Supporting Information

Zinc-based radioenhancers to activate tumor radioimmunotherapy by PD-L1 and cGAS-STING pathway

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Methods

Preparation of ZnO-Au@mSiO$_2$

ZnO-Au@mSiO$_2$ nanocomposites were prepared through a template method. Firstly, Au@mSiO$_2$ NPs were synthesized following a previously reported method[1]. Typically, 1.2 mL of 0.5 M NaOH solution was added into 48 mL of cetyl trimethyl ammonium bromide (CTAB, 0.10 g) aqueous solution, and the solution was heated to 80 °C for 15 min with vigorous magnetic stirring. Then, 2.0 mL of formaldehyde solution (3.7 wt%) was added, followed by the addition of 2.0 mL of $HAuCl_4$ (5 mg/mL) aqueous solution. After 10 min, 2 mL of TEOS (20 wt % ethanol solution) was quickly injected into the above heated solution, and the mixture was further heated for 1 h. After the solution was cooled down to room temperature, the product (SiO_2@Au) was collected by centrifugation and washed with water for one time and washed with ethanol for two times. To obtain meso-porous SiO_2, CTAB micelles were removed by an ion-exchange method. SiO_2@Au was dispersed in 20 mL of ammonium nitrate (0.38 M) solution and stirred at 60 °C for 12 h at reflux to remove the template. The obtained product Au@mSiO$_2$ NPs were collected by centrifugation and washed with water and ethanol for three times. The obtained Au@mSiO$_2$ was functionalized with amino and carboxyl groups. In brief, Au@mSiO$_2$ was redispersed in ethanol (20 mL), and then added 200 μL APTES. After stirring for 12 h at room temperature. Au@mSiO$_2$-NH$_2$ was obtained and washed by centrifugation with water and ethanol for three times. Then, the Au@mSiO$_2$-NH$_2$ was dissolved in DMSO, and added 80 mg succinic anhydride and 1.6 mL triethylamine at room temperature, and then stirred for 12 h to obtain Au@mSiO$_2$-COOH.

Then, APTES-modified ZnO QDs (ZnO-NH$_2$) were synthesized. Briefly, 30 mL of ethanol was added into a 100 mL round-bottom flask, followed by the addition of Zinc acetate (219.50 mg) and Magnesium acetate (21.45 mg). The mixture was heated to 70 °C and refluxed under vigorous magnetic stirring for 0.5 h. Subsequently, after cooled down to room temperature, 8 mL of NaOH (0.16 M) solution in ethanol was added dropwise and stirred for 4 h to obtain the ZnO QDs. For functionalization of ZnO

QDs, 5 mL of APTES (2% ethanol solution) was added to above ZnO QDs suspension and stirred for another12 h. The solution was purified with cyclohexane. The obtained ZnO-NH_2 was redispersed in ethanol and it emitted yellow fluorescence when irradiated with a 365 nm UV lamp.

To prepare ZnO-Au@$mSiO_2$ nanocomposites, aqueous solution of EDC (75 mg/mL, 1 mL) and NHS (30 mg/mL, 1 mL) was added into 20 mL Au@$mSiO_2$-COOH (1 mg/mL ethanol solution). After 2 h, ZnO-NH_2 was added, and stirred at room temperature for another 6 h, and centrifuged for several times and the ZnO-Au@$mSiO_2$ nanocomposites were obtained. In order to improve the water solubility of ZnO-Au@$mSiO_2$, polyacrylic acid (PAA) was added in the solution at a ratio of 1:3, stirred at room temperature for 4 h. Afterwards the precipitate was centrifuged, washed several times with H_2O, and the ZnO-Au@$mSiO_2$ nanocomposites were obtained and stored at 4 ℃ for the following experiments.

***In vitro* release of Zn^{2+}**

In vitro profiles of the release of Zn^{2+} from ZnO-Au@$mSiO_2$ at different pH (7.4, 6.5 and 5.8) were measured. Briefly, ZnO-Au@$mSiO_2$ suspension was sealed and shaken different time at 37 ^{o}C. At the designated time points, the amount of Zn^{2+} released in the medium was centrifuged and detected by Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, PerkinElmer, USA).

Cleavage efficiency of DNAzyme

To investigate the effect of Zn^{2+} on cleavage efficiency, different concentrations of Zn^{2+} ions were co-incubated with the mixture of DNAzyme (5 μM) and substrate (5 μM) for 1 h at 37 °C. Then the samples were mixed with loading buffer and addded into the freshly prepared 4 % gel. Electrophoresis was performed at a constant potential of 120 V in 1×TBE buffer for 40 minutes. Finally, the images was obtained by using a imaging system (Bio-Rad ChemiDoc XRS System, Bio-Rad，USA).

Cell uptake and cytotoxicity

The cytotoxicity of ZnO-Au@$mSiO_2$ was evaluated using a standard MTT assay. Lewis lung tumor cells (LLC) were seeded and incubated for 24 h in 96-well plates.

The cells were treated with ZnO-Au@$mSiO_2$ and Au@$mSiO_2$ (0–400 μg/mL) for 12 h, then irradiated with X-ray (8 Gy), and cultured for another 12 h. The MTT assay was then performed, and the absorbance was measured at 490 nm using a microplate reader.

To further identify the uptake effect of ZnO-Au@$mSiO_2$, LLC cells (1×10^5 cells per well) were seeded in 24-well plates and cultured for 24 h. Then, the cells were incubated with ZnO-Au@$mSiO_2$ for different times (0, 2, 4 and 8 h), respectively. All the samples were collected and measured by flow cytometry (FCM, ACEA Biosciences, USA).

ROS generation in Cells

To monitor ROS generation in cells, LLC cells (2×10^5 cells per well) were planted into confocal dishes and 12-well plates for detecting by Laser Scanning Confocal Microscopy (CLSM, Zeiss LSM780, Germany) and FCM (ACEA Biosciences, USA). Then, the cells were irradiated with X-ray (8 Gy) after incubation with ZnO-Au@$mSiO_2$ (200 μg/mL) for another 12 h. After 2 h incubation, LLC cells were stained with a ROS probe DCFH-DA (10 μM). The generation of ROS was evaluated by the change of fluorescence intensity.

Assessment of intracellular apoptosis

Cell apoptosis was evaluated by FCM using the Annexin V-FITC/PI Apoptosis Detection Kit (BD Biosciences). LLC cells (1×10^5 cells per well) were plated 24-well plates. After 24 h, LLC cells were incubated with ZnO-Au@$mSiO_2$ (200 μg/mL) and Au@$mSiO_2$ (200 μg/mL) for another 12 h. Then, the cells were irradiated with X-ray (8 Gy). 12h later, LLC cells were stained with FITC Annexin V and PI for 15 min and measured using FCM (ACEA Biosciences, USA). The apoptotic rate was calculated via the percentage of early apoptotic cells (FITC+/PI-) and late apoptotic cells (FITC+/PI+).

Assessment of mitochondrial damage

To evaluate the change of mitochondrial membrane potential after different treatment, LLC cells were incubated with ZnO-Au@$mSiO_2$ (200 μg/mL) and Au@$mSiO_2$ (200 μg/mL) for 12 h. Then, they were irradiated with X-ray (8 Gy). After another 12 h, LLC cells were stained with 20 μM JC-1 for 15 min. After washing with

PBS, the mitochondrial membrane potential was monitored by FCM and CLSM (JC-1 Aggregates, Ex/Em: 585/590 nm; JC-1 Monomer, Ex/Em: 510/527 nm).

To further assess the mitochondria activity, the treated LLC cells were stained with 10 μM Mito-Tracker Green, a mitochondria-specific dye, and detected by FCM (ACEA Biosciences, USA).

Lipid peroxidation assay

The cellular oxidative stress response after treated with ZnO-Au@$mSiO_2$ and X-ray was also evaluated by the detection of lipid peroxidation. LLC cells were incubated with ZnO-Au@$mSiO_2$ (200 μg/mL) and Au@$mSiO_2$ (200 μg/mL) for 12 h, then, were irradiated with X-ray (8 Gy). 12h later, LLC cells were stained with 20 μM BODIPY™ 581/591 C11 (Lipid Peroxidation Sensor) for 15 min. After washing with PBS, the cells were tested by FCM (ACEA Biosciences, USA).

Cell cycle distribution

Cell cycle and proliferation were investigated using fluorescein Cell Cycle and Apoptosis Analysis Kit (BD Pharmingen). To assess the effect of the ZnO-Au@$mSiO_2$ on cell cycle distribution, LLC cells (1×10^4 cells per well) were planted into 24-well plates, and treated with ZnO-Au@$mSiO_2$ (200 μg/mL) and PBS for 12 h. Then, the cells were irradiated with X-ray (8 Gy). 12h later, the treated cells were collected and treated with PI, and analyzed using FCM (ACEA Biosciences, USA).

Western blotting

LLC cells and DC 2.4 cells were co-cultured with ZnO-Au@$mSiO_2$ for 24 h. Then the treated cells were collected for western blotting. LLC cells were used to detect the expression of PD-L1 and ICD related factors, and DC2.4 cells was used to detect the expression of cGAS-STING signal path related factors. Western Blotting were performed according to standard protocols. The primary antibodies used anti-mouse anti-PD-L1 antibody (Abcam, 1:1000), anti-TBK1 Polyclonal antidody (Baijia, 1:1000), anti-TBK1/NAK (phospho-Ser172) antidody (Baijia, 1:1000), anti-IFN-β Polyclonal antibody (Baijia, 1:1000), anti-HMGB1-antibody (Baijia, 1:500), anti-Calretinin-antibody (Baijia, 1:1000), anti-GAPDH antibody (CST, 1:1000) and anti-β-actin-

antibody (Boster, 1:1000). The blots were developed with BeyoECL Plus substrate (Beyotime, P0018AS), and imaged using a chemiluminescence imaging system (Bio-Rad ChemiDoc XRS System, Bio-Rad，USA).

Quantitative PCR (qPCR) analysis

LLC cells and DC 2.4 were seeded on a 6-well (5×10^5 cells per well) and co-cultured with ZnO-Au@mSiO_2 for 24 h. Then, cells were washed by PBS and collected for real-time quantitative PCR (q-PCR). Total RNA was isolated by Trizol reagent. Reverse transcription was performed to obtain cDNA using SPARKscript II RT Plus Kit with gDNA Eraser. q-PCR analysis was performed using the SYBR Green qPCR Mix. The program was as follows: 94 ℃ for 3 min, 94 ℃ for 10 s for40 cycles, and 60 ℃ for 30 s. Relative mRNA expression levels of the target genes were normalized to control gene GADPH. The primer sequences are listed in Supplementary Table 3. LLC cells were used to detect the expression of PD-L1 mRNA and ICD related factors, and DC 2.4 was used to detect the expression of cGAS-STING signal path related factors.

The qPCR data were used to calculate the gene expression based on the cycle threshold (CT) value obtained from the amplification curve. Using the GAPDH gene as an internal reference, the expression of the target gene in the experimental group was calculated as the multiplicity relative to the PBS group, and expressed as $2^{-\triangle\triangle CT}$ value.

$\triangle$CT = CT (target gene per group) - CT (internal reference gene per group)

$\triangle\triangle$CT = $\triangle\triangle$CT (experimental group) -$\triangle\triangle$CT (PBS group)

Clone formation

To evaluate the long-term proliferate ability of treated cells, LLC cells (2×10^5 cells per well) were firstly planted in 6-well plates for 24 h. Then LLC cells were incubated with PBS and ZnO-Au@mSiO_2 in different concentrations (50, 100 and 200 μg/mL) for another 12 h, irradiated with X-ray (8 Gy) and cultured for another 12 h. Then, the cells were collected and re-seeded into 6-well plate (1000 pcs/well) for 10 days. Finally, the LLC cells were fixed and stained with crystal violet and imaged.

Biosafety assay

For hemolysis assay, fresh blood sample collected from a healthy mouse was used to separate red blood cells (RBCs) by diluting with PBS and centrifugation (3000 rpm, 10 min). After washing with PBS for several times, the RBCs (2 %) was treated with saline (negative control), H_2O (positive control) and various concentrations of ZnO-Au@mSiO_2 (20 mg/kg), and incubated at 37 °C for 3 h. The mixtures were centrifuged (3000 rpm, 10 min). Finally, the absorbance of the supernatant at 540 nm was recorded to calculate the percent hemolysis.

For biochemical analysis, healthy C57BL/6 mice (n = 3) were intravenously injected with ZnO-Au@mSiO_2 (20 mg/kg). The blood samples were collected and centrifuged on 7-, 14- and 21- days post-injection. The obtained serum was used to detect the level of AST, ALT, ALP, ALB, and UREA for biochemical analysis.

***In vivo* evaluation of targetability and distribution**

C57BL/6 mice bearing with LLC tumor (60-100 mm^3) were injected with CY5.5-labelled ZnO-Au@mSiO_2 (20 mg/kg) and monitored by IVIS imaging systems (Series III, PerkinElmer, USA) to obtain the fluorescence images at various time points (0 h, 3 h, 7 h, 12 h, and 24 h). 24 h later, the mice were sacrificed and the major organs and tumors were collected and analyzed by IVIS imaging systems.

The blood was also collected at different times after administration and detected Zn content by to evaluate the blood circulation profiles and metabolism. The blood was treated with nitric acid and hydrogen peroxide. ICP-MS was applied for quantitative test finally.

***In vivo* anti-tumor therapy**

To further confirm the anti-tumor effects of synergistic enhancement strategy of ZnO-Au@mSiO_2 *in vivo*, we established a subcutaneous LLC tumor model in C57BL/6 mice and randomly divided into four groups (n = 3): PBS (Group 1), PBS + X-ray (Group 2), ZnO-Au@mSiO_2 (Group 3), ZnO-Au@mSiO_2 + X-ray (Group 4). The injection dose was 20 mg/kg. After 12 h intravenous injection, the tumors of group 2 and 4 were treated with X-ray (4 Gy). On Day 7, the same procedure was repeated. The tumor volumes and body weight of mice were measured every two days. Tumor volume

was calculated by $L \times W^2/2$, where L and W refer to the length and width of the tumor, respectively. After the treatment period of 14 days, mice were sacrificed and tumors were obtained for hematoxylin-eosin (H&E) staining.

***In vivo* antitumor immune responses of ZnO-Au@mSiO$_2$**

After 14th day different treatment, spleens and lymphonodus were harvested digested, and resuspended to examine immune cells by FCM (Beckman Cytoflex LX, Beckman Coulter, USA). The single cells suspensions were collected according to the manufacturer's protocols (Dakewei, catalogue no. 7211011). Briefly, to analyse cytotoxic T cells, the single cells from spleens were stained with antibodies against CD8a-APC (BioLegend, catalogue no. 100712), CD3-FITC (BioLegend, catalogue no. 100206) and CD4-PE (BioLegend, catalogue no. 100411). To analyse DC maturation, the single cells were stained with antibodies against CD80-APC (BioLegend, catalogue no. 104714), CD86-PE (BioLegend, catalogue no. 159204), CD11c-FITC (BioLegend, catalogue no. 117306).

Elisa analysis

Serum was collected from each group after different treatment and measured the expression level of TNF-α and Granzyme by an Elisa kit according to the manufacturer's instructions.

Statistical analysis

Data were presented as means ± standard deviation. For multiple comparisons, one-way ANOVA was performed. Statistical significance was indicated by *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Reference

[1] Zhang, Y.,Guo, C.,Liu, L.,Xu, J.,Jiang, H.,Li, D.,Lan, J.,Li, J.,Yang, J.,Tu, Q.,Sun, X.,Alamgir, M.,Chen, X.,Shen, G.,Zhu, J.,Tao, J., ZnO-based multifunctional nanocomposites to inhibit progression and metastasis of melanoma by eliciting antitumor immunity via immunogenic cell death. *Theranostics* **2020,** *10* (24), 11197-11214.

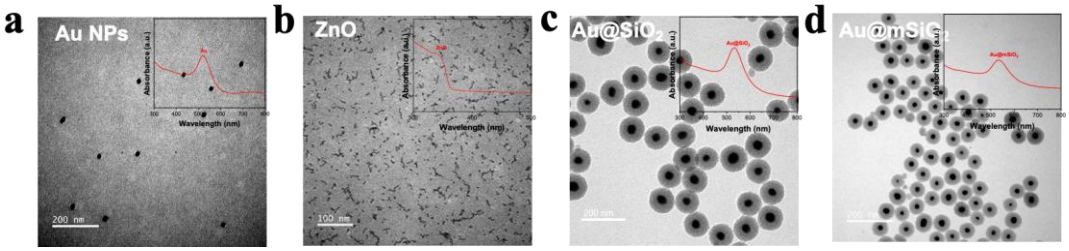

Figure S1. TEM images of (a) Au NPs, (b) ZnO, (c) $Au@SiO_2$ and (d) $Au@mSiO_2$.

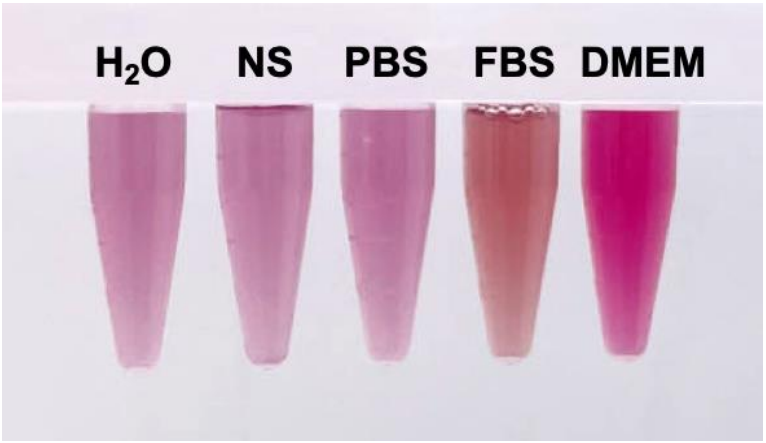

Figure S2. Photographs of ZnO-$Au@mSiO_2$ after being incubated in H_2O, NS, PBS (7.4), serum (FBS) and cell medium (DMEM) for 12 h.

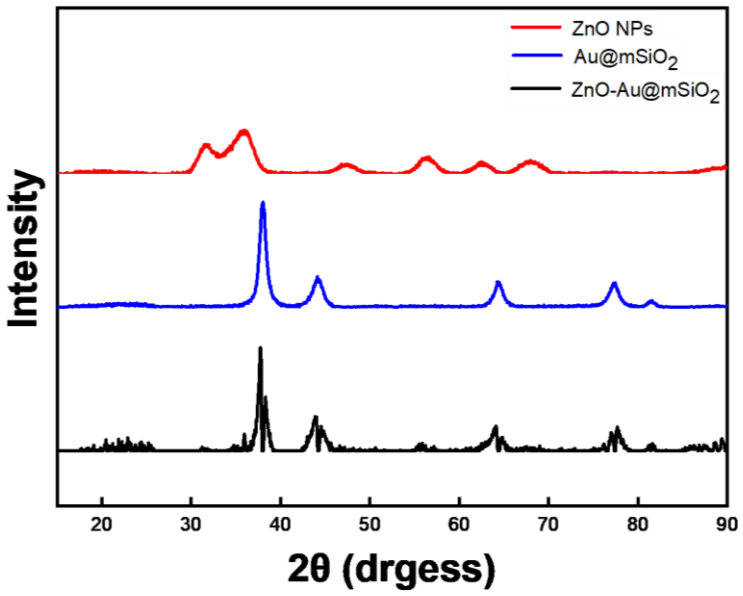

Figure S3. Powder XRD patterns of ZnO nanoparticles, $Au@mSiO_2$, and ZnO-$Au@mSiO_2$.

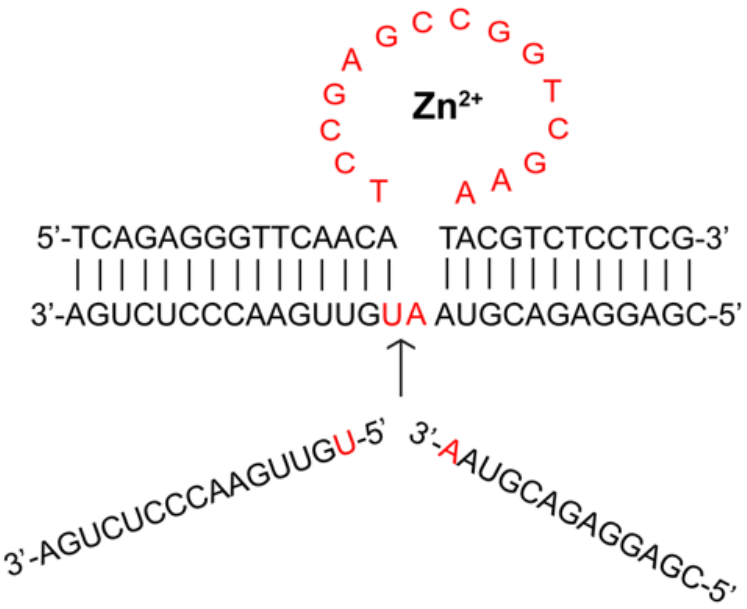

Figure S4. Schematic diagram of DNAzyme cutting PD-L1mRNA. Majuscule marked in red on the mRNA chain is the recognition and cutting site of DNAzyme.

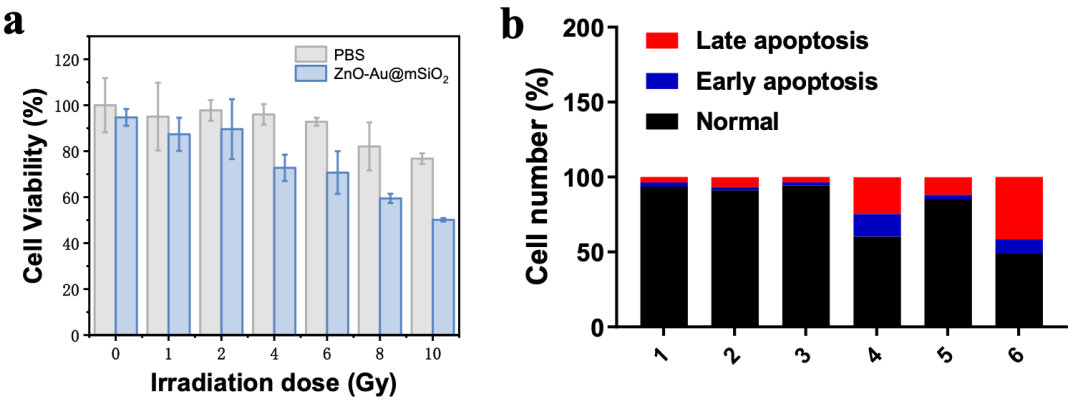

Figure S5. (a) The cytocompatibility assessment of ZnO-Au@mSiO$_2$ on LLC-luc cells under various radiation doses by MTT assay. (b) Early apoptosis and late apoptosis of the LLC cells after treatment with different therapeutic groups.

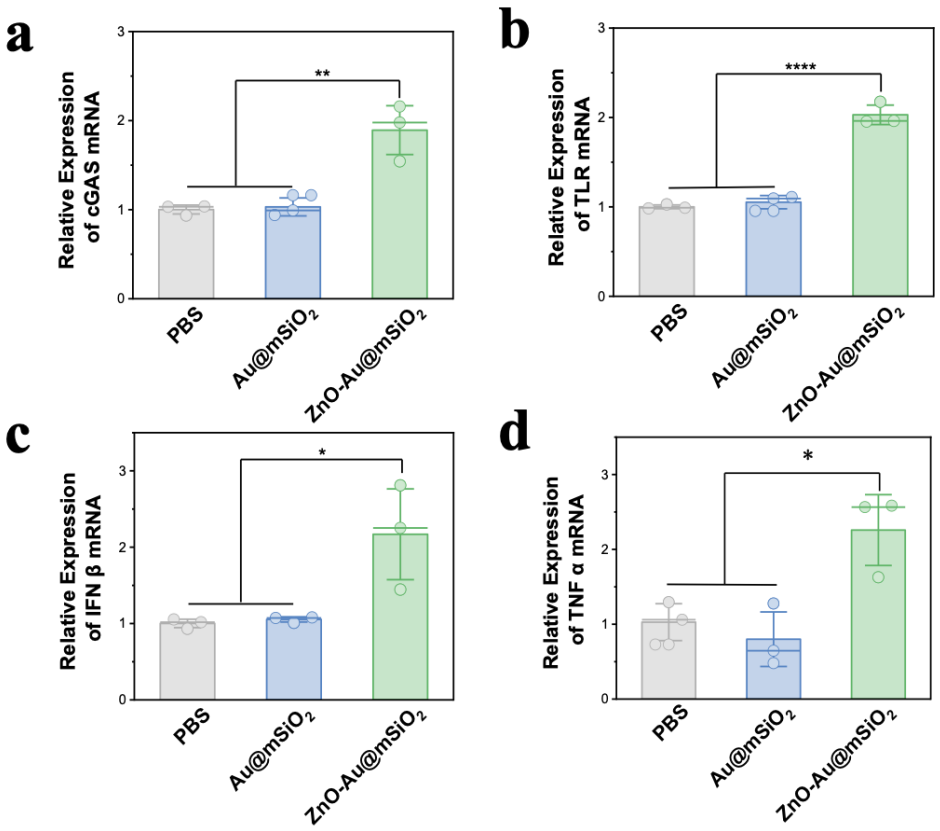

Figure S6. qPCR assay measuring the cGAS mRNA, TLR mRNA, IFN β mRNA and TNF mRNA. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 represent significant difference.

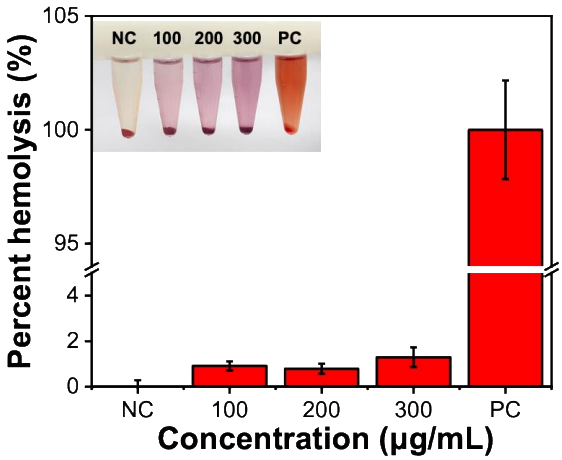

Figure S7. Hemolytic photographs and analysis of red blood cells with different treated.

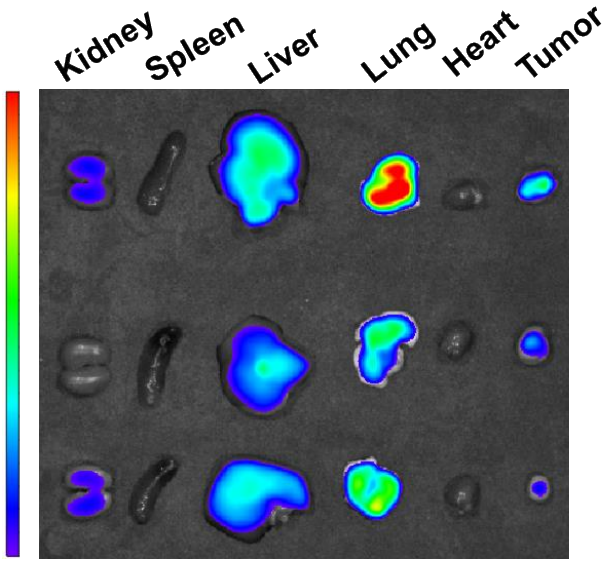

Figure S8. Ex vivo fluorescence images of the organs harvested in C57BL/6 tumor-bearing mice at 24 h post-injection of ZnO-Au@mSiO$_2$-Cy5.5 (Heart, Liver, Lung, Spleen, Kidney and Tumor).

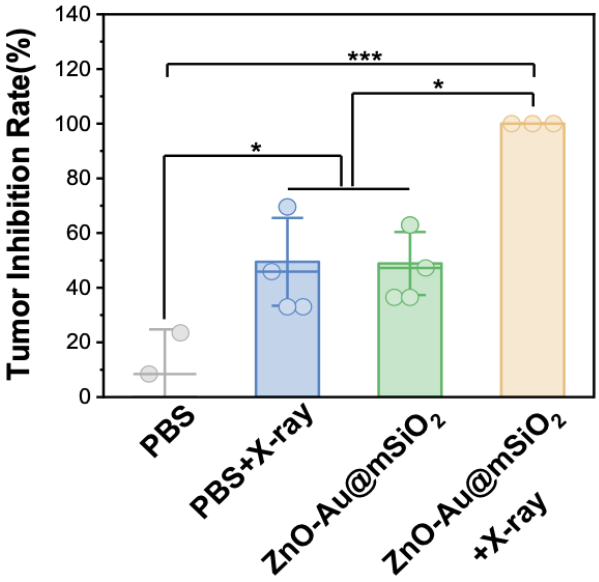

Figure S9. Tumor inhibition rate. *p < 0.05, **p < 0.01, ***p < 0.001 represent significant difference.

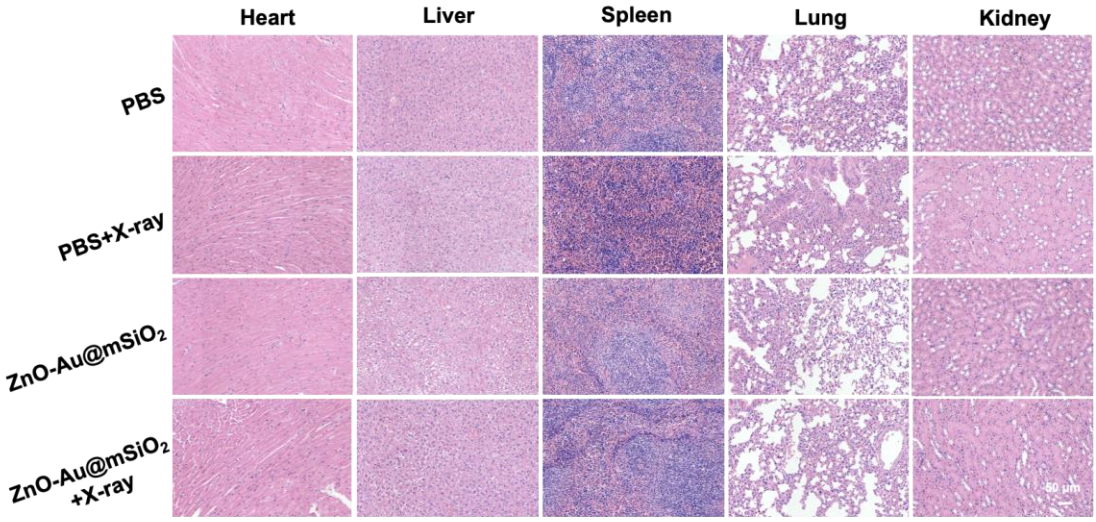

Figure S10. H&E staining images of major organs (Heart, Liver, Spleen, Lung and Kidney) collected on Day 15 after different treatments (Scale bar, 50 μm).

Table 1. Blood biochemistry and hematology analysis of mice on day 0, day 7, day 14 and day 21 (n = 3).

Project abbreviation	Day 0	Day 7	Day 14	Day 21
WBC（*10^9/L）	5.63±0.72	8.1±1.75	7.8±1.79	11.6±1.76
HGB（g/L）	149±13.54	126.25±11.15	127±6.78	136.25±6.08
RBC（*10^{12}/L）	9.04±0.74	8.14±0.54	7.72±0.18	8.58±0.39
HCT（%）	45.63±4.02	40.35±2.85	41.05±1.67	43.03±1.39
MCV（fL）	50.5±0.48	49.65±0.58	53.23±1.19	50.25±0.81
MCH（pg）	16.43±0.25	15.48±0.48	16.43±0.51	15.85±0.54
MCHC（g/L）	326±3.16	312±6	308.75±7.63	316±9.93
MPV（fL）	6.05±0.82	6.55±1.1	6.93±1.01	5.83±0.21
PDW	15.58±1.59	16.05±1.81	16.55±1.45	14.33±0.1
ALT（U/L）	9.13±2.17	12.3±1.99	12.33±1.5	8.65±1.31
AST（U/L）	24.9±6.16	22.68±1.56	16.73±19.76	32.8±9.85
ALP（U/L）	87.48±5.47	80.38±7.95	77.15±12.91	92.08±14.58
ALB（g/L）	9.08±0.84	8.78±0.74	9.35±1.45	9.65±0.44
UREA（mmol/L）	4.49±0.44	4.6±0.71	3.95±0.92	4.45±0.35

Table 2. The nucleotide sequence required for the experiment (5’-3’)

Name	Sequence（5’-3’）
DNAzyme	TCAGAGGGTTCAACA TCCGAGCCGGTCGAA TACGTCTCCTCG
PD-L1-mRNA Substrate	CGAGGAGACGTA/rA/GCAGTGTTGAACCCTCTGA

Table 3. QPCR primer sequence (5'-3')

Primer Name	Sequence (5'-3')
PD-L1 F	CTACGGTGGTGCGGACTACAA
PD-L1 R	GGATAACCCTCGGCCTGACATA
CALR F	GCAGACCCTGCCATCTATTTC
CALR R	TCGGACTTATGTTTGGATTCGAC
HMGB1 F	GCTGACAAGGCTCGTTATGAA
HMGB1 R	CCTTTGATTTTGGGGCGGTA
STING F	ATATCTGCGGCTGATCCTGC
STING R	GGTCTGCTGGGGCAGTTTAT
IFN β F	ACTGCCTCAAGGACAGGATG
IFN β R	AGCCAGGAGGTTCTCAACAA
TLR F	CTGCCACATGACCATCGAG
TLR R	GGACAGGGATATGAGGGATTTGG
TNF α F	GCTGCACTTTGGAGTGATCG
TNF α R	TCACTCGGGGTTCGAGAAGA

Table 4. Flow cytometric antibodies list.

Antibody name	Product code	Source
FITC anti-mouse CD3	100203	Biolegend
PE anti-mouse CD8a	100707	Biolegend
APC anti-mouse CD4	100411	Biolegend
PE anti-mouse CD11c	117307	Biolegend
APC anti-mouse CD80	104713	Biolegend
FITC anti-mouse CD86	100712	Biolegend
PD-L1 antibody	ab213480	Abcam
IFN-β antibody	IPB3809	Baijia
TBK1 antibody	IPB6368	Baijia
p-TBK1 antibody	IPH0271	Baijia
HMGB1 antibody	BMB1829	Baijia
CRT antibody	IMB0350	Baijia