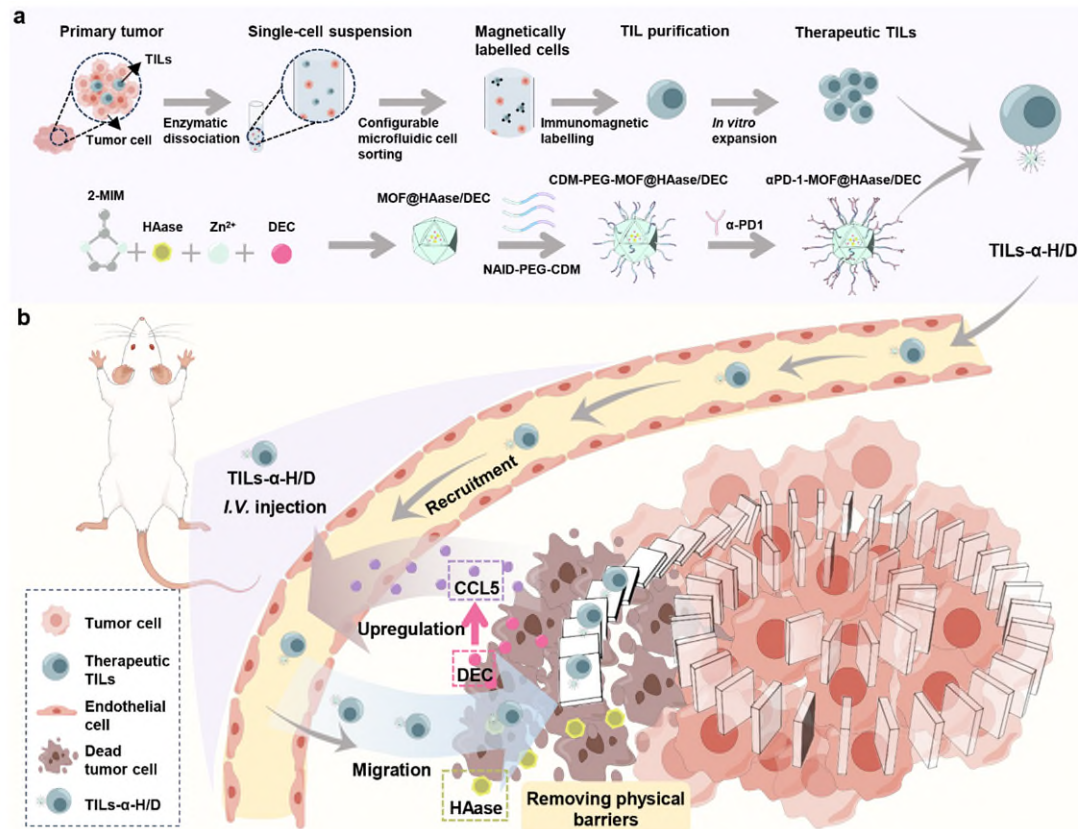


Fig. 1 Schematic illustration of the preparation of nanodrug-carrying TILs (TILs-α-H/D) and their antitumor effect. (a) The preparation process of TILs and the nanodrug α-H/D, including the synthesis of TILs-α-H/D. (b) The antitumor mechanism of TILs-α-H/D, wherein TILs utilize their natural tumor-homing properties to deliver the nanodrug into the tumor. Upon reaching the tumor microenvironment, the nanodrug releases HAase, which degrades hyaluronic acid, facilitating TILs infiltration into the tumor. Simultaneously, DEC upregulates CCL5 expression within the tumor, further enhancing the recruitment of TILs-α-H/D into the tumor. Abbreviations: TIL: Tumor-infiltrating lymphocytes; HAase: Hyaluronidase; DEC: Decitabine; NAID-PEG-CDM: 1-(3-aminopropyl)imidazole)-(polyethylene glycol)-(2,5-Dihydro-4-methyl-2,5-dioxo-3-furanpropanoic acid); α-PD-1: Anti-PD-1 antibody; MOF: Metal-organic framework; CCL5: Chemokine (C-C motif) ligand 5; α-H/D: αPD-1-MOF@HAase/DEC; TILs-α-H/D: TILs-αPD-1-MOF@HAase/DEC.

Figure 2:

- Missing figure legend for b and f.

Response: We have now added the figure legends for panels b and f in the revised manuscript (see the **Fig. 2** legend).

- b and c are wrongly named. Should be c and d.

Response: Thank you for pointing this out. We have corrected the naming convention of the figure in the revised manuscript (see the **Fig. 2** legend).

- h. “Zata” should be “Zeta”

Response: We apologize for the typographical error. The term "Zata" has been corrected to "Zeta" in panel h and in the figure legend of the revised manuscript (see the **Fig. 2** legend).

- k, l, m: look very similar. Would be clearer to have a title in each graph.

Response: We appreciate this suggestion. To make the differences between panels k, l, and m clearer, we have added individual titles to each of the graphs in the revised manuscript (see the **Fig. 2** legend).

Figure 3

- Abbreviations needed for CFSE and CLSM

Response: We have added the full abbreviations for CFSE (Carboxyfluorescein diacetate succinimidyl ester) and CLSM (Confocal Laser Scanning Microscopy) in the figure legend (see the **Fig. 3** legend).

- i. scalebar not visible

Response: We have increased the size of the scale bar and clearly indicated it in the figure legend (see the **Fig. 3** legend) as “scale bar: 200 μm .”

- k. x-axis = “TILs distribution”, not very clear. What is the difference between the dots on the left side of the graph to the once on the right?

Response: Thank you for your comment. The x-axis, labeled as "TILs distribution," represents the horizontal position of TILs within the tissue slice obtained from

immunofluorescence images in Fig. 3k, with cells positioned from the left to the right side of the image. The data points on the left side of the graph correspond to TILs located closer to the left edge of the fluorescent images, while the points on the right represent those closer to the right edge. The y-axis shows the infiltration depth of each TIL, indicating how far the cells have penetrated into the tissue. We have included this clarification in the revised manuscript (see the **Fig. 3** legend). Regarding the use of the term "TILs distribution" on the x-axis, we understand your concern and have revised it to "TILs position along the tissue slice" to more clearly reflect the concept of spatial positioning from left to right within the slice.

Figure 4

- b, n. You show the mouse on its back, and it has high expression from the right side. However, I would expect the high signal should be coming from the pancreas on the left side?

Response: Thank you for your comment. In fact, Fig.4b and 4n show the mouse's abdomen, not its back. We observed that the high signal is indeed on the left side, which is consistent with your expectation. When the mouse is in the supine position, the pancreas is located on the right side. However, our *in vivo* imaging system requires the mouse to be placed prone on the imaging platform, and the camera is positioned beneath the platform. Therefore, the strong signal observed in the images is from the left side of the mouse, indicating the tumor aggregation in the pancreatic region. Additionally, *ex vivo* images (Fig. 4d and 4p) further confirm the high expression of fluorescence signal at the pancreatic tumor site.

- m. very unclear figure. What is x-axis? What do you mean by Gray value? Also, no white lines in Figure i as stated in the figure text.

Response: We apologize for the confusion caused by a typographical error. The "white line" in the figure should actually refer to the orange arrow, and we have updated the figure legend accordingly. The x-axis represents "distance", which corresponds to the length indicated by the orange arrow in Fig. 4l. We have added this clarification in the revised manuscript. Additionally, the term "Gray value" refers to the grayscale intensity

(fluorescence intensity), and to enhance clarity, we have updated the y-axis label to “Relative intensity”.

- Are there no statistical analysis done for any of the figures? If so, the info is missing in the figure text.

Response: Thank you for your suggestion. We have performed statistical analysis for Fig. 4i, and the relevant information has been added to the figure legend (see the **Fig. 4** legend).

Figure 5

- d. No mention of cyclophosphamide. How was it given and in what dose?

Response: Thank you for your suggestion. The information regarding cyclophosphamide has been added to Fig. 5d. Additionally, as indicated in the revised manuscript (see the *Animal model* section), cyclophosphamide was administered via intraperitoneal injection at a dose of 200 mg kg⁻¹.

- k and n are missing statistics

Response: As suggested, we have added the statistical analysis for figures k and n in the revised manuscript (see **Fig. 5**). The statistics for these figures were calculated using a student's t-test.

- m. missing scalebar in the enlargement images

Response: Thank you for your feedback. We have added the scale bar in the enlarged images for **Fig. 5m**.

- g. make sure all the text is visible.

Response: Thank you for your comment. We have increased the font size to ensure that all the text is visible in **Fig. 5g**.

Figure 6

- c. would be good to have also a figure of the size of the tumour when the treatment started as a baseline.

Response: Thank you for your suggestion. In fact, the tumor size at the start of treatment is already represented in Fig. 6b (MRI), which provides the relevant information. In Fig. 6b, the tumors are outlined using dashed lines, and we can observe that by day 5, the tumor sizes across different groups are similar, which we believe serves as a suitable baseline. The purpose of Fig. 6c is to compare the effects of different treatments on tumor growth at the end of the study, after treatment has been administered. Therefore, the data in Fig. 6b sufficiently addresses the baseline tumor size.

Figure 7

- k. More info about the markers in the figure text, what is each measuring?

Response: We have added detailed functional descriptions for each serum biochemical marker in the revised figure legend for Figs. 7k-n: alanine aminotransferase (ALT) measures hepatocellular injury, total bilirubin (TBIL) reflects hepatobiliary function, urea (UREA) indicates renal filtration and metabolic status, and creatinine (CREA) assesses glomerular filtration rate.

- e. images of liver don't seem to be from the same liver lobe. What liver lobes were collected? Where all of them sectioned? Where the sections throughout to make a 3D analysis?

Response: Thank you for your comment. In this study, liver sections were obtained from the left lateral lobe of the liver for histological analysis. Only this specific lobe was sectioned to maintain consistency across all samples. The liver was not sectioned at multiple sites for 3D analysis; instead, a single standardized site was chosen for sectioning to allow for 2D histological comparison between treatment groups. This information has been added to the *Methods* section (see the *Histological analysis* section) of the revised manuscript.

Supplementary information:

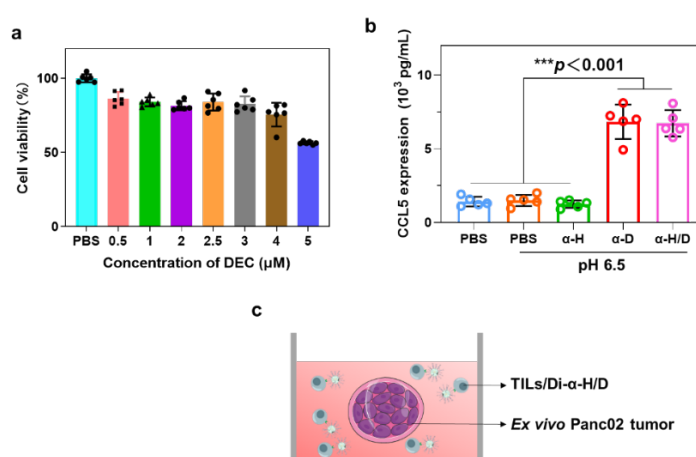
Fig. S6

- Abbreviation of ROS and MSCs are missing

Response: We sincerely apologize for the inadvertent inclusion of the abbreviations reactive oxygen species (ROS) and mesenchymal stem cells (MSCs) in the original Fig. S6 legend. These terms were erroneously retained from an earlier draft template and do not pertain to any data or methodology in our study. The legend has been fully corrected in the revised manuscript as follows: “Supplementary Figure 6 TILs incubated with α -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.”

- Why two PBS groups? What’s the difference between them?

Response: We thank the reviewer for raising this important clarification. These two PBS groups in Fig. S7b (original) represent distinct experimental conditions: the left group represents PBS treatment under standard culture conditions, while the right group reflects PBS treatment in an acidic tumor-mimetic microenvironment (pH~6.5). In the revised manuscript, we have added explicit labels "PBS (pH 6.5)" to this figure (Supplementary Figure 10).



Supplementary Figure 10 (a) Cytotoxicity was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, mean $\pm$ SD, $n = 6$ independent cell samples per group. (b) Quantification of CCL5 in the supernatant 48 h after the treatment, mean $\pm$ SD, $n = 5$ independent cell samples per group. Statistical

significance was calculated using a one-way analysis of variance (ANOVA) test, *** $p < 0.001$. (c) Illustration of an *ex vivo* tumor-coculture model to evaluate the infiltration capabilities of TILs/Di- α -H/D.

Point-by-point response to reviewer comments

Reviewer #2

In their revised version of their manuscript, authors have successfully replied to most of my concerns.

Response: We sincerely thank the reviewer for the positive evaluation of our revised manuscript. We greatly appreciate the reviewer's constructive comments and suggestions during the review process, which have helped us to further improve the quality and clarity of our work.

There are only two remaining minor points:

- First, in supplementary figure 11, graphics still display high differences between TILs/Di cells and TILs/Di- α -H/D whereas they should display similar amounts.

Response: We would like to thank the reviewer for the detailed observation regarding the data presented in Supplementary Figure 11. In this experiment, all cells isolated from the tumor tissue were collected and used for flow cytometric analysis. The difference in total event counts between the TILs/Di and TILs/Di- α -H/D groups that the reviewer noted reflects the biological effects induced by the treatment. At the starting point of the experiment (Day 0), the total cell numbers in both groups were nearly identical, indicating that the experimental setup and initial conditions were comparable. However, by Day 7 and Day 11 post-treatment, the TILs/Di- α -H/D treatment group exhibited significantly higher total cell numbers compared to the control group.

We believe this observation is closely related to the characteristics of the NCG mouse model and the mechanisms of the treatment. Since NCG mice lack T, B, and NK cells but retain functional myeloid cells, the increase in total cell number in the treated group is likely a result of the anti-tumor effects triggered by the treatment. After TILs/Di- α -H/D treatment effectively kills tumor cells, the ensuing inflammatory response recruits host-derived innate immune cells (such as neutrophils and macrophages) into the tumor tissue, thereby increasing the total number of cells in the tumor microenvironment.

- Second, in the figure R1 for reviewers, on one out of three tumors has a real acidic pH of 6.47. The two others are pretty close to 7 which is not so acidic!

Response: Thank you for your valuable suggestions. We fully understand your concern regarding the variation in tumor pH values. As you pointed out, the pH values of the three tumor regions measured with the fluorescent probe were 6.47, 6.85, and 6.92, which indeed show some variability. This observation is consistent with the known characteristics of the TME. Due to lactic acid accumulation caused by the Warburg effect, solid tumors typically exhibit a weakly acidic environment (*Annu Rev Physiol.*2020.10:82:103-126.). However, this acidity is not uniformly distributed, and there is considerable heterogeneity within the tumor. As noted in existing reviews (*Annu Rev Physiol.*2020.10:82:103-126), the pH of tumor regions typically fluctuates between 6.0 and 7.0, and our measured values are well within this reported range. Regarding the detection methods, our protocol is based on previously published literature (*Adv Mater.* 2023;35(21): e2300216), which reported tumor pH values ranging from 6.8 to 6.9. These values are very similar to our measurements of 6.85 and 6.92, further validating the reliability of our method.

We understand that the reviewer's concern might stem from our *in vitro* experiments, where drug release behavior was tested under a pH of 6.5. Therefore, there may be concerns about whether the drug can still be released in the tumor microenvironment with a pH of 6.8/6.9. To clarify, we elaborated on the design principles of our nanodrug delivery system here: the presence of the nitrogen atom in imidazole ring makes 2-methylimidazole can be regarded as base, with pKa value above 7.4 (*J. Phy. Chem.* 1975, 79, 17, 1775-1780; *J. Analy. Chem.* 2003, 58, 2, 129-134; *J. Analy. Chem.* 2000, 55, 2, 142-145). Thus, under acidic conditions (pH < 7.4), the nitrogen atom would be protonated, which increases the hydrophilicity of 2-methylimidazole and subsequently leads to the degradation of ZIF-8. Though the pH of 6.85 and 6.92 are pretty close to 7, they are still below the pKa of 2-methylimidazole, suggesting that protonation is likely to occur, which would trigger ZIF-8 degradation and subsequent drug release.

For the pH sensitive CDM moiety, a critical feature of this part is that the amide bond can be cleaved at the acidity of a tumor environment (pH < 7.0) (*Angew. Chem., Int. Ed.* 2010, 49, 3621-3626). In this environment, the carboxylate group adjacent to the amide can easily attack the carbonyl group, leading to the formation of a tetrahedral intermediate and subsequent cleavage of the bond (Fig. R1A). Therefore, at mildly

acidic pH (pH < 7.0), CDM can be cleaved to release α PD-1. All the pH values we measured, including 6.85 and 6.92, fall within the weakly acidic range. Thus, from a theoretical standpoint, the acidity of the tumor microenvironment is sufficient to trigger drug release.

To directly address the reviewer's concern, we have conducted additional release experiments at different pH conditions to verify the drug release performance under higher pH values (Fig. R1B, C). Both DEC and α PD-1 could be released from the nanodrug at pH 6.9 and 6.7, although the release rates were slightly slower compared to pH 6.5 (Fig. 2k, m), likely due to the less acidic environment. Importantly, our *in vivo* anti-tumor experiments demonstrated significant therapeutic effects of TILs-NPs delivery system (Fig. 5). This indicates that, even with pH heterogeneity in the tumor, the overall weakly acidic TME is sufficient to drive adequate drug release, ultimately resulting in significant therapeutic outcomes.

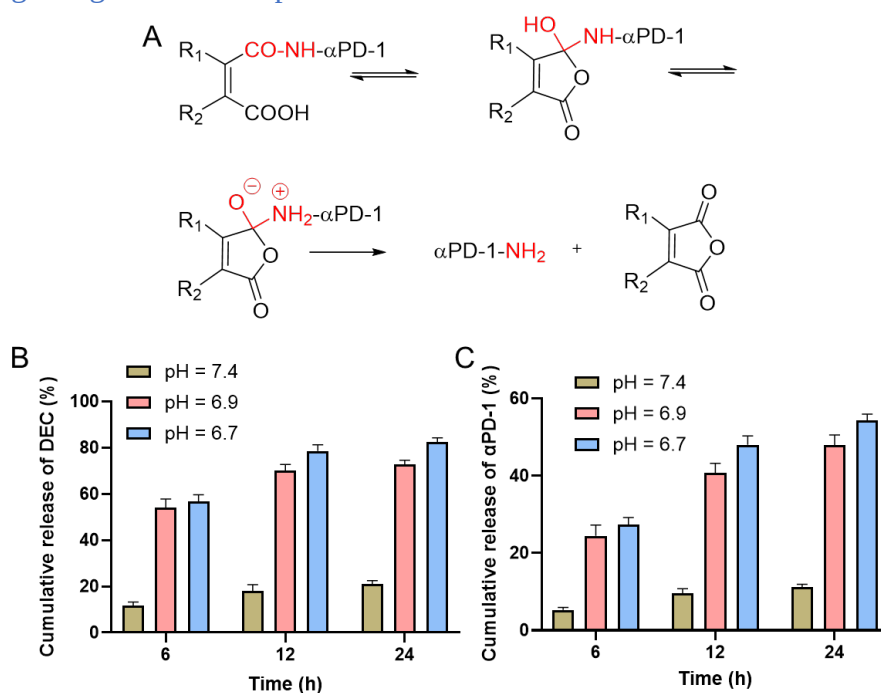


Fig. R1 (A) Schematic illustration of CDM cleavage. Release profiles of DEC (B) and α PD-1 (C) at pH 7.4, 6.9 and 6.7.