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Electronic Supplementary Information

Co-inhibition of TGF-β pathway and the PD-L1 checkpoint by pH-responsive clustered nanoparticles for pancreatic cancer microenvironment regulation and anti-tumor immunotherapy

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1. Materials

The 4th generation poly(amidoamine) dendrimers (PAMAM) were purchased from Weihai CY Dendrimers Technology Co., Ltd. (Shandong, China). Rhodamine B was purchased from Aladdin Industrial, Inc. (Shandong, China). LY2157299 was purchased from MedChemExpress (Shanghai, China). siRNA targeting PD-L1 mRNA (si*PD-L1*, antisense strand 5'-UUCAACACUGCUUACGUCUTT-3'), FAM-labeled siRNA and Cy5-labeled siRNA were synthesized by Suzhou Ribo Life Science (Kunshan, China). The negative control siRNA with a scrambled sequence (si*NC,* antisense strand 5'-ACGUGACACGUUCGGAGAATT-3') was synthesized by Suzhou GenePharma Co. (Suzhou, China). All the other reagents that were not mentioned were of analytical grade.

2. Synthesis of fluorescently-labeled polymers

Rhodamine B-labeled PAMAM ($PAMAM_{RhB}$) was synthesized as follows: rhodamine B and PAMAM with a molar ratio of 1:1 were dissolved in dimethyl sulfoxide (DMSO) and stirred for 24 h at room temperature (RT). Then, the solution was collected and dialyzed in a dialysis bag (Spectra/Por, molecular cutoff (MWCO) = 3500 Da, Spectrum, USA) against DMSO to remove the excess unreacted rhodamine B for 24 h and then lyophilized to acquire the product.

Rhodamine B-labeled PCL-CDM-PAMAM (PCL-CDM-$PAMAM_{RhB}$): PCL-CDM was synthesized according to our previously described method[1], where the molecular weight of PCL was 4000 g/mol. Then, PAMAM-RhB and PCL-CDM were dissolved in DMSO and stirred for 4 h at 60 °C. The molar ratio of PAMAM-RhB to PCL-CDM was 1:1. Then, five volumes of Milli-Q ultrapurified water (18.2 Ω, Millipore, Bedford, MA) was added and stirred for another 0.5 h. Then, the mixture was lyophilized to obtain PCL-CDM-$PAMAM_{RhB}$.

3. Preparation of $^{LY}iCluster_{siRNA}$

LY2157299-loaded nanoparticles ($^{LY}iCluster$) were prepared as follows: LY2157299 (20 mg/mL in DMSO, 9 μL), PEG-*b*-PCL(10 mg/mL in DMSO, 60 μL), PCL (10 mg/mL in DMSO, 60 μL) and PCL-CDM-PAMAM (10 mg/mL in DMSO, 60 μL) were placed into a round-bottomed flask. After stirring for 10 min to mix adequately at 60

°C and 1400 rpm, 1.9 mL of RNase-free water was added followed by stirring for another 10 min. Then, the mixture was collected for ultrafiltration to remove the organic solvent by using an Amicon Ultra-15 centrifugal filter unit (nominal molecular weight limit (NMWL) = 10 KDa, Millipore, Germany). The content of LY2157299 in ^{LY}iCluster was detected by measuring the ultraviolet absorption at $\lambda = 284$ nm using a UV-VIS spectrophotometer (UV-2600, Shimadzu, Japan). A standard LY2157299 solution was prepared by diluting LY2157299 (20 mg/mL in DMSO) with Milli-Q ultrapurified water to 50 μg/mL and then further diluting to 0.39 μg/mL by half-and-half. The drug loading content (DLC) and drug loading efficiency (DLE) were calculated as follows:

DLC (%) = Mass of LY2157299 in ^{LY}iCluster/Mass of ^{LY}iCluster × 100

DLE (%) = Mass of LY2157299 in ^{LY}iCluster/Mass of feed × 100

4. Agarose gel retardation assay

iCluster or ^{LY}iCluster was diluted to different concentrations for the desired N:P ratio, and mixed with siRNA at a final concentration of 5 nmol/mL. Then, 15 μL of the mixture was electrophoresed on a 1% agarose gel (Biofrox, Germany) in Tris-borate-EDTA (TBE) buffer at 100 V for 30 min. The siRNA bands were stained with GelRed™ (TsingKe, Beijing, China) and imaged with a Tanon 1600 gel imaging system (Tanon, Shanghai, China).

5. Cell lines and animals

The murine pancreatic ductal adenocarcinoma cell line Panc02 was kindly provided by Dr. Zhigang Zhang of State Key Laboratory of Oncogenes and Related Genes, Shanghai Cancer Institute, Shanghai Jiao Tong University, Shanghai, P.R. China. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, USA) containing 10% fetal bovine serum (FBS, ExCell Bio, Shanghai, China) in a humidified atmosphere containing 5% CO_2 at 37 °C. Panc02 cells stably expressing luciferase (Panc02-luc) were established by transfection with lentiviral vectors from Suzhou GenePharma Co. Ltd. (Suzhou, China) carrying the firefly luciferase gene.

C57BL/6 mice (6~8 weeks age) were purchased from Hunan Silaike Jingda Laboratory Animal Co Ltd. (Hunan, China).

6. The primers for qRT-PCR

The primer sequence for PD-L1: forward 5'-TGCTGCATAATCAGCTACGG-3', reverse 5'-CCACGGAAATTCTCTGGTTG-3'.

The primer sequence for GAPDH: forward 5'-GTGGACCTCATGGCCTACAT-3' and reverse 5'-TGTGAGGGAGATGCTCAGTG-3'.

All the primers were purchased from Beijing TsingKe Life Science Co. (Beijing, China).

7. Western blot analysis

Proteins extracted from these cells were separated with 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 90 min and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, USA) at 300 mA for 60 min. After blocking with PBS-Tween (PBST) containing 4% nonfat milk at 4 °C overnight, the membranes were incubated with anti-PD-L1 antibody (ab213524, Abcam, USA, 1:5000 dilution in PBST) or anti-GAPDH antibody (60004-1-Ig, ProteinTech, Chicago, USA, 1:10000 dilution in PBST) for 4 h at RT. Membranes were washed three times with PBST for 10 min and incubated with secondary antibody (goat anti-rabbit IgG HRP (BL003A, Biosharp, China, 1:5000 dilution in PBST) for anti-PD-L1 antibody and goat anti-mouse IgR-HRP (BL001A, Biosharp, China, 1:10000 diluted in PBST) for anti-GAPDH antibody) for 2 h at RT. After washing with PBST three times (10 min each), the membranes were imaged using the SuperSignal West Pico chemiluminescent substrate (Pierce, Thermo Fisher Scientific) on an Amersham Imager 600 (GE Healthcare Life Sciences, USA).

8. $CD8^+$ cytotoxic T lymphocyte (CTL) isolation and culture

$CD8^+$ T cells were separated from the spleens of C57BL/6 mice and cultured in RPMI-1640 medium (Gibco, Grand Island, USA) in the presence of 1 μg/mL anti-CD3 mAb (clone: 145-2C11, Biolegend, USA), anti-CD28 mAb (clone: 37.51, Biolegend, USA) and 10 ng/mL IL-2 (Peprotech, USA) for 24 h.

9. PSCs isolation and culture

C57BL/6 mice were sacrificed and soaked in 75% alcohol to maintain sterility. Each pancreas was removed, washed 3 times with sterile PBS, soaked in FBS containing 1%

penicillin/streptomycin (HyClone, Logan, UT, USA) and cut into small pieces (0.5~1.0 mm^3). After centrifugation for 5 min at 1000 rpm, the cell pellets were resuspended in 5 mL of DMEM containing 1 mg/mL collagenase IV (Sigma-Aldrich, St. Louis, USA) and digested at 37 °C for 20 min with shaking. Cells were then collected by filtration through a 200-mesh sieve and centrifugation at 1000 rpm for 5 min at 4 °C. Then, the cells were resuspended in 5 mL of DMEM containing 15% FBS and cultured in culture dishes (Corning Incorporated, USA) for 3 days.

10. Immunohistochemical analyses

Tumors after the last treatment were fixed in 4% formaldehyde and embedded in paraffin. Tumor sections (4 μm) were subjected to immunohistochemical staining. Slices were dewaxed, rehydrated and boiled for 15 min in 0.01 M sodium citrate buffer (pH 6.0) in a microwave oven for antigen retrieval. After pretreatment for 30 min with goat serum, slices were incubated with the respective primary antibody overnight at 4 °C, and after washing in PBST, the corresponding secondary antibody was added for incubation for 50 min (for α-SMA or CD8) or 15 min (for collagen I) at RT. After washing again, the slides were counterstained with hematoxylin (Sangon Biotech, China) and imaged with a digital pathology scanning system (Leica Aperio CS2, Germany). $CD8^+$ T cells were counted and statistically analyzed. Primary antibody: anti-α-SMA antibody (ab5694, Abcam, USA), anti-collagen I antibody (ab34710, Abcam, USA) or anti-CD8α rabbit pAb (GB11068, Servicebio, Wuhan, China). Secondary antibodies: goat anti-rabbit IgG HRP (G1215-3, Servicebio, Wuhan, China) for the anti-α-SMA antibody or anti-CD8α rabbit pAb; goat anti-mouse/rabbit IgG HRP (0013001010, Panovue, Beijing, China) for the anti-collagen I antibody.

11. Statistical analysis

The results are presented as the mean ± SD. Statistical analysis was performed using Student's *t*-test (two-tailed); $p < 0.05$ was considered statistically significant in all analyses (95% confidence level). $^*p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

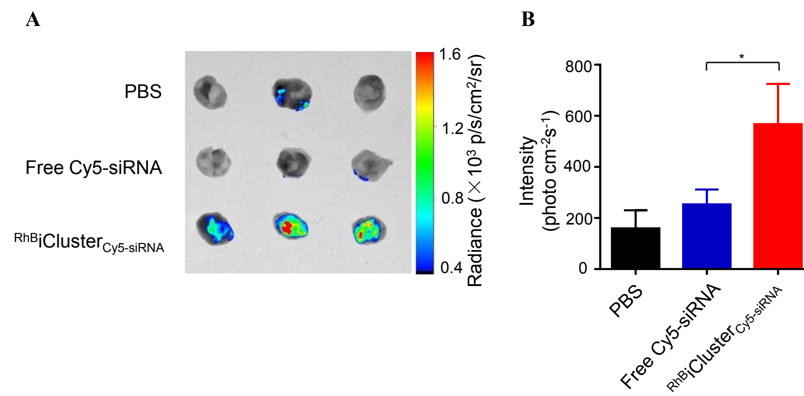

Fig. S1 *Ex vivo* image of RhB in ^{RhB}iCluster$_{Cy5\text{-}siRNA}$ in the tumors from C57BL/6 mice bearing Panc02 xenografts. λ_{ex} = 540 nm, λ_{em} = 600 nm.

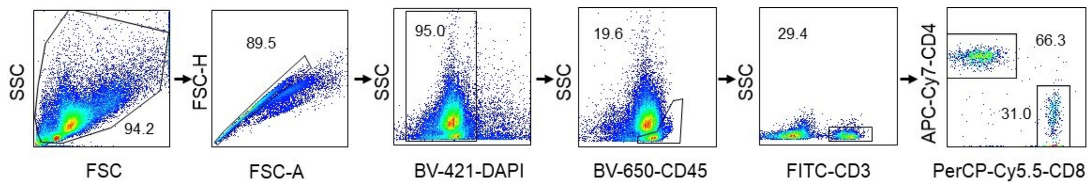

Fig. S2 Gating strategy for identification of CD4^{+} and CD8^{+} T cells by flow cytometry.

Reference

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