This Supplementary Information file replaces the version that was published on 12 June 2023. Minor typographical errors have been corrected and scale bars have been added to Figures S4 and S8.

Supporting information

Engineering Magnetotactic Bacteria MVs to Synergize Chemotherapy, Ferroptosis and Immunotherapy for Augmented antitumor Therapy

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Materials and Methods

Experimental materials and instruments

All starting chemical reagents and materials were purchased from chemical suppliers and used as received without further purification. Iron (II) acetate tetrahydrate was purchased from Meryer (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was obtained from Aladdin China. The bicinchoninic acid (BCA) protein quantitation kit and syringe filters were bought from Beytotime Biotechnology (Shanghai, China). Phosphate buffered saline (PBS) was obtained from Macgene (Beijing, China). RPMI 1640 cell medium was purchased from Hyclone (Logan, Utah, USA). Fetal bovine serum (FBS) was purchased from Gibco (California, USA). Hoechst 33342 was purchased from Sigma Aldrich (St. Louis, MO, USA). Recombinant mouse interferon-gamma was purchased from Novoprotein (Shanghai, China). JC-1 assay kit was bought from Yeasen Biotechnologies co., Ltd (Shanghai, China). PE anti-mouse IFN-gamma antibody, FITC anti-mouse CD80 antibody, and PE anti-mouse CD86 antibody were obtained from BioLegend, Inc (San Diego, USA). The sulfo-Cyanine5 NHS ester, antibodies for Calreticulin (CRT) were purchased from Abcam (Cambridge, UK). The cell counting kit-8 was purchased from Dojindo (Japan). The mouse TNF-α/ IFN-γ/ IL-6 ELISA kits were obtained from Solarbio Science & Technology Co. Ltd (Beijing, China). Tumor neovascularization targeting peptide of DSPE-PEG5000-cRGD used in the study was synthesized by Xi'an Ruixi Biological Technology Co., Ltd (Xi'an, China).

The morphologies of MVs were observed by using HT7700 transmission electron microscopy (Hitachi, Japan). The zeta potential and size of MVs were measured by a Zetasizer Nano ZS (Malvern Instruments, England). The enumeration analysis of isolated MVs was identified using the NanoSight NS300 system (Malvern Instruments, UK). To visualize the fluorescence signal of DOX in MVs, a super-solution imaging microscope (Leica SP8 STED 3X, Germany) was applied. UV/vis

spectra of MVs were measured by a Hitachi 5300 spectrophotometer. Inductively coupled plasma mass spectrometry was performed on an iCAP Q series ICP-MS instrument (Thermo, Waltham, USA). The XPS analysis was carried out on an EscaLab 250Xi X-ray photoelectron spectroscopy.

Cell lines. The murine breast cancer 4T1 cells were obtained from the National Collection of Authenticated Cell Cultures (Shanghai, China). The 4T1-Luc cell line and adriamycin-resistant cell line MCF-7/ADR were provided by iCell Biotechnology (Shanghai, China). All cells were incubated in the RPMI medium supplemented with 1 % penicillin-streptomycin and 10 % FBS (v/v) at 37 ℃ in a humidified atmosphere containing 5 % CO_2.

Animals. BALB/c mice and nude mice (6-8 weeks, female) used in the study were provided by Charles River Laboratories (Beijing, China). All experiments in this study were performed in compliance with the relevant laws and guidelines of the Institutional Animal Care and Use Committee of the National Center for Nanoscience and Technology, China (Ethical approval number: NCNST21-2206-0603) and also the Institutional Animal Care and Use Committee of the National Center for Nanoscience and Technology have approved the experiments. Informed consent was obtained for any experimentation with animal subjects.

Preparation of MVs

The membrane vesicles (MVs) were obtained through a facile method. To start with, the *Magnetospirillum gryphiswaldense* MSR-1 bacteria were grown in a modified LAY medium supplemented with 60 μM Fe (II) at 30 ℃ with shaking for 24 h, after which well-grown bacteria were observed. Subsequently, the DOX solution (0.1 mg/ μL) was transferred to the flask to a final concentration of 15 μg/mL. Then bacteria were cultured continuously with the stimulation of DOX for 48 hours. Afterward, the main procedures for MVs preparation were as follows: the bacteria cells were pelleted by centrifugation at 8,000 g for 5 min. Then they were

removed, and the obtained supernatant was filtered through a 0.45 μm syringe filter, followed by concentration using 100 kDa regenerated cellulose membrane. The resulting concentrated medium was then centrifuged at 14,000 g for 3 h at 4 ℃ by using an Optima XPN-100 ultracentrifuge (Beckman Instruments, Waltham, USA). To avoid the contamination from bacterial and cell debris, the pellets were filtered through the 0.45 μm filter membrane again. Ultimately, the MVs in pellets were harvested and resuspended in PBS for further studies. In the medium without DOX, original bacterial membrane vesicles were collected and referred to as BMV. Empty membrane vesicles (EMV) were extracted in the same procedure with the exception that no ferrous iron or DOX was added to the growth medium of MSR-1 bacteria. To modify the MVs with cRGD peptides, 1 mg MVs were first dispersed in the buffer and then the same amount of DSPE-PEG_{5000}-cRGD was added. After incubating the mixture at room temperature for 2 hours, the T-MVs were obtained.

Cell viability test

To evaluate the *in vitro* cell viability of MVs, a CCK-8 assay was carried out. Typically, 1×10^4 MCF-7/ADR cells were seeded in a 96-well plate and cultured for 24 h to reach 60% confluence. Then culture medium was replaced and MCF-7/ADR cells were incubated with same concentrations of free DOX, EMV@DOX, BMV@DOX, T-BMV@DOX. After further incubation of 24 h, the medium was discarded and replaced with a fresh culture medium containing 10% CCK-8 for 2 h. The absorbance of OD was measured at 450 nm using a microplate reader.

***In vitro* uptake of MVs by 4T1 cell line**

Sulfo-Cyanine5 NHS ester, a red-emitting fluorescent dye (excitation: 646 nm; emission: 662 nm), was used to label MVs via an amidation reaction. The 4T1 cells were seeded in confocal microscopy dishes at a density of 1×10^5 cells per dish for 24 h before the experiment. Then Cy5 labeled MVs and T-MVs at the same

concentration were added to the confocal dishes and incubated for different time spans at 37 ℃ in the 5 % CO_2 atmosphere. For confocal microscopy imaging, the nuclei of 4T1 cells were stained with Hoechst 33342 for 10 min. After washing three times with PBS, the cells were observed by using a confocal microscope (Zeiss LSM710, Germany).

In vitro DC stimulation experiment

Dendritic cells (DC 2.4) were incubated in RPMI medium containing 10 % FBS (v/v) and 1 % P/S at 37 ℃ in 5 % CO_2 environment. To confirm whether MVs could promote the maturation of DC cells, the *in vitro* DC stimulation experiment was conducted. Briefly, immature DCs with a density of 1×10^5 cells/mL were seeded in a 6-well plate overnight. After being treated with free DOX, Fe^{2+}, LPS, and T-BMVs (DOX at 2 μg/mL) for 24 h, cells were then stained with FITC anti-mouse CD80 antibody and PE anti-mouse CD86 antibody. After centrifugation at 200 × g for 5 min, the DCs were collected and resuspended in PBS, and their maturation state was detected by a BD Accuri C6 flow cytometry (BD Biosciences, USA).

Investigation of the intracellular levels of reactive oxidate species (ROS) in various treatment groups

4T1 cells were seeded in confocal dishes at a density of 10^5 cells/well and cultured following conditions as described above. When the cell confluence reached 70 %, the culture medium in the dishes was replaced with the fresh medium containing PBS, EMV@DOX, BMV, BMV@DOX with an equal concentration of DOX at 1 μg/mL, and incubated for 24 hours. Following the incubation, the drug-treated medium was gently removed, then the cells were incubated with a serum-free medium containing the DCFH-DA probe (10 μM). After incubation for 20 minutes at 37 ℃, cells were washed with the serum-free medium three times to remove excess unbound probes. To compare the ROS generation levels in each group, a laser

scanning confocal microscope (Zeiss 710, Germany) was used. For confocal settings, the excitation wavelength was 488 nm, and a wavelength of 525 nm was for the emission wavelength.

Detection of the changes in mitochondrial membrane potential

To prepare samples for observation, 4T1 cells were seeded in confocal dishes at a density of 10^5 cells/well and cultured in 1640 medium to reach 70 % confluence. Subsequently, the cells were co-incubated with PBS, EMV@DOX, BMV@DOX, and BMV@DOX+IFN-γ. After 12 hours of incubation, the cells in different groups were stained with a JC-1 probe according to the manufacturer's instructions and then observed by confocal imaging. For JC-1 monomers, the excitation and emission were 488 nm and 525 nm. For JC-1 aggregates, the excitation and emission were 525 nm and 590 nm.

Assessment of systemic inflammatory response in MVs-treated mice

For the assessment of systemic toxicity, 4T1 tumor-bearing female mice were randomly assigned into 6 groups and received an injection of PBS, DOX, T-EMV@DOX, T-BMV, BMV@DOX, T-BMV@DOX through the tail vein, respectively. The MV dosage was maintained at 4 mg/kg of total protein. At various time points during the study, the blood sample of each group of mice was taken and corresponding inflammation factors (TNF-α, IFN-γ, IL-6) were measured based on the standard sandwich ELISA protocol. The tumor tissues of mice in each group were harvested, and the IFN-γ expression was evaluated using immunofluorescence.

***In vivo* fluorescence imaging and distribution of MVs in mice**

To establish the tumor-bearing model, 4T1 cells (1×10^6) were subcutaneously injected into the flanks of female BALB/c mice. Membrane vesicles were fluorescently labeled with DiR by directly incubating with 1 μM DiR dye for 2 hours

and then washed with PBS. When the tumor volume reached ~300 mm^3, selected mice randomly divided into 2 groups (n=4) were individually injected with BMV@DOX-DiR and T-BMV@DOX-DiR via the tail vein. The injected concentration was adjusted by DiR at 0.3 mg/kg. At various time points after injection, the mice in each group were imaged by an IVIS Spectrum imaging system (Perkin Elmer, USA). For DiR signal detection, the wavelength of excitation and emission were set at 748 nm and 780 nm, respectively.

In vivo magnetic resonance imaging of MVs-treated mice

4T1 tumor-bearing mice were prepared and randomized into 3 groups, then PBS, BMV@DOX, and T-BMV@DOX at a DOX concentration of 1 mg/kg were intravenously injected into the mice. At scheduled time points, a BioSpec 70/20 USR animal MRI scanner (Bruker, Germany) was used to capture the T_2-weighted MR images of mice in each group. The magnetic field was 7.0 T, repetition time (TR) and echo time (TE) were 3500 ms and 40 ms, respectively.

Evaluation of anti-tumor efficacy of MVs in 4T1 tumor-bearing mice

By subcutaneous injection of 4T1 cells in the right flank of mice, the 4T1 tumor-bearing mice model was established. Upon reaching a tumor volume of ~100 mm^3, all mice were randomized into 6 groups (n=5). PBS, DOX, T-EMV@DOX, T-BMV, BMV@DOX, and T-BMV@DOX at the same dosage of 1 mg/kg DOX were given to the mice in each group by tail vein injection, respectively. The drug was administrated every 3 days until the end. Throughout the entire treatment period, the body weight and tumor size of the mice in each group were recorded every 2 days. When the tumor volume of mice in the PBS group reached 1800 mm^3, the experiment was terminated. The mice were euthanized and the tumors were excised, weighed, photographed, and embedded for both TUNEL and H&E staining analysis. Along

with the tumor tissues, the main organs of mice (heart, liver, spleen, lung, kidney) were also harvested and H&E stained.

Anti-metastasis study of MVs

To induce lung metastasis in breast cancer models, luciferase-labeled 4T1 (4T1-Luc) cells (1×10^6) were intravenously injected into the mice. Then the mice were randomly divided into 3 groups (n=4), receiving an injection of PBS, DOX, and T-BMV@DOX at the same DOX concentration of 1 mg/kg via the tail vein. 15 days later, each mouse was intraperitoneally injected with approximately 150 mg/kg D-Luciferin (Solarbio, Beijing). After being anesthetized with isoflurane gas, the mice were scanned by an IVIS Spectrum imaging system to obtain the *in vivo* and *in vitro* luciferase images in each group. For histopathology analysis, lung tissues of mice were sliced and H&E stained.

The therapeutic effect of MVs in MCF-7/ADR-resistant tumor model

The harvested MCF-7/ADR cells suspended in PBS were mixed with Matrigel at a ratio of 1:2 (v/v), then the mixture was injected into the female BALB/c nude mice via the tail vein. Weeks later, when the tumor reached about 100 mm^3 volume, MCF-7/ADR tumor-bearing mice were selected and randomly divided into 3 groups (n=4) for the injection of PBS, DOX and T-BMV@DOX via tail vein. For each group of mice, the drug administration was conducted at 3-day intervals and continued for 15 days. During the treatment, the body weight, as well as the tumor size of mice in each group were measured every 2 days. After the mice in each group were euthanized at the end of the treatment, tumors were carefully removed, photographed, and weighed. The release of CRT and HMGB1 levels were detected by using immunohistochemistry and immunofluorescence staining.

Statistical analysis

Data performed as indicated were presented as means ± SD, while error bars in all the bar graphs, line graphs and box graphs represented SD. Shapiro-Wilk test was employed to determine whether the data were normally distributed. The comparisons between the two groups were evaluated by an independent sample two-tails t-test. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

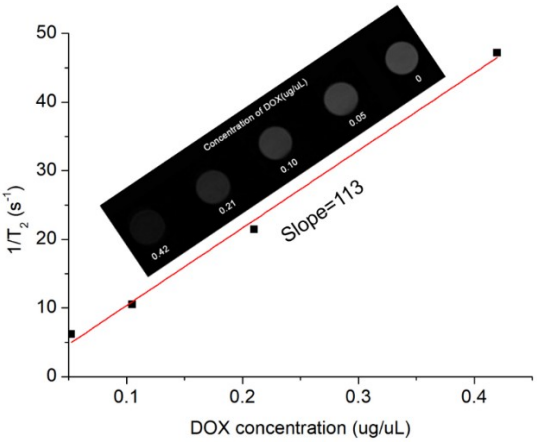

Fig. S1. The T_2 relaxation rate as a function of DOX concentrations and corresponding MR images of BMV@DOX.

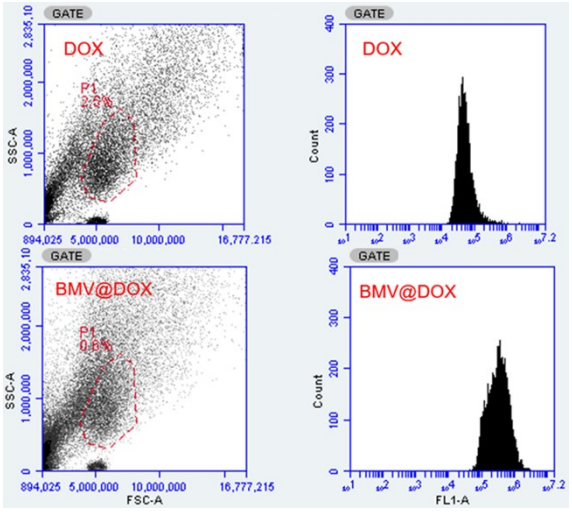

Fig. S2. Flow cytometric image of 4T1 cells after incubation with DOX (upper) and BMV@DOX (below) for 24 h.

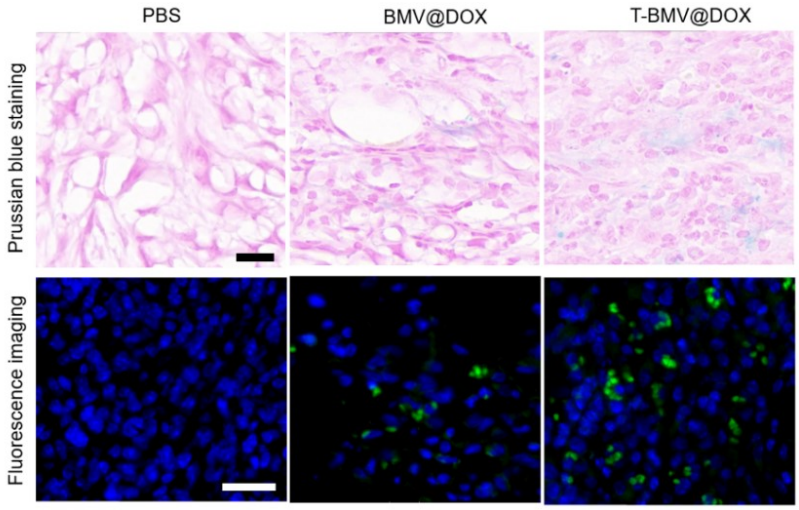

Fig. S3. The Prussian blue staining and fluorescence images of tumors from the mice after MR imaging. The blue and green colors of the bottom pictures show the nucleus and DOX signals, respectively. Scale bars: 25 μm.

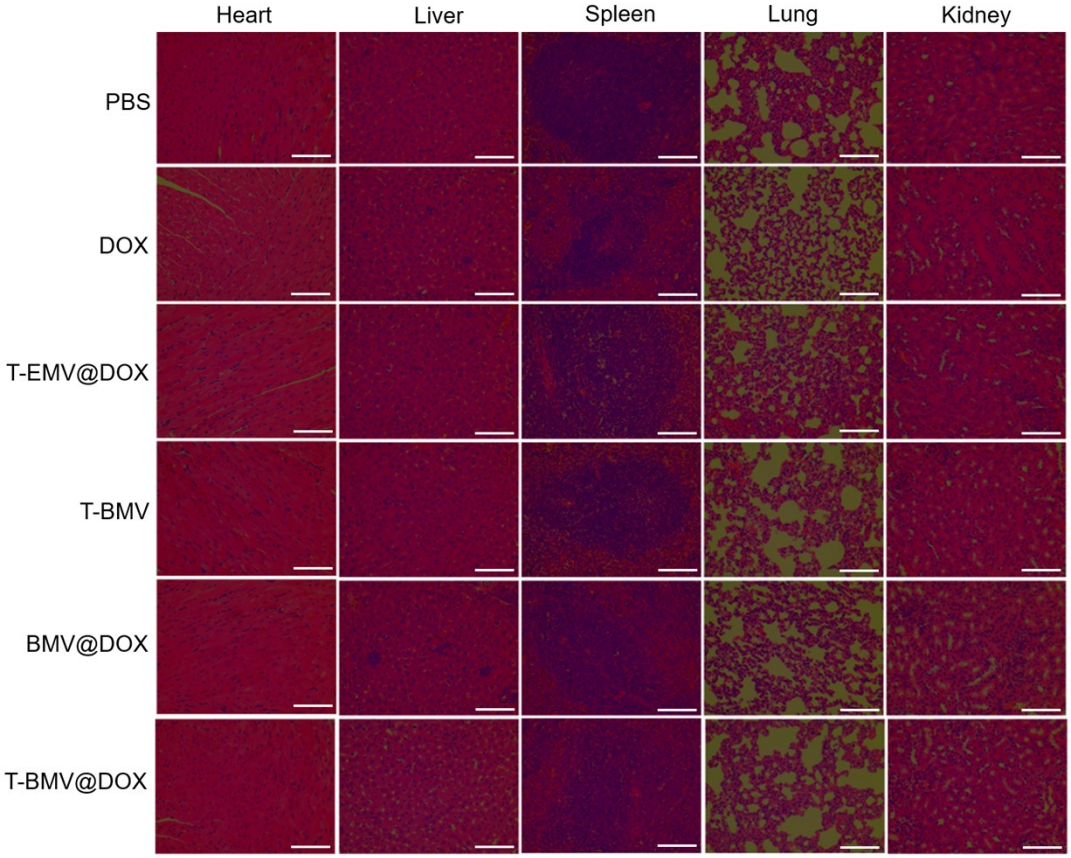

Fig. S4. H&E staining of major organs from 4T1 tumor-bearing mice after treatment with PBS, DOX, T-EMV@DOX, T-BMV, BMV@DOX, and T-BMV@DOX. Scale bars: 100 μm.

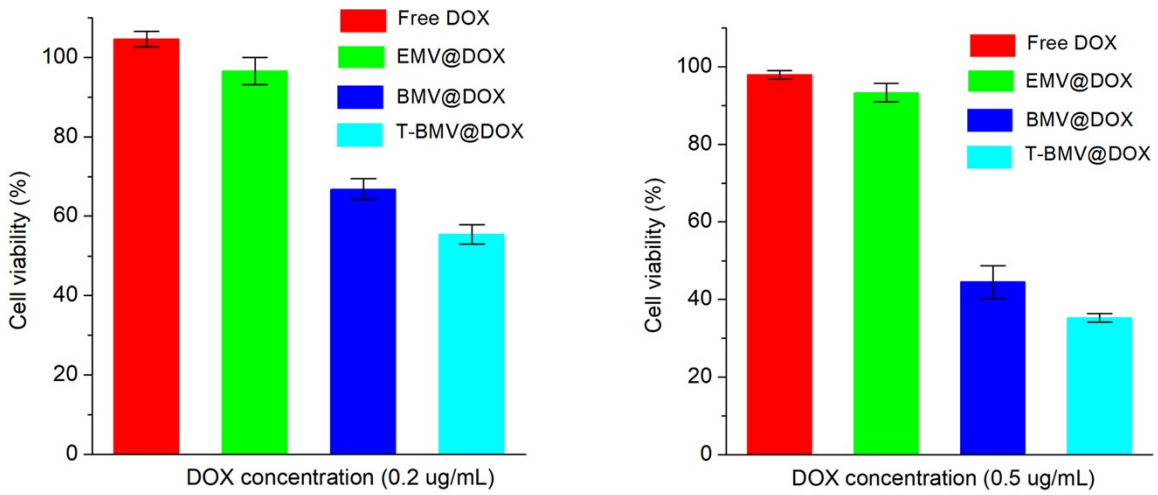

Fig. S5. The cellular cytotoxicity of free DOX and various MVs encapsulated DOX with the concentrations of DOX normalized to 0.2 (left) and 0.5 μg/mL (right) on MCF-7/ADR cells.

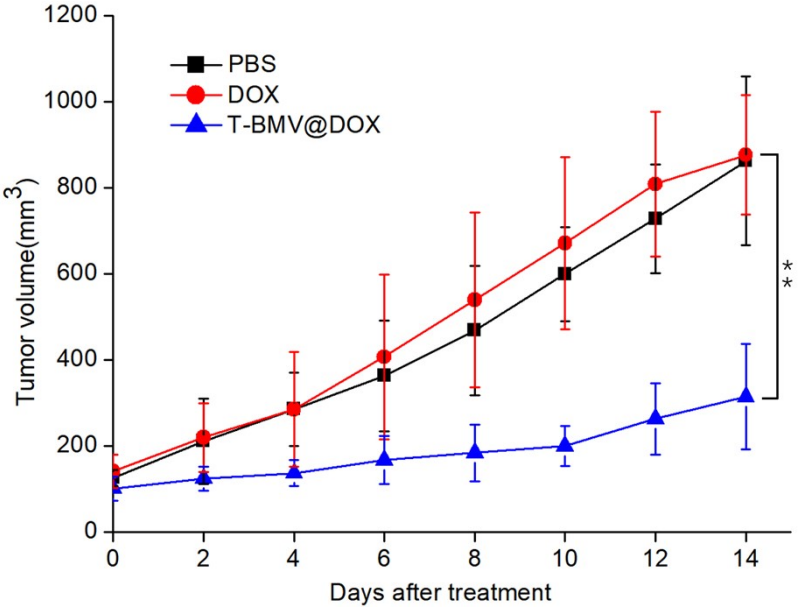

Fig. S6. Tumor growth curves of MCF-7/ADR tumor-bearing mice after different treatments (*n*=4).

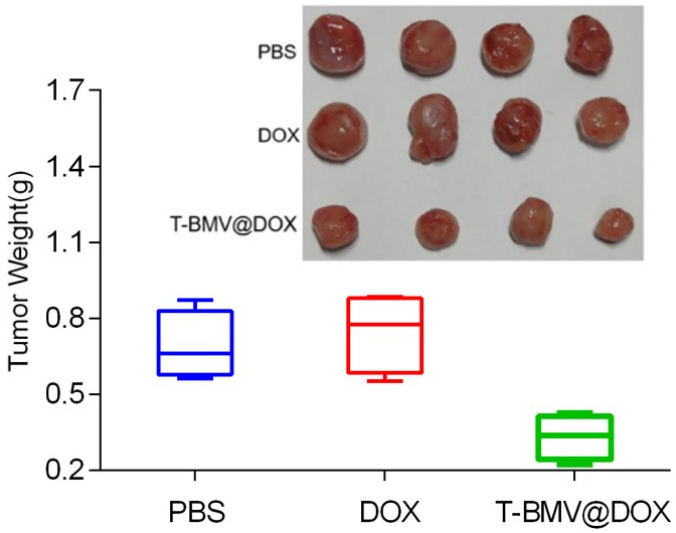

Fig. S7. *Ex vivo* weights and images of isolated tumors from the MCF-7/ADR tumor-bearing mice after treatment of PBS, DOX, and T-BMV@DOX (*n*=4).

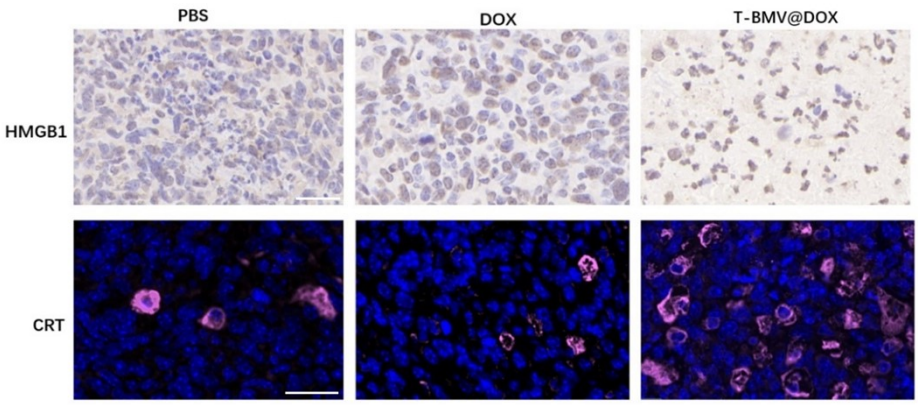

Fig. S8. Histology and immunofluorescence examination of tumors in MCF-7/ADR tumor-bearing mice after therapy. Scale bars: 30 μm.