Supporting Information

Biomimetic Nanoparticles with Red Blood Cell Membrane for Enhanced Tumor Photothermal and Immunotherapy

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Experimental

Materials. Dulbecco's modified eagle medium (DMEM) and Penicillin-streptomycin were obtained from Gibco-BRL (Burlington, Canada). Essential cell culture reagents such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), trypsine-EDTA, fetal bovine serum (FBS) and DAPI were supplied by Biomics Biotechnologies Co. Ltd. (Nantong, Jiangsu, China). Calcein/PI live/dead viability/cytotoxicity assay kit were provided by Beyotime Biotechnology (Shanghai, China). ELISA kits for the detection of TNF-α, IFN-γ, IL-10, and IL-6, as well as a panel of antibodies including anti-CD25, anti-CD8, anti-CD3, anti-CD4, anti-CD80, anti-CD86, and anti-CD11c antibodies were purchased by Dakewei Biotechnology (Shenzhen, China).

Fabrication of nanoparticles. DSPE-PEG-OH@IR780 (DIR780) was synthesized using the micro-emulsification technique. Specifically, a mixture of DSPE-PEG-OH (25 mg) in deionized water (3 mL) and IR780 (2.5 mg) in chloroform was prepared. The resulting mixture was subjected to sonication to obtain a uniform green solution, which was subsequently stirred at 50°C for 30 min until chloroform had completely evaporated, resulting in a clear solution. The solution was subsequently mechanically filtered with a 450 nm filter.

Red blood cells (RBCs) membrane-encapsulated DIR780. According to our published paper[1], fresh whole blood was obtained from mice using orbital blood sampling. RBCs were separated by centrifugation (3000 rpm, 5 min, 4 ℃) and washed three times with phosphate-buffered saline (PBS). The washed RBCs were gently combined with an excess of 1/4 × PBS (pH 7.4, 1.675 mM PO_4^{3-}) and exposed to low osmolarity conditions for 1 h to ensure adequate swelling of the erythrocytes. The resulting mixture was then subjected to centrifugation (14000 rpm, 15 min, 4 ℃) to collect the RBCs membrane. Finally, the RBCs membrane was mixed with

DIR780 and extruded through a 200 nm polycarbonate membrane at least ten times to obtain RDIR780.

Cellular uptake. The 4T1 breast cancer cells derived from mice were cultivated under controlled conditions of 37 ºC and 5% CO_2 in a laboratory incubator. The culture medium used for the cells consisted of Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and a 1% solution of penicillin-streptomycin. The 4T1 cells were then placed in a 24 well plate at a density of 5×10^4 cells per well and allowed to incubate for 24 h. Subsequently, the original medium was substituted with a mixture containing DIR780 and RDIR780 dissolved in DMEM. Following a 4 h incubation period, the cells were thoroughly washed with phosphate-buffered saline (PBS) to eliminate any residue of the solutions and then fixed using a 4% paraformaldehyde solution for 5 min. Staining with DAPI (blue dye) took place for 10 min, followed by two rounds of rinsing with PBS, and observation under confocal laser scanning microscopy (CLSM).

***In vitro* cell viability.** The cytotoxicity of DIR780 and RDIR780 was evaluated by utilizing the Cell Counting Kit-8 (CCK-8) assay. 4T1 cells were seeded in a 96 well plate at a density of 1×10^4 cells per well and incubated for 18 h. Subsequently, the cell culture medium was replaced with fresh medium containing the respective nanoparticles. Following a 4 h incubation, the cells were exposed to irradiation with or without an 808 nm NIR laser for 5 min at an intensity of 1.0 W/cm^2. After an additional 18 h incubation period, the medium was substituted with a CCK-8 solution. The absorbance at 450 nm was then measured using a microplate reader after a 1 h incubation with the CCK-8 solution.

Detection of immunogenic cell death (ICD) biomarkers. 4T1 cells were inoculated in a 12 well plate (1×10^5 cells per well) and incubated for 18 h. DIR780, RDIR780, and RDIR780 +

anti-PD-L1 antibody were added to 4T1 cells and incubated for 4 h with or without laser at 808 nm (1.0 W/cm^2, 5 min) and then incubated for 18 h. After incubation, the cells were washed with PBS, fixed using 4% paraformaldehyde for 5 min, and incubated with calreticulin (CRT) primary antibody for 30 min and then incubated with goat Alexa Fluor488 anti-rabbit IgG secondary antibody for another 30 min. Finally, the cells were stained with DAPI for 5 min, and observed by fluorescence microscope.

As for the quantitative analysis of CRT by flow cytometry. The treated cells were washed with PBS, fixed using 4% paraformaldehyde for 5 min, and incubated with CRT primary antibody for 30 min and then incubated with goat Alexa Fluor488 anti-rabbit IgG secondary antibody for another 30 min. Finally, scraping the cells off, the surface expression of CRT was evaluated by flow cytometry.

Immunofluorescent staining was also estimated the intranuclear High Mobility Group Box 1 (HMGB1). After fixing the cells with paraformaldehyde (4% *w*/*v*), the cells were washed with PBS and permeabilized with Triton X-100 (0.5% *w*/*v*) for 10 min. Subsequently, the cells were blocked with 5% FBS for 0.5 h, followed by incubation with PE anti-HMGB1 antibody for 1 h. Finally, the cells were stained with DAPI for 5 min, and observed by fluorescence microscope.

4T1 cells were inoculated in a 12 well plate (1×10^5 cells per well) and incubated for 18 h. DIR780, RDIR780, and RDIR780 + anti-PD-L1 antibody were added to 4T1 cells and incubated for 4 h with or without laser at 808 nm (1.0 W/cm^2, 5 min) and then incubated for 18 h. Adenosine triphosphate (ATP) assay was performed by collecting cell supernatant utilizing an ATP assay kit following the instructions.

***In vivo* fluorescence imaging.** In 4T1 tumor-bearing mice, DIR780 (60 μg/mL, 200 μL) or RDIR780 (60 μg/mL, 200 μL) was injected into the tail vein, and in different time points (1 h, 2

h, 4 h, 6 h, 8 h, 12 h, 24 h, 36 h, 48 h, 60 h, 72 h, 84 h, and 96 h), *in vivo* fluorescence imaging was performed using a fluorescence imaging system.

***In vivo* anti-tumor.** The mice were housed at the Laboratory Animal Center of Hangzhou Normal University with use license number SYXK (Zhejiang) 2020-0026, and cultivated in a pathogen-free environment with appropriate humidity and temperature. All animal experimental procedures were conducted in strict accordance with the protocols approved by the Institutional Animal Care and Use Committee and complied with the guidelines set forth by the State Department of Health. 4T1 cells (1×10^6 cells) were subcutaneously injected into the right back of BALB/c mice. After 7 days, 4T1 cells were then subcutaneously implanted into the left hind leg of the mice. The tumor-bearing mice were randomly divided into 7 groups (n = 8) and subjected to various treatments, including I. PBS, II. anti-PD-L1 antibody, III. DIR780, VI. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti-PD-L1 antibody + L (1.0 W/cm^2, 6 min). The body weight and tumor volume were recorded every two days.

Immunohistochemical analyses. To systematically investigate the anti-tumor immune response *in vivo*, mouse tumors were surgically removed and subsequently fragmented, collagenase at a concentration of 2 mg/mL was introduced to facilitate digestion, and the tumors were thoroughly digested in a water bath at 37 ºC for 4 h. The suspension was filtered twice through a 200-mesh nylon strainer, followed by the collection of cells *via* centrifugation (700 g, 5 min). Subsequently, the cells were co-stained with anti-CD11c FITC, anti-CD80 APC, and anti-CD86 PE for *in vivo* DCs maturation analysis.

Whole blood of mice was collected and divided into two. One portion of serum was taken to detect the level of cytokines (TNF-α, IFN-γ, IL-6, IL-10) in serum by ELISA, and the other portion was added with sodium heparin and RBCs were lysed by using RBCs lysing solution

then co-stained with lymphocytes by utilizing the anti-CD3-BV421, anti-CD4-PE, and anti-CD8-APC antibodies to analyze the lymphocytes in peripheral blood.

Lymphocytes were extracted from mouse spleens and collected into culture medium, and then lymphocytes were co-stained using anti-CD3-BV421, anti-CD4-PE, and anti-CD8-APC antibodies. Treg cells were also analyzed using co-staining with anti-CD3-BV421, anti-CD4-PE, and anti-CD25-APC. The remaining splenic lymphocytes were incubated in culture medium for 24 h to detect the level of cytokine (TNF-α, IFN-γ, IL-6, IL-10) in the supernatant by ELISA.

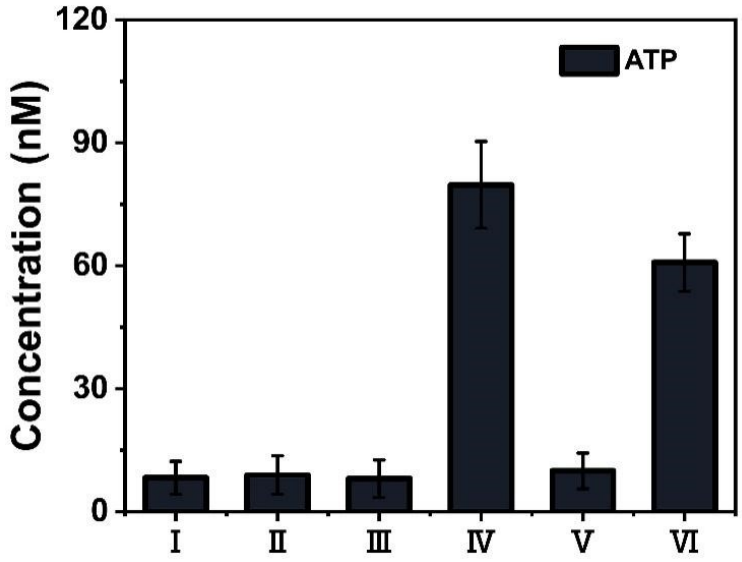

Figure S1. ATP secretion levels after different treatments (Group: I. PBS; II. PBS + L; III. DIR780; IV. DIR780 + L; V. RDIR780; VI. RDIR780 + L）

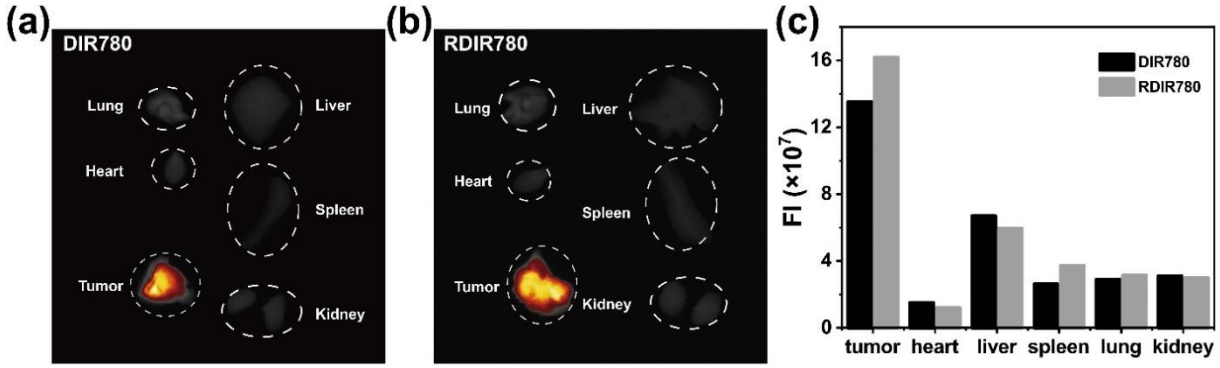

Figure S2. (a) The fluorescence imaging of tumor, heart, liver, spleen, lung, and kidney of mice 96 h after administration of DIR780. (b) The fluorescence imaging of tumor, heart, liver, spleen, lung, and kidney of mice 96 h after administration of RDIR780. (c) Quantitative statistics of fluorescence intensity of (a) and (b).

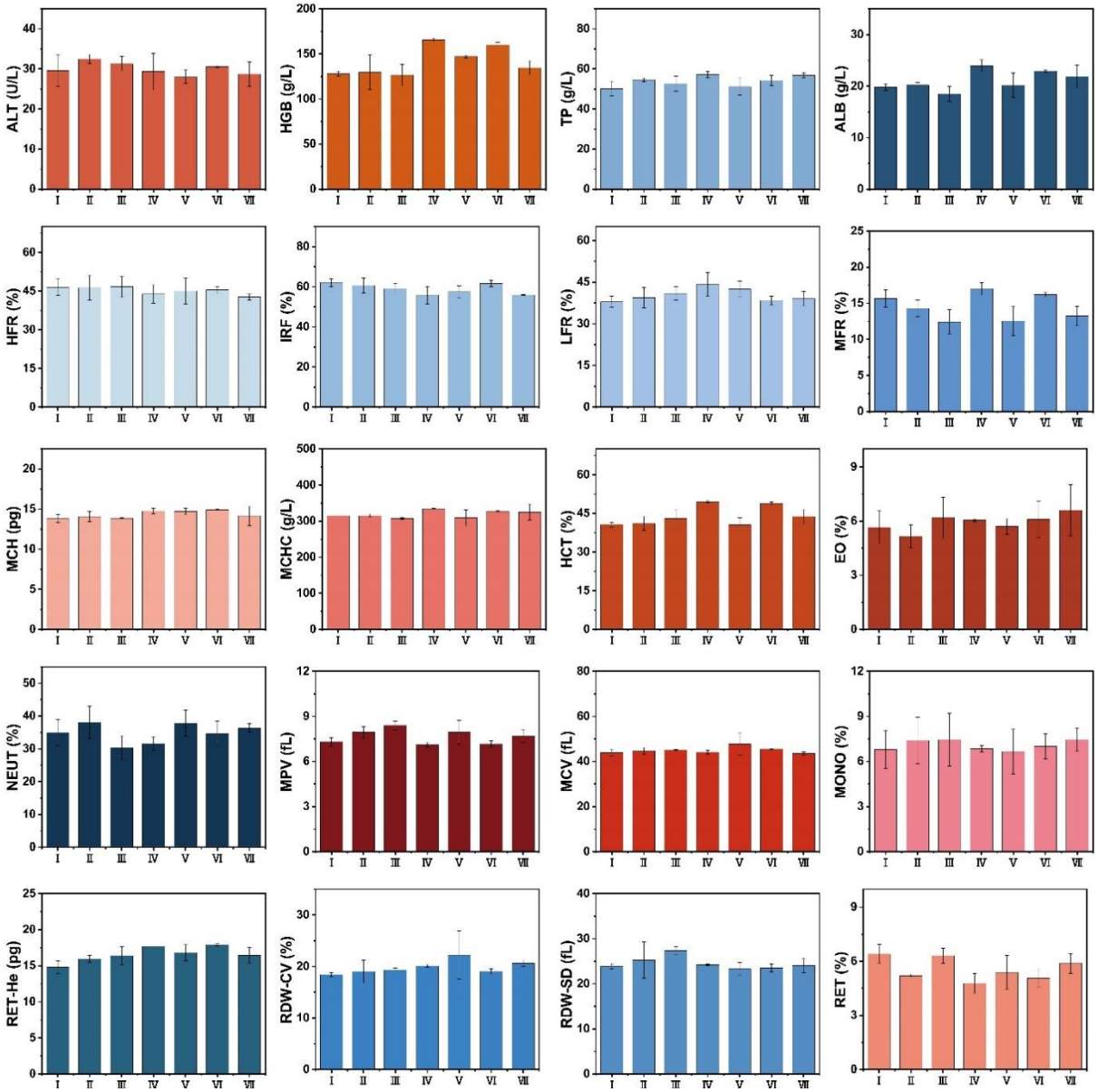

Figure S3. The related indexes of blood biochemistry and hematology analysis from experimental mice after different treatments. Group information: I. PBS, II. anti PD-L1 antibody,

III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

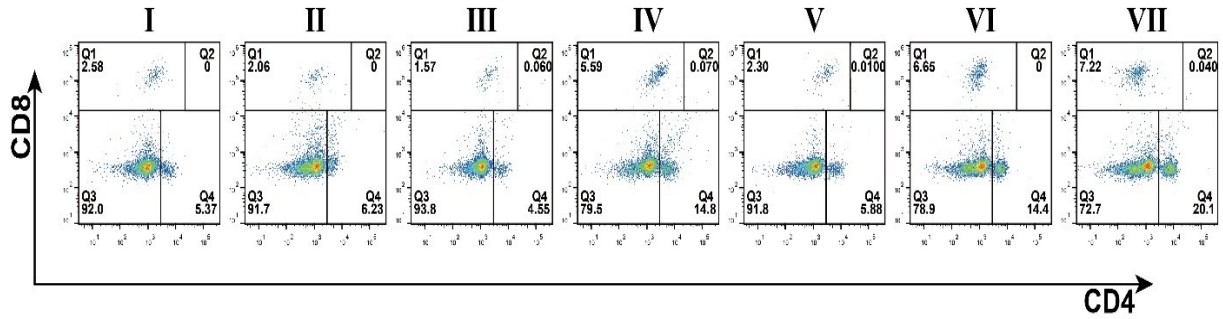

Figure S4. Flow cytometry analysis of the proportion of CD4$^+$/CD8$^+$ T cells in the blood. Group information: I. PBS, II. anti PD-L1 antibody, III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

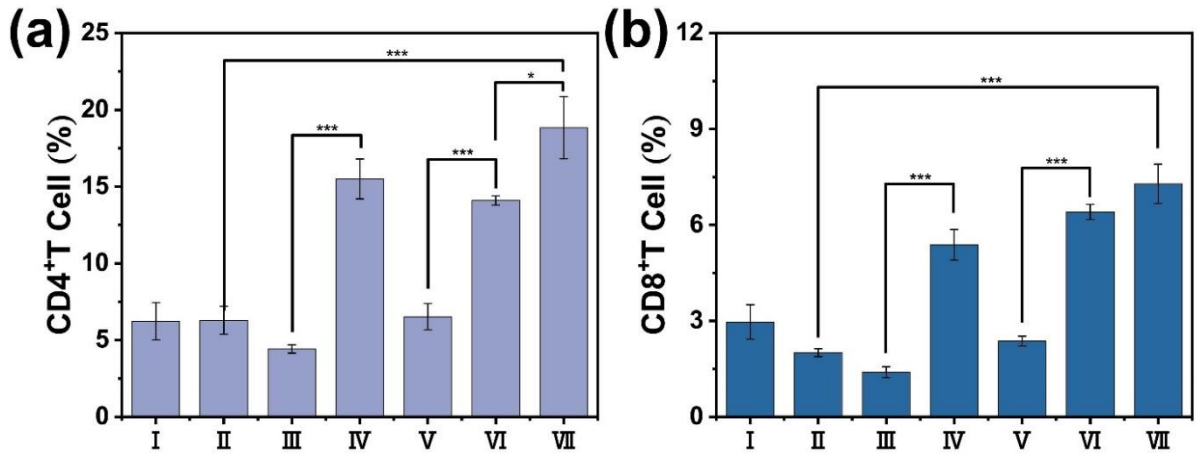

Figure S5. (a) The proportion of CD4$^+$ T cells in the blood. (b) The proportion of CD8$^+$ T cells in the blood. Group information: I. PBS, II. anti PD-L1 antibody, III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

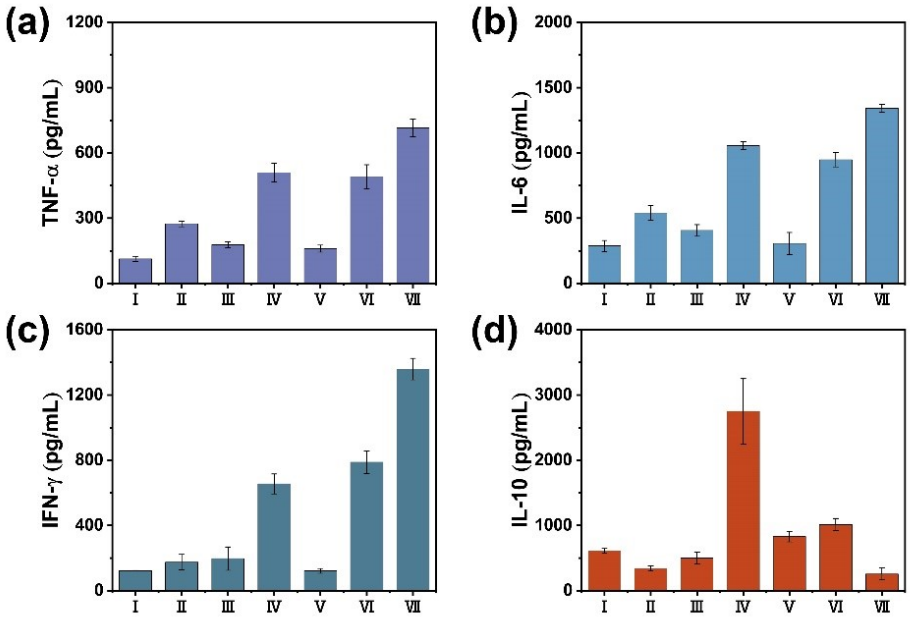

Figure S6. Secretion levels of (a) TNF-α, (b) IFN-γ, (c) IL-6, and (d) IL-10 in the spleen of the 4T1 tumor-bearing mice (n = 3). Group information: I. PBS, II. anti PD-L1 antibody, III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

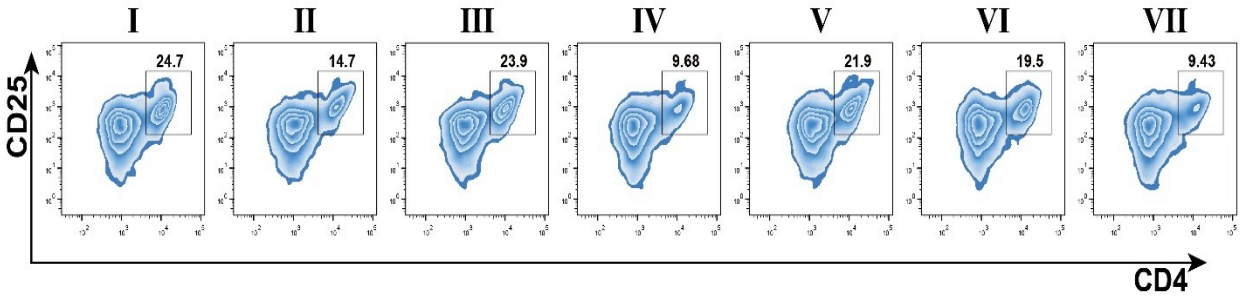

Figure S7. Flow cytometry analysis of the proportion of $CD4^+CD25^+$ T cells in the spleen. Group information: I. PBS, II. anti PD-L1 antibody, III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

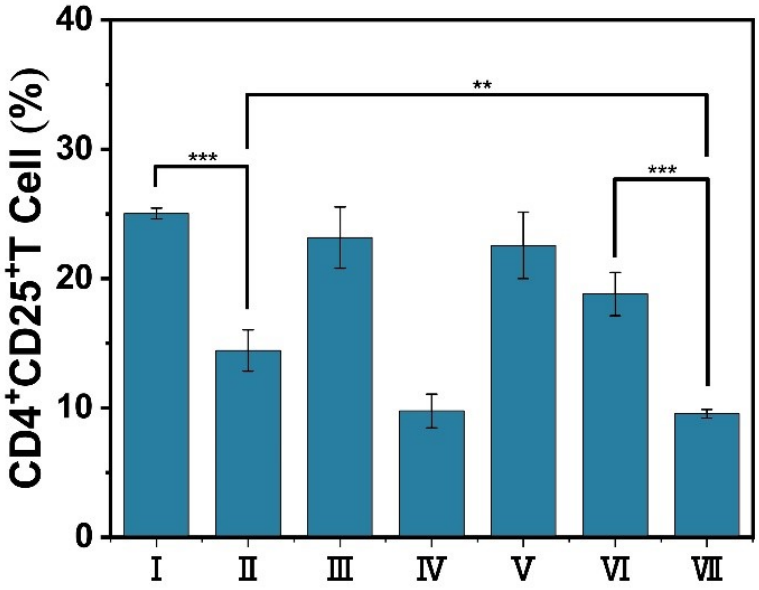

Figure S8. The proportion of $CD4^+$ T cells in the spleen. Group information: I. PBS, II. anti PD-L1 antibody, III. DIR780, IV. DIR780 + L (1.0 W/cm^2, 6 min), V. RDIR780, VI. RDIR780 + L (1.0 W/cm^2, 6 min), and VII. RDIR780 + anti PD-L1 antibody + L (1.0 W/cm^2, 6 min).

Reference

1. J. Ye, Y. Yu, Y. Li, B. Yao, M. Gu, Y. Li, S. Yin, *ACS Applied Mater. Interfaces* 2024, **16**, 34607.