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Synergic cancer chemo-immunotherapy comprising combined doxorubicin and siRNA targeting CD47 co-delivered by a bola-amphiphilic dendrimer

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Materials

Doxorubicin hydrochloride was purchased from Beijing Huafeng United Technology. Nile Red was purchased from Macklin (Shanghai, China). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), N-acetylcysteine (NAC), and poly(2-hydroxyethyl methacrylate) were purchased from Sigma-Aldrich (Shanghai, China). The CellROX® Orange reagent was purchased from Thermo Fisher Scientific (Carlsbad, CA, USA). Lysotracker Green was purchased from KeyGEN Biotechnology (Nanjing, China). Hoechst 33342 was purchased from Beyotime Biotechnology (Shanghai, China). siRNA targeting mouse CD47 mRNA (sense strand: 5'-GGACUUGGCCUCAUUGAATT-3', and antisense strand: 5'-UUCAAUGAGGCCAAGUCCTT-3') was synthesized by GenePharma Co., Ltd. (Shanghai, China). APC-Cy7 anti-CD45, BV421 anti-CD11b, Alexa Fluor 647 anti-F4/80, Alexa Fluor 488 anti-CD86, Alexa Fluor 700 anti-CD3, PE-Cy7 anti-CD11c, Alexa Fluor 594 anti-CD80, Alexa Fluor 488 anti-CD4, Alexa Fluor 647 anti-CD8, and PE anti-CD11c were from BD Biosciences (USA); CD86, CD80, and CD4 antibodies were from ABclonal (Hunan, China).

All the other reagents were of analytical grade and used directly from commercial sources without additional purification.

Method

Cell Culture.

Mouse breast cancer 4T1 cells, luciferase-tagged 4T1 cells (4T1-luc cells), and mouse melanoma B16F10 cells were obtained from the Cell Bank of the Chinese Academy of Sciences (Beijing, China). Mouse melanoma luciferase-tagged B16F10 cells (B16F10-luc cells) were kindly provided by Prof. Can Zhang (China Pharmaceutical University, Nanjing, China).

4T1 and B16F10 cells were cultured in RPMI-1640 medium (HyClone™, USA) supplemented with 10% fetal bovine serum (FBS; Gemini Bio-Products, USA). 4T1-

luc cells and B16F10-luc cells were cultured in RPMI-1640 with 10% FBS and puromycin. 4T1 + NAC cells and B16F10 + NAC cells (ROS-depleted cells) were maintained in RPMI-1640 with 10% FBS and 10 mM N-acetylcysteine (NAC). All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Transmission Electron Microscopy (TEM)

A 10 μL sample of the complex solution (N/P ratio of 5, siRNA at 10 ng/μL) was placed on a Formvar-coated copper grid and air-dried for 15 minutes. The sample was then stained twice with 2.0% (w/v) uranyl acetate (Sigma-Aldrich, St. Louis, USA) for 3 minutes each. TEM imaging was performed using a JEOL 2100F electron microscope (Tokyo, Japan) operated at 300 kV.

ROS Level Assessment in Different Cell Lines.

The ROS levels in cancer cells were measured following the methodology described in literature[1]. Cells were seeded into 3.5 cm dishes at a density of 3×10^5 cells per dish and incubated for 24 hours. Afterward, the cells were harvested and resuspended in 1.0 ml of complete growth medium. CellROX® Orange Reagent was added to the suspensions at a final concentration of 500 nM, followed by incubation at 37 °C for 30 minutes in the dark. The cells were then washed three times with PBS. Fluorescence intensity was measured with a flow cytometer. All experiments were performed in triplicate.

***In Vitro* Cell Uptake**

Flow cytometry: B16F10 or 4T1 cells were seeded in 24-well plates at a density of 1 × 10^5 cells per well and incubated overnight to allow adhesion. The medium was then removed and replaced with bola4A/DOX/Cy5-siRNA (DOX: 3.6 μM, siRNA: 50 nM, N/P ratio = 5) for time points of 1, 2, and 4 hours. After incubation, cells were harvested, washed three times with PBS, and analyzed with an Attune NxT acoustic focusing cytometer. All experiments were conducted in triplicate.

Confocal microscopy: B16F10 or 4T1 cells were seeded in confocal dishes at 1 ×

10^5 cells per dish and incubated overnight. The medium was discarded, and cells were treated with bola4A/DOX/Cy5-siRNA (DOX: 3.6 μM, siRNA: 50 nM, N/P ratio = 5) for 4 hours. Post-incubation, cells were washed three times with PBS, stained with Hoechst 33342 and LysoTracker Green for 15 minutes, then washed three additional times with PBS. Cells were then visualized using a Zeiss confocal microscope.

Evaluation of BMDCs Maturation and BMDMs polarization

To evaluate dendritic cell maturation, bone marrow-derived dendritic cells (BMDCs) were cocultured for 24 hours with supernatants from B16F10 and 4T1 tumor cells treated with various formulations: bola4A, DOX (3.6 μM), bola4A/DOX (3.6 μM), bola4A/DOX/scramble (3.6 μM DOX, 50 nM siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (3.6 μM DOX, 50 nM siRNA, N/P ratio = 5). Afterward, BMDCs were harvested and stained with Alexa Fluor 450-anti-CD11c, FITC-anti-CD80, APC-anti-CD86, and APC-eFluor™ 780-anti-I-A/I-E (MHC-II). Flow cytometry was used to analyze the expression of costimulatory molecules CD80, CD86, and MHC-II.

To assess macrophage polarization toward the pro-inflammatory M1 phenotype, bone marrow-derived macrophages (BMDMs) were incubated for 24 hours with supernatants from similarly treated tumor cells. These cells were then collected and labeled with Alexa Fluor 450-anti-CD11b, FITC-anti-CD80, APC-anti-CD86, and APC-eFluor™ 780-anti-I-A/I-E (MHC-II). The mean fluorescence intensity (MFI) of CD80, CD86, and MHC-II was measured by flow cytometry.

***In Vitro* Anticancer Activity Assay.**

MTT assays were conducted to evaluate the antiproliferative effect of bola4A/DOX with and without NAC. Cells were seeded in 96-well plates at a density of 5.0×10^3 cells per well and incubated overnight to allow for adhesion. Subsequently, they were treated with various formulations, including DOX and bola4A/DOX, at concentrations from 0.001 to 5 μM for 24 hours. Control cells were cultured in complete medium alone. Post-treatment, 10 μL of 10 mg/mL MTT solution was added to each

well, followed by a 4-hour incubation. The medium was then carefully replaced with 100 μL of DMSO. After gentle shaking, absorbance was read at 590 nm using a multimode microplate reader.

MTT assays assessed the antiproliferative effects of various formulations. Cells were seeded in 24-well plates at a density of 5.0 × 10^4 cells per well and incubated overnight for adhesion. They were then treated with DOX, bola4A/DOX, and bola4A/DOX/siRNA (3.6 μM DOX, 50 nM siRNA, N/P ratio = 5) for 24 hours. Control cells were grown in complete medium without treatment. Afterward, 10 μL of 10 mg/mL MTT solution was added to each well, and incubation continued for 4 hours. The medium was carefully removed, and 100 μL of DMSO was added to dissolve the formazan crystals. Gentle shaking ensured thorough mixing, and absorbance was measured at 590 nm using a multimode microplate reader.

Establishment of Cancer Cell Xenograft Mouse Models.

Female BALB/c and C57BL/6 mice, all female, 6 weeks old, were obtained from Shanghai Xipuer-Bikai Experimental Animal Co., Ltd. All experiments involving animals received approval from the Animal Ethics Committee of China Pharmaceutical University (Approval numbers YSL-20251121, 2023-12-026, and 2023-12-027) and adhered to national animal protection guidelines. For the 4T1 orthotopic triple-negative breast cancer model, BALB/c mice were injected in the fourth mammary fat pad with 2×10^5 4T1-luc cells suspended in PBS. Tumor-bearing mice were used for in vivo anti-cancer testing one week after inoculation. To create the B16F10 subcutaneous melanoma model, C57BL/6 mice received a subcutaneous injection of 1×10^6 B16F10-luc cells in PBS into the right flank. One week later, these mice were used for further in vivo anti-tumor evaluation.

***In Vivo* Biodistribution**

Animal experiments received approval from the Animal Ethics Committee of China Pharmaceutical University (approval No. YSL-20251121) and conducted in

accordance with national guidelines for animal protection guidelines. The bola4A/DOX/DiR and bola4A/DOX/DiR/Cy5-siRNA nanosystems were prepared using the same method described earlier. An orthotopic 4T1 breast cancer model was created, and once the average tumor size reached around 500 mm^3, mice were randomly divided into six groups (n = 3 per group). Each mouse received an intravenous injection of 100 µL PBS, free Cy5-siRNA (1 mg/kg), free DiR (40 µg/kg), bola4A/DOX/DiR (DiR: 40 µg/kg, DOX: 2.5 mg/kg), bola4A/Cy5-siRNA (Cy5-siRNA: 1 mg/kg), or bola4A/DOX/DiR/Cy5-siRNA (DiR: 40 µg/kg, DOX: 2.5 mg/kg, Cy5-siRNA: 1 mg/kg, N/P ratio = 5). Twelve hours after injection, the accumulation of nanoassemblies in organs and tumors was observed using an in vivo imaging system (Cambridge Research & Instrumentation, USA).

***In Vivo* Toxicity.**

Following treatment, serum samples from the mice were collected. The levels of liver enzymes, Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST), as well as kidney function markers like creatinine (CREA) and blood urea nitrogen (UREA), were measured. Additionally, blood triglyceride (TG) and cholesterol (TCHO) levels were assessed using an SABA-18 Automatic Biochemical Analyzer (Analyzer Medical System).

Results

Figure S1. Intracellular reactive oxygen species (ROS) levels in the absence and presence of N-acetylcysteine (NAC). (A) ROS levels in B16F10 cells and B16F10 cells treated with 10 mM NAC. (B) ROS levels in 4T1 cells and 4T1 cells treated with 10 mM NAC. Data are shown as mean ± standard deviation (n = 3). One-way ANOVA followed by Tukey's multiple comparisons test was used for statistical analysis. ***$p < 0.001$.

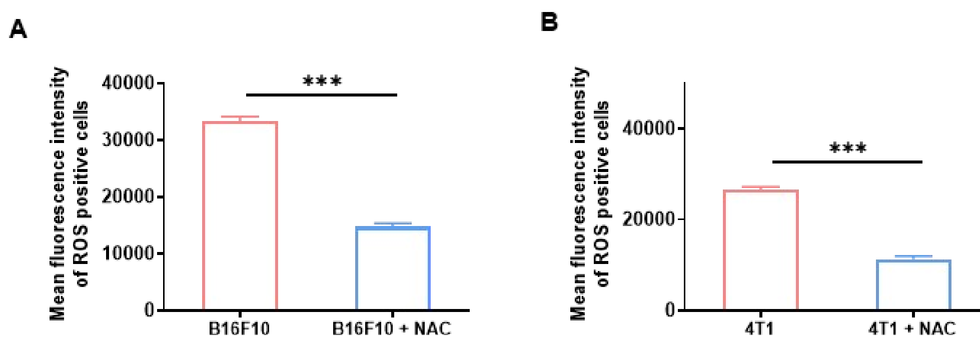


Figure S2. TEM image of the bola4A/DOX/siRNA nanosystem. (scale bar = 50 nm).

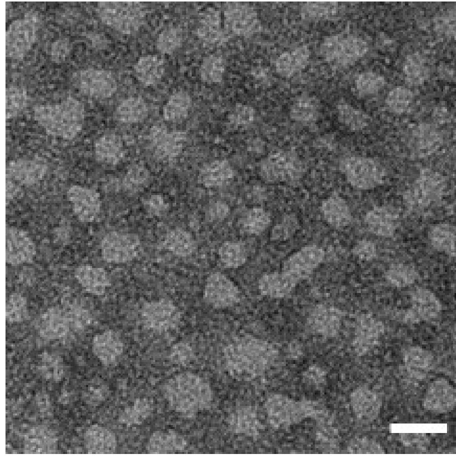


Figure S3. Cellular uptake of bola4A/DOX/Cy5-siRNA in B16F10 and 4T1 cells. (A) Flow cytometry analysis showing cellular uptake after treatment with bola4A/DOX/Cy5-siRNA (DOX: 3.6 μM, siRNA: 50 nM, N/P ratio of 5). (B) Confocal microscopy images of B16F10 and 4T1 cells after 4 h incubation with bola4A/DOX/Cy5-siRNA (DOX: 3.6 μM, siRNA: 50 nM, N/P ratio of 5). The red channel indicates DOX; the green channel represents Cy5-siRNA; and the blue channel shows nuclei stained with Hoechst 33342. The scale bar equals 20 μm.

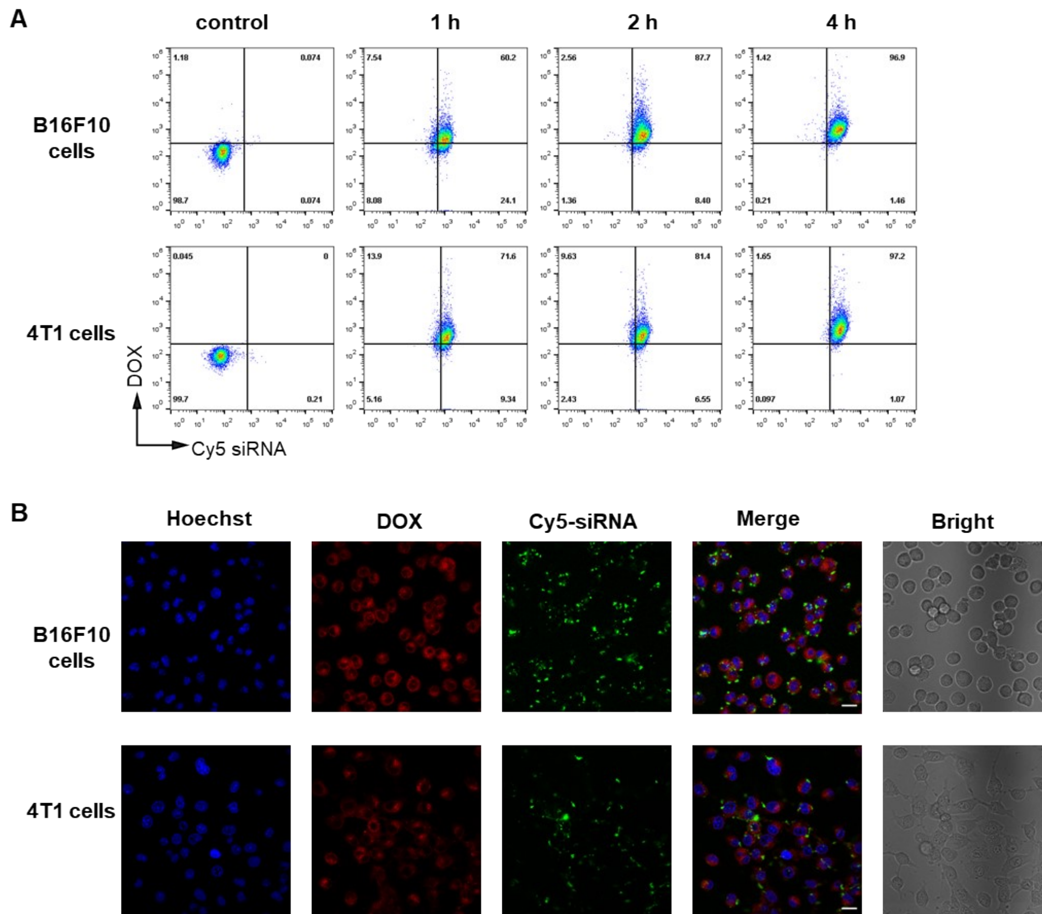


Figure S4. In vivo biodistribution of the nanosystem in mice bearing orthotopic 4T1-luc tumors. The mice received treatments of PBS, free Cy5-siRNA (1 mg/kg), free DiR (40 μg/kg), bola4A/DOX/DiR (DiR: 40 μg/kg, DOX: 2.5 mg/kg), bola4A/Cy5-siRNA (Cy5-siRNA: 1 mg/kg, N/P ratio = 5), or bola4A/DOX/DiR/Cy5-siRNA (DiR at 40 μg/kg, DOX at 2.5 mg/kg, Cy5-siRNA at 1 mg/kg, N/P ratio = 5).

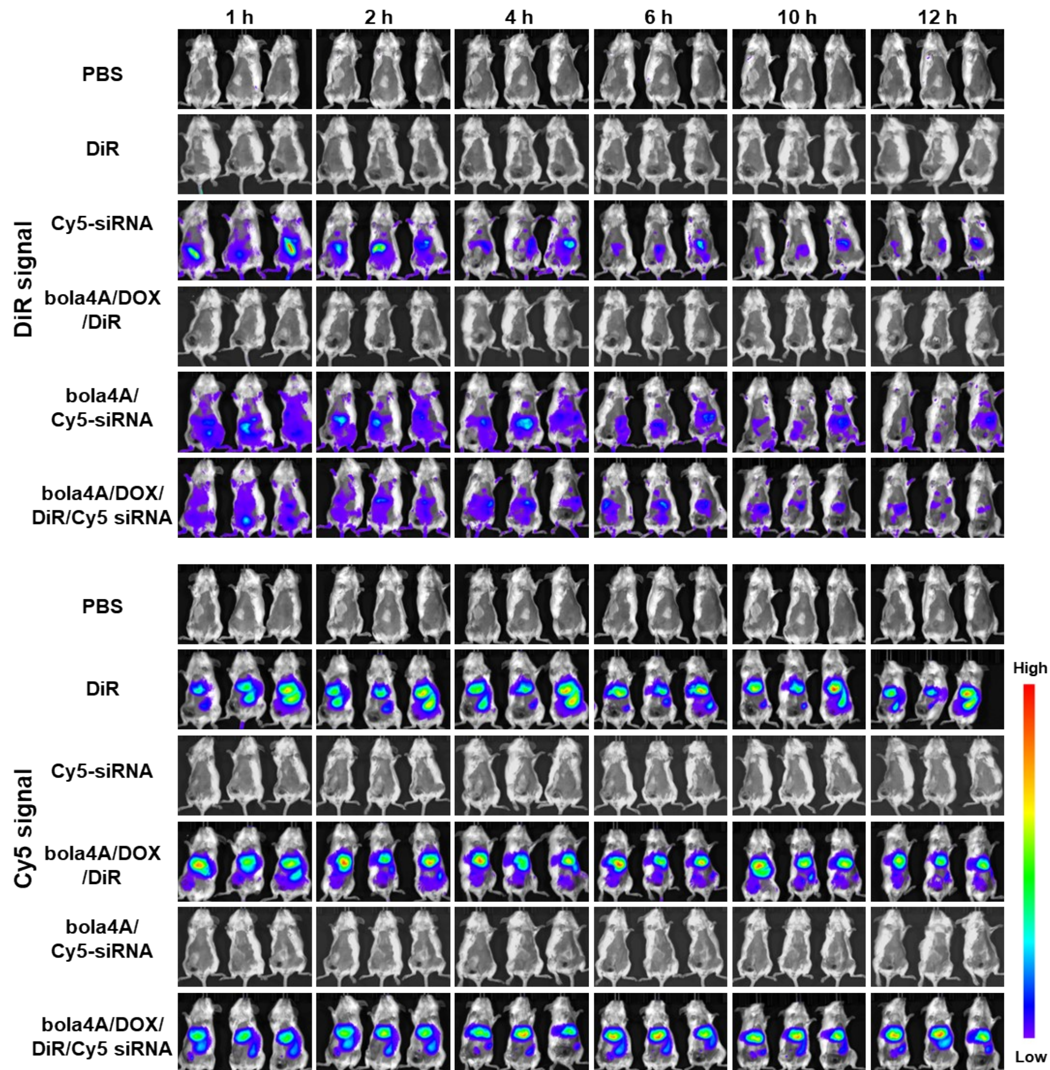


Figure S5. Tumor accumulation of the nanosystem in orthotopic 4T1-luc tumor-bearing mice. The mice received various treatments: PBS, free Cy5-siRNA (1 mg/kg), free DiR (40 µg/kg), bola4A/DOX/DiR (DiR: 40 µg/kg, DOX: 2.5 mg/kg), bola4A/Cy5-siRNA (Cy5-siRNA: 1 mg/kg, N/P ratio = 5), or bola4A/DOX/DiR/Cy5-siRNA (DiR: 40 µg/kg, DOX: 2.5 mg/kg, Cy5-siRNA: 1 mg/kg, N/P ratio = 5). (A) Fluorescence imaging of tumors with signals from Cy5 (siRNA) and near-infrared DiR. (B) Quantification of tumor fluorescence intensity (mean ± SD, n=3, ***$p < 0.001$). (C) Displays fluorescence images of tumor sections: DOX (red), Cy5-siRNA (green), and nuclei stained with DAPI (blue). The scale bar measures 50 µm.

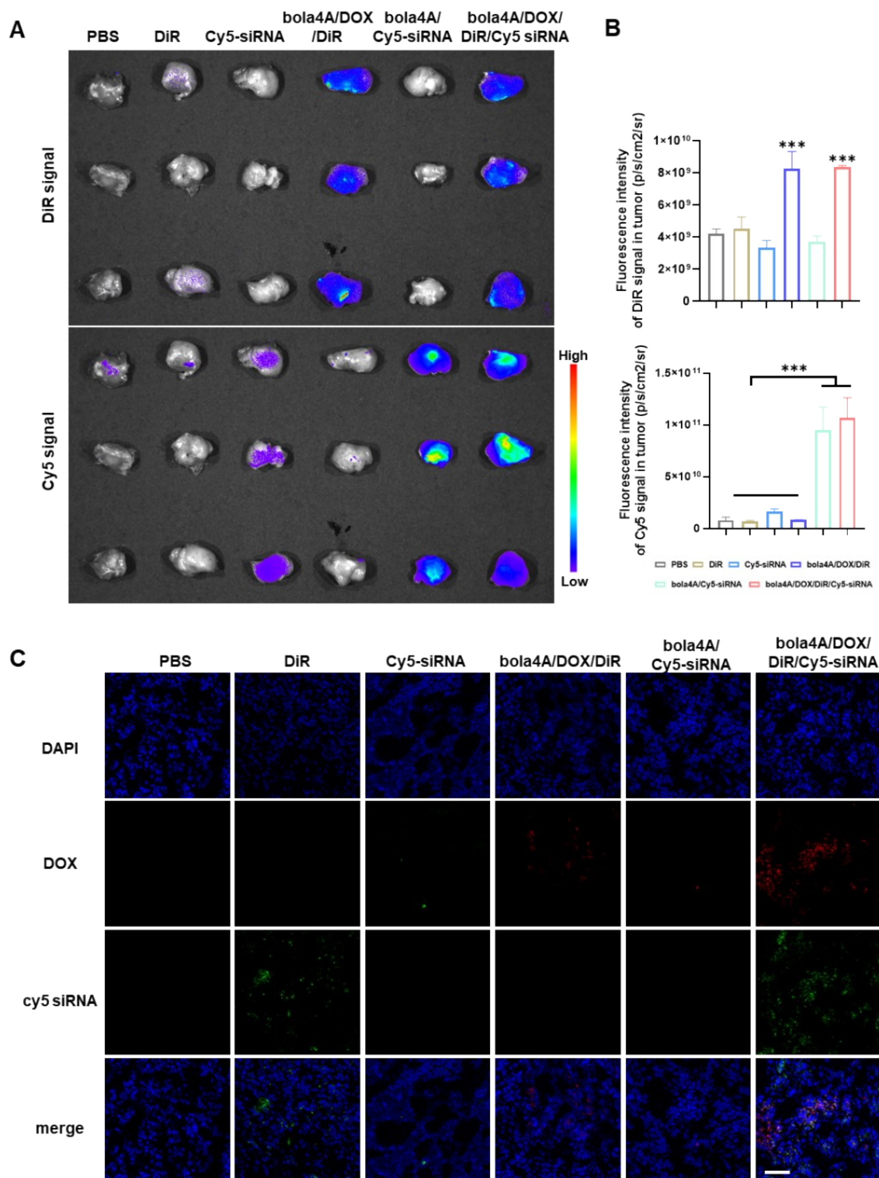


Figure S6. The bola4A/DOX/siCD47 nanosystem downregulates CD47 expression in B16F10 and 4T1 cells. (A) Surface CD47 expression on B16F10 and 4T1 cells treatment with the nanosystem (DOX: 3.6 μM, siRNA: 50 nM, N/P ratio = 5) assessed by flow cytometry (mean ± SD, n = 3, *** $p < 0.001$). (B) Confocal images showing CD47 expression (green) and nuclei stained with Hoechst33342 (blue) following various treatments. Scale bar = 50 μm.

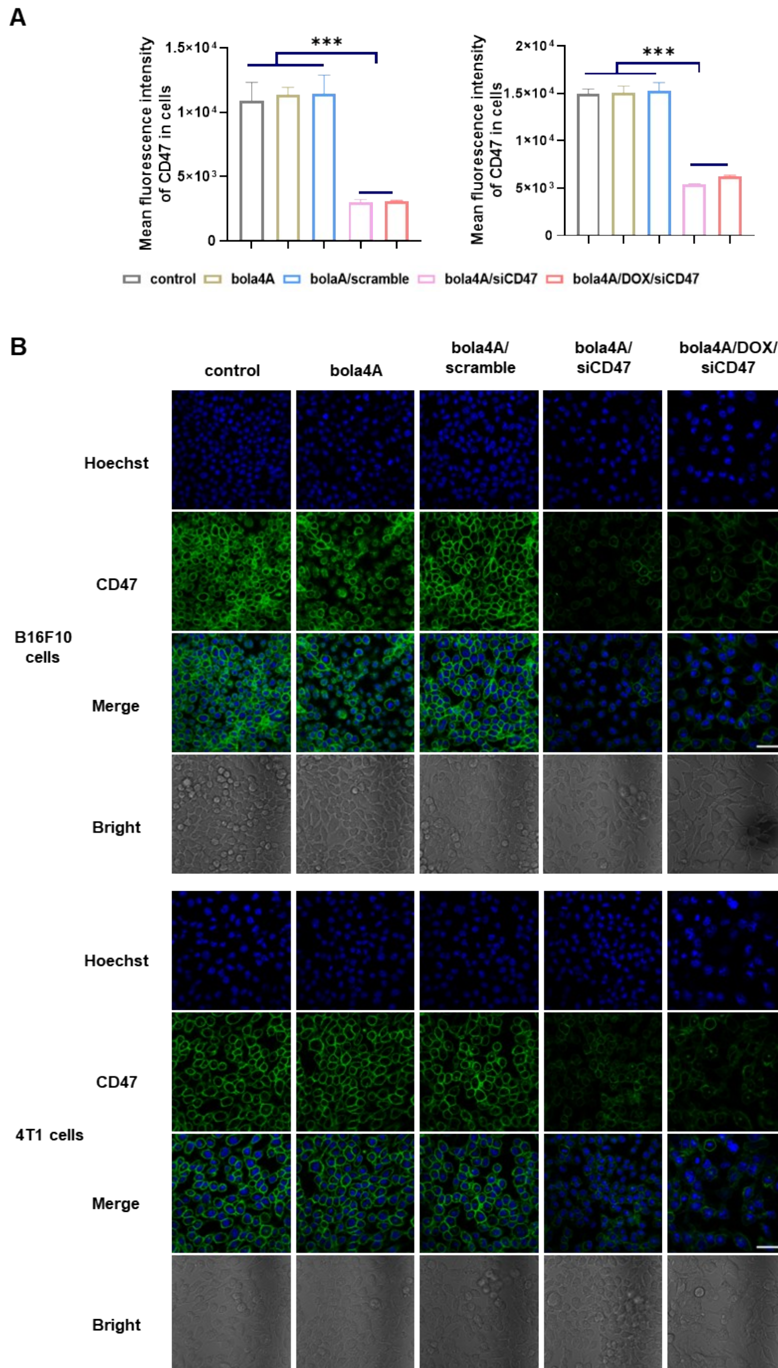


Figure S7. Flow cytometry analysis of MHC-II, CD80, and CD86 expression on immune cells. Surface marker expression was assessed on CD11b^{+} bone marrow–derived macrophages (BMDMs) in (A) B16F10 and (B) 4T1 models, and on CD11c^{+} bone marrow–derived dendritic cells (BMDCs) in (C) B16F10 and (D) 4T1 models following treatment. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test ($n = 3$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

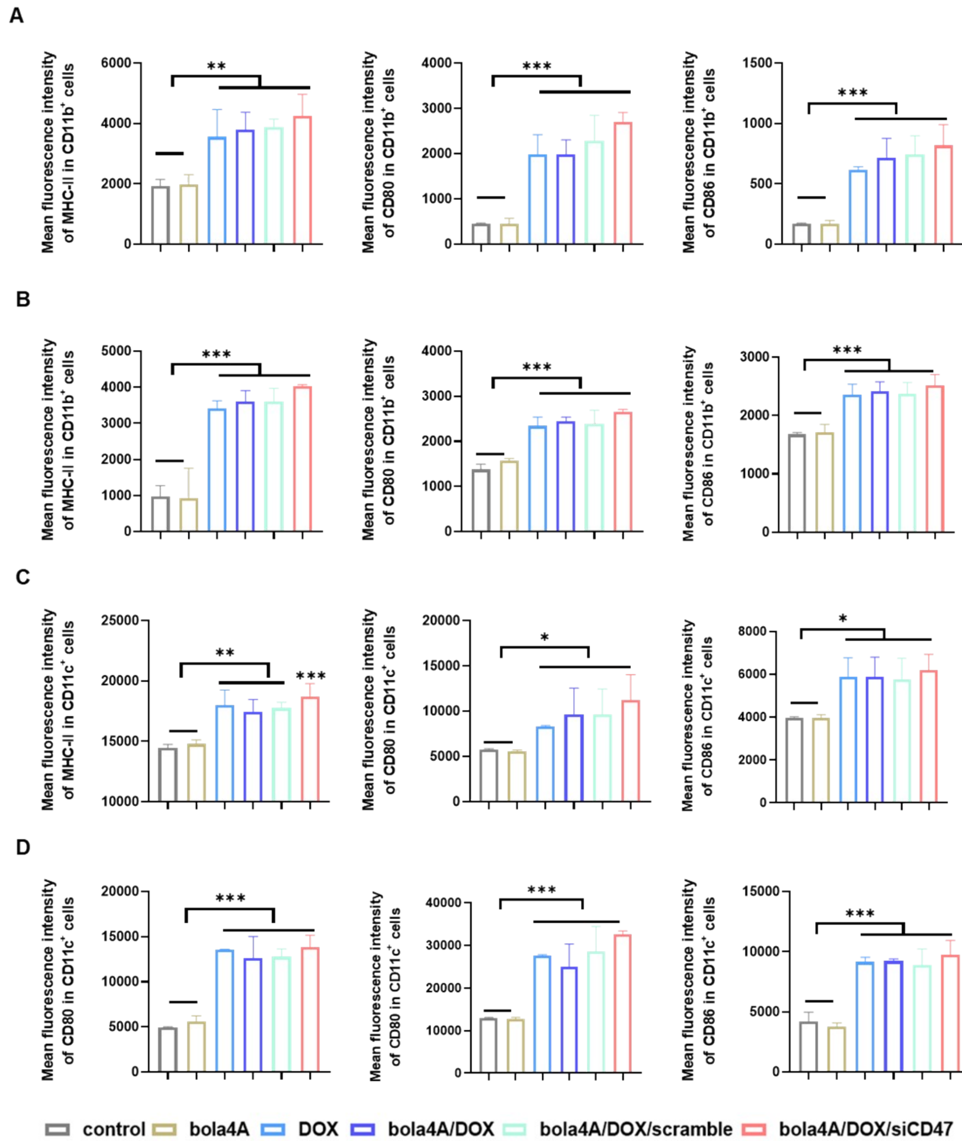


Figure S8. Photographs of isolated tumor tissues collected after the final treatment in the orthotopic 4T1-luc tumor mouse model. Tumors were harvested following the last administration of PBS, free DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg DOX), bola4A/siCD47 (1 mg/kg siRNA), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5).

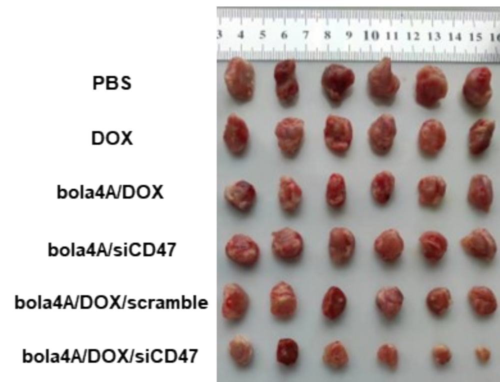


Figure S9. Quantification of (A) Ki67 expression from immunohistochemistry images and (B) TUNEL assay in mice bearing orthotopic 4T1-luc tumors. The mice received treatments including PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg DOX), bola4A/siCD47 (1 mg/kg siRNA, N/P ratio = 5), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). Expression levels were measured with ImageJ software.

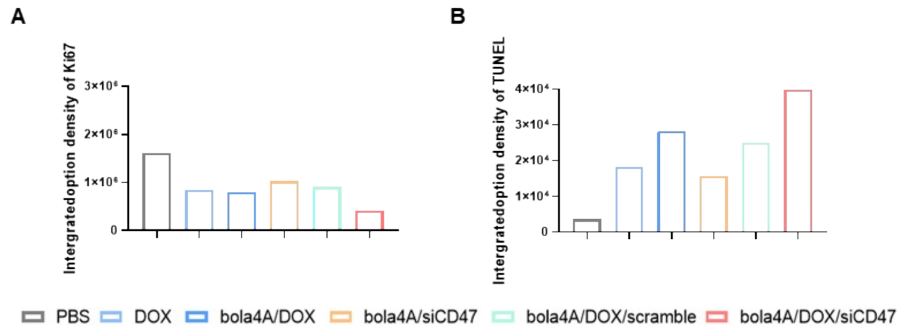


Figure S10. Toxicity assessment following treatment in the orthotopic 4T1-luc tumor mouse model. Mice were administered with PBS, free DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg), bola4A/siCD47 (1 mg/kg), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). (A) The body weight of mice was monitored throughout the study. (B) Histological analysis of major organs (heart, liver, spleen, lung, and kidney) from female BALB/c mice post-treatment (Scale bar = 200 μm). (C) Blood biochemical tests evaluating liver and kidney function. Results are shown as mean ± standard deviation (n = 6).

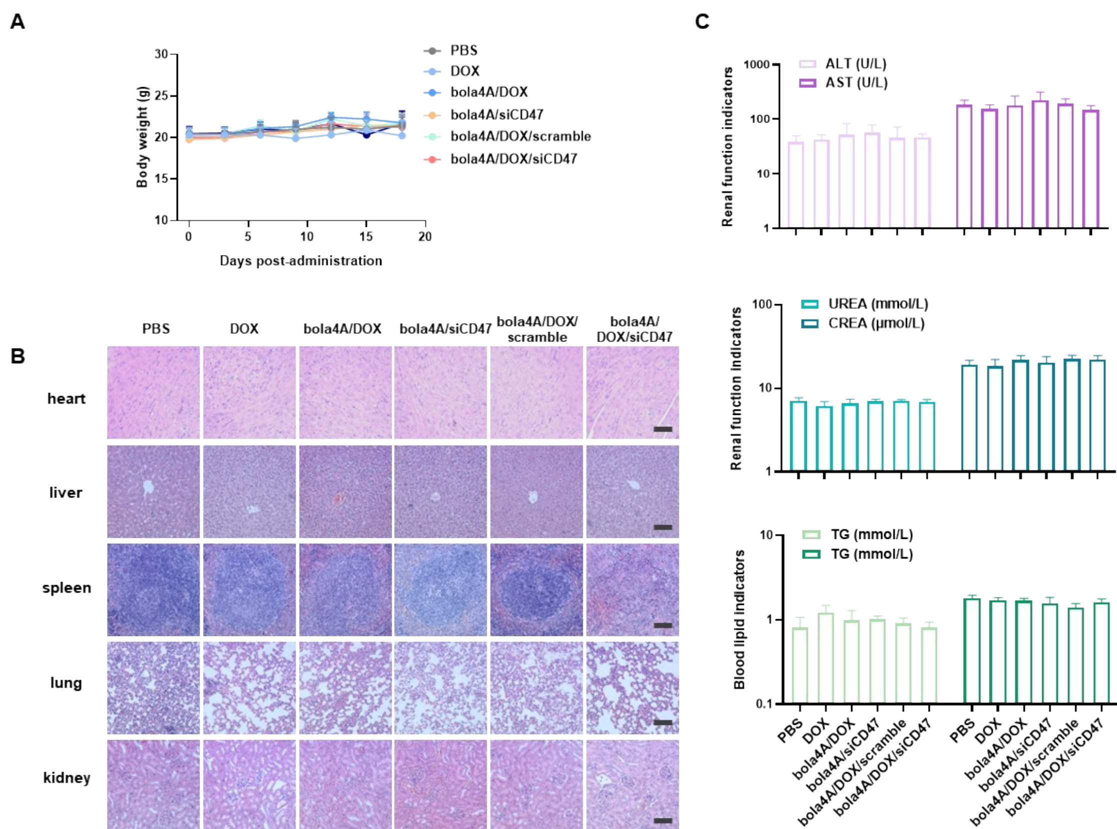


Figure S11. Quantification of (A) CD47 and (B) calreticulin in immunohistochemistry images from the orthotopic 4T1-luc tumor mouse model. Mice received treatments with PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg), bola4A/siCD47 (1 mg/kg siRNA), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). Expression levels were measured using ImageJ software.

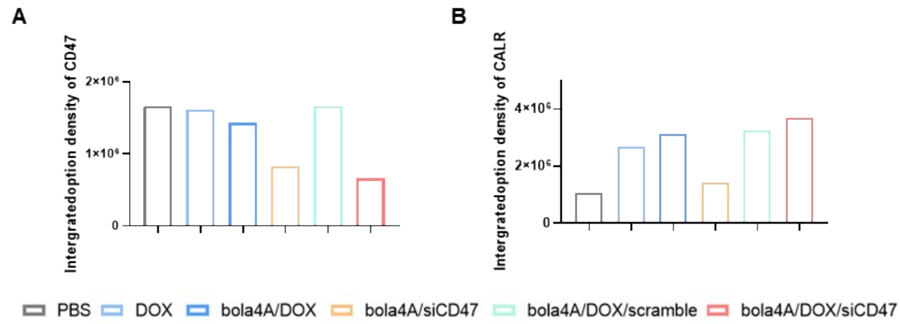


Figure S12. In vivo anticancer efficacy of the bola4A/DOX/siCD47 nanosystem in the B16F10 subcutaneous tumor mouse model. Mice were treated with PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg DOX), bola4A/siCD47 (1 mg/kg siRNA), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). (A) Tumor growth inhibition rates post-treatment. Statistical analysis was performed using one-way ANOVA with Tukey's multiple comparisons test, with n=6, where *p < 0.05, **p < 0.01, ***p < 0.001. (B) Immunohistochemical detection of Ki67 in tumor tissues to evaluate cell proliferation, accompanied by quantitative analysis of Ki67 staining. (C) TUNEL assay images illustrating apoptotic cells at the end of treatment (Scale bar, 50 μm), with quantification of TUNEL-positive cells. (D, E) Quantitative analysis of Ki67 immunohistochemistry (D) and TUNEL assay (E) using ImageJ.

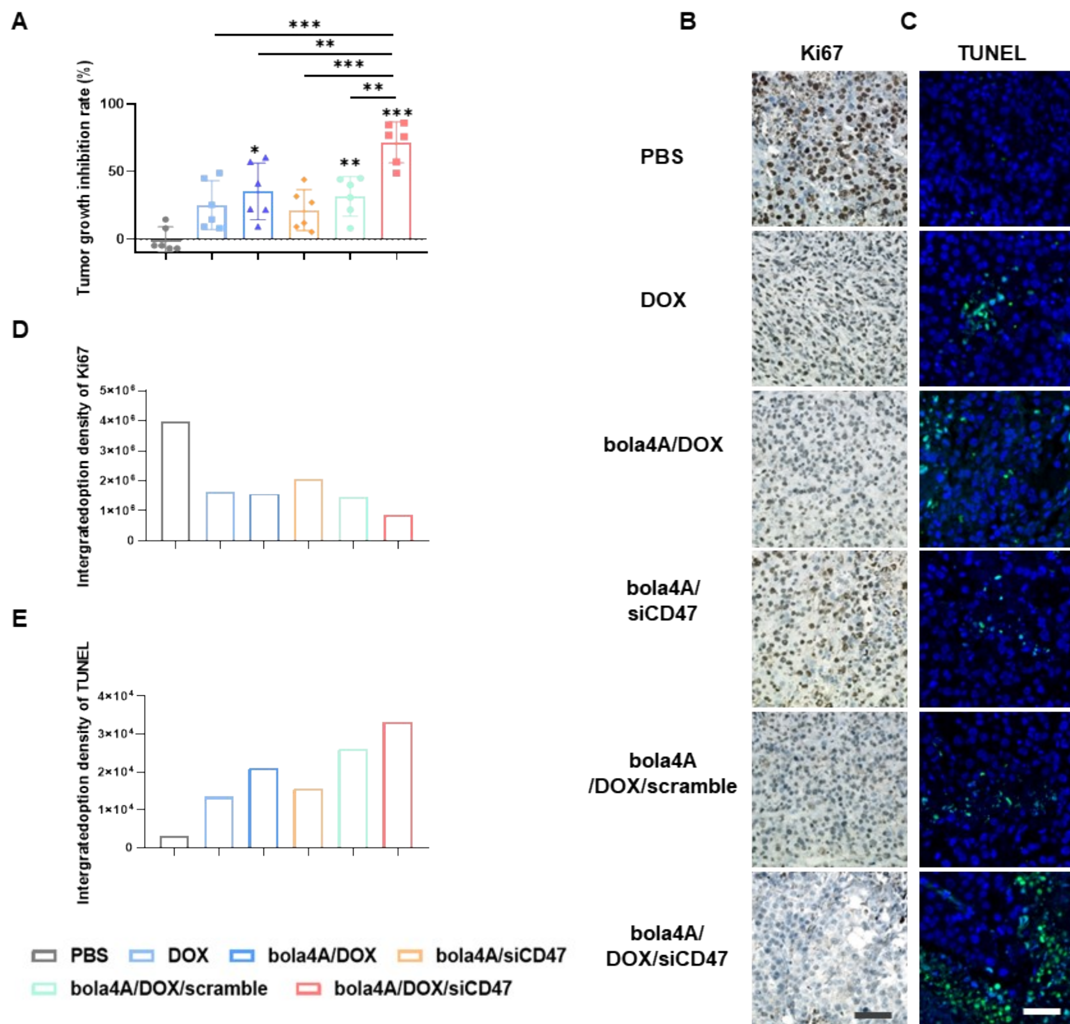


Figure S13. Evaluation of toxicity following treatment in the B16F10-luc subcutaneous tumor mouse model. Mice were treated with PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg), bola4A/siCD47 (1 mg/kg), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), or bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). (A) Body weight of female C57BL/6 mice tracked during treatment. (B) Histopathological examination of major organs post-treatment, including the heart, liver, spleen, lung, and kidney (scale bar, 200 μm). (C) Blood biochemistry tests measuring liver and kidney functions. Data are presented as mean ± standard deviation (n = 6).

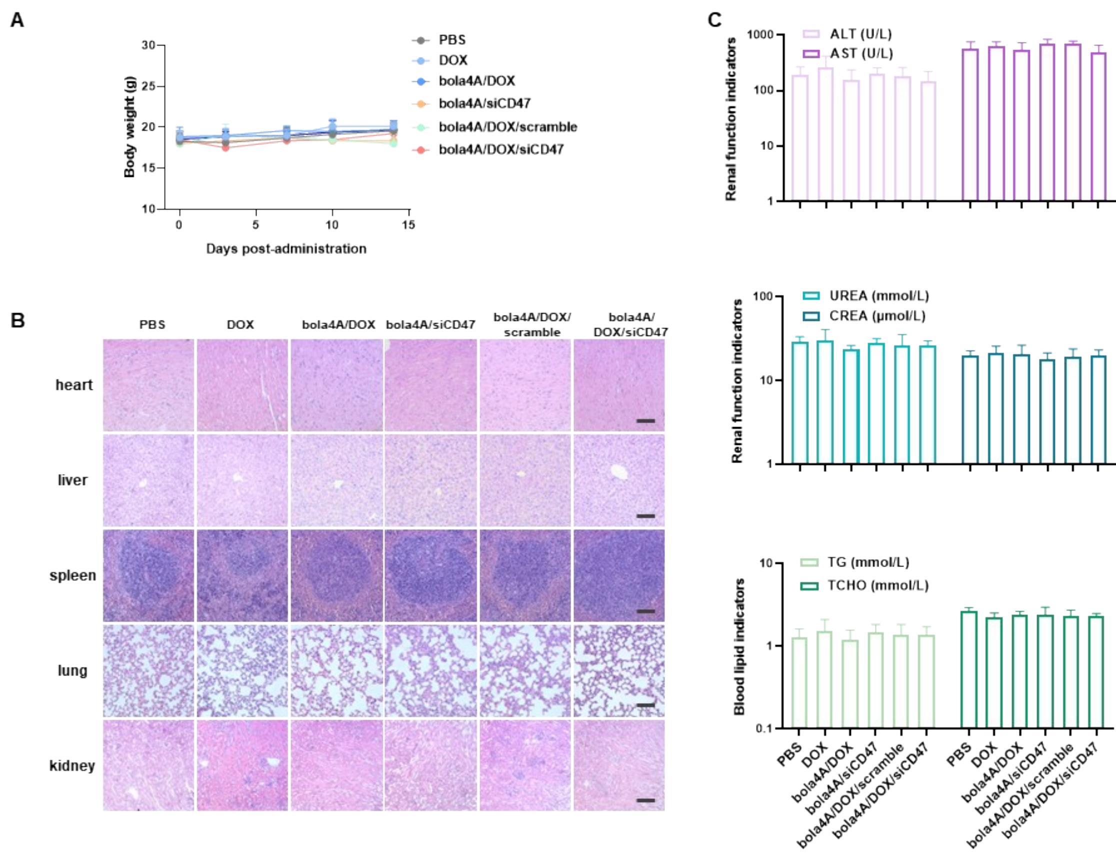


Figure S14. CD47 expression and CALR induction following bola4A/DOX/siCD47 treatment in a B16F10 subcutaneous tumor mouse model. Mice were treated with PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg), bola4A/siCD47 (1 mg/kg), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). (A) Western blot analysis of CD47 protein expression in tumor tissues from B16F10 xenograft mice. (B, C) Immunohistochemical staining of tumor tissues, highlighting CD47 (B) and CALR (C) protein expression (Scale bar = 50 μm).

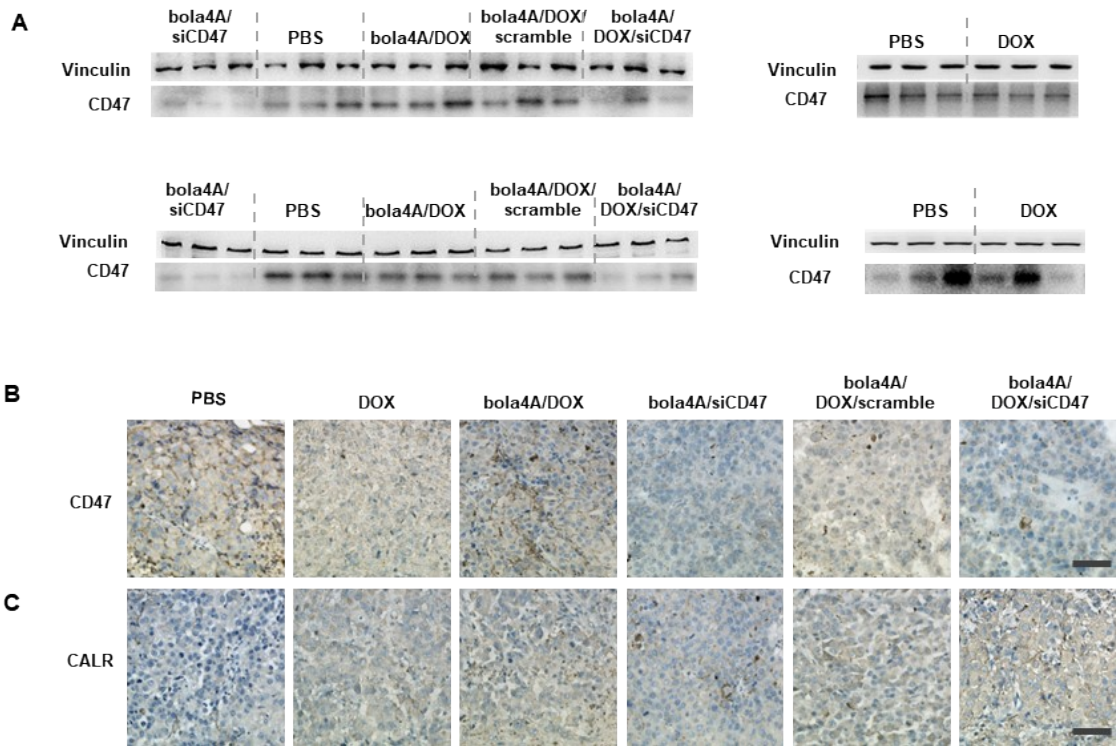
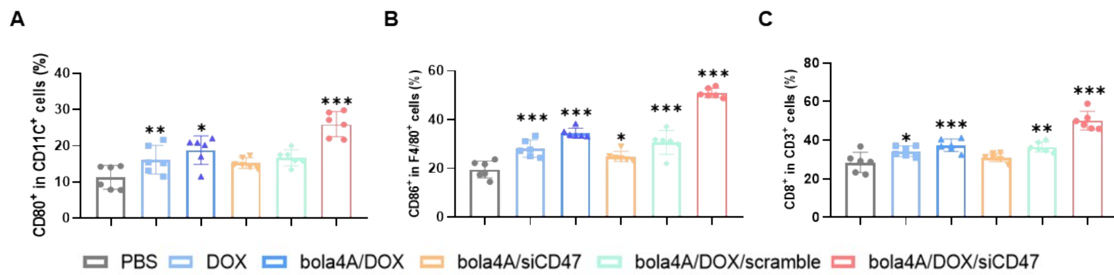


Figure S15. Flow cytometry analysis of tumor-infiltrating immune cells in a B16F10 subcutaneous tumor mouse model. The mice received treatments with PBS, DOX (2.5 mg/kg), bola4A/DOX (2.5 mg/kg), bola4A/siCD47 (1 mg/kg), bola4A/DOX/scramble (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5), and bola4A/DOX/siCD47 (2.5 mg/kg DOX, 1 mg/kg siRNA, N/P ratio = 5). Tumor tissues were analyzed by flow cytometry to determine: (A) M1-like macrophage phenotype (CD86+ cells within CD45+ F4/80+ cells), (B) mature DC percentage (CD80+ cells within CD45+ CD11c+ cells), and (C) CD8+ T cell percentage (within CD45+ CD3+ cells). Statistical significance was assessed using one-way ANOVA with Tukey's test (n=6), with *p < 0.05, **p < 0.01, and ***p < 0.001.



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