Electronic Supplementary Information

Neutrophil Membrane Functionalized Black Phosphorus Riding Inflammatory Signal for Positive Feedback and Multimode Cancer Therapy

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Experimental Procedures

Materials. Bulk BP (99.998%) was purchased from Xianfeng Nanomaterials Technology Co., LTD (Nanjing, China). PEI (1.8K) were obtained from Sigma-Aldrich (St. Louis, MO, USA). LY364947 (99.79%) was purchased from Selleck Co., LTD (Shanghai, China). Percoll was bought from GE Healthcare Bio-Sciences Co., LTD (Uppsala, Sweden). DCFH-DA, Annexin V-FITC Apoptosis Detection Kit, Mouse IL-6 ELISA Kit and Mouse TNF-α ELISA Kit were purchased from Beyotime Biotechnology (Shanghai, China). MTT and Cy7 were obtained from Sigma-Aldrich, Co. LTD (USA). FITC and DAPI were offered by Aladdin Reagent Database Inc. (Shanghai, China). FITC-labeled Gr-1 and PE-labeled MAIR-IV antibodies were purchased from Biolegend (USA). Other primary antibodies and Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from SantaCruz Biotechnology (Santa Cruz, CA, USA).

Preparation of BP nanoflakes. The BP nanoflakes were prepared according to the

reported liquid exfoliation.[1, 2] In brief, 20 mg of BP powder was dispersed in 50 mL of ultrapure water bubbled with nitrogen to eliminate dissolved oxygen. The sample was sonicated in ice water with a sonic tip at a power of 950 W for 4 h and sequentially with an ultrasonic bath at 300 W for another 6 h. The resulted brown suspension was centrifuged at 2000 g for 10 min to remove the unexfoliated bulk. The BP nanoflakes were obtained by lyophilization of the brown supernatant.

Preparation of BP-PEI-LY. To synthesize BP-PEI-LY, 20 mg of LY in DMSO was firstly added into 10 mg of PEI aqueous solution and stirred at room temperature (R.T.) for 24 h.[3] The mixture was then dropped into 10 mg of BP suspension for another 4 h agitation and 1 h sonication with a sonic tip at 200 W. The BP-PEI-LY was obtained by centrifugating the suspension in Amicon tube (10 kDa) to remove the solvent, repeated water rinsing and freeze drying. BP-PEI as control was fabricated similar to BP-PEI-LY without loading of LY.

Preparation of NG/BP-PEI-LY. NEs from peripheral blood were isolated through Percoll gradient method described by Boxio et al.[4] Mice blood collected from eyeball into anticoagulant tube was centrifugated (800 g, 10 min, 4℃) for blood cells sedimentation. After removing serum, the buffy coat and upper part of blood cells containing leukocytes was drawn and diluted in PBS buffer. The cells were subsequently laid on the top of a three-layer Percoll gradient of 52%, 69%, and 78%, followed by centrifugation (1500 g, 30 min, R.T.) for separation. The 69%/78% interface and the upper part of the 78% layer enriched with NEs were harvested and submitted to flow sorter for further isolation. For isolating NEs membrane,[5] NEs after

4 h of activation by LPS were homogenized in cold hypotonic lysing buffer with protease inhibitor cocktail for 50 strokes using a tight pestle equipped homogenizer. Then, the homogenate was centrifuged (10000 g, 10 min, 4℃) to discard the unbroken cells and intracellular substance. The supernatant was centrifuged again (20000 g, 1 h, 4℃) to collected the NEs membrane containing pellet. The NGs was obtained by extruding NEs membrane sequentially through an 800 nm and a 400 nm polycarbonate membrane for 11 passes for each membrane by a mini-extruder and freeze-dried for further use.

To prepare NG/BP-PEI-LY, 2.6 mg of NGs mixed with 10 mg of BP-PEI-LY was dispersed in distilled water and sonicated with a sonic tip at 200 W for 3 min. The mixture was then extruded sequentially through an 800 nm and a 400 nm polycarbonate membrane for 11 passes for each membrane by a mini-extruder. NG/BP-PEI-LY was received by collecting the pellet after centrifugation (20000 g, 50 min, 4°C). RG/BP-PEI-LY as control were prepared by similar protocols as NG/BP-PEI-LY but replaced NG with RG.

Characterization of NG/BP-PEI-LY. The morphologies and elements distributions of the prepared samples were observed by transmission electron microscope (TEM, Hitachi, Japan, 80 kV), scanning electron microscope (SEM) and energy dispersive spectrometer mapping (EDS mapping, FEI, USA, 300 kV). Hydrodynamic diameters and zeta potentials of different samples were determined by dynamic lights scattering (DLS) using a Malvern Zetasizer (Nano ZS-90, Malvern instruments, UK, 25℃ with 90° scattering angle). Raman spectra were performed by confocal micro-Raman

spectroscopy (LabRam HR800, France). The chemical composition of the samples was confirmed by X-ray photoelectron spectroscopy (XPS). To reveal the amount of LY and PEI on BP-PEI-LY, optical density (OD) value of LY at 279 nm was obtained by ultraviolet spectrophotometer and the absorbance of FITC-labeled PEI was determined by fluorophotometer.

Coomassie blue staining was employed to verify the inheritance of membrane associated protein on NG/BP-PEI-LY. Homogenized NE, NG and NG/BP-PEI-LY were lysed in a RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS) and centrifuged (12000 g for 5 min at 4℃) to remove the pellet. The total protein in supernatant was quantified by BCA protein assay, followed by heating in SDS loading buffer at 100℃ for 5 min. Equal amount of protein per sample was subjected to an SDS-PAGE electrophoresis assay. The images were displayed through staining protein blots with Coomassie blue fast staining solution. To investigate the major adhesion molecules on NG/BP-PEI-LY, proteins from each sample were performed by a standard western-blot analysis to detect L-selectin, LFA-1, Mac-1 and β1 expression. The bands were analyzed with an Odyssey Scanning System (LI-COR Inc., USA).

Stability study. The dispersion stabilities of different samples were investigated in distilled water and BPS buffer, respectively. The suspensions were stood at R.T. for 24 h. At predetermined time, the absorbance of the suspensions at 459 nm were obtained by a UV-vis spectrophotometer. For photothermal stabilities, samples suspended in water were exposed to ambient at R.T. for 5 days. At predetermined time, the

temperature changes of suspensions containing equal amount of BP under 808 nm laser irradiation (1.0 W/cm^2, 10 min) were revealed by a thermal infrared imaging camera (Flir C2, USA).

ROS generation study. 1, 3-diphenylisobenzofuran (DPBF) in ethanol (20 μg/mL) were stirred with different samples (containing 10 μg/mL of BP) in dark for 100 min to reach the adsorption/desorption equilibrium prior to the test. The samples were then subjected to a 650 nm laser irradiation (0.5 W/cm^2, 10 min) and detected by UV−Vis spectrophotometer at 412 nm. The concentration of NaN_3 for ROS elimination was 50 μg/mL.

In vitro **drug release.** To establish the release curve of LY from NG/BP-PEI-LY, samples were dispersed in water at different pH (pH 7.4 and pH 5.5) with or without laser irradiation (650 nm, 0.5 W/cm^2, 10 min or 808 nm, 1.0 W/cm^2, 10 min). A bit of the samples was drawout and centrifuged (12000 g, 30 min) at pre-set time points to detect the OD values of supernatants at 279 nm. The release percentages of LY were determined as: the amount of LY in supernatant/the total amount of LY in NG/BP-PEI-LY × 100%.

Cell experiments. Mouse breast carcinoma cell 4T1 and human umbilical vein endothelial cell HUVEC was purchased from the Cell Bank of Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2.

To investigate the cellular uptake of the prepared samples, FITC labeled PEI was

used to prepare preparations. 4T1 cells in confocal dishes with 80% cell confluence were incubated with different formulations containing 20 μg/mL of BP for 4 h. The cells were then fixed and stained with DAPI (10 μg/mL), followed by visualizing by CLSM. Cells suffered from 4 h cellular uptake were also harvested for flow cytometry analysis (BD FACS Calibur, USA). The cellular uptake efficiencies were determined by FITC fluorescence intensities.

For intracellular ROS measurement, 4T1 cells and HUVEC pretreated with different formulations containing 20 μg/mL of BP were incubated with H_2DCFH-DA (0.03 mg/mL) and irradiated with 650 nm laser (0.5 W/cm^2, 10 min). The cells washed with PBS buffer were imaged under CLSM and harvested for flow cytometry analysis.

To demonstrate *in vitro* safety, PTT and PDT efficiencies, 4T1 cells and HUVEC with 80% cell confluence were incubated with different formulations containing 20 μg/mL of BP for 4 h and exposed to an 808 nm laser (1.0 W/cm^2, 10 min) or/and a 650 nm laser (0.5 W/cm^2, 10 min). The cells were additionally cultured for 6 h for apoptosis detection in accordance with the kit manufacturer's protocol by flow cytometry analysis. The cells additionally cultured for 24 h were measured by a standard MTT assay to examine cell viabilities.

To reveal the *in vitro* positive feedback effect of NG/BP-PEI-LY, a two-dosing experiment was carried out. HUVEC with 80% cell confluence were suffered from a 4 h of cellular uptake of RG/BP-PEI-LY or NG/BP-PEI-LY containing 20 μg/mL of BP. For laser irradiation groups, cells were exposed to an 808 nm laser (1.0 W/cm^2, 10 min) or/and a 650 nm laser (0.5 W/cm^2, 10 min) followed by another 12 h incubation. For

TNF-α activation groups, cells were activated by TNF-α (25 ng/mL) for 6 h and pre-saturated with anti-ICAM-1. The cells were then treated with FITC labeled RG/BP-PEI-LY or NG/BP-PEI-LY and PE labeled ICAM-1 to display the amount of preparations in cytoplasm and ICAM-1 on cell membrane revealing by CLSM and flow cytometry. To further verify the positive feedback effect of NG/BP-PEI-LY, a parallel plate flow chamber described in previous literature[6] was used in *in vitro* adhesion assay. Cells suffered from a first round of treatment as describe before were then incubated in the flow containing FITC labeled RG/BP-PEI-LY or NG/BP-PEI-LY (containing 20 μg/mL of BP) at a rate of 0.1 dyn/cm^2 for 2 h. The amount of RG/BP-PEI-LY or NG/BP-PEI-LY adherent to the HUVEC was visualized by CLSM and quantified by Image J.

The real-time cellular uptake study of NG/BP-PEI-LY with and without laser irradiation were performed by FITC-labeled NG/BP-PEI-LY in the two rounds of administrations. The fluorescence intensities of HUVEC at different time points were detected by CLSM and quantified by FCM.

***In vivo* positive feedback effect of NG/BP-PEI-LY.** Female BALB/c mice (4-5 weeks, 18-20 g) were obtained from Shanghai Laboratory Animal Center (SLAC, China) and used under protocols approved by the University Ethics Committee for the use of experimental animals. To establish 4T1 orthotropic transplantation tumors, 4T1 cells (5×10^5) were injected into mammary fat pad of each mouse. When tumors reached 150 mm^3, Cy7 labeled preparations containing equal BP (5 mg/kg) were administrated *via* tail vein. 24 h after injection, tumors were irradiated by 808 nm laser

(1.0 W/cm^2, 10 min) or/and 650 nm laser (0.5 W/cm^2, 10 min), followed by a second round of injection of Cy7 labeled preparations at 24 h post-irradiation. The NIR fluorescent images were acquired at pre-set time points by an *in vivo* imaging system (FX PRO, Kodak, USA). Subsequently, mice were sacrificed and tumors as well as major organs (heart, liver, spleen, lung and kidney) were excised for *ex vivo* imaging for quantitative analysis. Besides, tumors were collected and sectioned for staining with anti-CD31 and anti-ICAM-1 for CLSM examination. To demonstrate PDT or/and PTT induced inflammation, cytokines level of IL-6 and TNF-a in both serum and tumor tissue were also determined by a standard immunosorbent assay (ELISA) before and after laser irradiation.

***In Vivo* Therapeutic Efficacy of NG/BP-PEI-LY.** 4T1 tumors were inoculated into mammary fat pad of each mouse as above mentioned. When the average tumor volume reached about 150 mm^3, mice were administrated various preparations *via* tail vein at day 0, 2, 4 and 6. Meanwhile, 808 nm laser (1.0 W/cm^2, 10 min) or/and 650 nm laser (0.5 W/cm^2, 10 min) were projected onto tumors at day 1, 3, 5 and 7. The infrared thermographic maps of mice under first two rounds of 808 nm laser irradiation were recorded. The tumors were measured by vernier caliper every other day until day 16 and the tumor volumes were calculated as 0.5 × length × width^2. The body weights of mice were recorded every other day as well. At day 16, mice were sacrificed and lungs were isolated carefully. After fixed in Bouin's solution, each of the five pulmonary lobes was separated, and surface metastatic nodules were counted under a dissecting microscope. Subsequently, the lungs were sectioned for histological examinations to

further investigate metastatic lesions. The survival periods of the mice received different treatments were monitored.

Arousal of tumor immune response. Tumor bearing mice were received different treatment as above mentioned. At day 16, the amount of intratumoral CD8+ and CD4+ T cells were examined. Tumors were harvested for frozen section and stained with FITC labeled anti-CD8 and PE labeled anti-CD4 antibodies for CLSM observation. The quantitative data was obtained by counting T cells in 5 randomly selected fields of each section. For quantitative analysis of the activation of tumor immune, tumors and draining lymph nodes were digested by collagenase IA (Sigma) at 37 °C for 30. The resulting tissue suspensions were passed through a 70 μm tissue filter and the cells were washed twice with PBS containing 1% FBS. To analyze activated CD8+ T cells, cells were incubated with PE labeled anti-CD8 (Biolegend) and FITC labeled anti-IFN-γ (Biolegend) antibodies. To analyze activated CD4+ T cells, cells were incubated with PE labeled anti-CD4 (Biolegend) and FITC labeled anti-IFN-γ (Biolegend) antibodies. To analyze Treg cells, cells were incubated with PE labeled anti-CD4 (Biolegend) and FITC labeled anti-Foxp3 antibodies. After washing to remove uncombined antibodies, cells were examined by flow cytometry.

Statistical analysis. All the data were expressed as mean ± SD from triplicate experiments performed in a parallel manner unless otherwise noted. Statistical analysis was performed by two-tailed Student's t-test and statistical significance was indicated as $*P < 0.05$, $**P < 0.01$ or $***P < 0.001$.

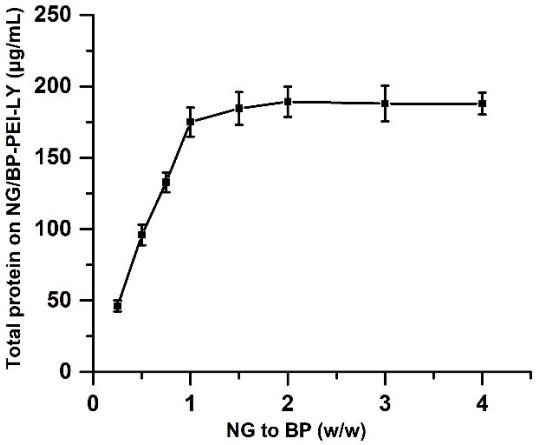

Fig. S1 Quantification of total protein on NG/BP-PEI-LY by BCA assay. The amount of protein on NG/BP-PEI-LY prepared under different NG to BP feeding ratio reflected the coating efficiency of NG on BP-PEI-LY. Data were expressed as mean ± S.D. (n = 3)

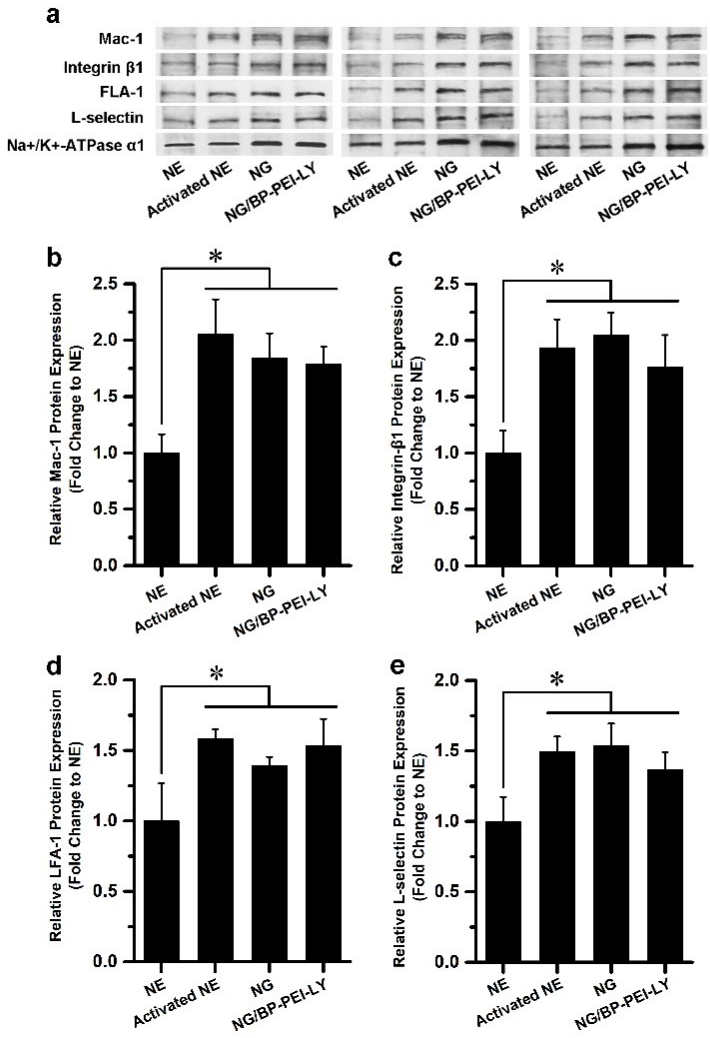

Fig. S2 Western blotting assay. (a) Western blotting assay of major adhesion molecules on NE, LPS activated NE, NG and NG/BP-PEI-LY. Quantitative analysis of (b) Mac-1, (c) Integrin-β1, (d) LFA-1 and (e) L-selectin on NE, LPS activated NE, NG and NG/BP-PEI-LY. The Na+/K+-ATPase α1 was set as internal control and the results were normalized to NE groups. Quantitative data were expressed as mean ± S.D. (n = 3), *$P < 0.05$.

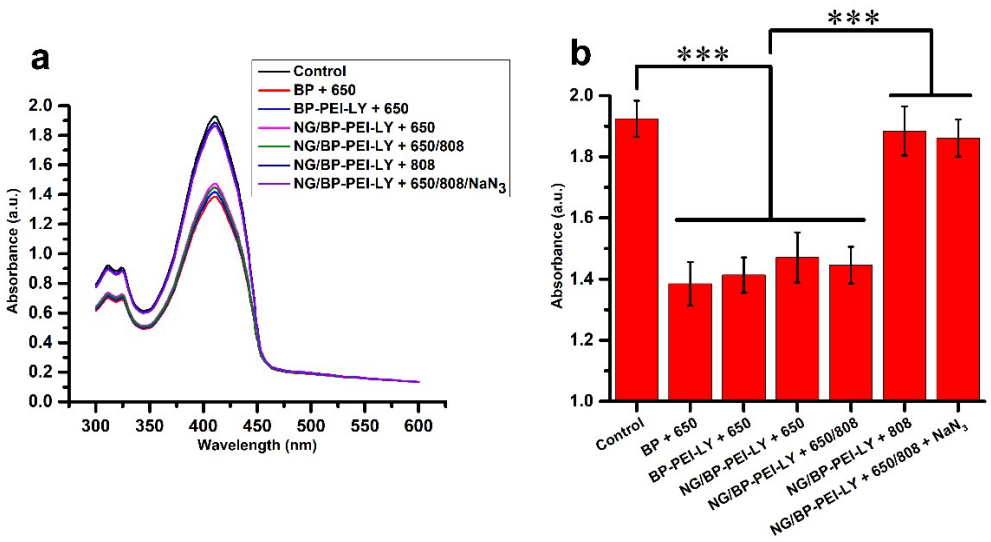

Fig. S3 Characterization of ROS generation ability of NG/BP-PEI-LY. (a) The UV-Vis absorption spectra of the DPBF with nanoparticles with different laser irradiations. (b) The quantitative analysis of UV-Vis absorption spectra at 412 nm of the DPBF with nanoparticles with different laser irradiations. Quantitative data were expressed as mean ± S.D. ($n = 3$), ***$P < 0.001$.

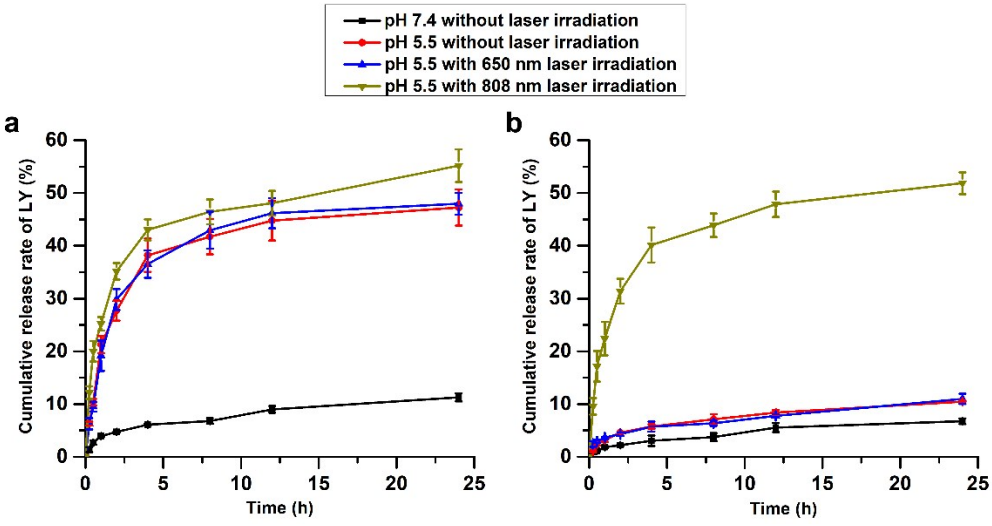

Fig. S4 *In vitro* LY release study. Cumulative release curve of LY from a) BP-PEI-LY and b) NG/BP-PEI-LY at different pH (pH 7.4 and pH 5.5) with or without laser irradiation (650 nm and 808 nm laser irradiation). Data were expressed as mean ± S.D.

(n = 3)

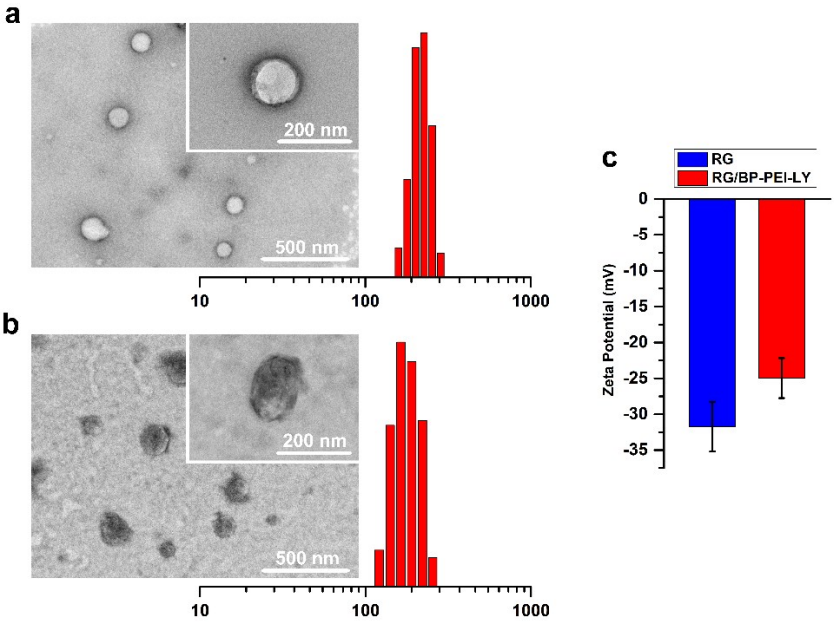

Fig. S5 Characterization of RG/BP-PEI-LY. TEM images and particle size distribution of a) RG and b) RG/BP-PEI-LY. c) Zeta potentials of RG and RG/BP-PEI-LY. Data were expressed as mean ± S.D. (n = 3)

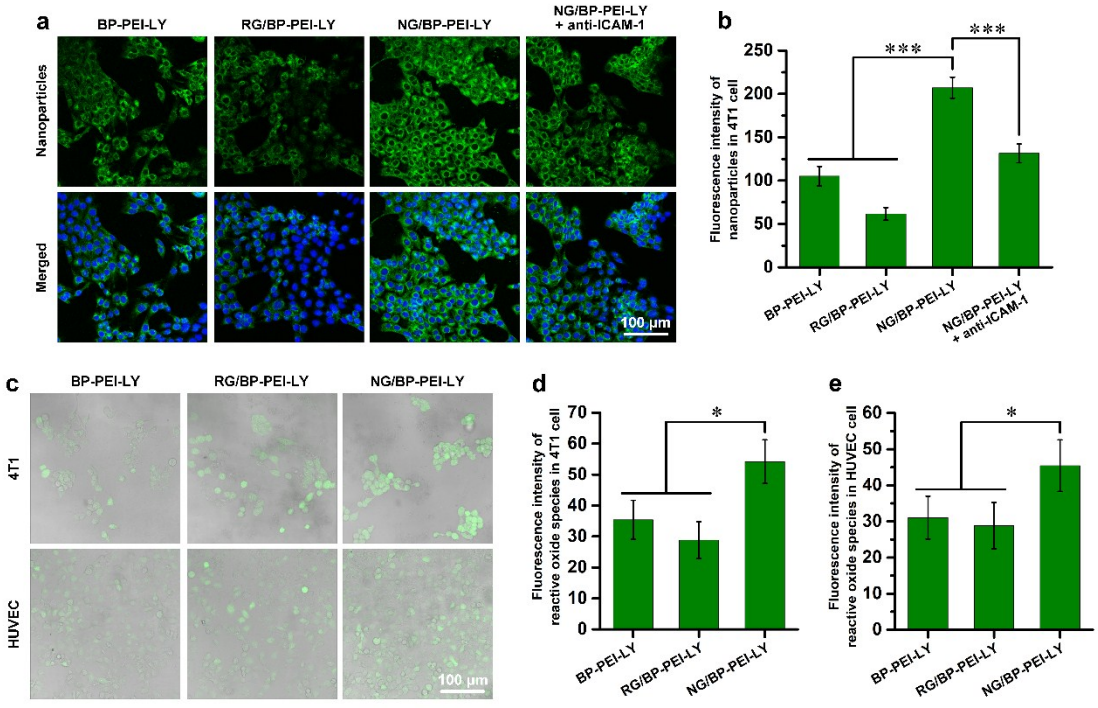

Fig. S6 Cellular uptake and intracellular ROS study. a) CLSM images and b) flow cytometry quantification of cellular uptake of FITC labeled preparations containing 20 μg/mL of BP for 4 h. c) CLSM images of ROS induced fluorescence in 4T1 and HUVEC after PDT. Quantitative data of ROS induced fluorescence intensities in d) 4T1 and e) HUVEC after PDT. Data were expressed as mean ± S.D. (n = 3), *P < 0.05 and ***P < 0.001.

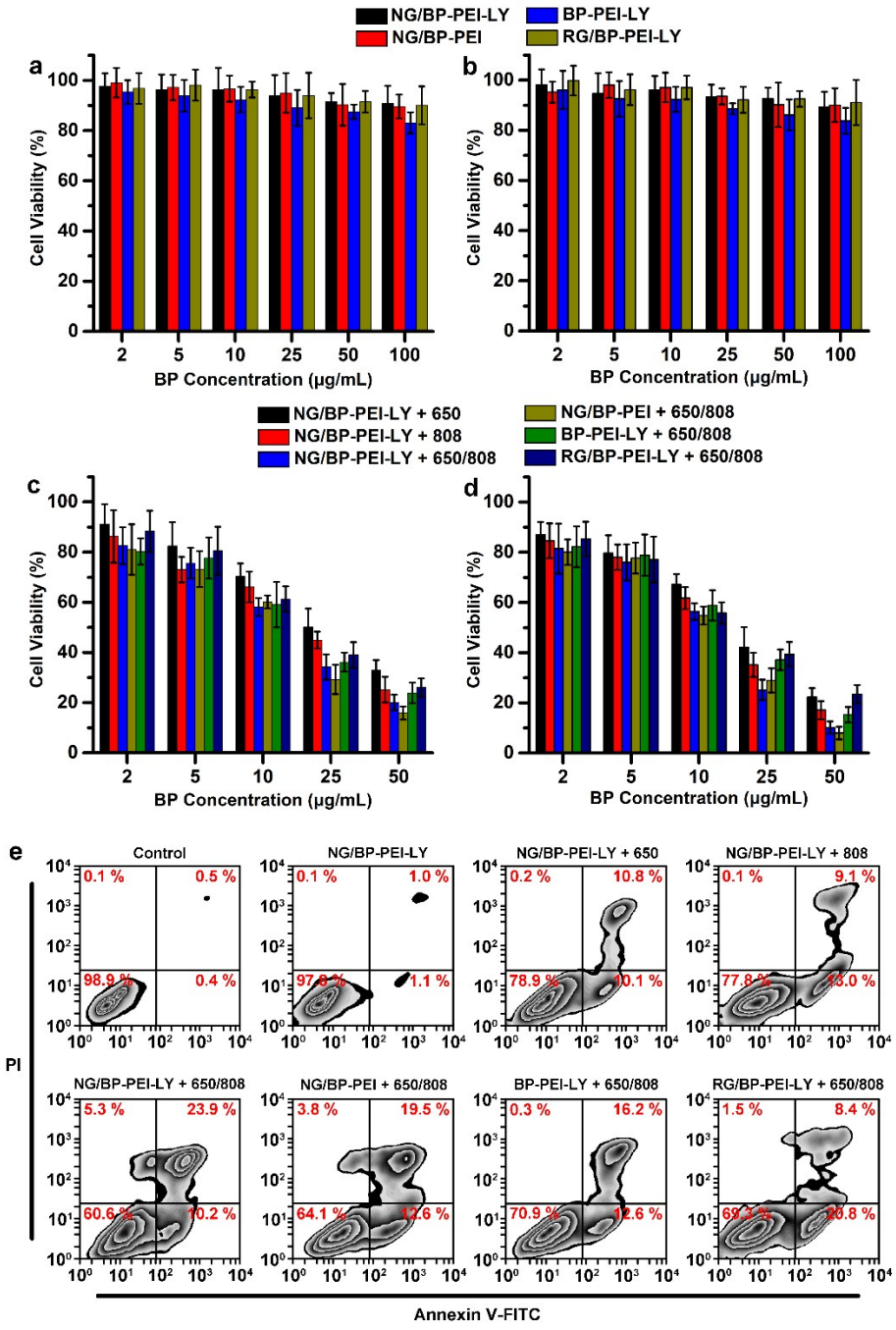

Fig. S7 *In vitro* safety and therapeutic efficiency study. Cell viabilities of a) HUVEC and b) 4T1 treated with different preparations without laser irradiation. Cell viabilities of c) HUVEC and d) 4T1 treated with different preparations with different laser irradiation. e) Flow cytometry analysis of 4T1 cell apoptosis induced by various treatments. Data were expressed as mean ± S.D. (n = 3)

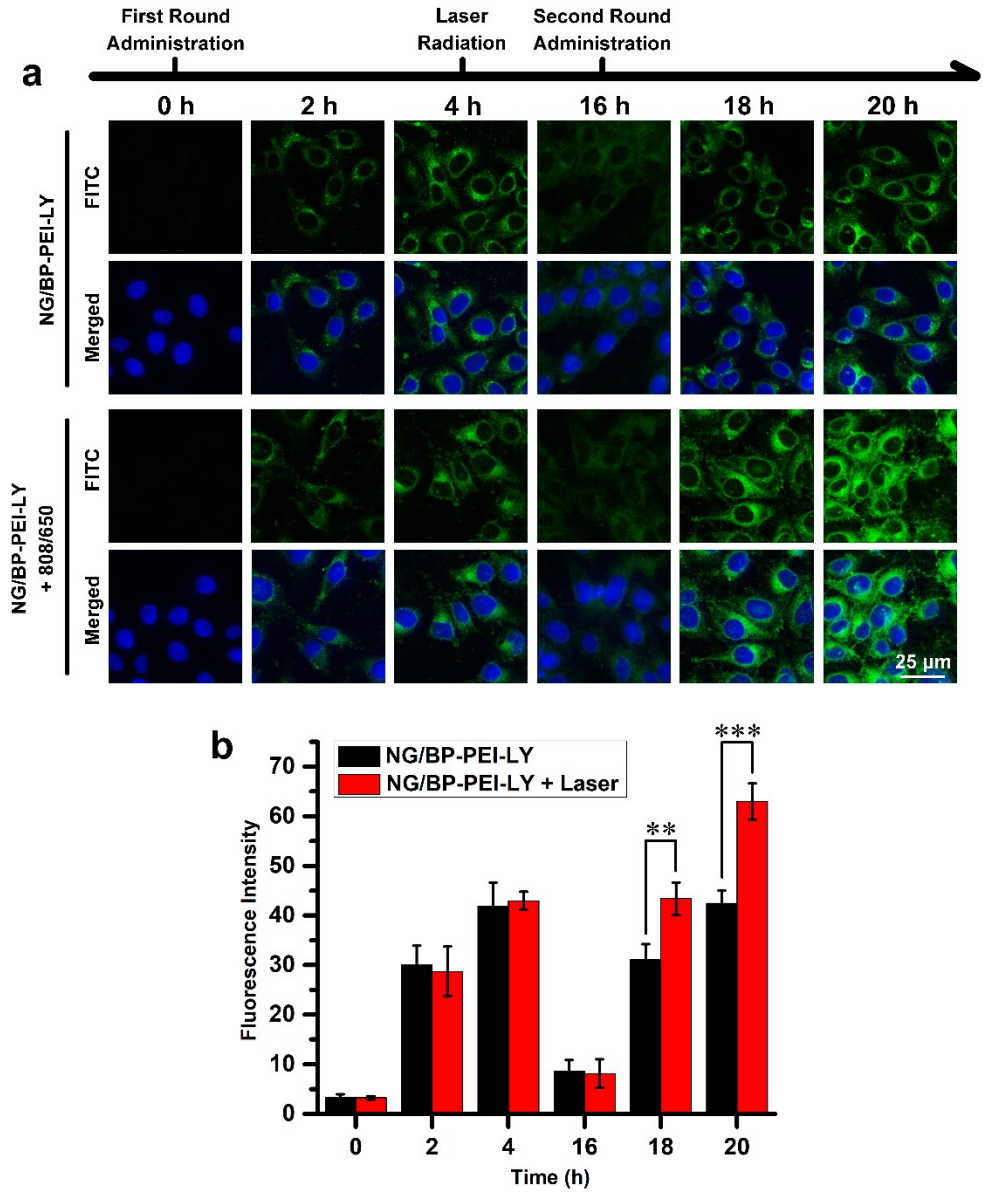

Fig. S8 *In vitro* positive feedback study. (a) Time lapse CLSM images of HUVEC undergo two rounds of cellular uptake of NG/BP-PEI-LY (green) with or without laser irradiation. The nucleuses were staining with DAPI (blue). (b) The quantitative analysis of time lapse fluorescence intensities of NG/BP-PEI-LY in cells by FCM. Quantitative data were expressed as mean ± S.D. (n = 3), $^{**}P < 0.01$ and $^{***}P < 0.001$.

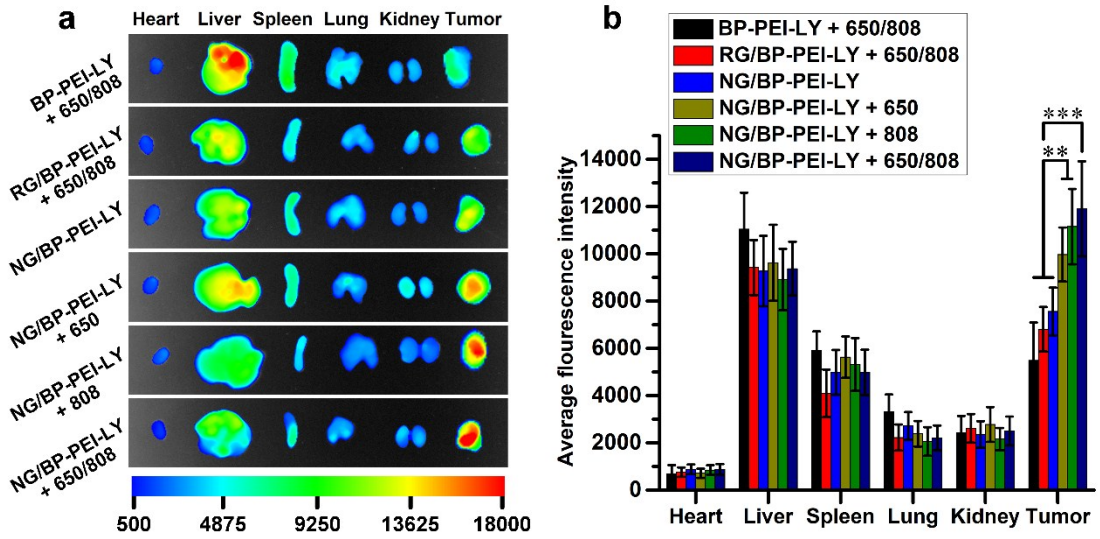

Fig. S9 a) The *ex vivo* NIR fluorescence images and b) quantification of average fluorescence intensities of dissected main organs and tumors at 72 h post first round of injection. Results were expressed as mean ± S.D. ($n = 5$), $^{**}P < 0.01$ and $^{***}P < 0.001$.

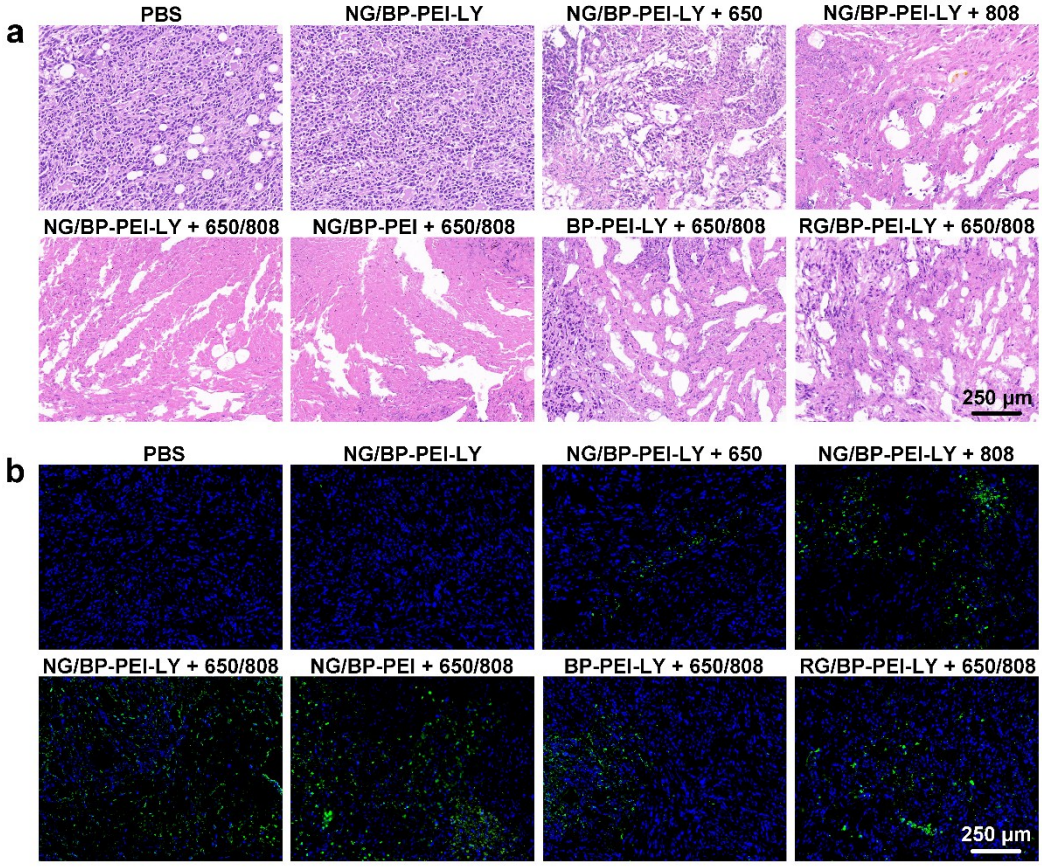

Fig. S10 a) Optical images of H&E staining and b) CLSM images of TUNEL staining

of *ex vivo* tumor from mice received different treatments at day 16.

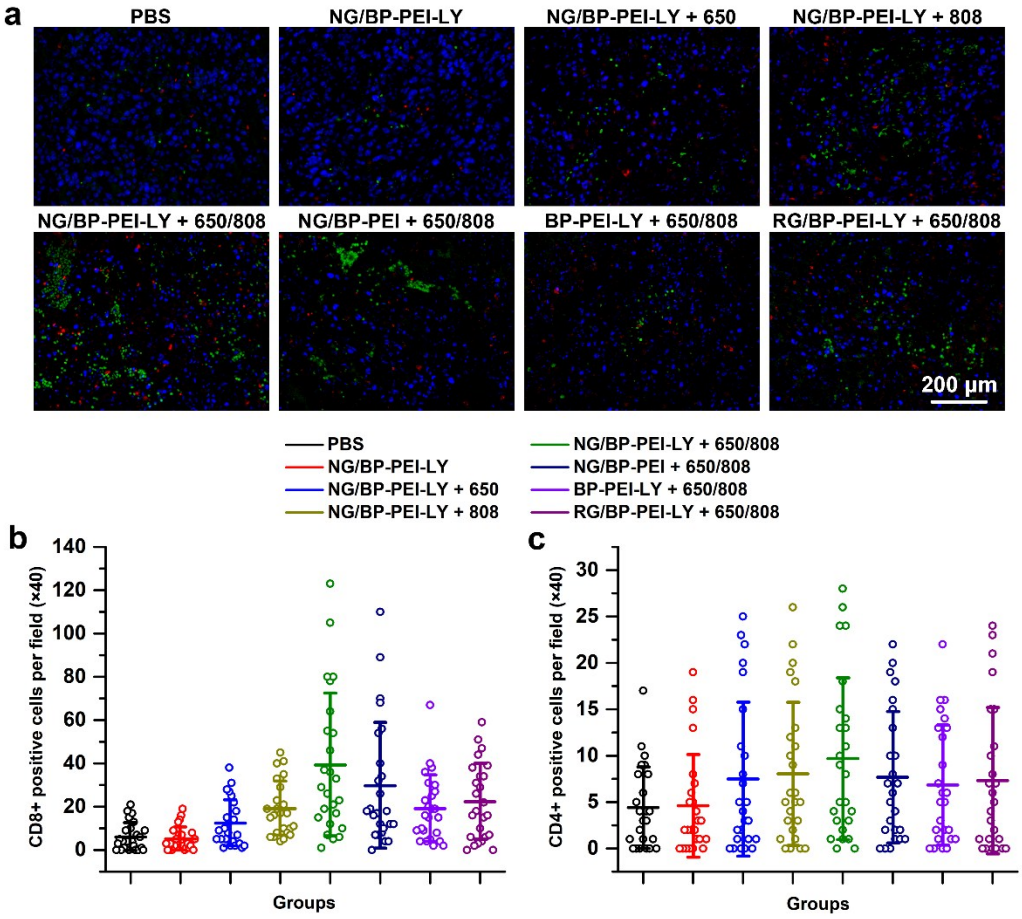

Fig. S11 Intratumoral T cells study. a) CLSM images of CD8+ T cells (green) and CD4+ T cells (red) in tumors harvested form mice received different treatments at day 16. Quantitative data of average fluorescence intensities of b) CD8+ T cells and c) CD4+ T cells in tumors obtained from 5 randomly selected fields from each of the 5 tumors in each group. Data were expressed as mean ± S.D. (n = 5).

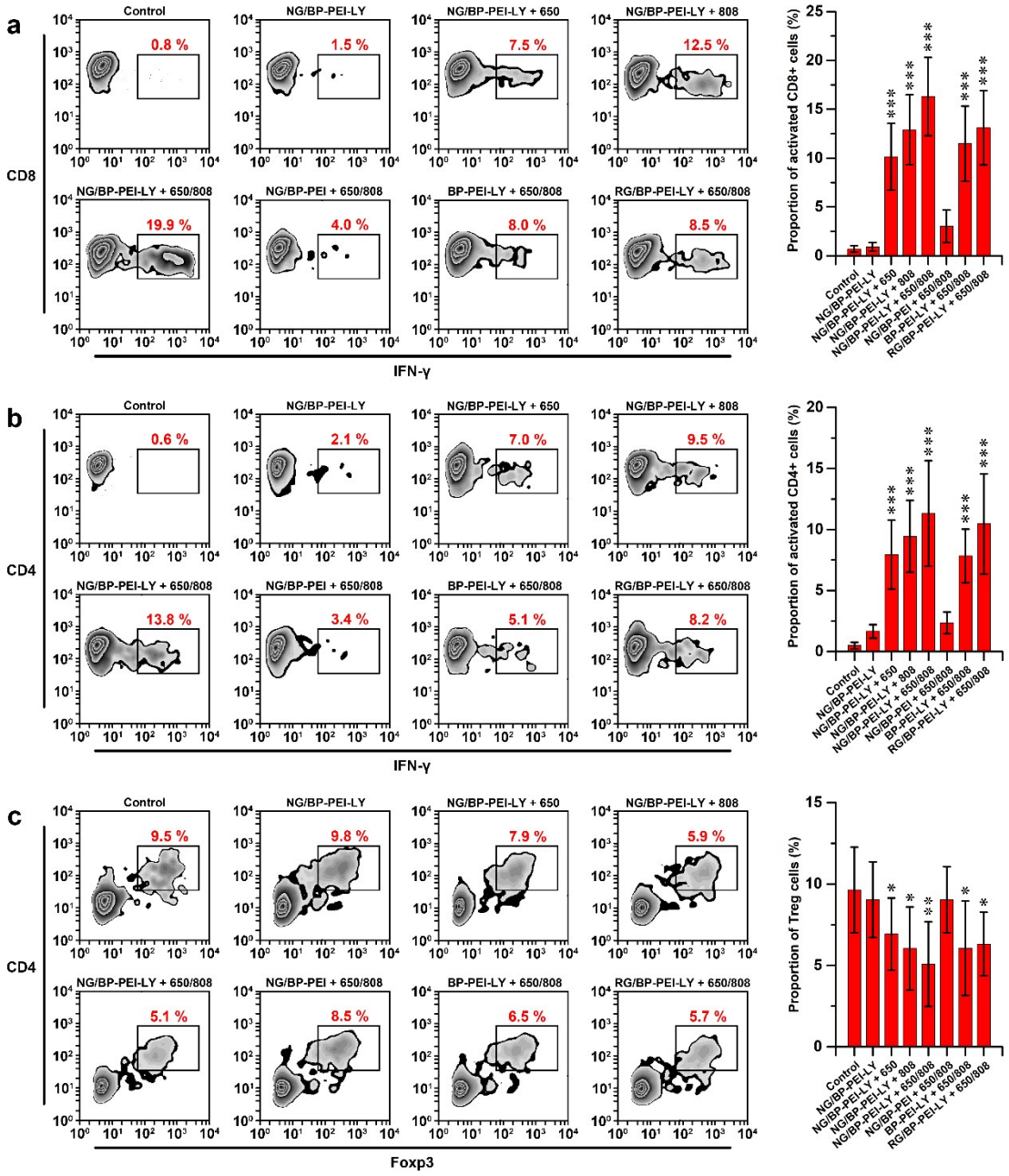

Fig. S12 Activation of tumor immune. Flow cytometry analysis and quantification of a) activated CD8+ T cells, b) activated CD4+ T cells and c) Treg cells in tumors from mice received different treatments at day 16. Data were expressed as mean ± S.D. (n = 5), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

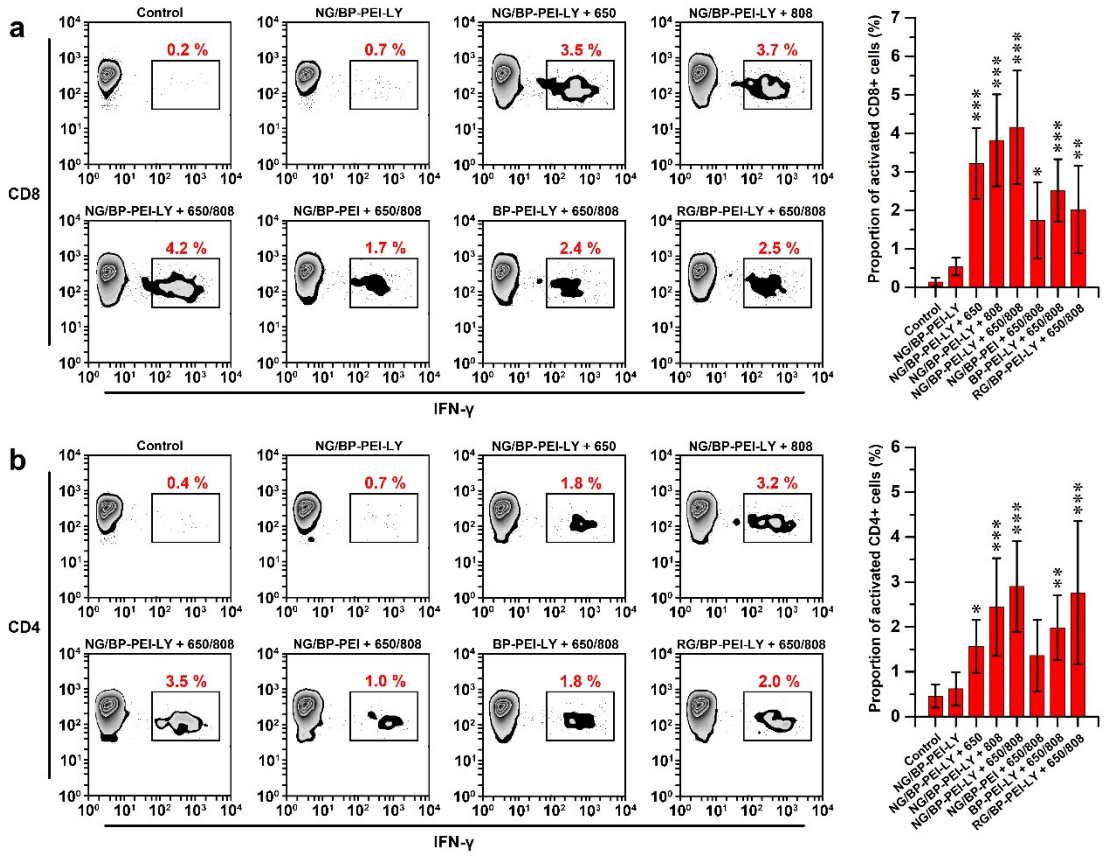

Fig. S13 Activation of tumor immune. Flow cytometry analysis and quantification of a) activated CD8+ T cells and b) activated CD4+ T cells in draining lymph nodes from mice received different treatments at day 16. Data were expressed as mean ± S.D. (n = 5), *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$.

Notes and references

1. H. Wang, X. Z. Yang, W. Shao, S. C. Chen, J. F. Xie, X. D. Zhang, J. Wang and Y. Xie, *J Am Chem Soc*, 2015, **137**, 11376-11382.
2. W. Tao, X. B. Zhu, X. H. Yu, X. W. Zeng, Q. L. Xiao, X. D. Zhang, X. Y. Ji, X. S. Wang, J. J. Shi, H. Zhang and L. Mei, *Adv Mater*, 2017, **29**.
3. H. Meng, Y. Zhao, J. Y. Dong, M. Xue, Y. S. Lin, Z. X. Ji, W. X. Mai, H. Y. Zhang, C. H. Chang, C. J. Brinker, J. I. Zink and A. E. Nel, *Acs Nano*, 2013, **7**, 10048-10065.
4. R. Boxio, C. Bossenmeyer-Pourie, N. Steinckwich, C. Dournon and O. Nusse, *J Leukocyte Biol*, 2004, **75**, 604-611.
5. J. M. Suski, M. Lebiedzinska, A. Wojtala, J. Duszynski, C. Giorgi, P. Pinton and M. R. Wieckowski, *Nat Protoc*, 2014, **9**, 312-322.
6. R. Palomba, A. Parodi, M. Evangelopoulos, S. Acciardo, C. Corbo, E. de Rosa, I. K. Yazdi, S. Scaria, R.

Molinaro, N. E. T. Furman, J. You, M. Ferrari, F. Salvatore and E. Tasciotti, *Sci Rep-Uk*, 2016, **6**, 34422.