Electronic Supplementary Information

Diselenide-Crosslinked Nanogels Laden with Gold Nanoparticles and Methotrexate for Immunomodulation-Enhanced Chemotherapy and Computed Tomography Imaging of Tumors

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Part of experimental details:

Materials. N-vinylcaprolactam (VCL), sodium borohydride ($NaBH_4$) and hydrogen peroxide (H_2O_2) were obtained from J&K Scientific (Shanghai, China). Methotrexate (MTX) and acetylacetoxyethyl methacrylate (AAEM) were purchased from Aladdin (Shanghai, China). Diselenide (SeSe) crosslinker was from Ruixi Biotechnology Co., Ltd. (Xi'an, China). 2, 2-Azobis [N-(2-carboxyethyl)-2-methylpropionamidine] (ACMA) was from Wako Pure Chemical Industries (Osaka, Japan). Sodium dodecyl sulfate (SDS) was from Sigma-Aldrich (St. Louis, MO). Au (III) chloride trihydrate ($HAuCl_4 \cdot 4H_2O$) and all the other chemicals were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm. Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 8000-14000 were acquired from Fisher (Pittsburgh, PA). B16F10 cells (a mouse melanoma cell line), L929 cells (a mouse fibroblast cell line), and RAW 264.7 cells (a mouse macrophage cell line) were acquired from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), RPMI-1640 medium, penicillin, streptomycin, fetal bovine serum (FBS) and trypsin (0.25%) were from Hangzhou Jinuo Biomedical Technology (Hangzhou, China). Cell Counting Kit-8 (CCK-8), Cell Cycle and Apoptosis Analysis Kit and recombinant murine Interleukin-4 (IL-4) were from Beyotime Biotechnology (Shanghai, China). Annexin V- fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit was acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). Anti-CD86-phycoerythrin (PE), anti-CD206-fluorescein isothiocyanate (FITC), anti-CD4-allophycocyanin (APC), anti-FOXP3-FITC and anti-CD25-PE were from Thermo Fisher Scientific (Waltham, MA). All chemicals and materials were used as received without further purification.

Preparation of PVCL NGs. Poly(N-vinylcaprolactam) (PVCL) nanogels (NGs) were first synthesized using a precipitation polymerization approach according to the previous reports.[1-3] In

brief, VCL (469.5 mg) and SDS (20 mg) were co-dissolved in 49 mL of water, then SeSe crosslinker (5 mg, in 0.5 mL DMSO) was added into the mixture under stirring at 70 ℃ for 30 min with N_2 protection to obtain a homogenous solution. Then, AAEM as a comonomer (42 mg, in 0.5 mL water) was dropped into the above mixture, and ACMA (17.5 mg, in 0.5 mL water) was added to initiate the polymerization. The sequential addition of chemicals was under N_2 protection, and the whole reaction was then maintained at 70 ℃ for 4 h under N_2 protection. Afterward, the reaction mixture was cooled down to room temperature (RT) and the obtained white dispersion was dialyzed against water using a dialysis membrane with an MWCO of 8000-14000 for 3 days (2 L, 9 times) to remove the unreacted monomers. Finally, the dialysis liquid was subjected to lyophilization to obtain the powder of PVCL NGs.

Preparation of Au@PVCL NGs. The PVCL NGs were used as nanoreactors to absorb Au(III) ions through interaction with the β-diketone groups of AAEM, and then the Au(III) ions were rapidly reduced with $NaBH_4$ to form Au NPs.[4] In short, an $HAuCl_4 \cdot 4H_2O$ solution (30 mg/mL, 2 mL in water) was added to a dispersion of PVCL NGs (10 mg/mL, 10 mL in water). The mixture was stirred intensively under ice bath for 1 h in order to let the Au(III) ions diffuse into the NGs network. Following this, 1 mL of a freshly prepared $NaBH_4$ solution (22 mg/mL, in icy water) was added quickly to the above mixture to reduce the Au(III) ions, and the mixture was further stirred overnight. A significant color change from yellow to dark purple indicated the formation of AuNPs in the NGs. The sample was dialyzed at RT against water through a dialysis membrane with an MWCO of 8000-14000 for 3 days (2L, 9 times) and subjected to lyophilization to obtain the powder of Au@PVCL NGs.

Characterization techniques. Dynamic light scattering (DLS) and zeta potential measurements were carried out using a Malvern Zetasizer Nano ZS model ZEN3600 (Worcestershire, UK) equipped with a standard 633-nm laser. The morphology of the MTX/Au@PVCL NGs was observed using scanning electron microscope (SEM, S-4800 analytical electron microscope, Tokyo, Japan) at a voltage of 15 kV. A typical sample was prepared by dropping an NG suspension (100 μg/mL, 5 μL)

onto silicon wafer, air dried and sputter coated with a gold film with a thickness of 10 nm. Transmission electron microscopy (TEM) imaging was performed using a JEOL 2100F electron microscope (Tokyo, Japan) at an operating voltage of 200 kV. One drop of the MTX/Au@PVCL NGs in water (100 μg/mL) was deposited onto a carbon-coated copper grid and air-dried before measurements. The particle size distribution was measured using Image J 1.40 G software (http://rsb.info.nih.gov/ij/download.html). For each sample, at least 100 NGs were randomly selected from SEM or TEM images and analyzed. UV-vis spectroscopy was carried out using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Waltham, MA). The Au concentration and the cellular uptake of the NGs in terms of Au contents were determined with inductively coupled plasma-optical emission spectroscopy (ICP-OES, Leeman Prodigy, Hudson, NH).

Stimuli-responsive release of MTX from the NGs. To test the H_2O_2-responsive MTX release, MTX/Au@PVCL NGs (5 mg) were dispersed in 1 mL of phosphate buffer under the following conditions: (1) pH = 7.4, (2) pH = 6.5, (3) pH = 7.4 + H_2O_2 (0.1 mM), or (4) pH = 6.5 + H_2O_2 (0.1 mM). Each solution was put in a dialysis bag (MWCO = 8000-14000), and then submerged into 9 mL of the corresponding buffer medium. At each scheduled time interval, 1 mL of the buffer medium was pipetted out and 1 mL of the same fresh buffer was replenished. The MTX concentration was determined through UV-vis spectrometry to quantify the MTX absorption at 303 nm. The experiment was performed in triplicate for each sample.

X-ray attenuation property. The X-ray attenuation property and computed tomography (CT) phantom study of MTX/Au@PVCL NGs were studied at different Au concentrations (5, 10, 30, 40 or 50 mM) using a dual-source SOMATOM Definition Flash CT system (iCT 256, Philips Medical Systems, Amsterdam, The Netherlands) at 120 kV and a slice thickness of 1.00 mm. For each sample, the X-ray attenuation intensity was determined in Hounsfield units (HU) by a standard display program.

Cytotoxicity assay. CCK-8 assay was performed to analyze the cytotoxicity of different materials. Firstly, B16F10 cells were seeded into 96-well plates at a density of 1×10^4 cells per well in 100 μL of DMEM under 5% CO_2 at 37 ℃ overnight to allow the attachment of cells. The next day,

the medium of each well was replaced with 100 μL fresh medium containing PVCL NGs or Au@PVCL NGs at different NG concentrations (0, 10, 50, 100, 200 and 300 μg/mL, respectively). The cells were incubated for 24 h. After that, 100 μL medium containing 10% CCK-8 agent was added to each well, and cells were incubated under regular culture conditions for 4 h. Then, the optical density of each well at 450 nm was determined using a Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). Meanwhile, the cytotoxicity of MTX and MTX/Au@PVCL NG on B16F10 cells was also evaluated at different MTX concentrations (0, 1, 5, 10, 20 and 40 μg/mL, respectively) under the same experimental conditions.

To confirm the biosafety of the MTX/Au@PVCL NGs, L929 cells as the representative normal cell line were also cultured in RPMI-1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 5% CO_2 and 37 ℃. Next, the L929 cells were incubated with medium containing PVCL NGs or Au@PVCL NGs at different NG concentrations (0, 10, 50, 100, 200 and 300 μg/mL, respectively) or with medium containing MTX or MTX/Au@PVCL NGs at different MTX concentrations (0, 1, 5, 10, 20 and 40 μg/mL, respectively) for 24 h to measure the cell viability through CCK-8 assay as described above.

Cellular uptake assay. The uptake of MTX/Au@PVCL NGs by B16F10 cells was quantified by ICP-OES. In brief, B16F10 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well with 1 mL of medium and cultured overnight. Afterwards, the medium was replaced with 1 mL of medium containing Au@PVCL NGs at different Au concentrations (0, 2, 10, 20, 40 and 80 μM, respectively) and cells were incubated for 6 h. Then, the cells were washed with phosphate buffered saline (PBS), trypsinized, and collected. After cell counting, the cells were digested by *aqua regia* solution (nitric acid/hydrochloric acid, v/v = 1: 3) for 4 h, diluted with water, and analyzed by ICP-OES to determine the cellular Au content. For each sample, the measurement was repeated for 3 times.

Cell apoptosis assay. Cell apoptosis assay was performed using an Annexin V-FITC/PI apoptosis detection kit by flow cytometry. B16F10 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL DMEM overnight, followed by incubation with fresh medium

containing PVCL NGs, Au@PVCL NGs, MTX or MTX/Au@PVCL NGs ([MTX] = 40 μg/mL, and the corresponding concentrations of PVCL, Au@PVCL and MTX/Au@PVCL NGs are 227 μg/mL) for 12 h. Subsequently, the cells in each well were washed, collected, and resuspended in 195 μL of binding buffer, followed by addition of 5 μL of Annexin V-FITC and 5 μL of PI. Then, the samples were incubated for 15 min at room temperature in the dark before flow cytometry analysis. Cells treated with PBS were used as control. For each sample, 1×10^4 cells were counted and each measurement was repeated for 3 times.

Cell cycle analysis. B16F10 cells were seeded in 6-well plates at a density of 2×10^5 cells per well overnight in 2 mL DMEM. Then, the cells in each well were incubated with 2 mL of complete DMEM containing PVCL NGs, Au@PVCL NGs, MTX or MTX/Au@PVCL NGs ([MTX] = 40 μg/mL, and the corresponding concentrations of PVCL, Au@PVCL and MTX/Au@PVCL NGs are 227 μg/mL) for 12 h. Cells treated with PBS were set as control. After that, cells in each well were washed with PBS, trypsinized, collected through centrifugation, and fixed with 70% precooled ethanol at 4 ℃ for 12 h. The cells were then washed with PBS thrice and stained with a mixture solution containing 1% Triton X-100, 0.01% RNase and 0.05% PI for 30 min at 37 ℃ in the dark. Flow cytometry was employed to quantify the DNA content to estimate the percentages of the cell population in different phases of each cell cycle. Modfit software (Verity Software House, Topsham, ME) was used for data fitting analysis.

Animals and tumor model construction. All animal experiments were approved by the Ethical Committee for Animal Care and Use of Donghua University, and were also performed in accordance with the guidance of the National Ministry of Health of China. Female C57BL/6 mice of 4-6 weeks old (body weight of 15-20 g) were obtained from the Shanghai Slac Laboratory Animal Center (Shanghai, China). The xenograft tumor model was established by injection of 100 μL PBS solution containing 2×10^6 B16F10 cells into the right thigh of each C57BL/6 mouse.

Hemolysis assay. Hemolysis assay was performed to evaluate the hemocompatibility of the MTX/Au@PVCL NGs according to the literature.[5] In brief, 1.5 mL of blood collected from the inner canthus vein plexus of mice was diluted with 3.5 mL of PBS, and then the pure red blood cells

(RBCs) were obtained *via* repeated centrifugation/redispersion processes (2000 rpm, 10 min, 3 times). The RBCs were then diluted with 5 mL of PBS. Thereafter, 100 μL of the obtained RBC suspension was mixed with 900 μL water (positive control), PBS (negative control) and MTX/Au@PVCL NGs dispersed in PBS at various concentrations (10-300 μg/mL). After 2 h incubation at 37 ℃, each sample was centrifuged at 13000 rpm for 15 min. UV-vis spectrometry was used to record the absorbance of the supernatant at 540 nm.

***In Vivo* CT imaging of tumors**. For *in vivo* CT imaging, each tumor-bearing mouse was anesthetized by pentobarbital sodium solution (1%, w/w), and intratumorally injected with the MTX/Au@PVCL NGs ([Au] = 50 mM, in 100 μL of PBS), and CT images of tumors were collected at different time points (0, 15, 30, 45, 60 and 90 min, respectively) post-injection by a CT system (iCT 256, Philips Medical Systems, Amsterdam, The Netherlands) at 120 kV and 97 mA. The CT value in Hounsfield unit (HU) was determined by a standard display program.

Histological and immunofluorescence analysis of tumor slices. The tumor-bearing mice after different treatments of PBS, PVCL NGs, Au@PVCL NGs, MTX or MTX/Au@PVCL NGs were sacrificed at 11 days post-treatment, and tumors were extracted, fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned for H&E staining, TdT-mediated dUTP Nick-End Labeling (TUNEL)-staining, ki-67 staining, Arg/iNOS and CD4+/CD8+ T cell immunofluorescence staining according to standard protocols reported in the literature.[6, 7]

Spleen immune cells analysis. To confirm the immunological effect induced by treatment with MTX/Au@PVCL NGs, the T cell activation in spleen was examined in different groups. In brief, the tumor-bearing mice were sacrificed by cervical dislocation and immersed in 75% alcohol for 2-5 min, and the spleen of each mouse in different groups was collected under sterile conditions and stored in icy PBS. To extract the spleen-infiltrating immune cells, the spleen was ground in a 400-mesh sieve to obtain the lymphocyte suspension, which was then filtered by the nylon wool column for 6-8 times to obtain the T cells. Further, the T cells were resuspended in PBS and stained with anti-CD4-FITC/anti-CD8-PE or anti-CD4-APC/anti-FOXP3-FITC/anti-CD25-PE before flow

cytometry assays. For each sample, 1×10^4 cells were counted and each measurement was repeated for 3 times.

Biosafety tests. For biosafety evaluation, the tumor-bearing mice after different treatments of PBS, PVCL NGs, Au@PVCL NGs, MTX or MTX/Au@PVCL NGs were sacrificed at 11 days post-treatment, and the vital organs (heart, liver, spleen, lung, and kidney) were extracted, fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned for H&E staining. In addition, blood routine tests of red blood cells (RBC), white blood cells (WBC), platelet (PLT), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular volume (MCV), hemoglobin (HGB), lymphocyte (Lymph) and percentage of neutrophils (Gran) were further used to verify the biosafety of the above materials. Blood samples were obtained by removing the mice eyeball after the mice were sacrificed at 11 days post-injection (n = 3 for each group) and analyzed by Mindray BC-2800vet Hematology Analyzer (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

Statistical analysis. One-way analysis of variance statistical analysis was performed using GraphPad Prism 7.00 software (San Diego, CA) to evaluate the significance of the experimental data. A p value of 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. The encapsulation efficiency and loading capacity of MTX in the MTX Au@PVCL NGs under different NG/MTX mass ratios.

Feeding ratio (Au@PVCL NGs: MTX, w/w)	Encapsulation Efficiency (%)	Loading Capacity (%)
1: 0.10	95.88	8.94
1: 0.25	68.84	15.18
1: 0.50	59.67	23.94

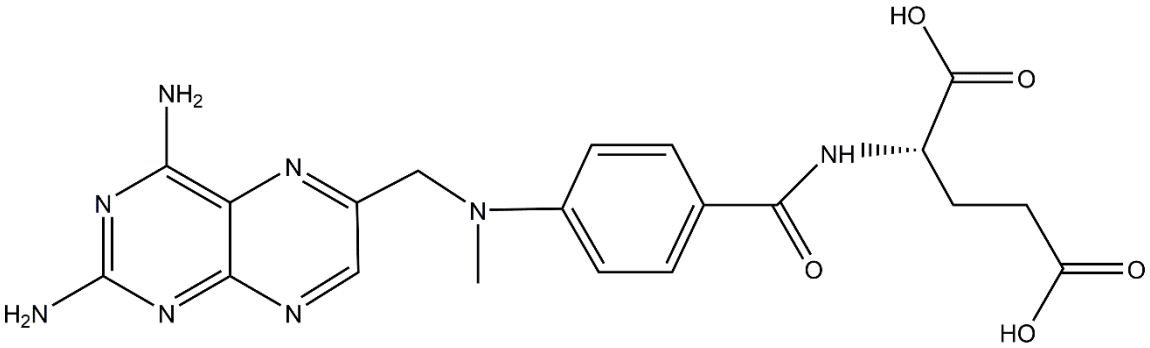

Figure S1. The chemical structure of Methotrexate (MTX).

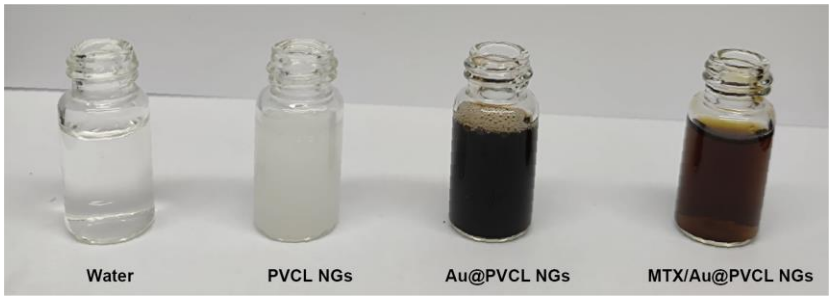

Figure S2. Photographs of water and three different NG dispersions.

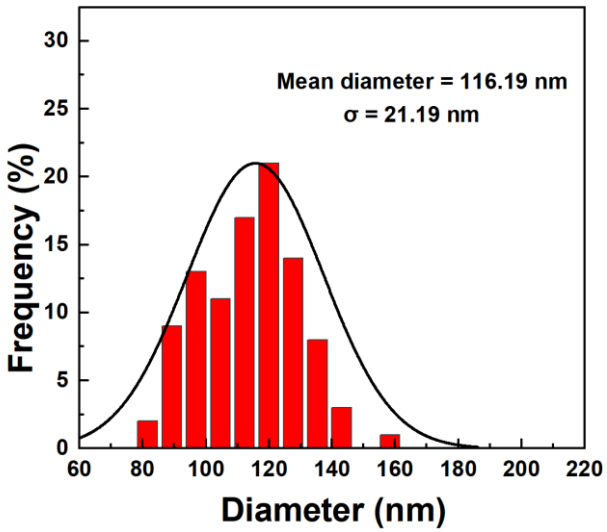

Figure S3. Size distribution histograms of the MTX/Au@PVCL NGs.

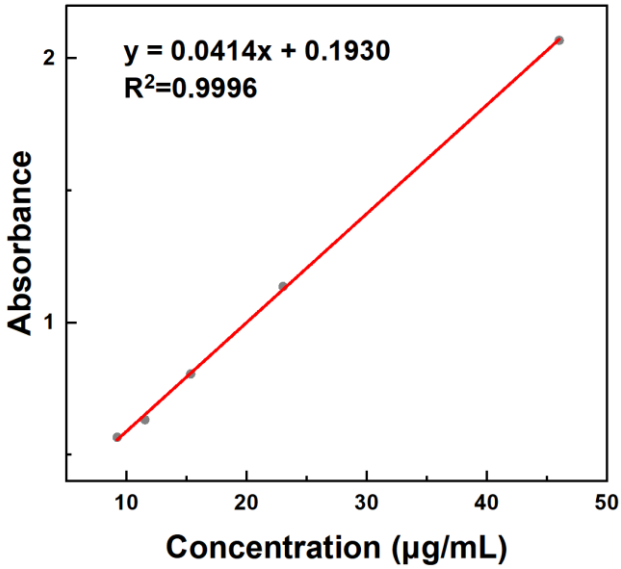

Figure S4. The calibration curve of MTX absorption at 303 nm versus concentration in methanol solution.

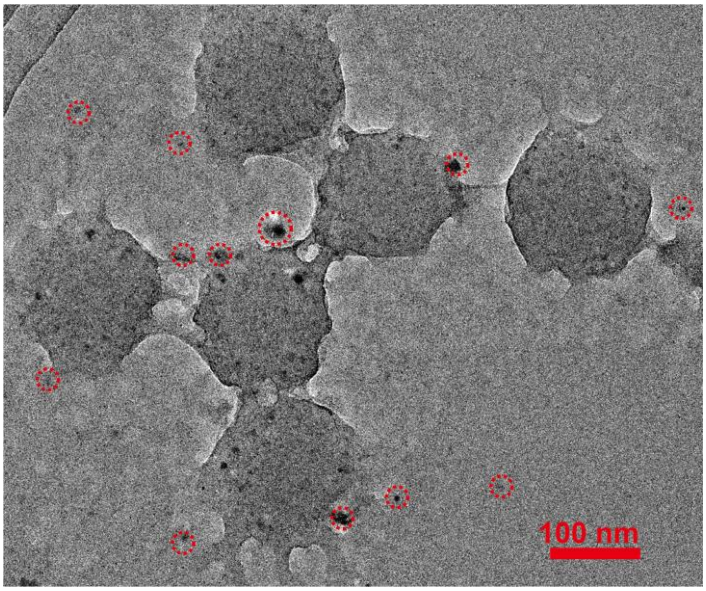

Figure S5. TEM image of MTX/Au@PVCL NGs after stimuli-responsive MTX release.

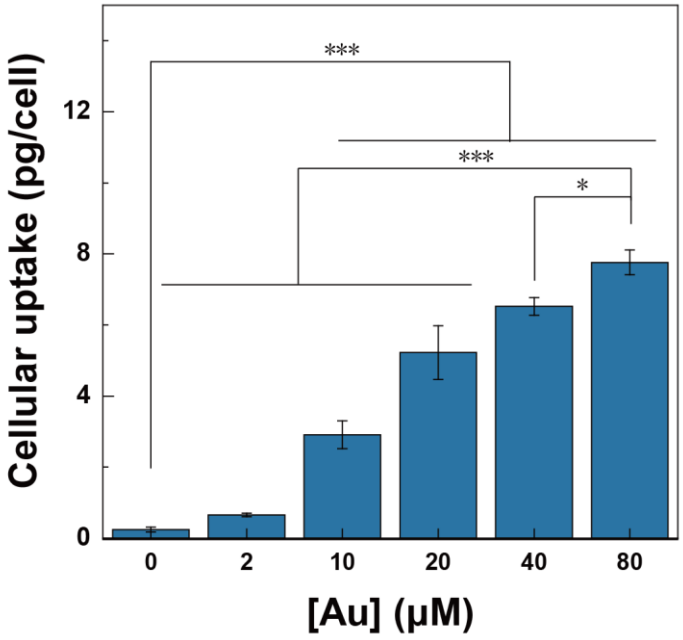

Figure S6. Cellular uptake of MTX/Au@PVCL NGs at different Au concentrations after incubation for 6 h (n = 3). * for $p < 0.05$ and *** for $p < 0.001$, respectively.

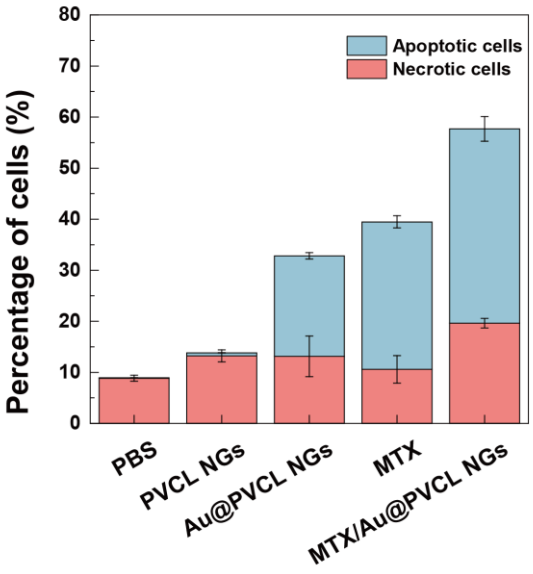

Figure S7. Flow cytometry quantification of apoptotic and necrotic B16F10 cells after treatment with PBS, PVCL NGs, Au@PVCL NGs or MTX/Au@PVCL NGs for 12 h (n = 3).

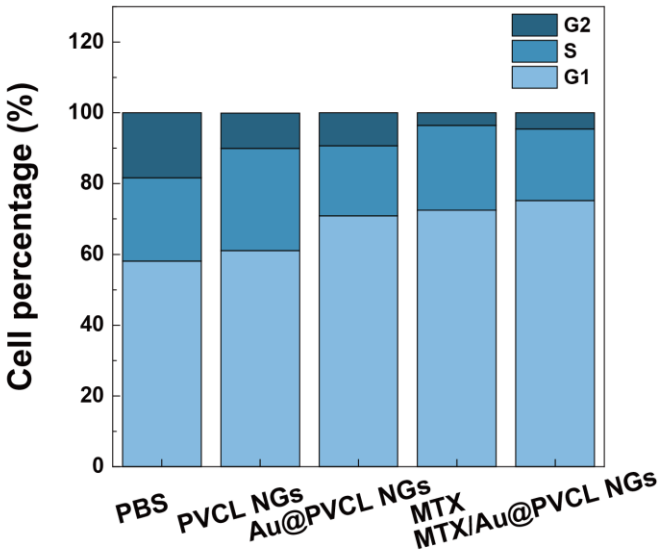

Figure S8. Percentage of B16F10 cells at different cell cycle phases after incubation with PBS, PVCL NGs, Au@PVCL NGs or MTX/Au@PVCL NGs for 12 h.

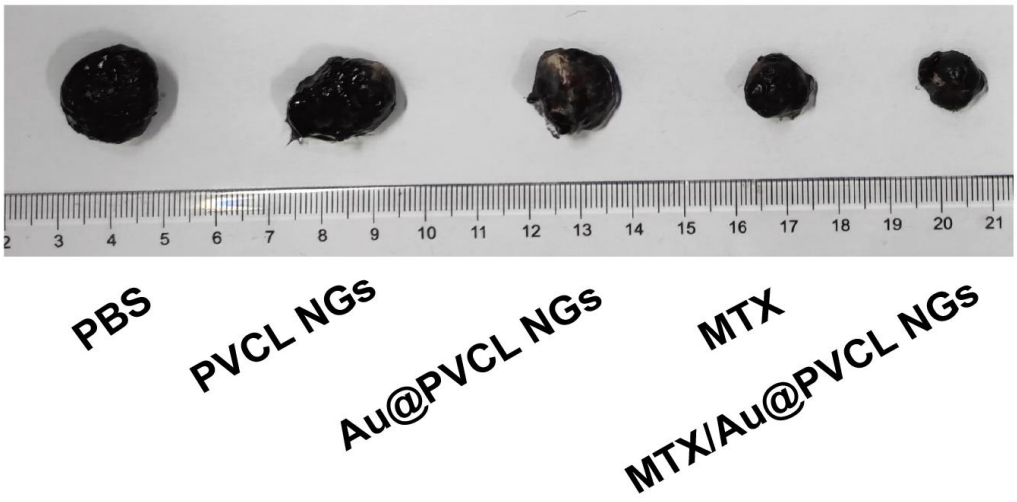

Figure S9. Representative mouse tumor photographs in different treatment groups of PBS, PVCL NGs, Au@PVCL NGs, MTX and MTX/Au@PVCL NGs.

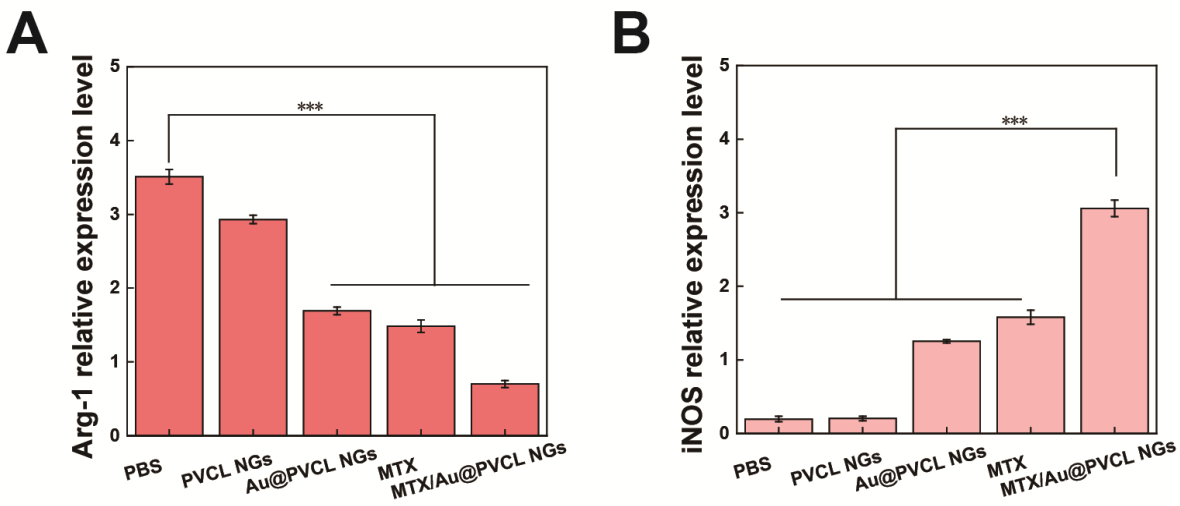

Figure S10. Quantification of (A) Arg-1 and (B) iNOS relative expression levels in tumor slices of different treatment groups including PBS, PVCL NGs, Au@PVCL NGs, MTX and MTX/Au@PVCL NGs (n = 3). For A and B, *** is for $p < 0.001$.

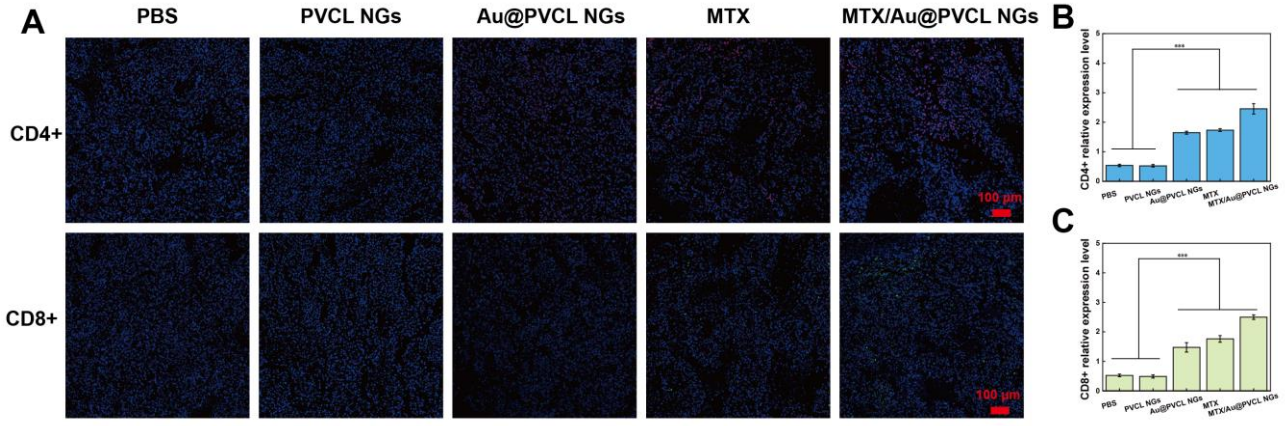

Figure S11. (A) Immunofluorescence staining of CD4+ T cells and CD8+ T cells in B16F10 tumors in different treatment groups (PBS, PVCL NGs, Au@PVCL NGs, MTX and MTX/Au@PVCL NGs) for 11 days (Scale bar = 100 μm for each panel), and the corresponding percentage of (B) CD4+ and (C) CD8+ expression levels (n = 3 for each measurement). For B and C, *** for $p < 0.001$.

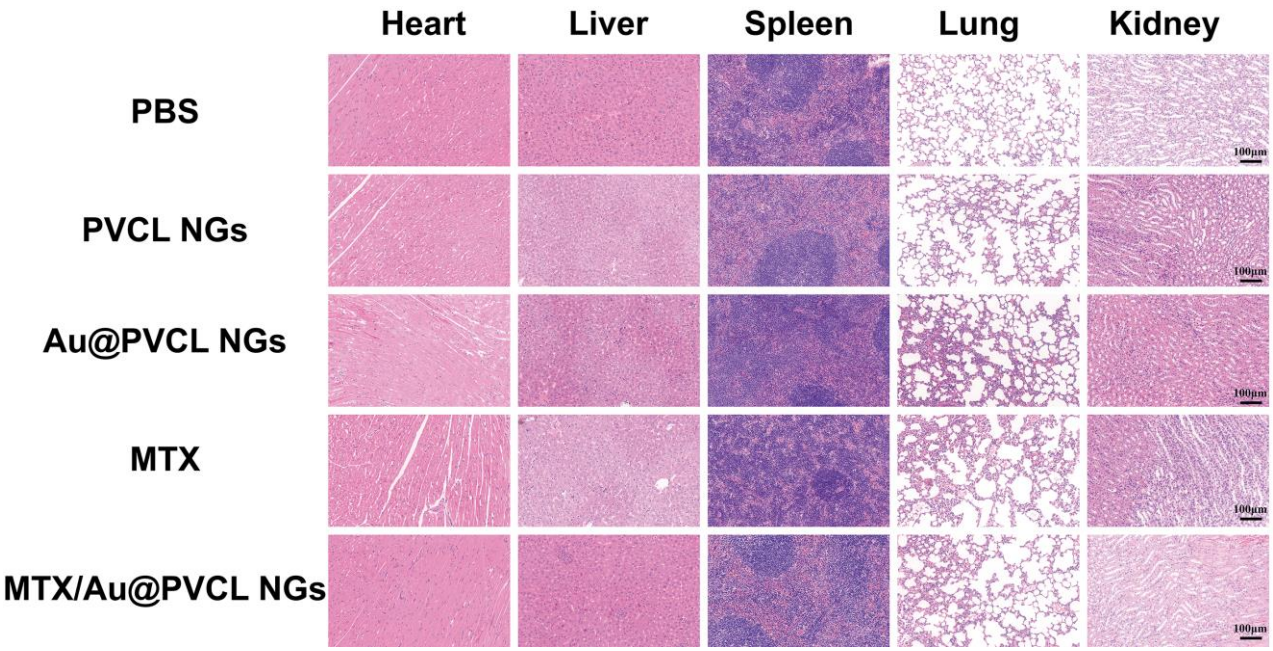

Figure S12. H&E staining of major organ sections of tumor-bearing mice after different treatments of PBS, PVCL NGs, Au@PVCL NGs, MTX and MTX/Au@PVCL NGs for 11 days (Scale bar = 100 μm for each panel).

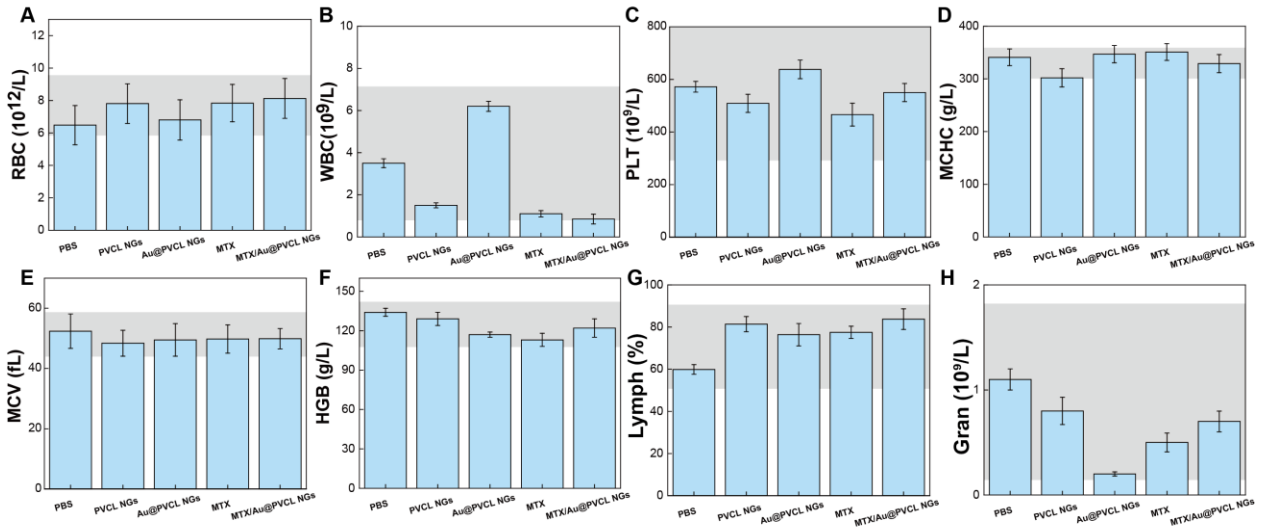

Figure S13. The blood routines including levels of (A) RBC, (B) WBC, (C) PLT, (D) MCHC, (E) MCV, (F) HGB, (G) Lymph and (H) Gran of tumor-bearing mice on the 11th day of different treatments of PBS, PVCL NGs, Au@PVCL NGs, MTX and MTX/Au@PVCL NGs (n = 3).

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