Electronic Supplementary Material (ESI) for ChemComm.

Electronic Supplementary Information

Photothermal therapy-induced immunogenic cell death based on natural melanin nanoparticles against breast cancer

Yanhua Li‡, Xiaohan Liu‡, Wei Pan, Na Li and Bo Tang**

Experimental details:

Materials. Cuttlefishes were purchased from market. 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma (Shanghai, China). Protease inhibitor was purchased from Medchemexpress (MCE), USA. The INCB24360 analogue, 4-amino-N-(3-chloro-4-fluorophenyl)-N'-hydroxy-1,2,5-oxadiazole-3-carboximidamide (IDOi) was purchased from Medchemexpress (MCE), USA. Penicillin/streptomycin were purchased from Gibco. Roswell Park Memorial Institute medium (RPMI 1640) was purchased from KeyGEN biotechnology (Nanjing, China). Fetal bovine serum (FBS) was purchased from Biological Industries, Israel. Trypsin-EDTA was purchased from Gibco, USA. The mouse breast cancer cell line (4T1) was purchased from Shanghai AOLU Biological Technology Co. Ltd, China. PBS buffer was purchased from Sangon Biotech Co., Ltd., China. The Mouse IL-12 Mini ELISA Kit and Mouse IL-6 ELISA Kit were purchased from Boster Biological Technology Co., Ltd. Anti-CRT-Alex 647 was purchased from Abcam, England. Anti-CD3 and CD8 were purchased from Boster Biological Technology Co., Ltd. Antibody diluent was purchased from Beijing Solarbio Science & Technology Co., Ltd. Glass Bottom dishes were purchased from Cellvis. 96-well plates were purchased from Hangzhou Xinyou Biotechnology Co., Ltd. Plastic centrifuge tubes were purchased from GeneBrick Bioscience LLC. All the aqueous solutions used in experiments were prepared using deionized water (18.2 MΩ cm) obtained from a Milli-Q water purification system. All chemicals were of analytical grade and were used without further purification.

Instruments. Transmission electron microscopy (TEM) was carried out on a JEM-100CX II electron microscope. UV-Vis absorption spectra were measured on pharmaspec UV-1700 UV-Visible spectrophotometer (Shimadzu, Japan). MTT assay was performed in a microplate reader (Synergy 2, Biotek, USA). Dynamic light scattering and zeta potential measurements were conducted with Malvern Zeta Sizer Nano (Malvern Instruments). Confocal fluorescence imaging assay were performed with a TCS SP8 confocal laser scanning microscopy (Leica,

Germany). Photoacoustic imaging was performed using an Endra Nexus 128 (Ann Arbor, Michigan). Western Blotting was performed by ChemiDoc XRS gel imaging system (bio-rad, Hercules, CA, USA).

Cell lines and animals. 4T1 cells were cultured in RPMI 1640 medium containing 10% FBS and 100 U mL^{-1} of 1% antibiotics (penicillin/streptomycin) and were maintained at 37 °C in a 5% CO_2/95% air humidified incubator (SANYO). All animal procedures were carried out according to the Principles of Laboratory Animal Care (People's Republic of China) and were approved by the Animal Care and Use Committee of Shandong Normal University (Jinan, China).

The preparation of melanin nanoparticles. The ink sac was extracted from cuttlefish. Then melanin nanoparticles were collected from the ink sac by differential centrifugation. In detail, melanin nanoparticles were firstly collected from the ink sac and dispersed in deionized water. The above solution was firstly centrifugated (500 rpm, 23 rcf, 10 min) to remove the largest particles. Then the supernatant was centrifugated (1000 rpm, 94 rcf, 10 min) to remove the second largest particles. Next, the supernatant was centrifugated (5000 rpm, 2348 rcf, 10 min) to remove the third largest particles. Eventually, the supernatant was centrifugated (12000 rpm, 13523 rcf, 10 min) to obtain the required nanoparticles. The obtained nanoparticles were dried in vacuum drying chamber for further used. To study the morphology of nanoparticles, 300 μg M NPs or M@C NPs was uniformly dispersed in 2 mL water and then dripped onto the carbon supported copper mesh for obtaining TEM images on a HT7700 electron microscope. 300 μg M NPs or M@C NPs was also uniformly dispersed in 2 mL water to be characterized by DLS, Zeta potential, UV-Vis. For the FI-IR characterization, melanin nanoparticles were dried at 40 ℃ in the oven.

The preparation of melanin nanoparticles coated with the membrane of cancer cells (M@C). When the 4T1 cell density reached 80% ~ 90%, the culture medium was wiped off. Then 2 mL of PBS was added to remove remaining medium and 1 mL of trypsin containing

0.25% EDTA was used to digestive cells for 3 min in the incubator. 2 mL RPMI 1640 complete medium was added to terminate digestion. Then the 4T1 cells were centrifuged (1000 rpm, 3 min) and washed twice with Tris-HCl buffer (pH 7.4). Finally, the Tris-HCl buffer with 1% protease inhibitor was added to resuspend the cells. Membrane fragments were achieved by mechanical membrane disruption. After sonication, membrane fragments and melanin nanoparticles mixture was stirred for 24 h in an ice-water bath to obtain melanin nanoparticles coated with membrane of 4T1 cells (M@C). Then M@C nanoparticles were characterized by TEM, DLS, Zeta potential and UV-Vis.

The photothermal performance measurement of materials. Different concentrations of melanin nanoparticles were prepared (0, 50, 100, 150 μg/mL). The measurement was performed under the irradiation 808 nm laser at 2 W/cm^2 for 500 s. The change of temperature was determined by infrared thermal image instrument per 20 s. The photographs were taken at the steady state temperature.

The photothermal stability of materials. In order to determine the photothermal stability of the nanoparticles, melanin nanoparticles solution (150 μg/mL) was irradiated under 808 nm laser (2 W/cm^2). Infrared thermal image instrument recorded the temperature every 20 s until reaching the steady state temperature. Then the solution was cooled naturally to the initial temperature with infrared thermal image instrument record temperature every 20 s record, too. Subsequently, the measurement was repeated.

Western blotting. Cancer cell membrane protein was characterized by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method. To obtain the protein sample, 4T1 cells, cell membrane extracted from 4T1 cells and M@C nanoparticles were lysed with RIPA lysis buffer. The concentration of protein was quantified using BCA protein assay kit. SDA-PAGE protein loading buffer was mixed with the protein samples and the solution was heated with boiling water for 10 min in order to make the protein fully denatured. Subsequently, samples with equal protein amount were added into the wells with 10% SDS-PAGE gel, and

SDS-PAGE gel electrophoresis was used to separate protein. Afterwards, the proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were blocked with western blocking buffer shaked slowly on a shaker at room temperature for 60 min and incubated with primary antibody at 4 °C. Then horseradish peroxidase-labeled goat anti-rabbit IgG (H + L) was added with incubation for 1 h. The signals of protein were checked by chemiluminescence enhancement method. The record information was obtained by the imaging system.

Cytotoxicity assay. The cells were planted into two 96-well plates, and incubated with different concentrations (0, 5, 25, 50, 75, 100, 150, 200, 400, 800, 1000 μg/mL) of M@C nanoparticles. After incubated for another 24 h, the cell culture medium was removed and cells were washed with PBS buffer for three times. Then DMSO was directly added to one 96-well plate and the absorbance at 490 nm was measured. These background signals were named as B1, B2, B3, ….; Then 200 μL MTT (0.5 mg/mL) was added into the other 96-well plate and DMSO was used to dissolve formazan crystal 4 h later. The absorbance at 490 nm was also measured. These experimental signals were named as E1, E2, E3, …. The final absorbance results were obtained by the E signals minus the corresponding B signals, so the interference of melanin nanoparticles was eliminated.

Phototoxicity assay. 4T1 cells were cultivated in 96-well plates in a humidified atmosphere with 5% CO_2 at 37 °C. After 24 h, they were treated with PBS (Control); Laser (L); M@C; M@C+L for 4 h. The groups needed laser were irradiate under 808 nm (2 W/cm^2) laser for 5 min. After incubated for another 24 h, 200 μL MTT (0.5 mg/mL) was added into the wells and treated for 4 h. DMSO was used to dissolve formazan crystal. The absorbance at 490 nm was measured by microplate reader. The interference of melanin nanoparticles was also eliminated according to the above procedure.

In vitro PA imaging (PAI) assay. For PAI in vitro, different concentrations (0, 10, 25, 50, 100, 150, 200 μg/mL) of M@C NPs were prepared. The PA signals were measured by PA imaging system.

In vivo PA imaging assay. 4T1 tumor-bearing mice were prepared for PA imaging in vivo. When the tumor volume was approximately 100 mm^3, the mice were treated with 100 μL (4 mg/mL) Melanin (M) and M@C solution injected intravenously. PA imaging was conducted at different time intervals (0, 1, 4, 8, 12, 24 h) after injection.

Photothermal imaging of mice. Mice were intravenously injected with PBS, M, and M@C, respectively. After 12 h, the tumors of mice were irradiated with 808 nm (2 W/cm^2) laser for 5 min, and the pictures were taken at different time.

Evaluation of calreticulin (CRT). The immunofluorescence staining was performed to study the calreticulin (CRT) of 4T1 cells with different treatments. 4T1 cells were divided into five groups: M@C+L, M+L, M@C, only laser (L), PBS. Then the cells were fixed and staining with anti-CRT-Alex 647 and Hochest 33342. Finally, the cells were studied by confocal fluorescence imaging.

Cell invasion assay. Firstly, 4T1 cells in the cell-culture dishes (35 mm × 10 mm) were treated with M@C+L, M+L, M@C (150 μg/mL melanin in 1 mL medium), L and PBS, respectively. 24 h later, the invasion ability of 4T1 cells were studied. In this assay, invasive chamber coated with matrigel was applied to simulate the natural matrix membrane structure. 5×10^4 suspended 4T1 cells were planted into the upper compartment. After incubated for 24 h, the noninvasive cells on the upper surface of matrigel were removed and the invasive cells were fixed with 4% paraformaldehyde and further stained with 0.2% crystal violet for 30 min before photographing. After washing with PBS, images were acquired.

Evaluation of immunologic factors. 4T1 cells were inoculated subcutaneously into female Balb/c mice. And the mice were divided into six groups: PBS, only laser (L), M@C+L+IDOi, M@C+L, M+L+IDOi, M@C+IDOi. Subsequently, blood samples were collected at 12 h after

treatment. Then serum samples were isolated by centrifugation for evaluation of immunologic factors by enzyme-linked immunosorbent assay (ELISA).

Evaluation of in vivo antitumor efficacy. 4T1 tumor-bearing Balb/c mice were divided into six groups: PBS, only laser (L), M@C+L+IDOi, M@C+L, M+L+IDOi, M@C+IDOi. The mice with "L" groups were irradiated with 808 nm (2 W/cm^2) laser for 5 min at 12 h after injection intravenously. The temperature changes were recorded with infrared thermal image instrument and the photographs were taken every minute. After 12 h, IDOi was treated with intraperitoneal injection. Tumor volumes and body weights were observed every other day. On day 15, the main organs were collected for histological sectioning and H&E staining.

Data availability. All relevant data are available from the authors, and/or are included within the manuscript and supplementary data.

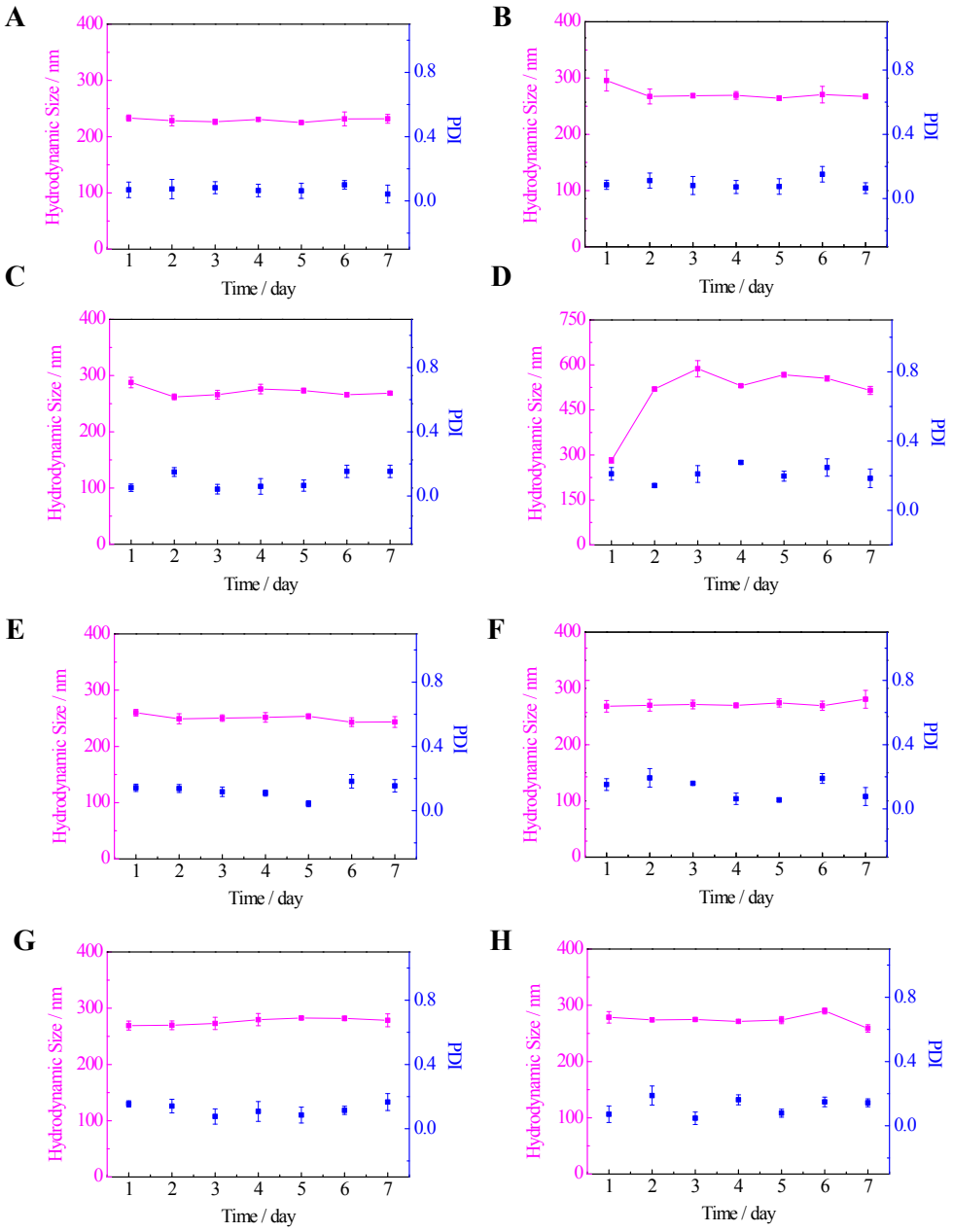

Figure S1 The hydrodynamic size and PDI of M NPs in water (A), 1640 medium (10% serum) (B), cell lysis buffer (C) and PBS (D); The hydrodynamic size and PDI of M@C NPs in water (E), 1640 medium (10% serum) (F), cell lysis buffer (G) and PBS (H).

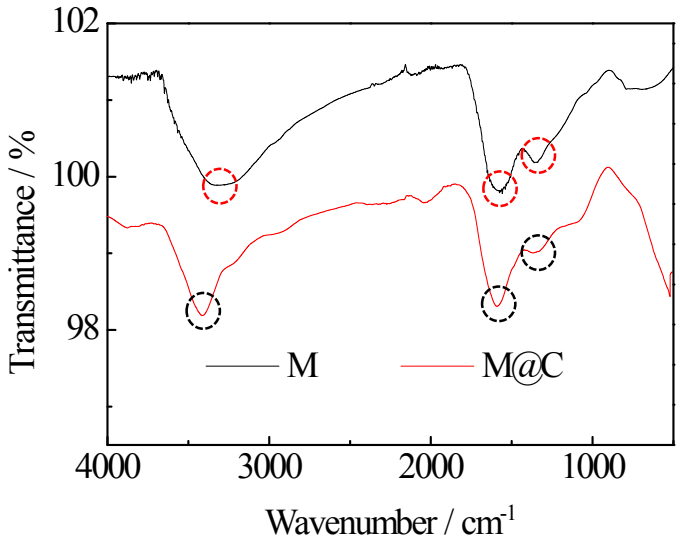

Figure S2 The FT-IR of M NPs and M@C NPs.

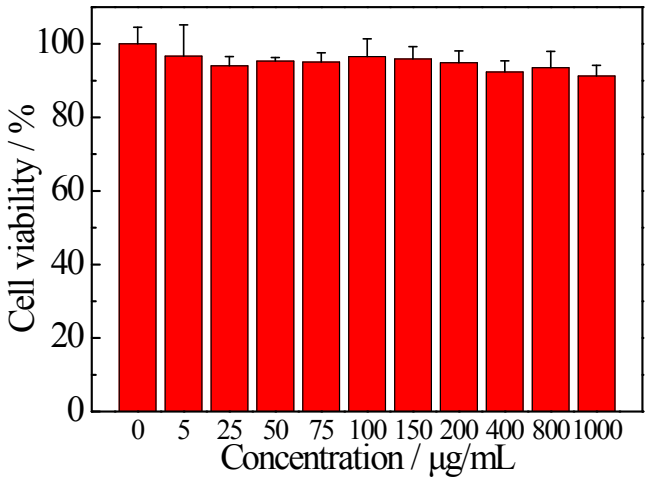

Figure S3 The cytotoxicity of M@C NPs.

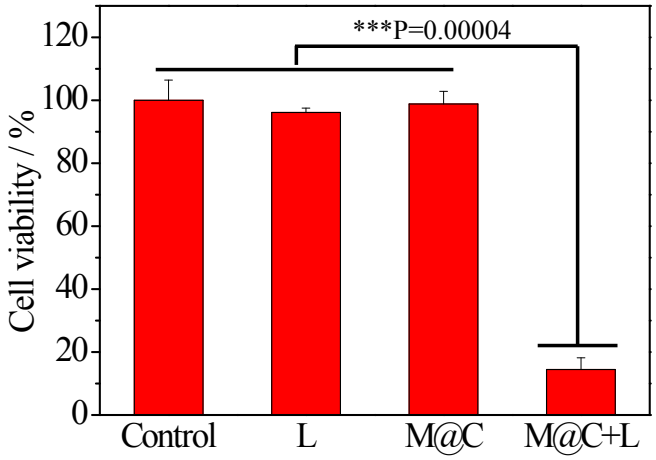

Figure S4 The cytotoxicity of laser (L); M@C NPs; and M@C NPs with laser (M@C+L). The concentration of M@C NPs was 150 μg/mL and the laser power density was 2 W/cm^2. P values were calculated using the t-test (***P < 0.001, **P < 0.01, *P < 0.05).

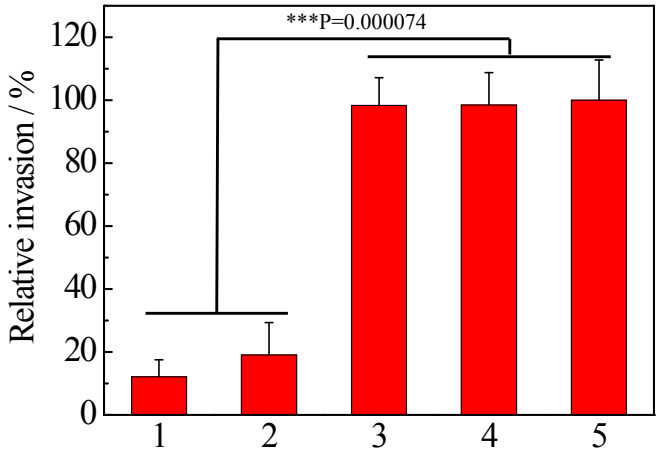

Figure S5 Quantitative results of invading cells (1) M@C+L, (2) M+L, (3) M@C, (4) L, (5) PBS. P values were calculated using the t-test (***P < 0.001, **P < 0.01, *P < 0.05).

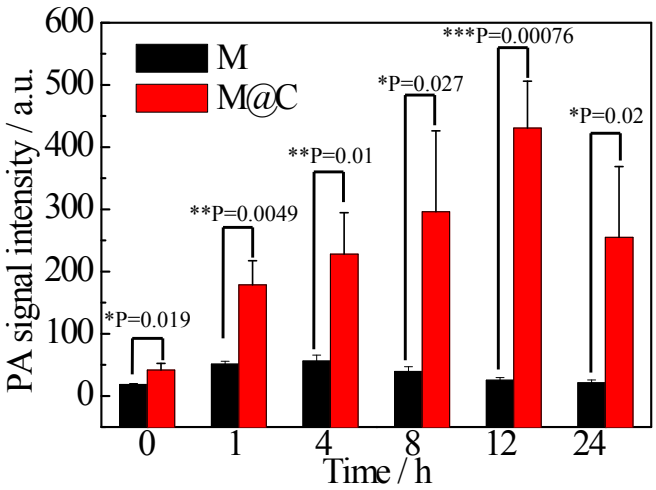

Figure S6 Quantification of photoacoustic signals in tumors. P values were calculated using the t-test (***P < 0.001, **P < 0.01, *P < 0.05).

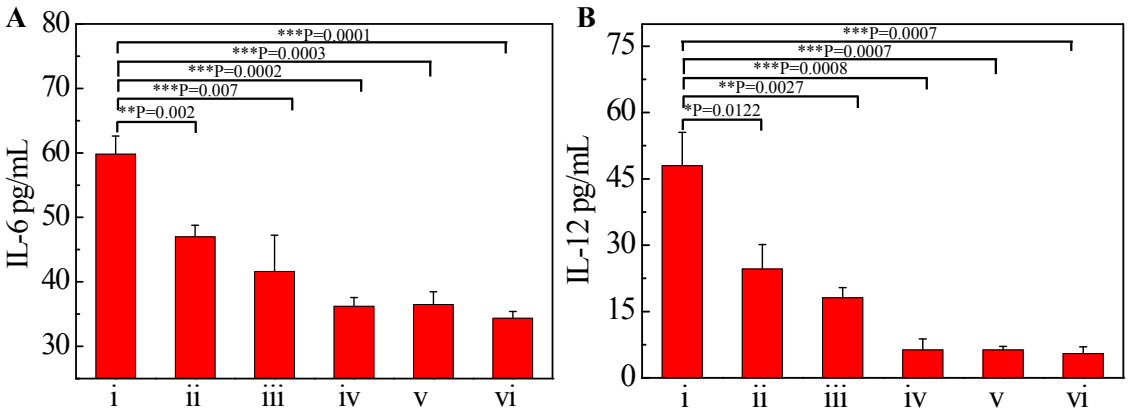

Figure S7 ELISA analysis of IL-6 (A) and IL-12 (B). i-vi represent mice were treated with: (i) M@C+L+IDOi, (ii) M@C+L, (iii) M+L+IDOi, (iv) M@C+IDOi, (v) L, (vi) PBS. P values were calculated using the t-test (***P < 0.001, **P < 0.01, *P < 0.05) to compare other groups with group i.

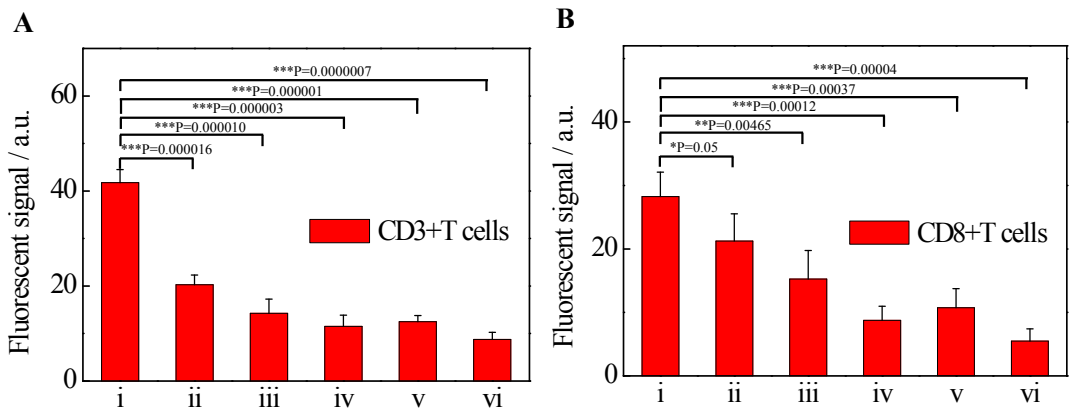

Figure S8 Quantification of fluorescent signals from immunofluorescent staining of CD3+ T cells (A) and CD8+ T cells (B) in tumors.

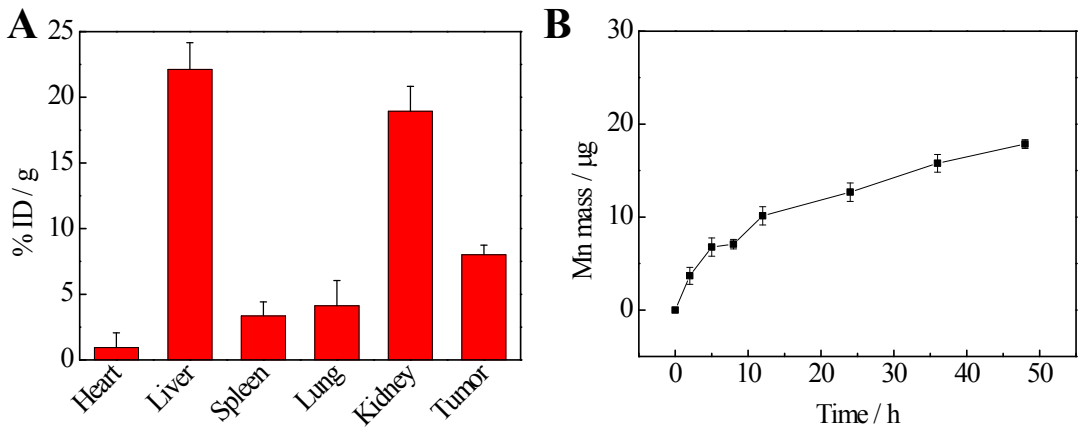

Figure S9 The distribution of M(Mn)@C NPs in heart, liver, spleen, lung, kidney and tumor at 12 h post intravenous injection (A). Mn amount in urine and feces of mice collected at various time points after injection (B).

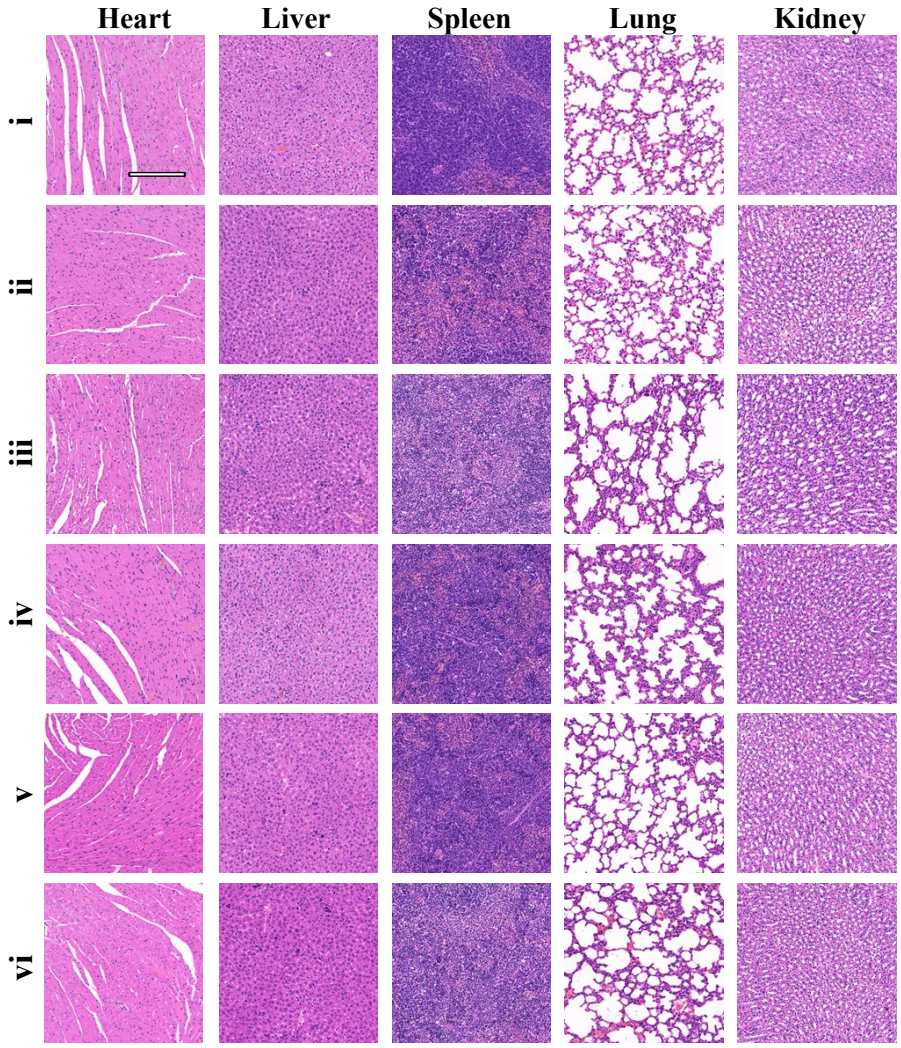

Figure S10 H&E stained images of major tissues obtained from different groups of mice: (i) M@C+L+IDOi (ii) M@C+L (iii) M+L+IDOi (iv) M@C+IDOi (v) L (vi) PBS. Scale bar = 200 μm.

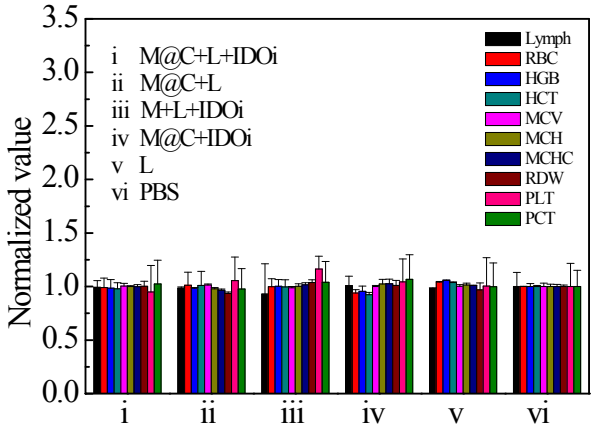

Figure S11 The routine blood test of mice after treatment with (i) M@C+L+IDOi (ii) M@C+L (iii) M+L+IDOi (iv) M@C+IDOi (v) L (vi) PBS.

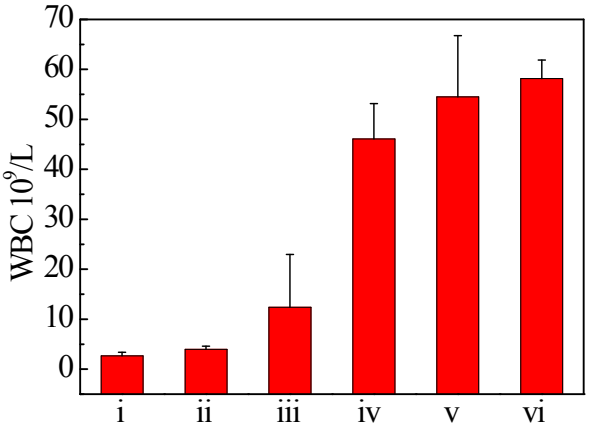

Figure S12 The white blood cell (WBC) of mice after treat with (i) M@C+L+IDOi (ii) M@C+L (iii) M+L+IDOi (iv) M@C+IDOi (v) L (vi) PBS. The normal range is 0.8-6.8^10^9/L.

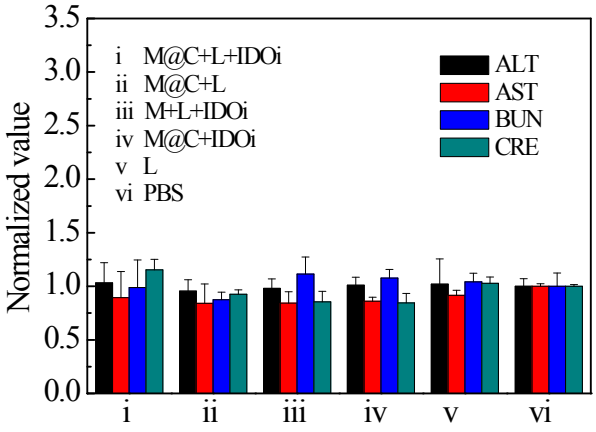

Figure S13 The blood biochemical indexes of mice after the treatment with (i) M@C+L+IDOi (ii) M@C+L (iii) M+L+IDOi (iv) M@C+IDOi (v) L (vi) PBS.

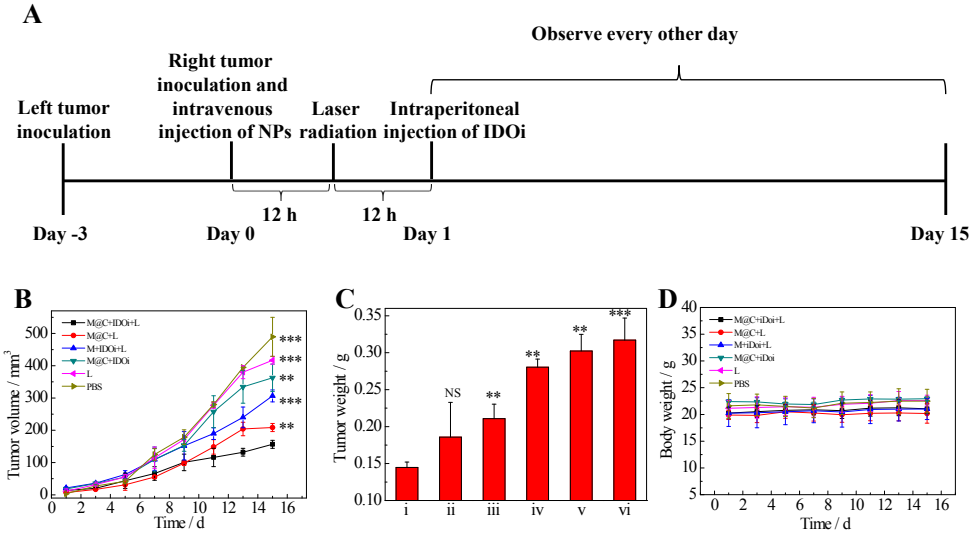

Figure S14 Photothermal therapy combined with immunotherapy to inhibit growth of distance tumors in vivo. Schematic illustration of M@C+L+IDOi to inhibit tumor growth at distant sites (A). The changes of distance tumors (B) tumor weights (C) body weights (D) of mice with different treatments: (i) M@C+L+IDOi (ii) M@C+L (iii) M+L+IDOi (iv) M@C+IDOi (v) L (vi) PBS. P values were calculated using the t-test (***P < 0.001, **P < 0.01, *P < 0.05, NS P > 0.05) to compare other groups with group i.