Type I Macrophage Activator Photosensitizer Against Hypoxic Tumor

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MATERIALS and METHODS

Materials. Chemicals were purchased from J&K, Sigma-Aldrich and TCI and used directly without further purification. 2,2,6,6-Tetramethyl-4-piperidone (TEMP) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were purchased from Dojindo Laboratories. 1,2-Distearoyl-*sn*-glycero-3-phosphoethano-lamine-*N* [methoxy (polyethylene glycol)-2000] (DSPE-PEG_{2000}) was purchased from Nanocs. Phosphate buffered saline (PBS, 1×) was purchased from Servicebio. Dulbecco's modified eagle medium (DMEM), Rosewell Park Memorial Institute (RPMI)-1640 medium and fetal bovine serum (FBS) were purchased from Gibco. Penicillin-streptomycin solution, and trypsin-EDTA (0.5% trypsin and 5.3 mM EDTA tetrasodium) were commercial products from ThermoFisher. TNF-α，IL-12 and TGF-β1 ELISA kits were purchased from 4A Biotech Co., Ltd. HMGB1 and HSP70 ELISA kits were brought from JINGMEI. NF-κB, p-NF-κB, β-actin antibodies for WB, F4/80, CD11c, HIF-1α antibodies and anti-rabbit IgG (FITC conjugate) for immunostaining, CD11c (FITC conjugate), CD4 (FITC conjugate) and CD8 for flow cytometric were brought from Cell Signaling Technology. CD206 antibody, goat anti-rabbit IgG, anti-rabbit IgG (Alexa-488, Alexa-555, APC conjugate) and goat anti-mouse IgG were obtained from Abcam. Immunostaining permeabilization buffer with triton X-100, Cell Counting Kit-8 and ATP kit were obtained from Beyotime Biotechnology. Anti-F4/80 MicroBeads UltraPure was brought from Miltenyi Biotec. ^{1}H and ^{13}C NMR spectra were recorded on the Bruker AV400 spectrometers using $CDCl_3$ as the solvent. High-resolution mass spectra were performed on Q-Exactive with Dionex Ultimate 3000. Time-resolved

fluorescence spectra were measured on PicoQuant with FluoTime 300. UV-Vis absorption spectra were recorded on a Shimadzu UV-2600 spectrometer. Photoluminescence (PL) spectra were recorded on the Hitachi F-4600 fluorescence spectrometer. The hydrodynamic diameters were determined using a Zetasizer Nano system (Malvern instrument) at room temperature. All animal experiments were performed in strict accordance with the guidelines for Care and Use of Laboratory Animals of SUSTech and approved by the Animal Ethics Committee of the Center for Experimental Animal Research of SUSTech (SUSTC-2018-115), China.

Synthesis of TPA-DCR. A mixture of 4-(diphenylamino)benzaldehyde (227 mg, 0.83 mmol), 2-(4-oxo-3-phenylthiazolidin-2-ylidene)malononitrile (200 mg, 0.83 mmol) and ammonium acetate (100 mg, 1.3 mmol) was added into acetic acid (8 mL) and refluxed at 120 °C for 12 h under Argon atmosphere. After cooling to the room temperature, the reaction was quenched by water. Then, the precipitate was filtered and washed three times with cold MeOH. The crude product was further purified by flash column chromatograph (silica gel, dichloromethane/EtOH = 15/1) as the eluent to afford a red solid (251 mg, 60% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.94 (s, 1H), 7.65 – 7.55 (m, 7H), 7.44 (t, J = 7.8 Hz, 4H), 7.23 (dd, J = 7.5, 6.0 Hz, 6H), 6.97 (d, J = 8.8 Hz, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 166.26, 165.86, 151.26, 145.75, 137.26, 132.91, 132.63, 131.72, 130.17, 129.86, 129.75, 128.78, 126.36, 125.48, 123.97, 119.89, 113.31, 111.63, 109.66, 56.94. HRMS (ESI): Calcd for: $C_{31}H_{21}N_4OS^+$ ($[M+H]^+$): 497.14306. Found: 497.14279.

Synthesis of AIEgens NPs. TPA-DCR (1.0 mg) and DSPE-PEG_{2000} (5 mg) were dissolved in THF (1 mL) by an ultrasonic dispersion, and then the homogeneous mixture was quickly added into ultrapure water (9 mL). After sonication using a probe sonicator at 20% output (VCX150, Sonics) for 2 min, the mixture was purified by dialysis (molecular weight cut-off 10 KDa) for 2 days to eliminate the THF. Then, the TPA-DCR NPs were collected and concentrated by centrifugal filters for further use.

QY measurements. The TPA-DCR NPs were dispersed in water and Rhodamine B was dissolved in ethanol at varied concentrations. The UV-Vis and PL spectra of TPA-DCR NPs and Rhodamine B were recorded. The integrated area of fluorescent profile was plotted against concentration to obtain the slope. QY_{ref} of Rhodamine B is 97% in ethanol, the refractive index of n_{sample} and n_{ref} was 1.333 and 1.003, respectively. The quantum yield was calculated according to the following equation:

$$QY_{sample} = QY_{ref}\frac{slope_{sample}}{slope_{ref}}\left(\frac{n_{sample}}{n_{ref}}\right)^2 = 97\% \times \frac{110278.7}{645371.8}\left(\frac{1.333}{1.003}\right)^2 = 29.3\%$$

Fluorescence decay measurements. The time-resolved fluorescence spectra were measured on PicoQuant with FluoTime 300 and Spectrofluorometer FS5 of EDINBURGH INSTRUMENTS. According to the time-resolved fluorescence spectra, the fluorescence lifetimes of TPA-DCR aggregates (H_2O/THF = 9/1) and NPs are 2.33 ns and 2.02 ns, respectively.

Total ROS generating ability test. 2',7'-Dichlorodihydrofluorescein (DCFH; 25 μL, 40 μM) as an indicator of total ROS was added into photosensitizers (TPA-DCR

aggregates, TPA-DCR NPs, Chlorin e6, 10 μM) of 1× PBS solution (1 mL). Upon irradiation with white light (40 mW cm^{-2}) for different times, the fluorescence spectra of 2',7'-dichlorofluorescein (DCF) were measured under excitation at 480 nm. The relative emission intensity ($I/I_0 - 1$) at 523 nm versus irradiation time was then plotted. In addition, AIEgens or DCFH alone were tested under the same experimental conditions as the control.

Singlet oxygen generating ability test. 2,2'-(Anthracene-9,10-diylbis(methylene)) dimalonic acid (ABDA; 20 μL, 10 mM in dimethyl sulfoxide) as an indicator of singlet oxygen was added into TPA-DCR or TPA-DCR NPs (10 μM) of 1× PBS solution (1 mL). Upon irradiation with white light (40 mW cm^{-2}) for different times, the absorption spectra of ABDA were measured. The relative absorption intensity (A/A_0) at 379 nm versus irradiation time was then plotted. In addition, ABDA with methylene blue (MB) or ABDA alone were tested under the same experimental conditions as the control.

Detection of •OH and $^1O_2$ in EPR. 2,2,6,6-Tetramethyl-4-piperidone (TEMP) and 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) were selected as spin-trap agents to detect $^1O_2$ and •OH, respectively. They were first mixed with TPA-DCR NPs (1 mg/mL according to the concentration of TPA-DCR) at 100 mM, and treated with white light irradiation (40 mW cm^{-2}) for 2 minutes, followed by recording the EPR spectra within 5 min. The EPR spectra of TPA-DCR NPs without light irradiation, TPA-DCR NPs free and spin-trap agents free were also measured for comparison.

Cell culture. RAW264.7 cells were maintained in DMEM medium with high

glucose and 4T1 cells were maintained in RPMI-1640 medium, supplemented with 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin and 10% heat-inactivated FBS. Cells were cultured at 37 °C in a 5% CO_2 incubator.

Cell viability test. The RAW264.7 cells were seeded in 96 well plates at a density of 1×10^5 cells per well for 12 h. TPA-DCR NPs at different concentrations were added into the cell culture medium, separately. The cells were then immediately irradiated by white light immediately for 3 min. After irradiation, the photosensitizer suspension was discarded and fresh medium was added to each well for further incubation for 24 h before testing the light cytotoxicity. On the other hand, the cells were incubated with different concentrations of photosensitizer for 24 h without washing and light irradiation to test the dark cytotoxicity. After treatment, the CCK-8 solution was added into medium for another 1 h incubation, and the absorbance of CCK-8 at 450 nm was measured. The cell viability was determined by the ratio of the absorbance of the cells incubated with different photosensitizers to that of the cells incubated with normal culture medium.

Measurement of TNF-α, IL-12p70 and TGF-β1. The RAW264.7 cells were pre-induced into a M2 phenotype by treatment with IL-4 (40 ng mL^{-1}) and IL-13 (20 ng mL^{-1}) for 48 h. RAW264.7 cells without further polarization (M0) and those induced into M2 phenotype (M2) were then seeded in the 24-well culture plates (2×10^5 cells per well), respectively. TPA-DCR NPs with increased concentrations (12.5, 25, 50, and 100 μM) were added into the wells, followed by irradiation with a white light (10 mW

cm^{-2}) for 3 min. After 24 h, the culture medium was collected, and the expression levels of various secreted cytokines were determined by enzyme-linked immunosorbent assay (ELISA), according to the manufacturer's instruction.

Immunofluorescence assay. After activation, macrophages were fixed in 4% paraformaldehyde for 10 min and incubated with anti-NF-κB p65 antibody (1:400) for 12 h at 4 ℃. Then the cells were washed three times with 1× PBS buffer and incubated with FITC secondary antibody (1:1000) for 1 h at room temperature. The cell nuclei were stained with DAPI for 5 min at room temperature. The location of NF-κB p65 was finally assessed using Lecia SP8 confocal fluorescence microscopy.

Protein expression measured by Western blotting. Western blotting method was used to measure the expression of p-NF-κB and CD206 in RAW264.7 cells after treatment. The total proteins of cell lysates were collected, and 25 μg of proteins were loaded on the SDS-PAGE and transferred to a PVDF membranes. Then the PVDF membranes were incubated with different primary antibodies and corresponding HRP-conjugated secondary antibodies subsequentially. Finally, the PVDF membranes were observed by a Tanon 5200Multi Chemiluminescence Imaging System.

Polarization of M0 and M2 macrophages by TPA-DCR NPS under hypoxic condition. The M0 cells were seeded in the 24-well plate (2×10^5 cells per well). The TPA-DCR NPs-supplemented culture medium was charged with argon to expel oxygen for 30 min before light irradiation. Then this anaerobic medium was immediately added to the 24-well plate, followed by irradiation with a white light (10 mW cm^{-2}) for 3 min.

During light irradiation, argon gas was used to ensure the hypoxic environment. After the light irradiation, the aerobic medium was replaced by normal culture medium and the cells were cultured for 24 h in normoxic condition before ELISA analysis.

Transwell assay. The M0 or M1 cells (activated by TPA-DCR NPs, 2×10^5) were seeded in the lower chambers of 24-well transwell chambers, and 4T1 cells (1×10^4) were seeded in the upper chambers. After 24 h, the 4T1 cell viability was determined by CCK8. Alternatively, the 4T1 cells (labeled by a green fluorophore BTPETD) were added in the lower chamber, and the M0 and M1 cells (labeled by a red fluorophore TPETPAFN) were seeded in upper chambers. After 24 h, the phagocytic effect of macrophages on 4T1 cells was then analyzed by confocal microscopy (BTPETD: E_x = 448 nm, E_m = 515-590 nm; TPETPAFN: E_x = 525 nm, E_m = 625-680 nm).

Measurement of ATP, HMGB1 and HSP70. The M0 or M1 (activated by TPA-DCR NPs) cells were seeded in the 24-well plates (2×10^5 cells per well), incubated with or without 4T1 for 24 h (1×10^5 cells per well). The culture medium was collected, and the ATP secretion levels of cells were determined by ATP Assay Kit, according to the manufacturer's instruction. Then, the total proteins of cell lysates were collected, and the expression levels of HMGB1 and HSP70 proteins were determined by ELISA, according to the manufacturer's instruction. Meanwhile, the total proteins of tumor cells lysates from tumor tissues were collected. The expression levels of ATP, HMGB1 and HSP70 were also tested by ATP Assay Kit and ELISA kits.

***In vivo* antitumor assessment.** To establish the 4T1 tumor-bearing mouse model,

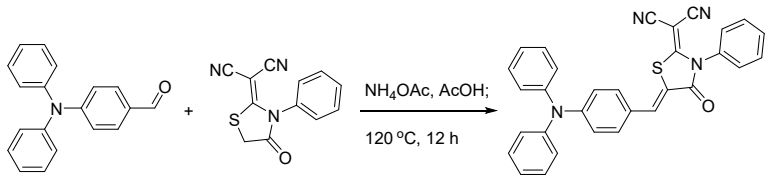
each female Balb/c mouse was subcutaneously inoculated with 2×10^5 4T1 cells in the left flank. When the tumor volume reached 45~55 mm^3, the mice (30 in total) were randomly divided into six groups (5 in each group), followed by intravenous injection of 1× PBS (200 μL) for Group i) and ii) and TPA-DCR NPs (200 μL, 1 mg mL^{-1}) for Group iii), iv), v) and vi). The clodronate liposomes (5 mg mL^{-1} in PBS, 50 μL) was intratumorally injected 1 day before the injection of TPA-DCR NPs or PBS in Group v) and vi). The IVIS Lumina *in vivo* imaging system (PerkinElmer) was utilized to image the fluorescence signal after intravenously injected with TPA-DCR NPs or PBS. After 12 h, only the tumors of Group ii), iv) and vi) were exposed to white light (300 mW cm^{-2}) for 5 min. The tumor sizes were then measured every other day to calculate the volumes. At the point of termination, the tumor tissues from all mice were collected to take photos.

Evaluation of *in vivo* photodynamic immune effect. The 4T1 cells were subcutaneously inoculated to establish the tumor-bearing mouse model. The tumor tissues were collected at 24 h after treatments to analyze the macrophage phenotypes by flow cytometry (BD FACSCanto SORP). Anti-F4/80 MicroBeads UltraPure was used to separate macrophages. The F4/80 antibody (1:200, APC) and CD11c antibody (1:500, FITC) were used to label M1 macrophages, while F4/80 (1:200, APC) antibody and CD206 antibody (1:400, Alexa 488) were used to label M2 macrophages in the samples. In addition, the spleens from different groups were also collected for detecting the counts of $CD4^+$ (1:500, FITC) and $CD8^+$ (1:500, Alexa 555) T cells by flow cytometry. The frozen section was used to analysis the macrophages and T cells in

tumor tissues by immunofluorescence staining. $CD4^+$ T cells were labeled with CD4 antibody (1:400, FITC), and $CD8^+$ T cells were labeled with CD8 antibody (1:400, Alexa 555).

***In vivo* biosafety assessment.** Healthy Balb/c mice (6 in total) were randomly divided into two groups (3 in each group) and intravenously injected with 1× PBS (200 μL) and TPA-DCR NPs (200 μL, 1 mg mL^{-1}), respectively. The blood samples were collected at day 7^{th} and the serum biochemistry data, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein, creatinine (CREA), and urea, were measured by an automatic biochemical analyzer MS-480 (Meikangshengde Biotechnology Co., Ltd, China). In addition, the white blood cell (WBC), red blood cell (RBC), blood platelet (PLT), lymphocytes (Lym), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and mean platelet volume (MPV), were measured by an automatic animal five classification blood cell analyzer DF-52Vet (Dymind Biotechnology Co., Ltd, China). The organs were also collected for H&E staining.

Statistics. Data are shown as Mean ± standard error of the mean (s.e.m.). All data were normally distributed. Statistical analysis was performed using Prism Software (GraphPad, USA). Student's t-test was performed, and the significance was defined as *P*-values < 0.05, 0.01 or 0.001, respectively.



Scheme S1. Design and synthesis of the photosensitizer TPA-DCR.

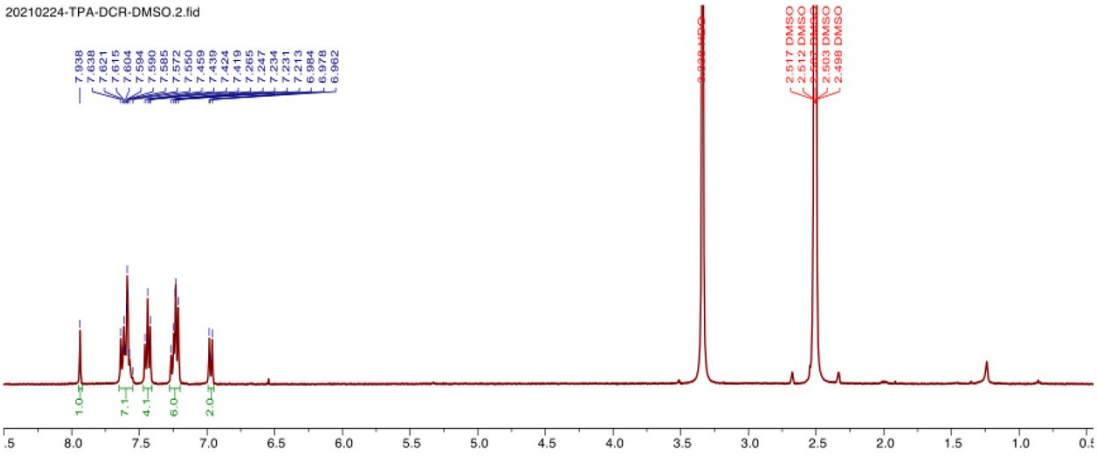


Figure S1. ^{1}H NMR spectrum of TPA-DCR.

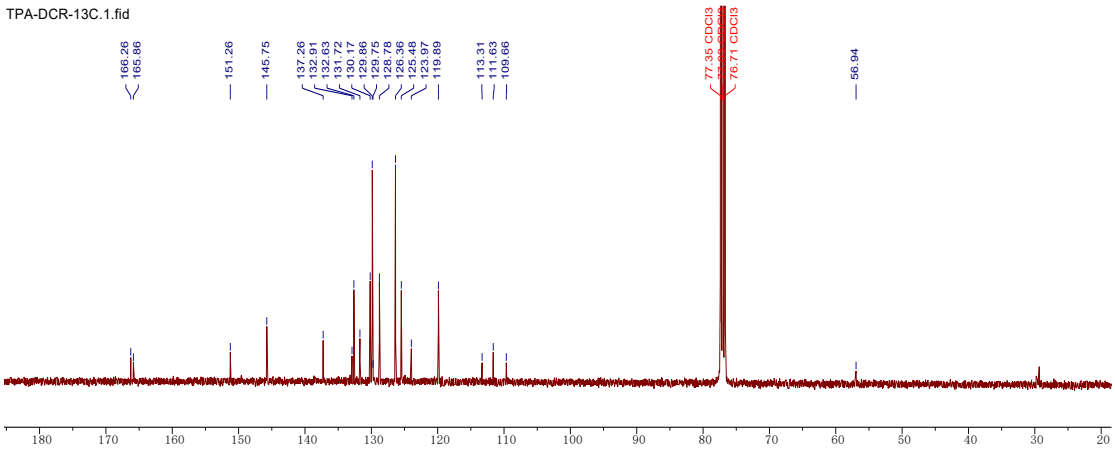


Figure S2. ^{13}C NMR spectrum of TPA-DCR.

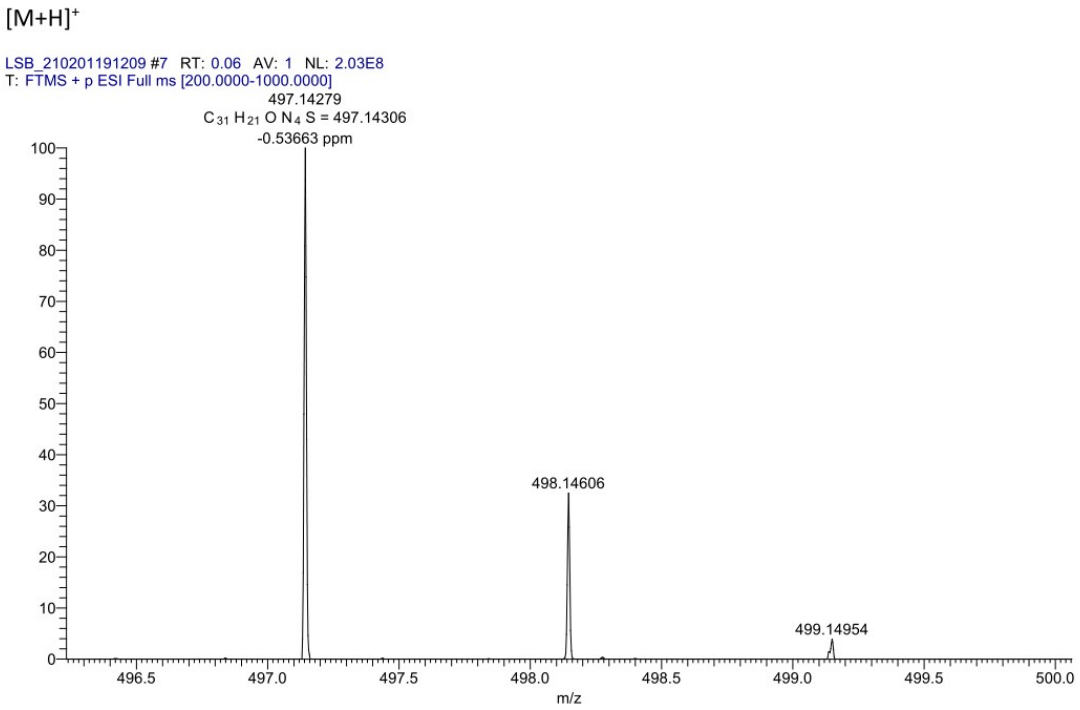


Figure S3. High resolution mass spectrum of $[M+H]^+$ (M = TPA-DCR).

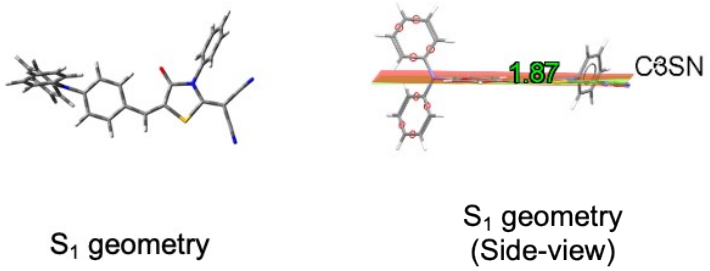


Figure S4. The geometries of TPA-DCR in THF.

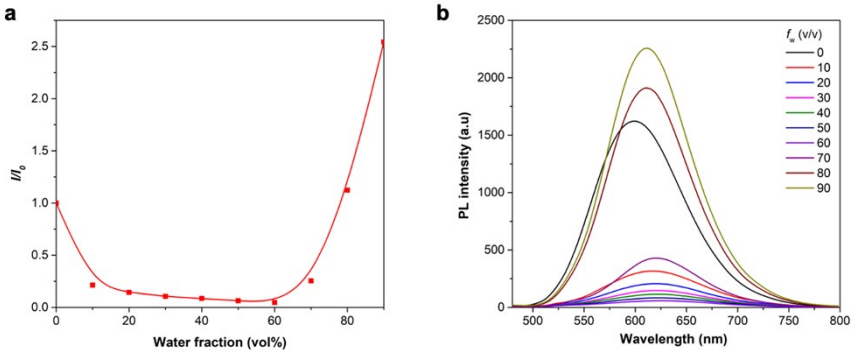


Figure S5. (a) Plots of AIE curves of TPA-DCR in THF solutions with different water fractions (vol%). (b) PL spectra of TPA-DCR in THF with different water fractions (0-90 vol%) at a concentration of 10 μM.

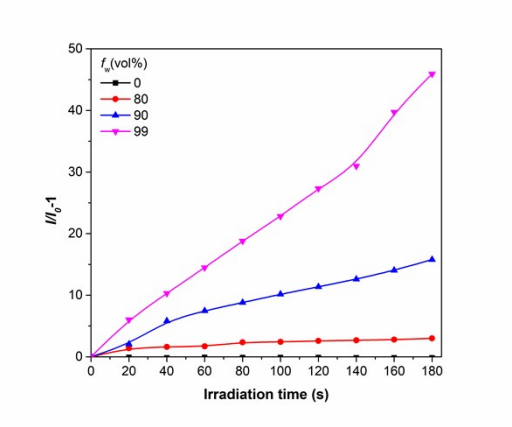


Figure S6. Time-dependent changes in fluorescence intensities (I/I_0-1) at 523 nm of DCFH/TPA-DCR in THF solutions with different PBS fractions upon white light irradiation (40 mW cm^{-2}). Concentrations: 10 μM for TPA-DCR and 1 μM for DCFH.

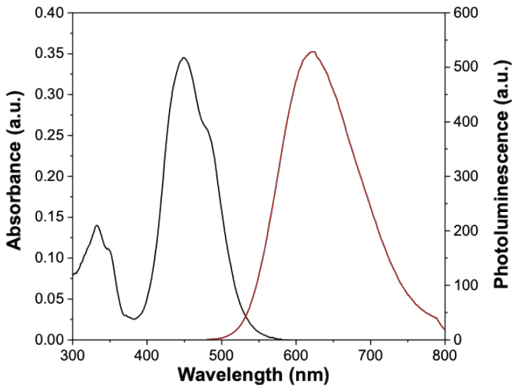


Figure S7. UV-Vis and PL spectra of TPA-DCR NPs in water.

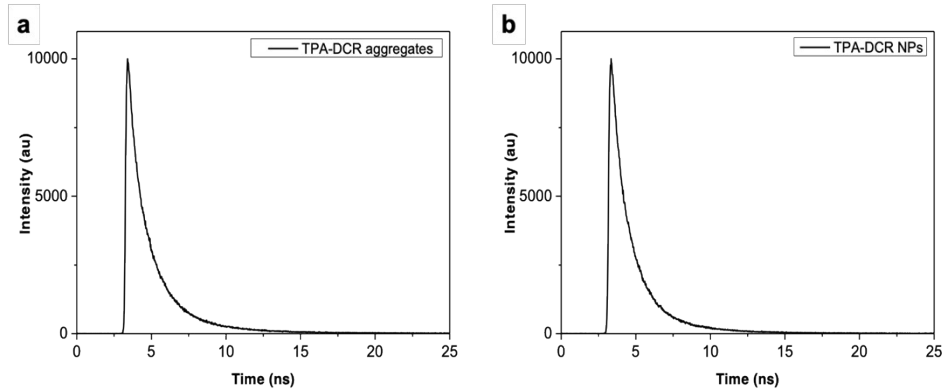


Figure S8. Fluorescence decay measurements of (a) TPA-DCR aggregates and (b) TPA-DCR NPs in water.

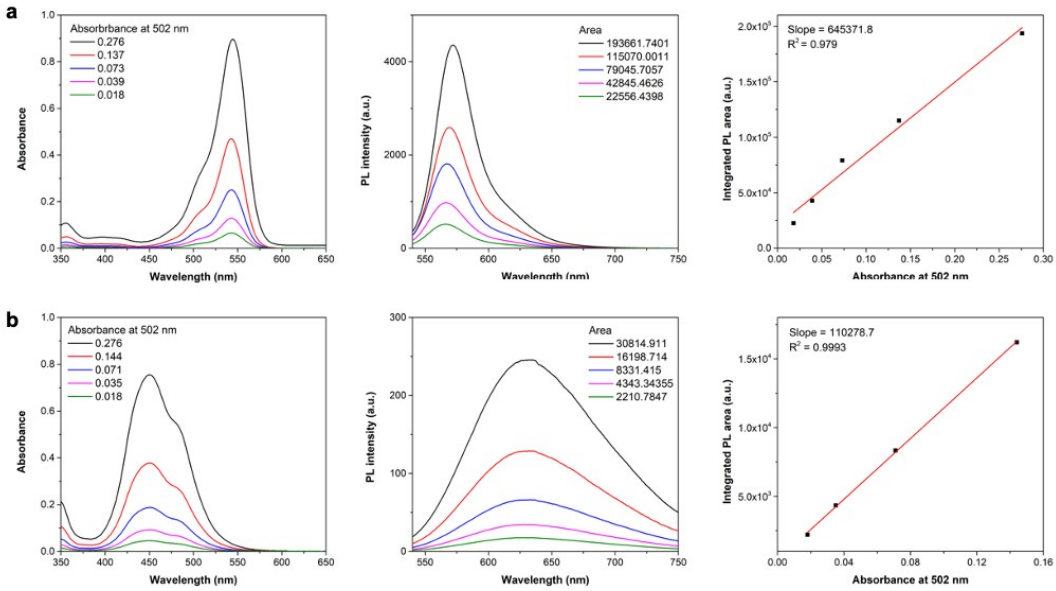


Figure S9. Quantum yield measurement of TPA-DCR NPs in water. UV-Vis, PL spectra and integrated emission intensities of (a) Rhodamine B and (b) TPA-DCR NPs in ethanol and water with increasing concentrations, respectively.

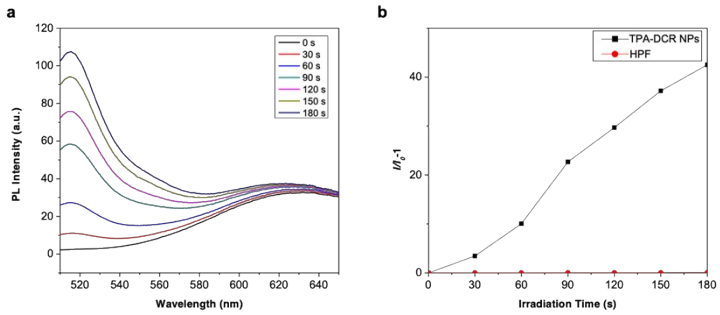


Figure S10. (a) PL spectra of HPF in presence of TPA-DCR NPs after continuous light irradiation for different times. (b) Time-dependent changes in maximum fluorescence intensities (I/I_0-1) of the mixture of HPF and TPA-DCR NPs. White light irradiation is 40 mW cm^{-2}, concentrations: 10 μM for TPA-DCR and 1 μM for HPF.

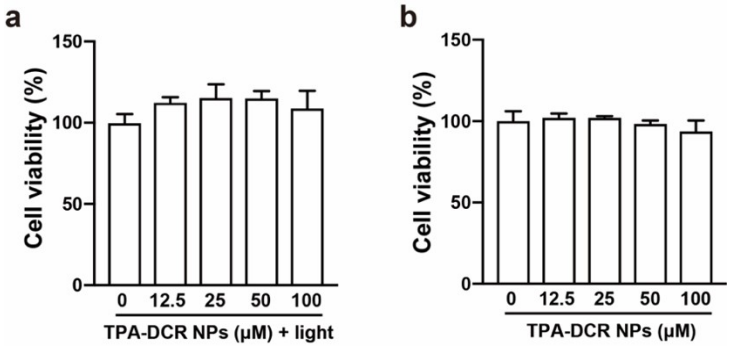


Figure S11. The light (a) and dark (b) cytotoxicity of RAW264.7 treated with TPA-DCR NPs were analyzed by CCK-8 assay after incubation for 24 h. For light toxicity evaluation, white light irradiation is 10 mW cm^{-2} for 3 min.

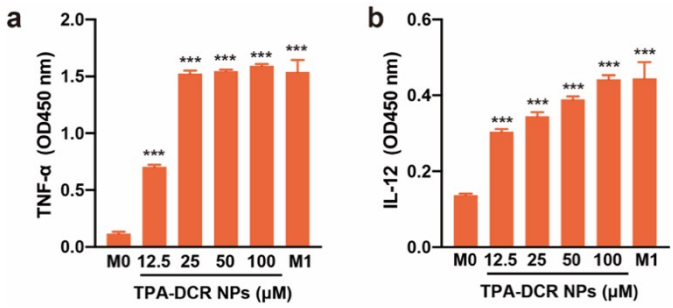


Figure S12. The expression level of pro-inflammatory cytokines of TNF-α (a) and IL-12 (b) secreted by M0 macrophages, after treatment with TPA-DCR NPs at varied concentrations and white light irradiation (***p<0.001 vs. the M0 group, n = 3).

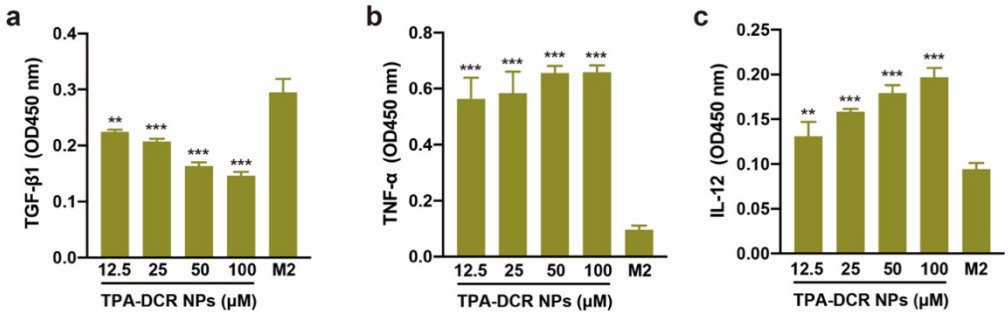


Figure S13. The expression level of anti-inflammatory cytokine of TGF-β1 (a) and pro-inflammatory cytokines of TNF-α (b) IL-12 p70 (c) secreted by M2 macrophages, after treating with TPA-DCR NPs at varied concentrations and white light irradiation (**p<0.01, ***p<0.001 vs. the M2 group, n = 3).

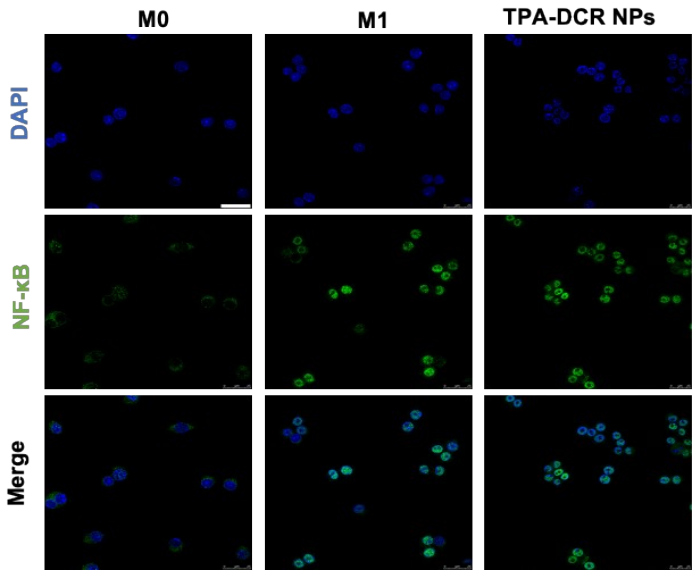


Figure S14. The localization of NF-κB subunits (green) and nuclei (blue) analyzed by confocal images after treating with 50 μM of TPA-DCR NPs and white light irradiation, 10 mW cm^{-2} for 3 min. The scale bar is 25 μm.

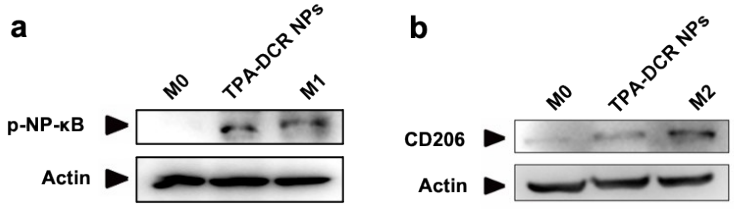


Figure S15. The Western blotting results of the protein expression of p-NF-κB (a) and CD206 (b) from macrophages treated with TPA-DCR NPs and white light irradiation. White light irradiation is at 10 mW cm^{-2} for 3 min.

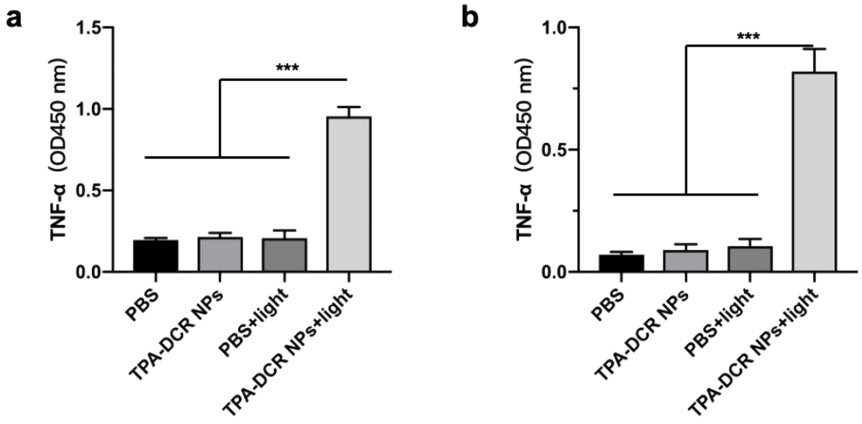


Figure S16. The expression levels of TNF-α secreted by (a) M0 macrophages and (b) M2 macrophages upon activation by TPA-DCR NPs (50 mM) and light (10 mW cm^{-2}), using the cells treated with PBS, TPA-DCR NPs, PBS+light as the control groups, ***p<0.001, n=3.

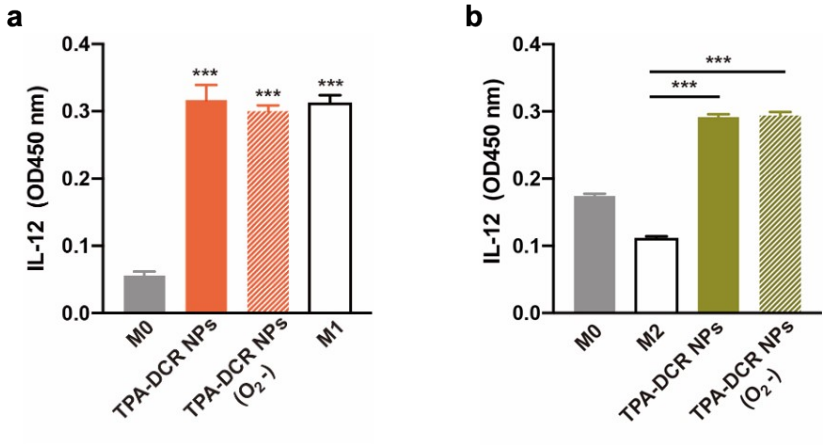


Figure S17. (a) The level of IL-12 secreted by M0 macrophages treated by TPA-DCR NPs and light under normoxic or hypoxic conditions, ***p<0.001 vs. the M0 group, n=3. (b) The level of IL-12 secreted by M2 macrophages treated by TPA-DCR NPs and light under normoxic or hypoxic conditions, ***p<0.001 vs. the M2 group, n=3.

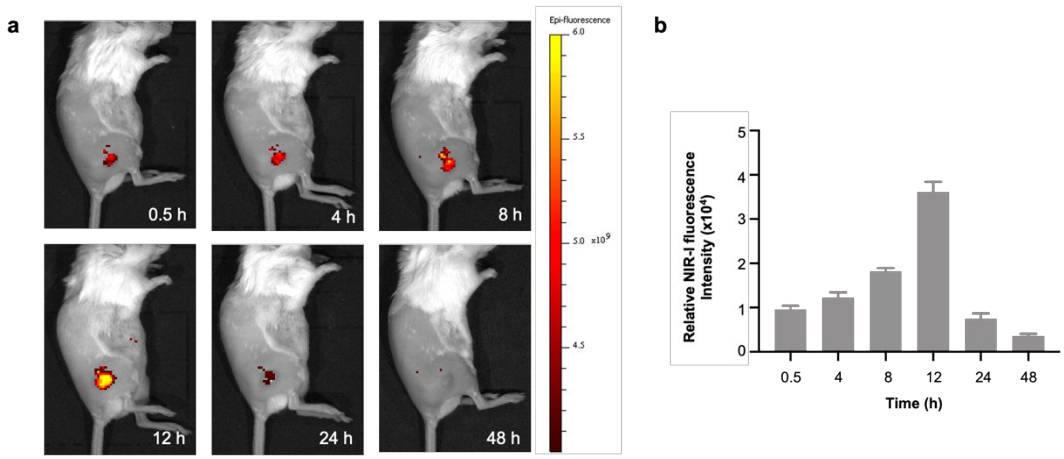


Figure S18. (a) Time-dependent NIR imaging of the representative 4T1 tumor-bearing mouse after intravenous injection of TPA-DCR NPs (10 mg/kg). (b) The quantitative analysis of fluorescence intensity of tumor. E_x = 460 nm, E_m = 620±25 nm.

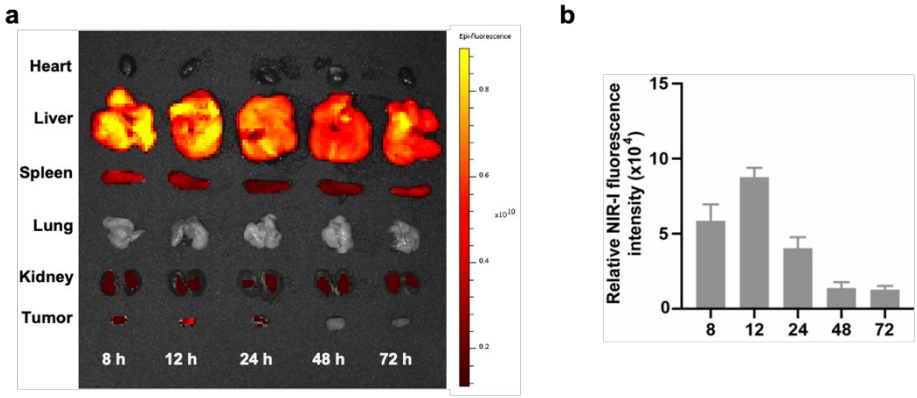


Figure S19. (a) Representative *ex vivo* fluorescence imaging of organs and tumor after intravenous administration of TPA-DCR NPs at different times. (b) The quantitative analysis of *ex vivo* fluorescence intensity of tumor tissues. E_x = 460 nm, E_m = 620±25 nm.

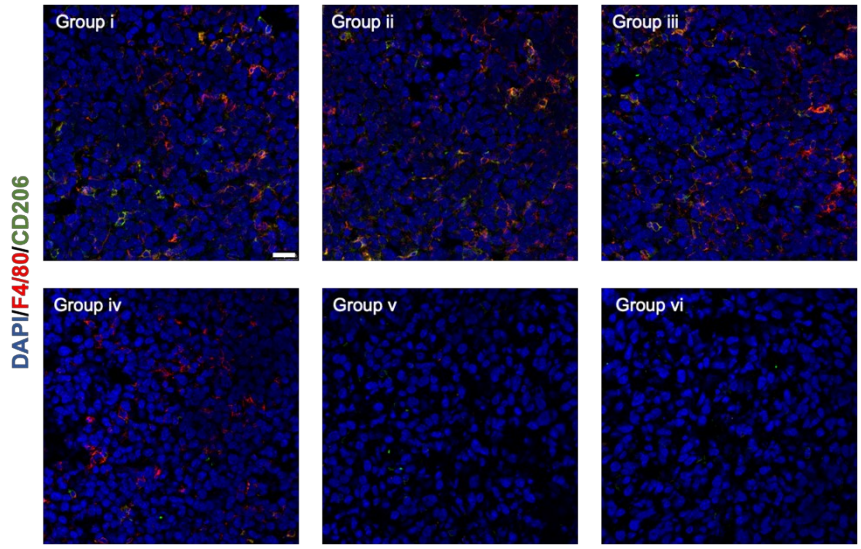


Figure S20. Representative images for co-staining of DAPI (blue), F4/80 (red), and CD206 (green) or in the tumor cells of tissues collected from 4T1-bearing mice after various treatments. Scale bar is 25 μm.

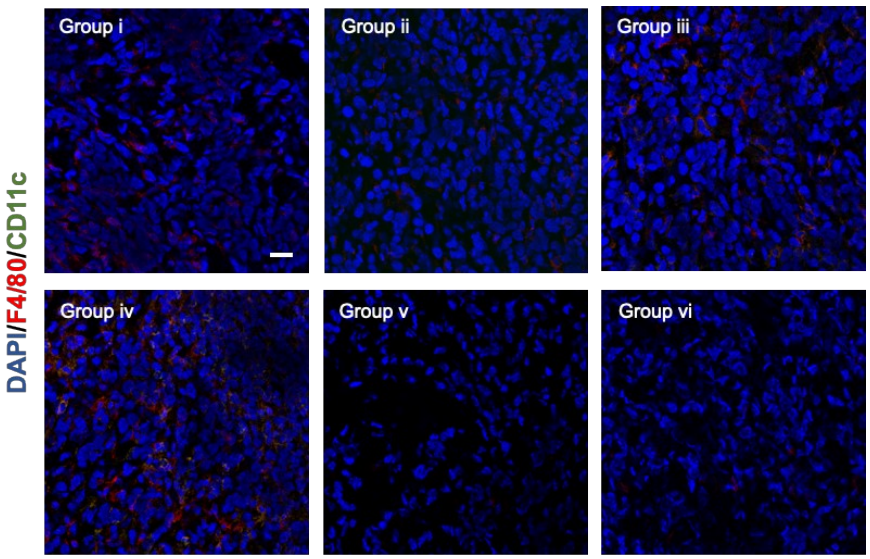


Figure S21. Representative images for co-staining of F4/80 (red) and CD11c (green) in the tumor tissues collected from 4T1-be aring mice after various treatments. The cell nuclei were counter-stained with DAPI (blue). Scale bar is 25 μm.

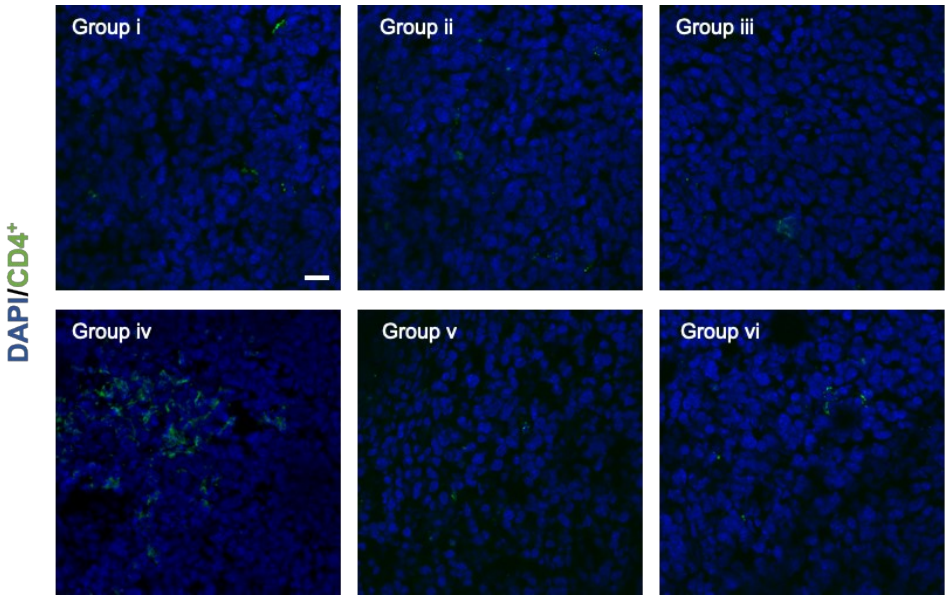


Figure S22. Representative images for co-staining of DAPI (blue) and $CD4^+$ (green) in the tumor tissues collected from 4T1-bearing mice after various treatments. Scale bar is 25 μm.

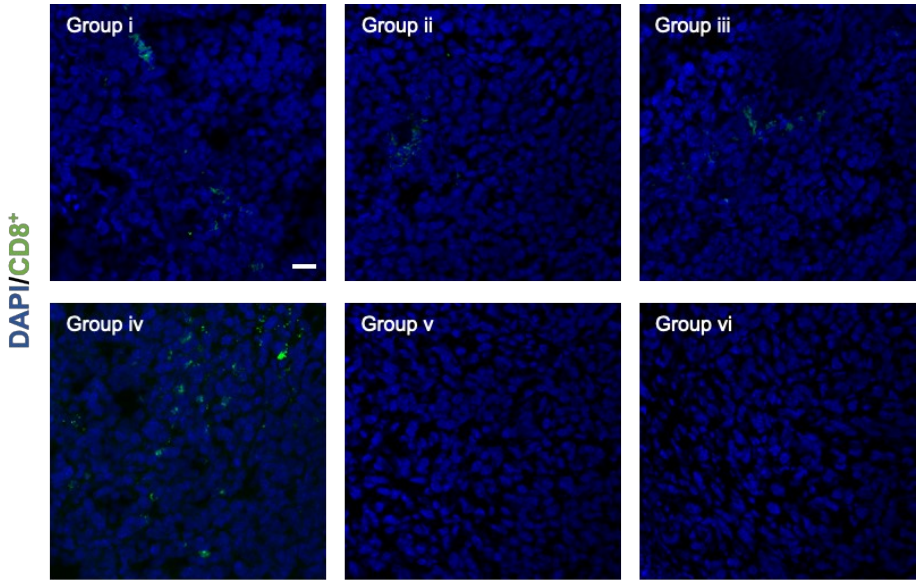


Figure S23. Representative images for co-staining of DAPI (blue) and $CD8^+$ (green) in the tumor tissues collected from 4T1-bearing mice after various treatments. Scale bar is 25 μm.

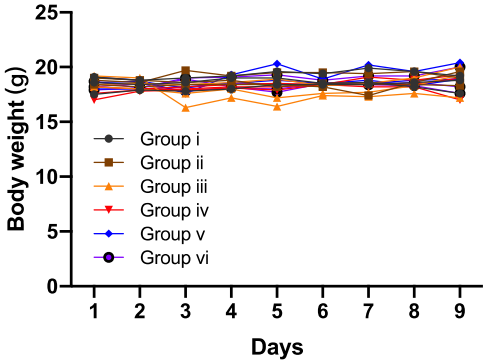


Figure S24. Body weights of the 4T1 breast-bearing mice in the six groups.

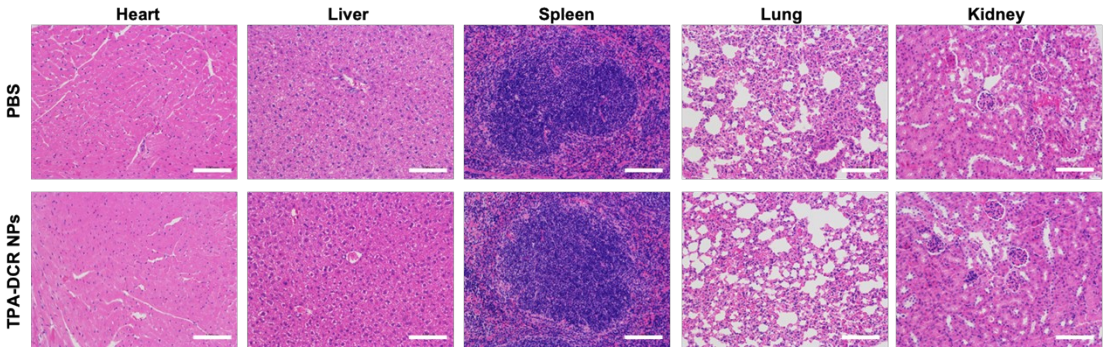


Figure S25. Hemotoxylin and eosin (H&E) staining of the sectioned tissues (heart, liver, spleen, lung and kidney) from mice injected with PBS or TPA-DCR NPs (10 mg/kg). The samples were collected at day 7 post injection. Scale bar is 100 μm.

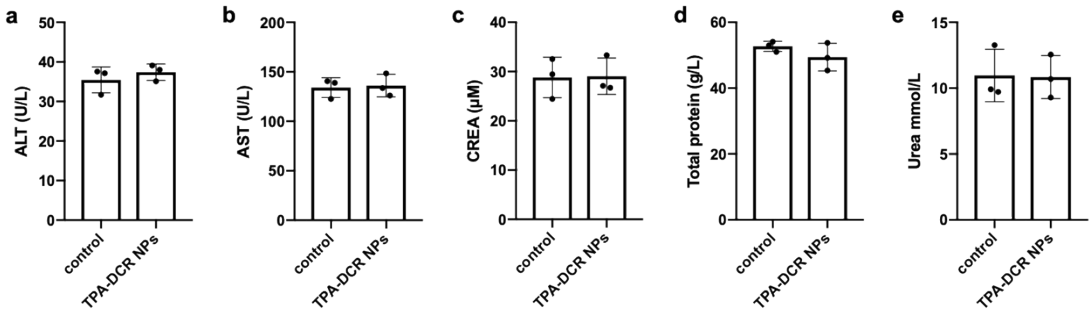


Figure S26. Serum biochemistry data. The levels of (a) ALT, (b) AST, (c) creatinine (CREA), (d) Total protein, and (e) Urea in samples collected from the control and TPA-DCR NPs treated mice (n = 3).

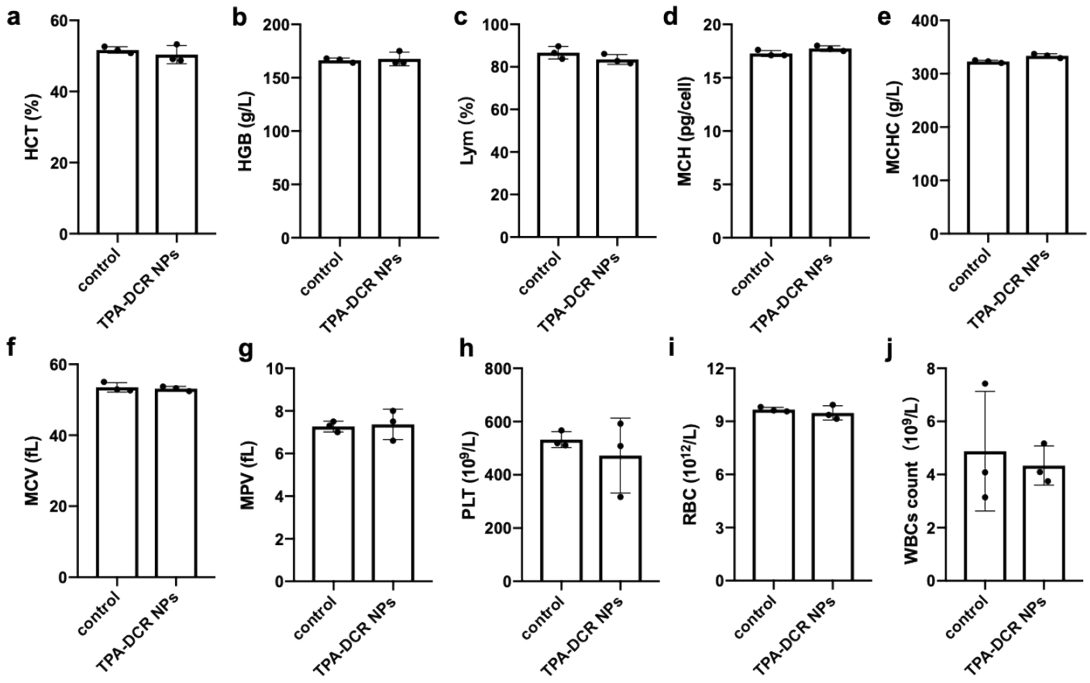


Figure S27. Blood routine analysis. The numbers of (a) HCT, (b) HGB, (c) Lym, (d) MCH, (e) MCHC, (f) MCV, (g) MPV, (h) PLT, (i) RBC, and (j) WBC in samples collected from the control and TPA-DCR NPs treated mice (n = 3).