Supplementary Information

NIR-II photothermal therapy combined with activatable immunotherapy against recurrence and metastasis of orthotopic triple-negative breast cancer

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Materials

1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl) and chloroform-d ($CDCl_3$) were purchased from Energy Chemical Co., Ltd.. 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-poly(ethylene glycol)$_{2000}$ (DSPE-PEG$_{2000}$) was purchased from Ponsure Biological Co., Ltd.. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Calcein-AM/PI Cell Viability/Cytotoxicity Assay Kit were purchased from Shanghai Beyotime Biological Technology Co., Ltd.. Collagenase, DNase I, and hyaluronidase were purchased from Shanghai Yuanye Bio-Technology Co., Ltd.. Hoechst 33258 were purchased from Jiangsu KeyGEN Biotechnology Co., Ltd.. Rb a Calreticulin, Ra a HMGB1, and Donkey Anti-Rabbit IgG H&L/FITC were purchased from Beijing Bioss Biotechnology Co., Ltd.. Antibodies for flow cytometry were purchased from Shenzhen DAKEWE Bio-engineering Co., Ltd.. Cell culture dishes were purchased from Guangzhou Jet Bio-Filtration Co., Ltd.. 96-Well plates were purchased from Wuxi NEST Biotechnology Co., Ltd.. Ultrapure water was prepared from a Milli-Q system (Millipore, USA). The other chemicals were used as obtained commercially. Analytical balance (XS105DU) and Rainin Pipettes from METTLER TOLEDO were used to quantify solid and liquid respectively. TEM and DLS results were determined by JEOL JEM-1011 electron microscope (acceleration voltage of 100 kV) and Malvern Zeta-sizer Nano. The flow cytometry measurement was performed on a Becton Dickinson FACS Aria sorting flow cytometer (Becton-Dickinson, USA). CLSM images were obtained from a Zeiss LSM 700 (Zurich, Switzerland).

Preparation of NPs

DSPE-PEG$_{2000}$ (10 mg) and PCD (5 mg) were dissolved in THF (5 mL), and then added into deionized water (5 mL) under stirring. After THF was fully evaporated, the mixture was dialyzed for 24 h, and PCD NPs were obtained. NLG NPs were obtained according to the same method.

For the preparation of PCDB NPs and NLGB NPs, BDP at a mass ratio of 3% to DSPE-PEG$_{2000}$ was dissolved in acetone, and then mixed with the THF solution of PCD/NLG and DSPE-PEG$_{2000}$. The subsequent steps are similar with the preparation method of PCD NPs. PCDI NPs were obtained according to the same method.

Photothermal effects of PCD NPs

PCD NPs at various concentrations (0-30 μg mL^{-1}) were subjected to 1064 nm laser irradiation (0.8 W cm^{-2}) for 10 min respectively. For PCD NPs at a fixed concentration of 30

μg mL^{-1}, the temperature of PCD NPs under 1064 nm laser irradiation of different power densities (0.2-1.0 W cm^{-2}) was also recorded. The photothermal conversion efficiency of PCD NPs was measured according to a previous method by recording the photothermal response of PCD NPs under 1064 nm laser irradiation (0.8 W cm^{-2}) and the temperature during the cooling period [1]. The photostability of PCD NPs upon 1064 nm laser irradiation was investigated by measuring the temperature variations of PCD NPs in water over 6 cycles of heating and natural cooling.

Cell lines and cell culture

4T1 (mouse breast cancer) cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) with 10% (v/v) FBS. Cells were cultured in a humidified incubator at 37 °C with 5% CO_2, and the culture medium was replaced once every day.

Cytotoxicity

The cytotoxicity of PCD NPs against 4T1 cells were examined via MTT assays. After 4T1 cells were seeded in 96-well plates and incubated overnight, the incubation media were discarded and replaced with PCD NPs diluted with the fresh media to desired concentrations. The cells were incubated for an additional 4 h at 37 °C. Then, 1064 nm laser irradiation (0.8 W cm^{-2}, 10 min) was performed on cells in PCD NPs + L group. After 24 h, 20 μL of MTT (5 mg mL^{-1} in PBS) was added, and the cells were incubated at 37 °C for another 4 h. Then, the culture medium supernatants were carefully removed, and 150 μL of DMSO was added to each well to dissolve the formed violet formazan crystals. Ultimately, the plates were shaken for 4 min, and the absorbance of violet product was determined by a microplate reader.

Cellular uptake of PCDB NPs and NLGB NPs measured by CLSM

The cellular uptake of PCDB NPs and NLGB NPs were evaluated by CLSM. First, 4T1 cells were seeded in six-well culture plates and allowed to adhere for 24 h. Then, the media were removed and replaced by PCDB NPs or NLGB NPs (5 μM BDP), and the cells were incubated at 37 °C for 0.5, 2, and 4 h, respectively. After being stained by Hoechst 33258 for 8 min to track cellular nuclei, the cells were imaged by CLSM.

Live-dead cell staining

4T1 cells were pretreated with PBS or PCD NPs (30 μg mL^{-1}), and 4 h later, the cells were irradiated with 1064 nm laser (0.8 W cm^{-2}, 10 min) for 10 min or not. After 24 h of

incubation at 37 °C, the cells were stained with calcein-AM/propidium iodide (PI) solution for 30 min at room temperature. Finally, the samples were imaged by a fluorescence microscope.

PTT-induced ICD

To determine PTT induced ICD of 4T1 cells in vitro, flow cytometry, immunofluorescence, and ELISA were used.

Calreticulin (CRT) expression was measured by flow cytometry. 6-Well plates were seeded with 2×10^5 4T1 cells per well, and the cells were incubated overnight. PCD NPs (30 μg mL^{-1}) were added to each well, and the cells were incubated for 4 h. Then, 1064 nm laser irradiation (0.8 W cm^{-2}, 10 min) was performed on cells in PBS + L group and PCD NPs + L group. After the cells were incubated for 6 h and harvested, PBS was used to wash the cells. The cells were then treated with anti-CRT antibody for 45 min before being rinsed twice with PBS. The cells were examined with flow cytometry.

The release of HMGB1 was detected via immunofluorescence. In 6-well plates, 1×10^5 4T1 cells per well were seeded on coverslips and incubated overnight. PCD NPs (30 μg mL^{-1}) were added to each well, and the cells were incubated for 4 h. Then, 1064 nm laser irradiation (0.8 W cm^{-2}, 10 min) was performed on cells in PBS + L group and PCD NPs + L group. After the cells were incubated for 12 h and harvested, PBS was used to wash the cells. The cells were fixed with 4% paraformaldehyde for 20 min, and permeabilized with 0.15% Triton X-100 for 10 min. After that, the cells were blocked with goat serum for 30 min before being treated with anti-HMGB1 antibody overnight (4 °C). The cells were then washed with PBS and stained with Hoechst 33258 for 5 min. CLSM was used to image the samples.

The secretion of ATP was detected by ELISA. 1×10^5 4T1 cells per well were seeded in 6-well plates with coverslips, and the cells were incubated overnight. PCD NPs (30 μg mL^{-1}) were added to each well and incubated for 4 h. Then, 1064 nm laser irradiation (0.8 W cm^{-2}, 10 min) was performed on cells in PBS + L group and PCD NPs + L group. After the cells were incubated for 6 h, the contents of ATP in the supernatants were detected with ELISA kit.

The detection of IDO-1 activity

The activity of IDO-1 was detected via measurement of the ratio of kynurenine (Kyn) to tryptophan (Trp) (Kyn/Trp). 4T1 cells were seeded in 6-well plates (5×10^4 per well) and incubated overnight. Then, the cells were stimulated with 100 ng mL^{-1} IFN-γ for 24 h and further incubated with NLG NPs for 36 h. Simultaneously, 100 μM tryptophan was added to amplify the experimental effect. Finally, 150 μL of the supernatant was collected from each

well, and 75 μL of 30% trichloroacetic acid was added to completely hydrolyze n-formylknurenine. Then, the same amount of Ehrlich's reagent (2% w/v p-dimethylaminobenzaldehyde/acetic acid) was added to the supernatant and left for another 10 min. Finally, the absorbance per well was measured by a microplate reader.

Maturation of DCs

6-Well plates were seeded with 2×10^5 4T1 cells per well, and the cells were incubated overnight. PCD NPs (30 μg mL^{-1}) were added to each well, and the cells were incubated for 4 h. Then, 1064 nm laser irradiation (0.8 W cm^{-2}, 10 min) was performed on cells in PCD NPs + L group. After the cells were incubated for 4 h, 1 mL of the supernatant was added to the six-well plate inoculated with DC2.4 cells, which were incubated overnight. Then, the cells were stained with CD80-APC and CD86-APC-Cy7 antibodies for 1 h. Cells were blocked with 5% BSA for 0.5 h, washed with PBS, and centrifuged at 1800 r min^{-1} for 10 min. The supernatant was discarded, and 0.5 mL of PBS was added to resuspend the cells. The expression of CD80 and CD86 on DCs was measured by flow cytometry.

Animal model

All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences (Approved No. 20220005). BALB/c mice (female, 20 g) were purchased from Liaoning Changsheng Biotechnology Co., Ltd.. 4T1 cells were injected into the right breast pad of mice to establish an orthotopic 4T1 tumor model. The tumor volume was determined using the following formula: volume = length × $width^2/2$. When the tumor volumes reached around 100 mm^3, therapies were initiated. The tumor volumes and body weights were measured every two days.

In vivo fluorescence imaging of PCDI NPs

To explore the distribution of NPs in tumors, PCD NPs were labeled with IR780 (PCDI NPs), and in vivo imaging was performed toward orthotopic 4T1 tumor-bearing BALB/c mice. The tumor-bearing mice were intravenously injected with PCDI NPs, and fluorescence imaging of the mice were conducted 0, 2, 6, 12, 24, and 36 h post injection. After administration for 36 h, the mice were sacrificed followed by the excision of the tumors and the main organs, and ex vivo fluorescence images were obtained.

Antitumor effects

To evaluate the antitumor effects of PCD NPs and NLG NPs, the mice bearing orthotopic 4T1 tumors were randomly divided into 6 groups, namely, PBS, PBS + L, PCD NPs, PCD NPs + NLG NPs, PCD NPs + L, and PCD NPs + NLG NPs + L. After injected with PCD NPs (10 mg kg^{-1}) and NLG NPs (10 mg kg^{-1}) for 12 h, the mice in PBS + L, PCD NPs + L, and PCD NPs + NLG NPs + L groups were irradiated with 1640 nm laser (0.8 W cm^{-2}) for 10 min. The temperature changes of the tumors during irradiation were recorded with an IR thermal imager every two minutes. After that, NLG NPs (10 mg kg^{-1}) were injected into the mice in PCD NPs + NLG NPs and PCD NPs + NLG NPs + L groups on the 3^{rd}, 6^{th}, 9^{th}, and 12^{th} day. The tumor dimensions (length and width) and body weights of the mice were measured every other day. 22 days later, the mice in 6 groups were all sacrificed followed by collection of blood, tumors, and main organs.

Flow cytometry assay

The tumor tissues were digested with collagenase (1 mg mL^{-1} in PBS with 10% FBS), DNase I (0.1 mg mL^{-1} in PBS with 10% FBS), and hyaluronidase (0.1 mg mL^{-1} in PBS with 10% FBS) to obtain single-cell suspensions. PBS (100 μL) was used to collect and disperse the cells. The cells were then treated with fluorescence-conjugated antibodies for 45 min before being washed twice with PBS. The cells were tested by flow cytometry. Table S2 shows the fluorescence-conjugated antibodies.

ELISA assay

The tumor tissues (100 mg) were homogenized with 1mL of PBS, and the mixtures were centrifuged to acquire the supernatants. TNF-α, IL-6, IFN-γ, and Granzyme B were measured in tumor samples using ELISA kits according to the manufacturer's instructions.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Significance analysis was performed using a two-way ANOVA. ns: not significant, *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$).

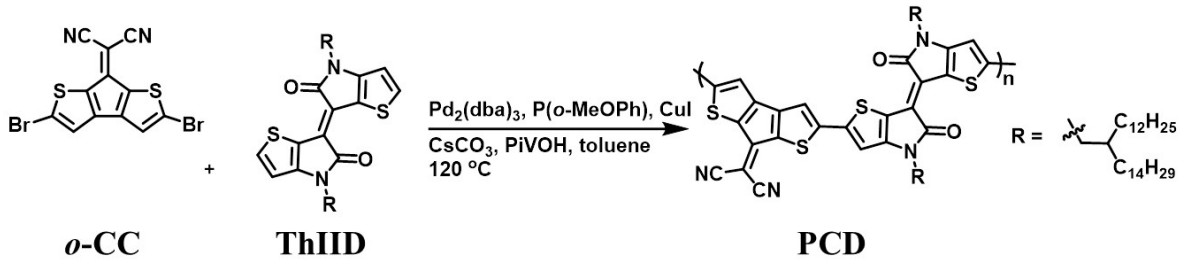

Fig. S1 Synthetic route of PCD.

Elemental	N (%)	C (%)	H (%)	S (%)
Measured	3.9	71.6	8.9	8.6
Theoretical	4.1	74.5	9.5	9.5
Mn (KDa)	10.3			
PDI	2.3			

Table S1 The results of elemental analysis and molecular weight of PCD.

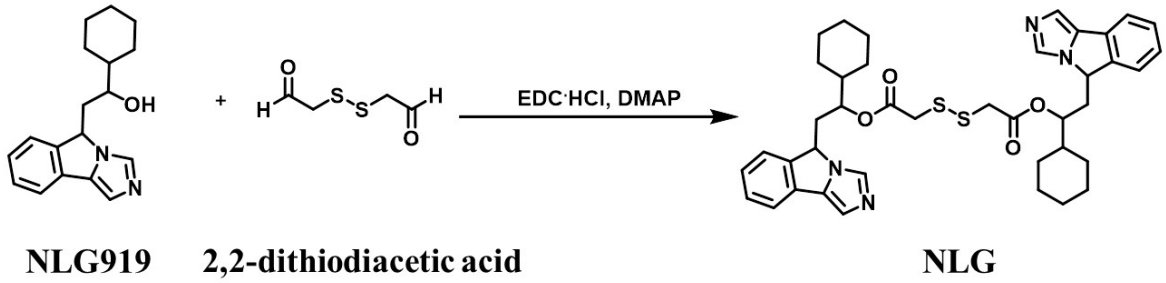

Fig. S2 Synthetic route of NLG.

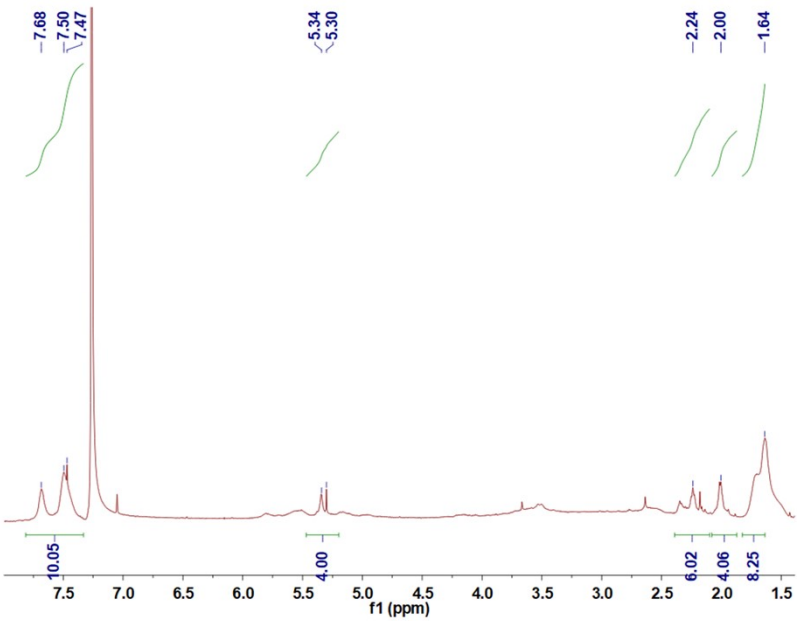

Fig. S3 ^{1}H NMR spectrum of NLG.

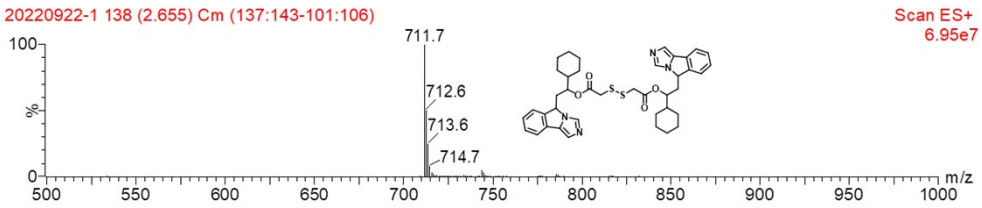

Fig. S4 Mass spectrum of NLG.

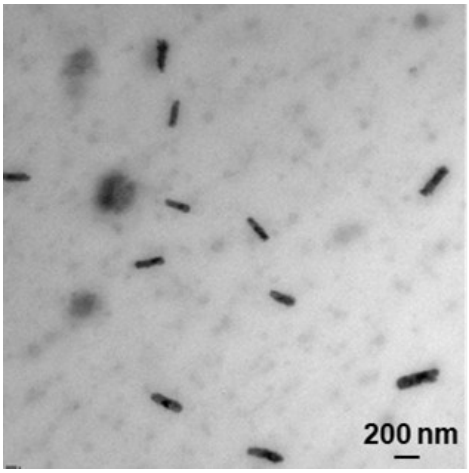

Fig. S5 TEM image of NLG NPs after storage for 7 days.

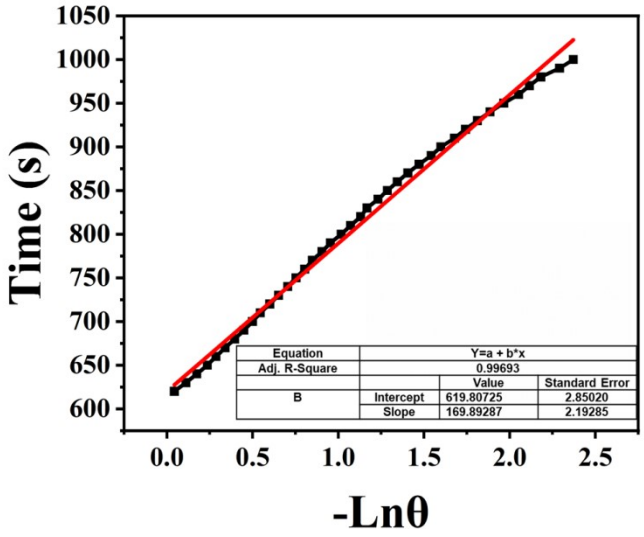

Fig. S6 The relationship between cooling period of time and the negative natural logarithm of temperature changes of PCD NPs.

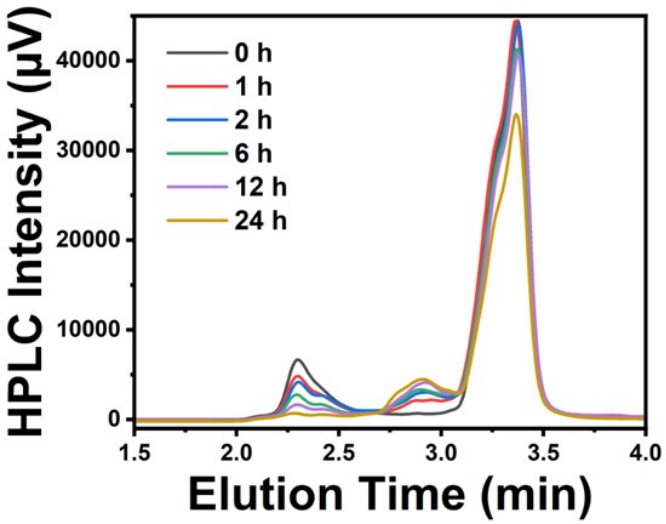

Fig. S7 Reduction responsiveness of NLG NPs in the presence of 10 mM DTT at 37 °C.

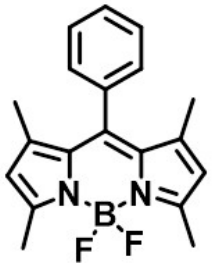

Fig. S8 Molecular structure of boron dipyrromethene probe.

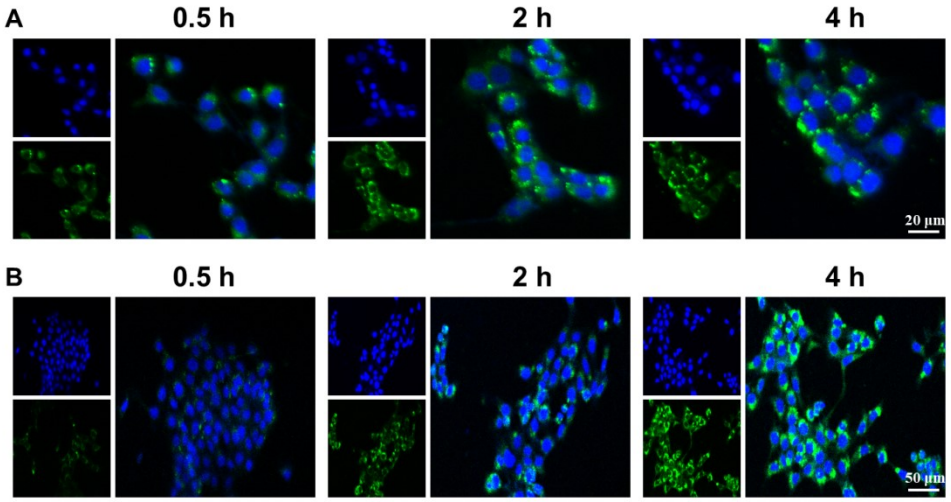

Fig. S9 CLSM images of 4T1 cells treated with (A) PCDB NPs and (B) NLGB NPs for 0.5, 2, and 4 h, respectively.

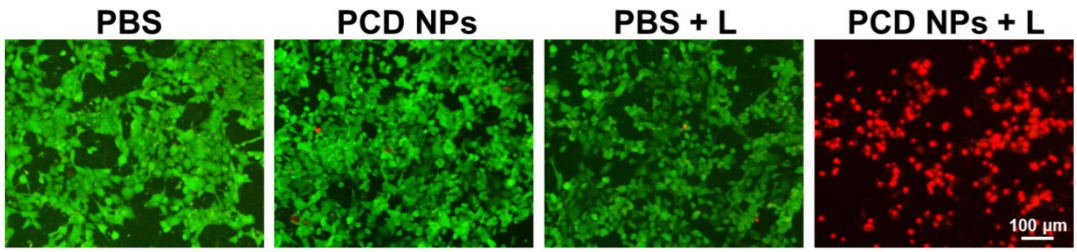

Fig. S10 Fluorescence images of calcein-AM/PI costained 4T1 cells subjected to different treatments.

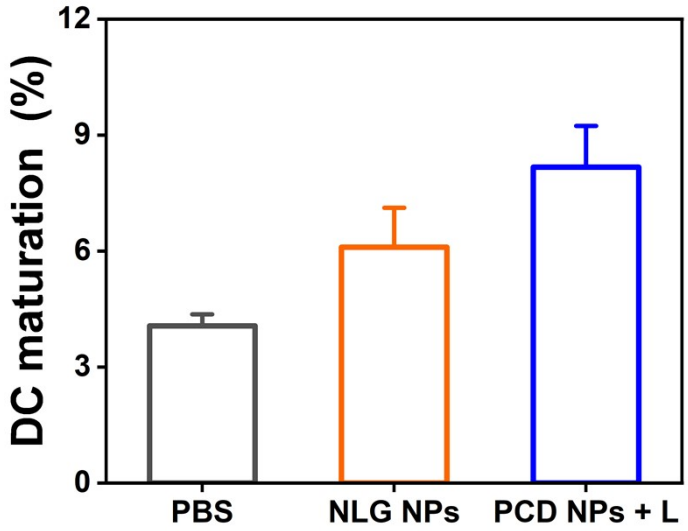

Fig. S11 Ratios of DC maturation under different conditions.

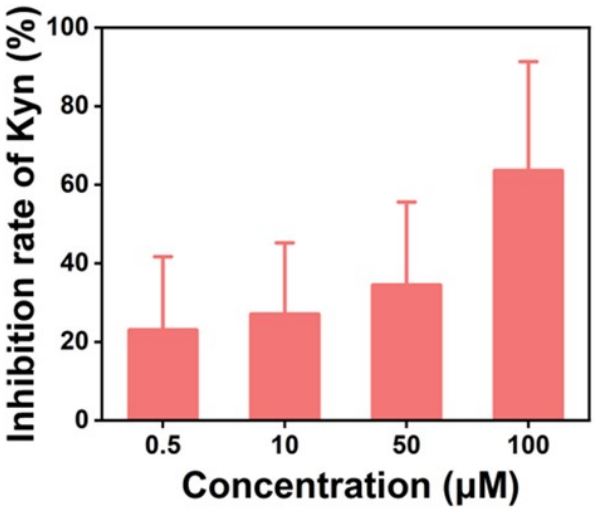

Fig. S12 Inhibition rates of Kyn in cells under treatments with different concentrations of NLG NPs.

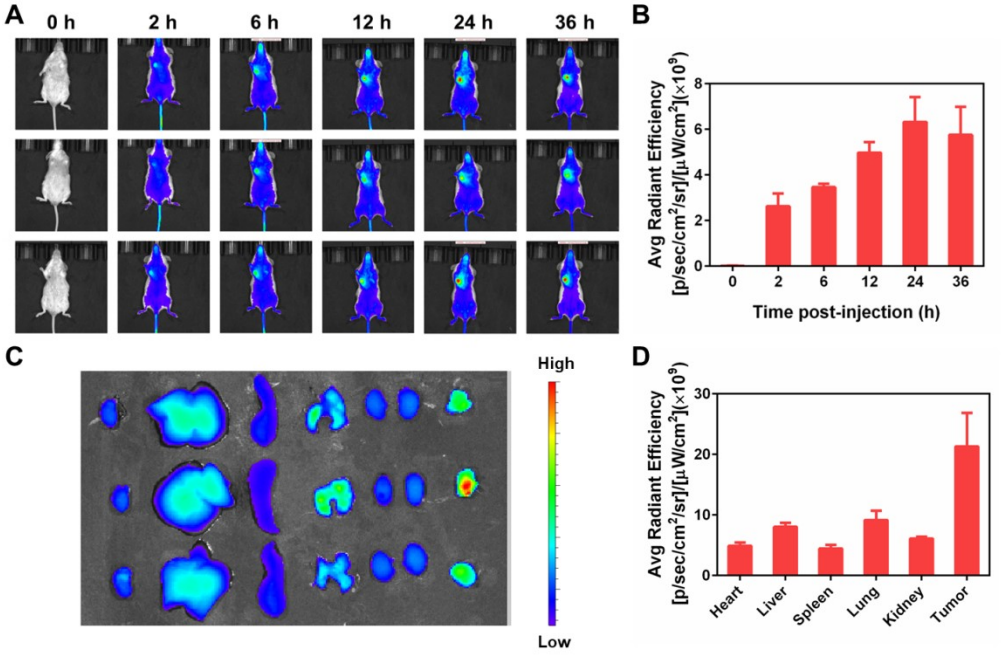

Fig. S13 (A) Fluorescence images of mice and (B) fluorescence intensity of tumors after intravenous injection of PCDI NPs for different times. (C) Fluorescence images and (D) fluorescence intensity of ex vivo organs and tumors of mice after intravenous injection of PCDI NPs for 36 h.

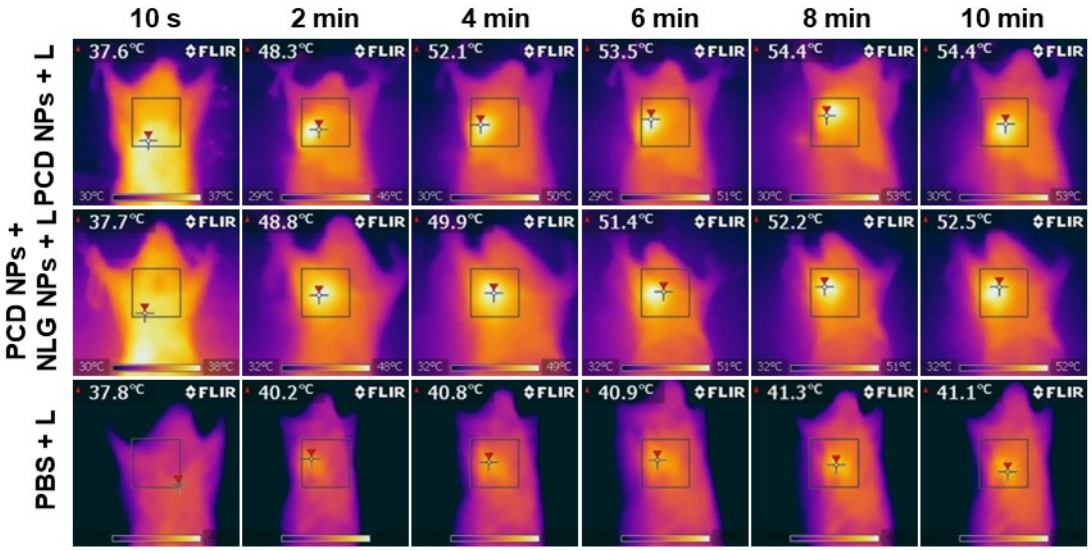

Fig. S14 Infrared thermal images of the mice in laser irradiation groups.

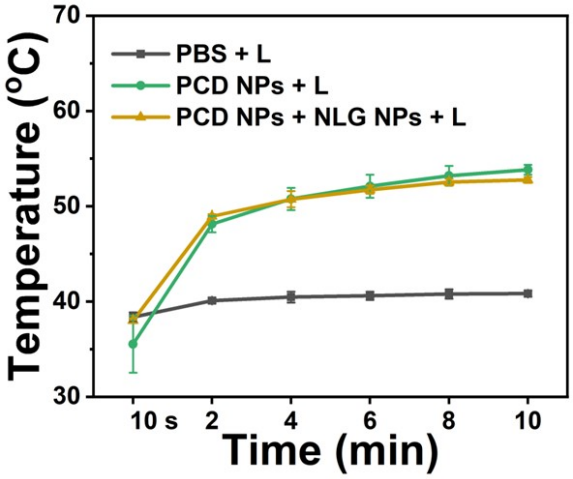

Fig. S15 Temperature changes of tumors with irradiation time.

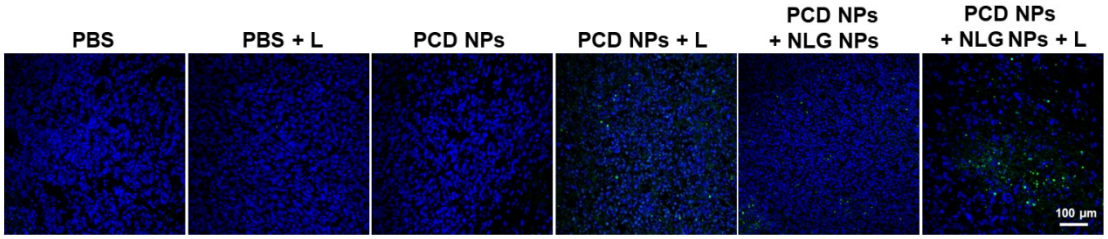

Fig. S16 TUNEL staining of tumor tissues.

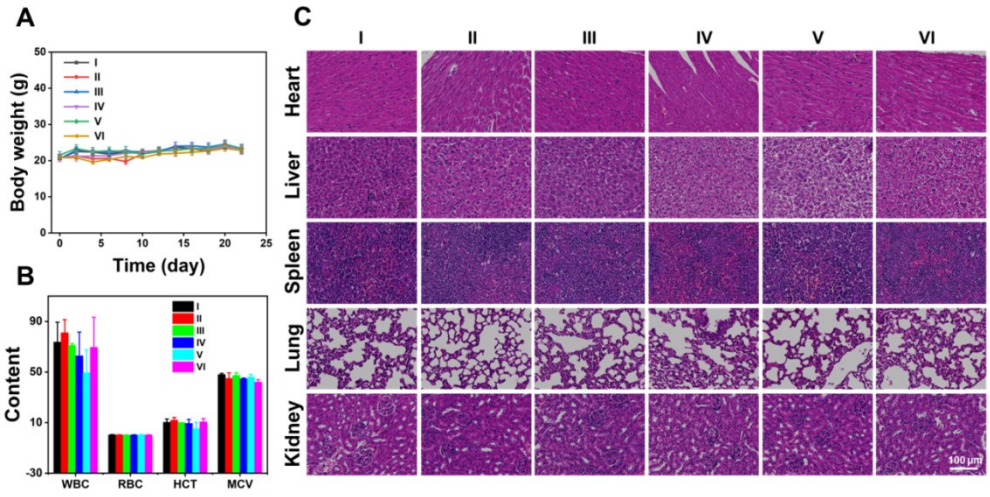

Fig. S17 (A) Body weights of the mice from day 0 to day 22. (B) Hematological parameters (WBC, white blood cell; RBC, red blood cell; HCT, hematocrit; MCV, mean corpuscular volume) of the mice after treatments for 22 days. (C) H&E staining of major organs in different treatment groups. I: PBS; II: PBS + L; III: PCD NPs; IV: PCD NPs + NLG NPs; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

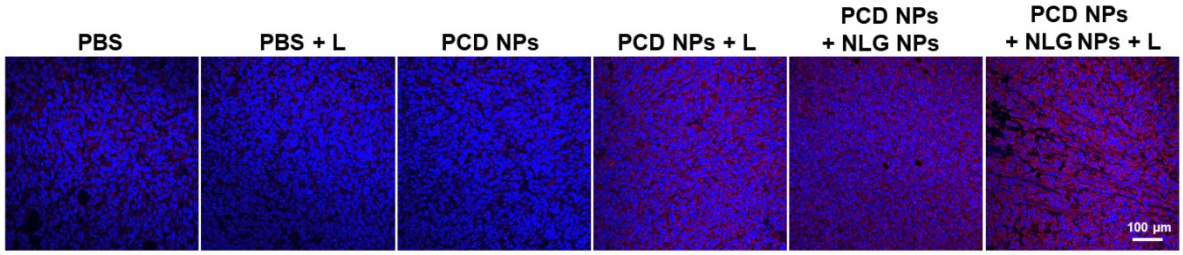

Fig. S18 Immunofluorescence staining of CRT in tumor tissues.

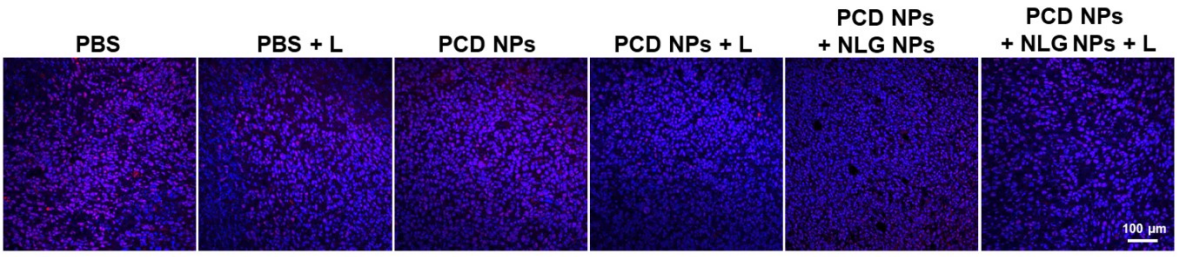

Fig. S19 Immunofluorescence staining of HMGB1 in tumor tissues.

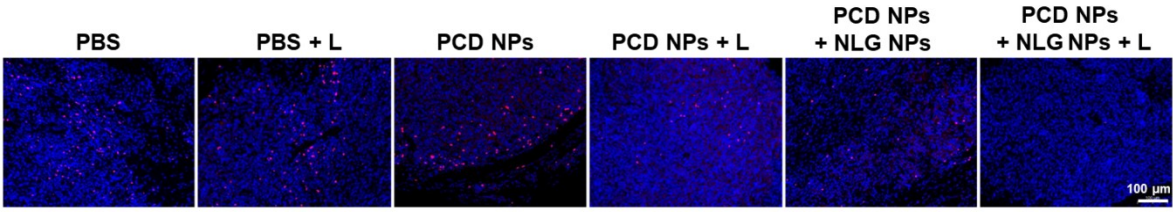

Fig. S20 Immunofluorescence staining of Tregs in tumor tissues.

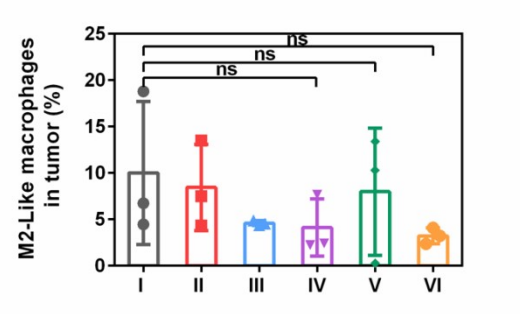

Fig. S21 Ratios of M2-like macrophages in tumors. I: PBS; II: PBS + L; III: PCD NPs; IV: PCD NPs + NLG NPs; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

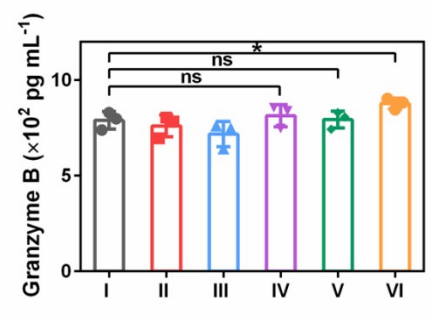

Fig. S22 Expression levels of Granzyme B in tumors characterized by ELISA. I: PBS; II: PBS + L; III: PCD NPs; IV: PCD NPs + NLG NPs; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

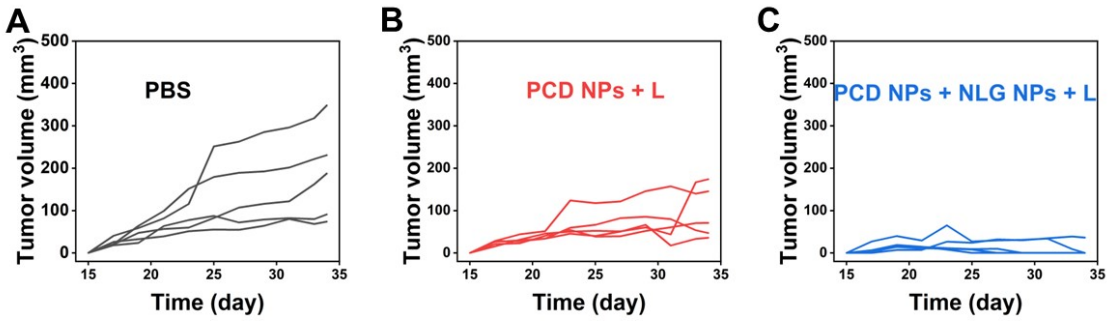

Fig. S23 Tumor growth curves of the recurrent tumors from day 15 to day 34 in (A) PBS, (B) PCD NPs + L, and (C) PCD NPs + NLG NPs + L groups.

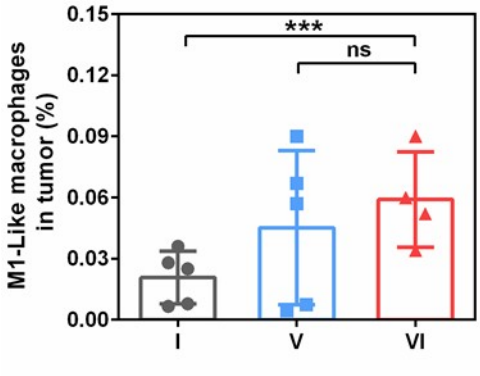

Fig. S24 The proportions of M1-like macrophages in the recurrent tumors. I: PBS; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

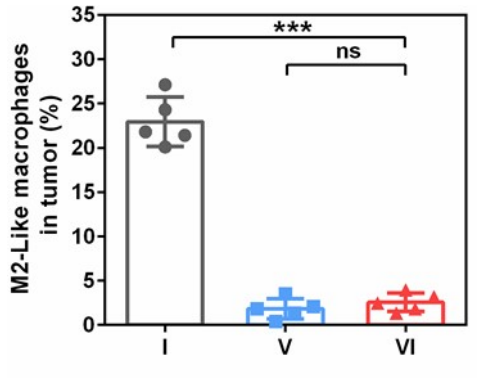

Fig. S25 The proportions of M2-like macrophages in the recurrent tumors. I: PBS; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

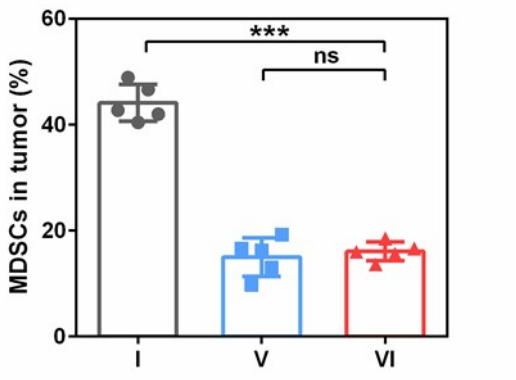

Fig. S26 The proportions of MDSCs in the recurrent tumors. I: PBS; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

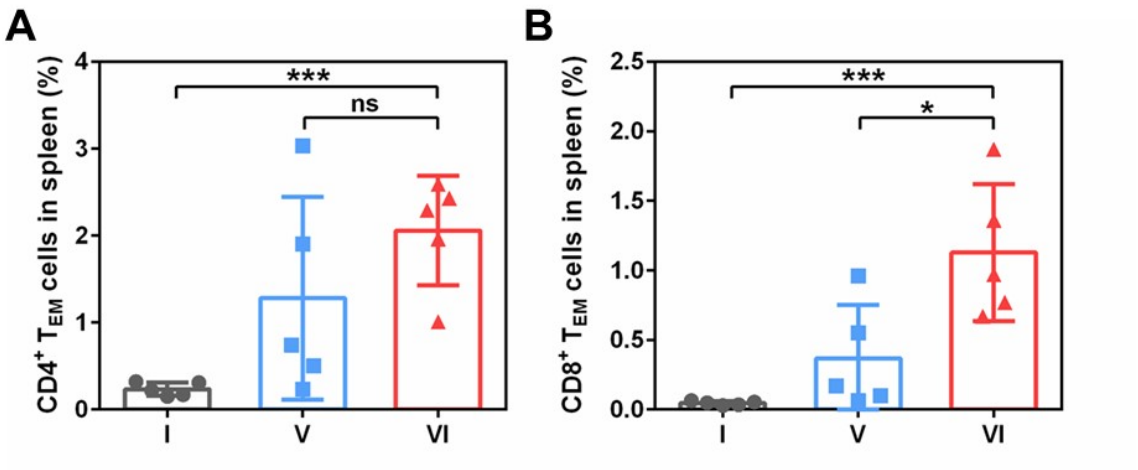

Fig. S27 (A) Ratios of $CD4^+$ and (B) $CD8^+$ T_{EM} cells in spleens. I: PBS; V: PCD NPs + L; VI: PCD NPs + NLG NPs + L.

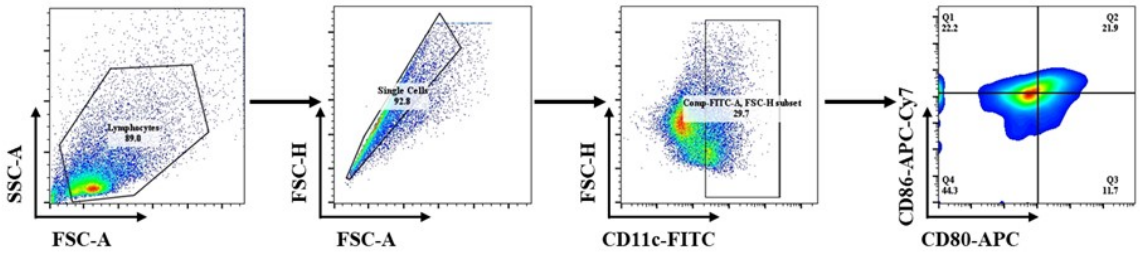

Fig. S28 Gating strategy for flow cytometry of DC maturation in TDLNs.

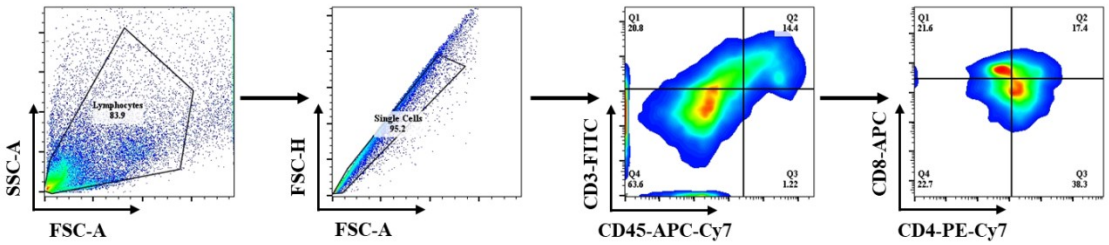

Fig. S29 Gating strategy for flow cytometry of $CD3^+$ T cells, $CD4^+$ T cells, $CD8^+$ T cells in tumors.

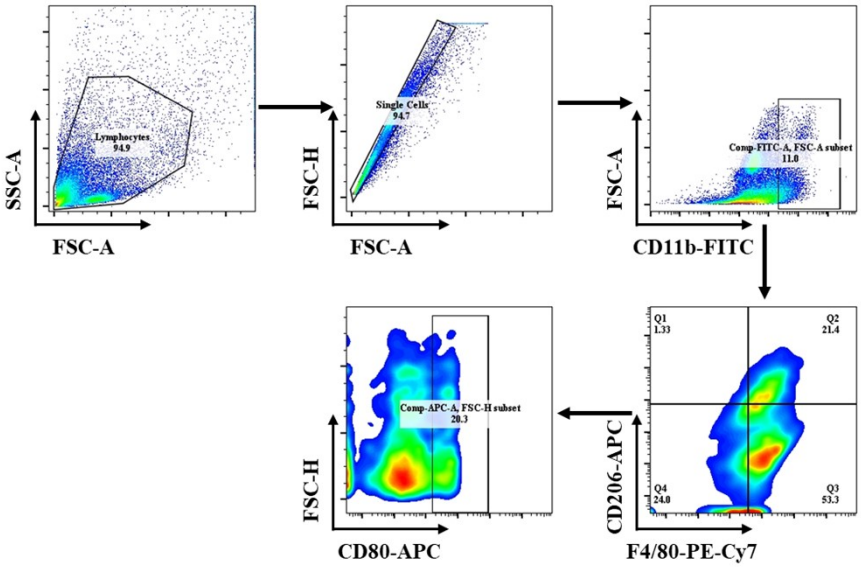

Fig. S30 Gating strategy for flow cytometry of M1-like macrophage and M2-like macrophage in tumors.

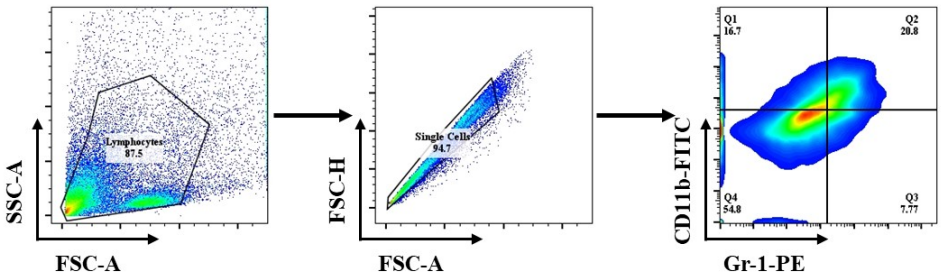

Fig. S31 Gating strategy for flow cytometry of MDSCs cells in tumors.

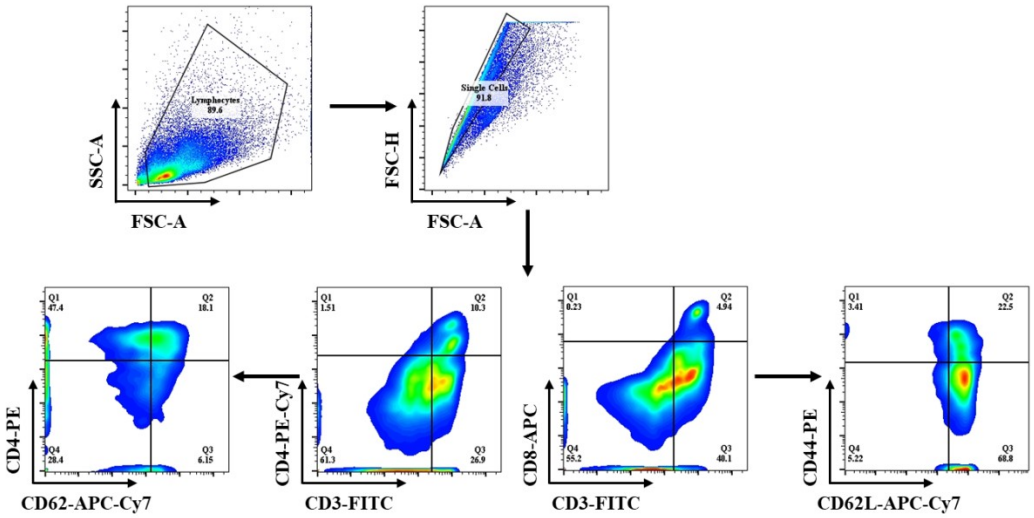

Fig. S32 Gating strategy for flow cytometry of $CD4^+$ T cells, $CD4^+$ T_{EM} cells, $CD4^+$ T_{CM} cells, $CD8^+$ T cells, $CD8^+$ T_{EM} cells, and $CD8^+$ T_{CM} cells in spleens.

Antibodies	Company	Catalog
CD45-APC-Cy7	Biolegend	103115
CD3-FITC	Biolegend	100203
CD4-PE/Cy7	Biolegend	100527
CD8-APC	Biolegend	100711
CD11b-FITC	Biolegend	101205
Gr-1-PE	Biolegend	108107
F4/80-PE-Cy7	Biolegend	123113
CD206-APC	Biolegend	141707
CD11C-FITC	Biolegend	117305
CD80-APC	Biolegend	104713
CD86-APC-Cy7	Biolegend	105029
CD44-PE	Biolegend	103023
CD62L-APC-Cy7	Biolegend	104427

Table S2 Antibodies for flow cytometry.

Reference

1 Y. Liu, K. Ai, J. Liu, M. Deng, Y. He and L. Lu, Dopamine-Melanin Colloidal Nanospheres: An Efficient Near-Infrared Photothermal Therapeutic Agent for In Vivo Cancer Therapy, *Adv. Mater.*, 2012, **25**, 1353-1359.