Supporting Information

Capsaicin-induced Ca^{2+} overload and ablation of TRPV1-expressing axonal terminals for comfortable tumor immunotherapy

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1. Materials.

Potassium permanganate ($KMnO_4$) was purchased from Sanhe Chemical Reagent Co., Ltd., Yantai (China). Capsaicin was purchased from Aladdin (Shanghai, China). Bovine serum albumin (BSA) and dopamine (DA) were purchased from Sigma-aldrich (St. Louis, MO, USA). Capsazepine was purchased from MedChemExpress (New Jersey, America). BAPTA-AM was purchased from Adooq Bioscience (Irvine, CA). Annexin V-FITC Apoptosis Detection Kit and AM/propidium iodide (PI) were obtained from Solarbio (Beijing, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Bogoo (Shanghai, China). Calcein 2′,7-dichlorodihydrofluorescein diacetate (DCFH-DA) was obtained from Shanghai Beyotime (S0033S). DAPI was purchased from Solarbio (Beijing, China). Rhod-2AM, Fura-4-AM and MitoTracker™ Deep Red FM were purchased from yeasen (Shanghai, China). Mitochondrial Membrane Potential Assay Kit with JC-1 (M8560) was purchased from Solarbio. Sulfo-Cy5.5,SE were obtained from Nanjing Goyoo biotech Co.,Ltd. The anti-mouse CRT antibody (EPR3924), TRPV1 antibody (BS397) and PGP9.5 antibody (Ab108986) were purchased from Abcam. Goat anti-rabbit IgG secondary antibody were purchased from Abbkine (A23320). The HMGB1 enzyme-linked immunosorbent assay Kit (SEKM-0145) and ATP Determination Kit (BC0300) were purchased from Solarbio. Lymphocyte Separation Kit (P8860) was purchased from Solarbio. Tunel (C1086) was purchased from Beyotime. Rabbit Anti-CGRP-1/2 antibody (bs-0791R) and Rabbit Anti-Substance P antibody (bs-0065R) were purchased from Bioss

(Beijing,China). CGRP and SP ELISA Kit (SP14060 and SP14205) was purchased from Spbio (Wuhan, China). VEGF antibody (YN5444) was purchased from Immunoway. ROS-ID®Hypoxia/Oxidative stress detection kit was purchased from Enzo (USA). PE anti-mouse CD25 antibody (cat no. 102007), anti-mouse FoxP3 antibody (cat no. 320013), APC anti-mouse CD86 antibody (105012), FITC anti-mouse CD86 (105109), PE anti-mouse CD11c antibody (117308), PerCP/Cyanine5.5 anti-mouse CD80 antibody (104722), APC anti-mouse CD3 antibody (100236), PE anti-mouse CD4 antibody (100512), FITC anti-mouse CD8a antibody (100706), APC anti-mouse CD206 (MMR) (141707), PerCP/Cyanine5.5 anti-mouse/human CD11b (101227), PE anti-mouse F4/80 (111704), Fixation Buffer (420801), Intracellular Staining Perm Wash Buffer (10X) (421002), TruStain FcX™(anti-mouse CD16/32) (101319), True-Nuclear™ Transcription Factor Buffer Set (424401), FITC anti-mouse CD45 (157214), PE anti-mouse Ly-6G/Ly-6C (Gr-1) (108407), FITC anti-mouse CD4 (100509), and PE anti-mouse CD8a (100707) were purchased from BioLegend (USA). The enzyme-linked immunosorbent assay (ELISA) kit against Interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α) and Interleukin-6 (IL-6) were purchased from liankebio (Hangzhou, China). Tunel staining was purchased from Beyotime (Shanghai, China). Hematoxylin-Eosin (H&E) Stain Kit were purchased from SolarBio (Beijing, China). Capsaicin was dissolved in DMSO to 100 μg/μL and stored at 4 °C protected from light. Capsazepine was dissolved in DMSO to a concentration of 20 mM and stored at 4 °C. BAPTA-AM was dissolved in DMSO to a stock concentration of 10 mM and stored at −20 °C. These stock solutions were

diluted before use. To be pointed out, the final solvent concentration did not exceed 0.1% (v/v) in any experiment.

2. Cell culture and mice.

293T cells and 4T1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) medium (GIBCO). All the cells were incubated at 37 ^{o}C with 5% CO_2 with the above-mentioned media supplemented with fetal bovine serum (10%, BI), penicillin (100 U/mL, Solarbio, China), and streptomycin (100 μg/mL, Solarbio, China). Healthy female BALB/c mice aged approximately six weeks were obtained from PengYue Co. Ltd (Jinan, China).

3. Characterisation of MBD&C NPs.

Dynamic light scattering (DLS; Malvern, Nano-ZS90, U.K.) and transmission electron microscopy (TEM; JEOL, Japan) were used to measure the size and morphology of the MBD and MBD&C NPs. X-ray photoelectron spectroscopy (XPS) spectra were obtained using a Thermo Fisher Scientific ESCALAB 250Xi XPS system. FT-IR were obtained using a Thermo Scientific™ Nico. A Shimadzu 2600 UV-vis-NIR spectrophotometer (Japan) was used to measure the UV absorption spectra. The loading efficiency of capsaicin in the final MBD&C NPs determined by the UV-vis spectrophotometer. First, a series of CAP ethanol solutions with different concentrations were prepared. A standard curve of CAP was prepared by linear fitting the function of CAP concentrations with the absorption intensity at 281 nm. Then, 1 ml of MBD&C solution was aspirated, 1.5 ml of anhydrous ethanol was added to it and mixed, centrifuged (5000 rpm, 5 min). The supernatant (2.5 ml in total) was

aspirated, and then 0.05 mL of the supernatant was pipetted for UV measurement and the absorbance was measured at 281 nm. Finally, the absorbance was calculated by inserting a standard curve. Eventually, the capsaicin content in the supernatant was subtracted from the total capsaicin content to obtain the capsaicin content in the nanoparticles. The drug loading content (DLC) and drug loading efficiency (DLE) were measure by UV-Vis. DLC and DLE were calculated according to the corresponding Eqs.

$$DLC(\%) = \frac{Weight\ of\ Loaded\ Drug}{Weight\ of\ Loaded\ Nanoparticle} \times 100\%$$

$$DLE(\%) = \frac{Weight\ of\ Loaded\ Drug}{Weight\ of\ Feeding\ Drug} \times 100\%$$

4. Drug release in vitro

MBD&C NPs (200 μg/mL) were dispersed in 10.0 mL of phosphate buffered saline (PBS) at pH 7.4 and 6.5 with 1 mM of H_2O_2 and stirred at 37 °C. The samples were then centrifuged to collect the supernatant. At specific time points, the supernatant of each sample was collected after centrifugation and then 5.0 mL of fresh PBS solution was added. Capsaicin release was quantified separately using UV-visible spectroscopy.

5. Synthesis MBD&C Loading Cy5.5-SE.

Briefly, MBD&C NPs (2 ml, 3mg ml^{-1}) and 10 μl of (cyanine5.5-SE) Cy5.5-SE were mixed in a flask and stirred magnetically in the dark for 12 hours. Remove and then mix with 1 ml of anhydrous ethanol, centrifuge (5000 rpm, 5 min), remove the supernatant and repeat once at the same speed and time to obtain pure MBD&C

loading Cy5.5-SE NPs.

6. Examination of cellular uptake efficiency

The MBD&C labeled Cy5.5-SE NPs were used to monitor cellular uptake in 4T1 cells. The medium containing MBD&C labeled-Cy5.5-SE NPs (200 μg, 3mg mL^{-1}) were co-cultured with 4T1 cells cultured in CLSM-specific dishes (2×10^5 cells/dish) at 37°C for 0.5 and 2 h. After the medium was removed, the CLSM-specific dishes were washed with PBS for 3 times. Then, 4 % paraformaldehyde was added and incubated for 15 minutes to fix the cells. At last, DAPI (500 μL, 5mg/mL) was added and co-cultured with the cells for 3 min. Confocal laser scanning microscopy (CLSM) was used to acquire the images.

7. Measurement of mitochondrial damage and mitochondrial distribution.

The mitochondrial damage was detected by CLSM and flow cytometry using 5,5‘,6,6’-Tetrachloro-1,1‘,3,3’-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) as a probe. Briefly, DMEM medium containing 200 μg mL^{-1} different samples were added to 4T1 cells that had been incubated in CLSM-special dishes (2×10^5 cells/dish) for 24 h. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. After another 12 h of incubation, the samples were washed with PBS for 3 times, followed by the addition of a culture medium containing 10 μg/mL JC-1 dye and incubated for 20 min at 37°C temperature. After removing the excess dye, mitochondrial damage was observed by CLSM and flow cytometry immediately.

The mitochondrial distribution was detected by MitoTracker™Deep Red FM using CLSM. Briefly, DMEM medium containing 200 μg/mL different samples were

added to 4T1 cells that had been incubated in CLSM-specific dishes (2×10^5 cells/dish) for 24 h. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. After 12 h of incubation, discard the medium and wash with PBS for 3 times and incubated with MitoTracker™Deep Red FM (final concentration: 200 nM) for 40 min. At last, discard the medium and wash with PBS for 3 times and the cell status under different treatments was observe with and CLSM immediately.

8. Antitumour effect *in vitro*

The in vitro cytotoxicity of different materials against 4T1 cells was measured by MTT assay. 4T1 cells were incubated on 96-well plates for about 12 h. Then, remove the DMEM medium and add various concentrations of DMEM medium containing MBD&C NPs or different materials. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. After 20 h of incubation, the cells were washed with 200 μL fresh DMEM medium. Then, 20 μL MTT (5 mg/mL) was added to each well and after 4 h of incubation, 150 μL of DMSO was added. Read the optical density (OD) of every well at 490 nm via a microplate reader. Calculate the relative cell survival rate according to the following formula: cell viability (%) = OD (sample) / OD (control)×100%. Similar procedures were implemented to assess the biocompatibility of 293T (human embryonic kidney cells).

The Live/dead was detected by Fluorescent microscope. 4T1 cells were cultured on 6-well plates for about 24 h. Then add different DMEM medium containing materials in it. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in

the above experiment. After incubating for 24 h, discard the medium and wash with PBS for 3 times and incubated with calcein-AM (final concentration: 1 μM) and PI (final concentration：3 μg/mL) for 20 min. At last, the cell status under different treatments was observe with and Fluorescent microscope.

The cell apoptosis was measured using flow cytometry. 4T1 cells were cultured on 6-well plates for about 24 h. Then add different DMEM medium containing materials in it. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. After incubating for 24 h, the cells were collected and double stained with FITC-conjugated annexin V and PI for 20 min. After staining, cells were subjected to flow cytometry analysis immediately.

9. ICD and dendritic cell maturation *in vitro*

Calreticulin (CRT) overexpression was assessed by immunofluorescence staining. After co-incubating 4T1 cells with MBD&C NPs (200 μg/mL) for 24 h. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. Then, PBS-Tween® (PBST) was employed to rinse the cells, followed by rinsing with 4% formaldehyde to fix the cells. Then, 5% FBS was supplemented to the cells and cultured at 37 °C for 30 min. Subsequently, rabbit anti-CRT antibody (1:100; Abcam, EPR3924) was added and incubated for 12 h at 4 °C. After washing with PBST three times, Dylight 594 and DAPI were used to stain the cells. CRT expression was also tested using CLSM.

The release of high-mobility group box 1 protein (HMGB1) and adenosine-5'-triphosphate (ATP) was tested using the HMGB1 enzyme-linked immunosorbent

assay (ELISA) kit and the chemiluminescence ATP determination kit (Solarbio, BC0300). Briefly, 200 μg mL^{-1} of MBD&C NPs were incubated with 4T1 cells for 24 h. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. The release level of HMGB1 in the cell culture medium was tested using the HMGB1 ELISA Kit. Similarly, the ATP ELISA kit was used to monitor ATP release.

To determine DC maturation, 4T1 cells were co-cultured with MBD&C NPs (200 μg mL^{-1}) for 12 h. CPZ+MBD&C group and BAPTA+MBD&C group were treated as in the above experiment. The supernatant from the 4T1 cell culture dish was removed and added to the culture dish seeded with DC cells (derived from BALB/c mouse bone marrow with GM-CSF stimulation) and then incubated for 24 h. The control group was cultured in fresh medium. Afterward, DCs were detected using flow cytometry after staining with anti-CD11c-PE, anti-CD80-PE/Cy5.5 and anti-CD86-APC.

10. Behavioral assessment

Mechanical allodynia (von Frey test). The mechanical allodynia was assessed by von Frey test. Briefly, mouse were placed in a clear plastic box (20*17*13 cm) with a wire mesh floor, 40 cm above the bench, and allowed to habituate to their environments for 15 min prior to testing. An electronic von Frey hair unit (IITC Life Science Inc., USA) was used: the withdrawal threshold was evaluated by applying forces ranging from 0 to 50 g with 0.2 g accuracy. Punctuate stimulus was delivered to the mid-plantar area of each hind paw from below the mesh floor through a plastic

tip and the withdrawal threshold was automatically displayed on the screen. The paw sensitivity threshold was defined as the minimum force required to elicit a robust and immediate withdrawal reflex of the paw. Voluntary movements associated with locomotion were not considered as a withdrawal response. Stimuli were applied to each hind paw at 5-minute intervals. Measurements were repeated 3 times.

Plantar Test Analgesia Meter (IITC Life Science Inc., USA). The mice were placed in a Plexiglas chamber (10*10*12 cm) with a glass heating base (29 °C). After accommodation for 30 min, the tests were performed on the plantar surface by focusing a focused, radiant heat light source on the top of the glass to create a 4.0 × 6.0 mm intense spot on the paw. The thermal withdrawal latency (TWL) was shown on the display until the withdrawal of hind paw. Each hind paw was measured three times, and the average was calculated. To protect the animals, the tests were performed with an interval of 5 min, and the cutoff time was set to 18 s.

11. Analgesic mechanism *in vivo*

After all treatments were completed, mice were anesthetized with sodium pentobarbital and perfused with normal saline (0.9%), followed by 4% paraformaldehyde. Following perfusion, the tumor tissues were removed and post-fixed overnight in fixative solution, then cryoprotected overnight in 30% sucrose in PBS. Frozen tumor tissues were embedded in Tissue-Tek O.C.T compound, then cut into 10.0 μm sections. The sections with different treatments were stained with TRPV1 and PGP9.5 for cell surface markers and imaged by CLSM.

12. Measurement of CGRP and SP content

Briefly, blood was taken from the eyeballs of mouse, added to the procoagulant tube, mixed, allowed to stand for 20 min, centrifuged at 1500 g for 20 min, and the upper serum layer was removed. The remaining steps were performed according to the CGRP and SP ELISA kit requirements.

13. Assessing the ability to inhibit angiogenesis.

The expression of angiogenesis was assessed by detection of VEGF. Paraffin sections of tumour tissue were deparaffinised to water and antigenically repaired. Then, PBS was employed to rinse the cells. Then, 5% FBS was supplemented to the cells and cultured at 37 ^{o}C for 30 min. Subsequently, rabbit anti-VEGF antibody (1:150; immunoway biotechnology, YN5444) was added and incubated for 12 h at 4 ^{o}C. After washing with PBS three times, Dylight 594 and DAPI were used to stain the cells. VEGF expression was tested using fluorescence microscopy.

14. Detection of *in vivo* immune activation by flow assay.

For DC maturation analysis, the lymph nodes of mice were gathered and filtered to gain single-cell suspension. Then, the cell suspensions stained with anti-CD86-APC, anti-CD11c-PE and anti-CD80-PerCP/Cyanine5.5 antibodies for flow cytometry assay. Briefly, the tumors and spleen of mice were dissected off and cut into small pieces with ophthalmic scissors, ground and filtered through a 70μm filters, separated with a lymphocyte isolation kit, and the lymphoid tissue was aspirated and analyzed. For macrophage analysis, the cells were first incubated with FcRblock (1g/test) at 4 ^{o}C for 5-10 min, followed by surface staining with anti-F4/80-PE, anti-CD86-FITC and anti-CD11b-PerCP/Cyanine5.5 antibodies at 4 ^{o}C for 30min. Then,

the cells were washed with PBS for 3 times, and then the cells were incubated with cell fixation buffer (Fixation buffer, 500 ul) at room temperature and protected from light for 30 min. Then centrifuged at 150g for 5 min to discard the fixation buffer and resuspend the cells by adding 10 x Intracellular Staining Permeabilisation Wash Buffer diluted 10-fold with ddH_20, and then centrifuged at 150g for 5 min to discard the supernatant, and repeated 3 times. Then, 100ul 1x Intracellular Staining Permeabilization Wash Buffer was used to resuspend the cells, anti-CD206 (MMR)-APC antibody (0.5 ug/test) was added, and the cells were incubated at room temperature and protected from light for 30 min. After incubation, 2 mL 1x Intracellular Staining Wash Buffer was used to wash the cells for 3 times, and 500ul cell staining buffer was added to resuspend the cells. For T cells activation analysis, the cell suspensions were stained with anti-CD3-APC, anti-CD4-FITC and anti-CD8a-PE antibodies. For Tregs analysis, firstly, the cells were labelled with anti-CD4-FITC, anti-CD25-PE antibodies, and the reaction was carried out at 4 ^oC of light protection for 30 min, then PBS was added and centrifuged at 1000 r/min for 5 min, and the supernatant was discarded. Add 500ul of 4 ^oC pre-cooled fixative, mix well, incubate for 30 min at 4 ^oC away from light, add 2 mL of membrane-breaking agent, 1500r/min, centrifuge for 5min, discard supernatant. Repeat once. Then, add anti-FoxP3-APC antibody and incubate for 30 min at 4 ^oC of light protection, add 1 mL of membrane breaker to resuspend the cells, centrifuge at 1000r/min for 5 min, and discard the supernatant. Add 0.3 mL flow-through wash solution to resuspend the cells on the machine for detection. For MDSCs analysis, the cell suspensions were

stained with anti-CD11b-PerCP/Cy5.5, anti-CD45-FITC and anti-Gr-1-PE antibodies.

Finally, cells were collected and analyzed by flow cytometry.

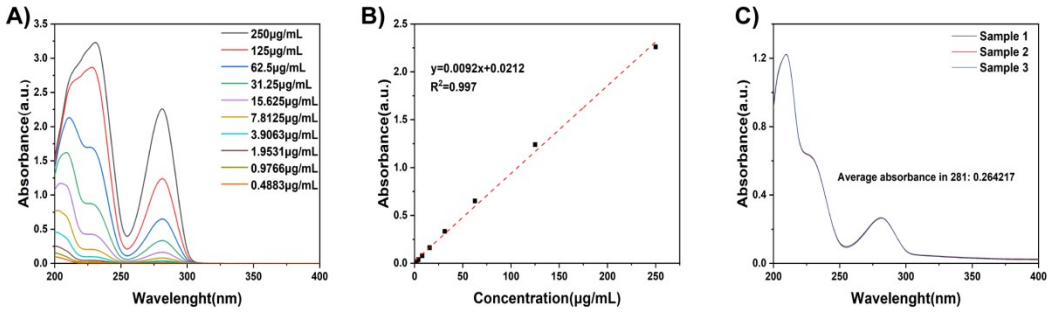

Fig. S1. (A) UV absorbance of different concentrations of capsaicin in anhydrous ethanol solution. (B) Standard curve of UV absorbance at 281 nm of different concentrations of capsaicin. (C) Concentration of capsaicin at 281 nm for 0.05 mL of supernatant, 2.5 mL of supernatant total.

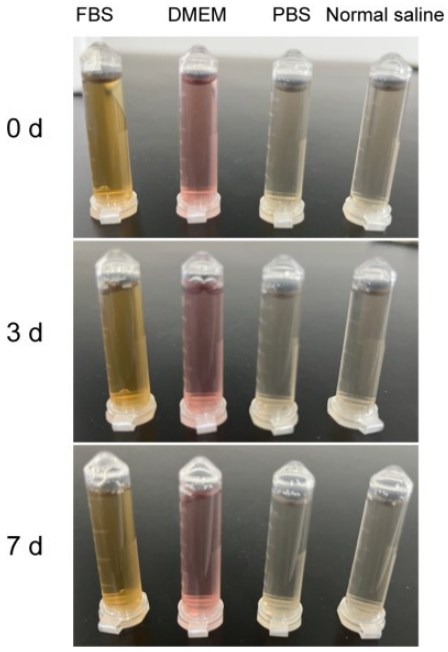

Fig. S2. Colloidal stability of MBD&C NPs (all at concentrations of 0.2 mg mL^{-1}) dispersed in different media (from left to right in each photo: FBS, DMEM, PBS and normal saline) over 7 days.

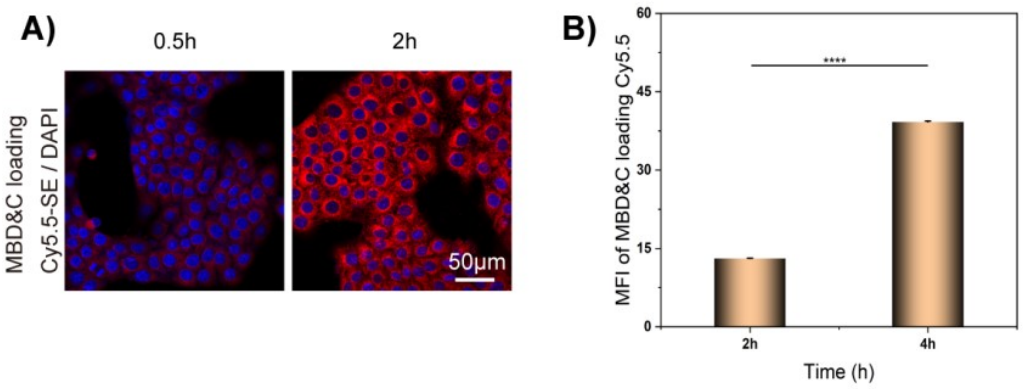

Fig. S3. (A) CLSM images of 4T1 cells treated with MBD&C loading Cy5.5-SE NPs for 0.5 h and 2 h. Scale bar =50 μm. (B) Quantification of the mean fluorescence intensity of MBD&C NPs in 4T1 cells. Data are means ± SD, (n=3). (****$p < 0.001$).

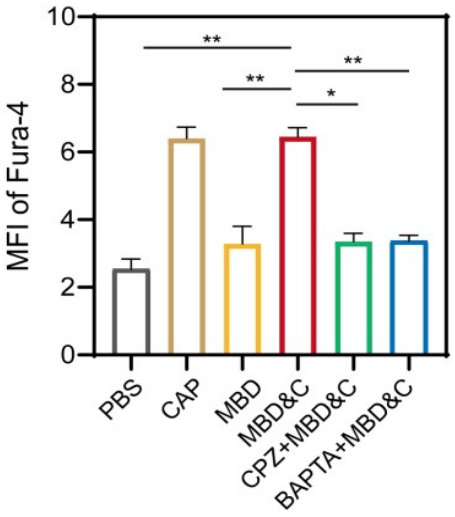

Fig. S4. Quantification of the mean fluorescence intensity of Fura-4. Data are means ± SD, (n=3). (*$p < 0.1$, **$p < 0.01$).

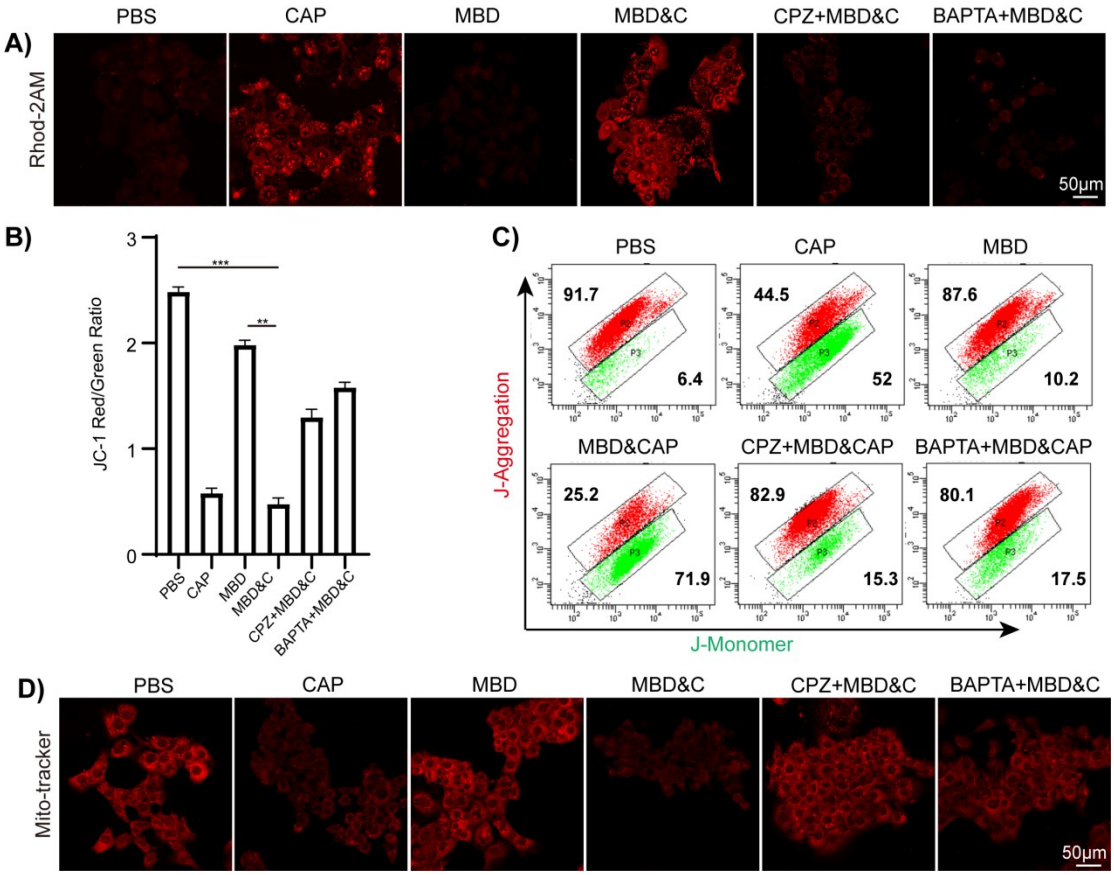

Fig. S5. (A) CLSM images of 4T1 cells being various treatments after incubated with various materials for 4 h and stained with an mitochondrial Ca^{2+} indicator, Rhod-2 AM (20.0 μM). (B) JC-1 Red/Greem ratio. (C) Quantification of mitochondrial membrane potential by flow cytometry. (D) Mitochondrial distribution. Scale bars = 50 μm.

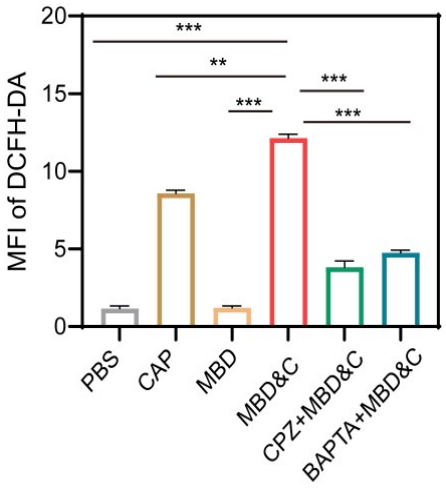

Fig. S6. Corresponding quantification of ROS fluorescence after various treatments in 4T1 cells. Data are means ± SD, (n=3). (**p < 0.01 and ***p < 0.001).

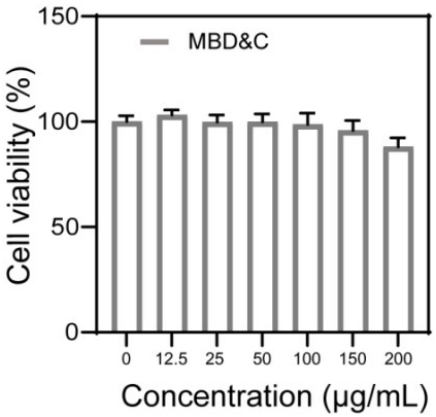

Fig. S7. Viability of 293T cells after being treated with MBD&C NPs for 24 h.

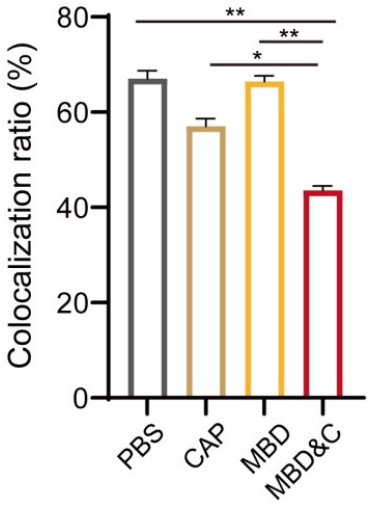

Fig. S8. The colocalization ratio of TRPV1 and PGP9.5. Data are means ± SD, (n=3). (*$p < 0.05$ and **$p < 0.01$).

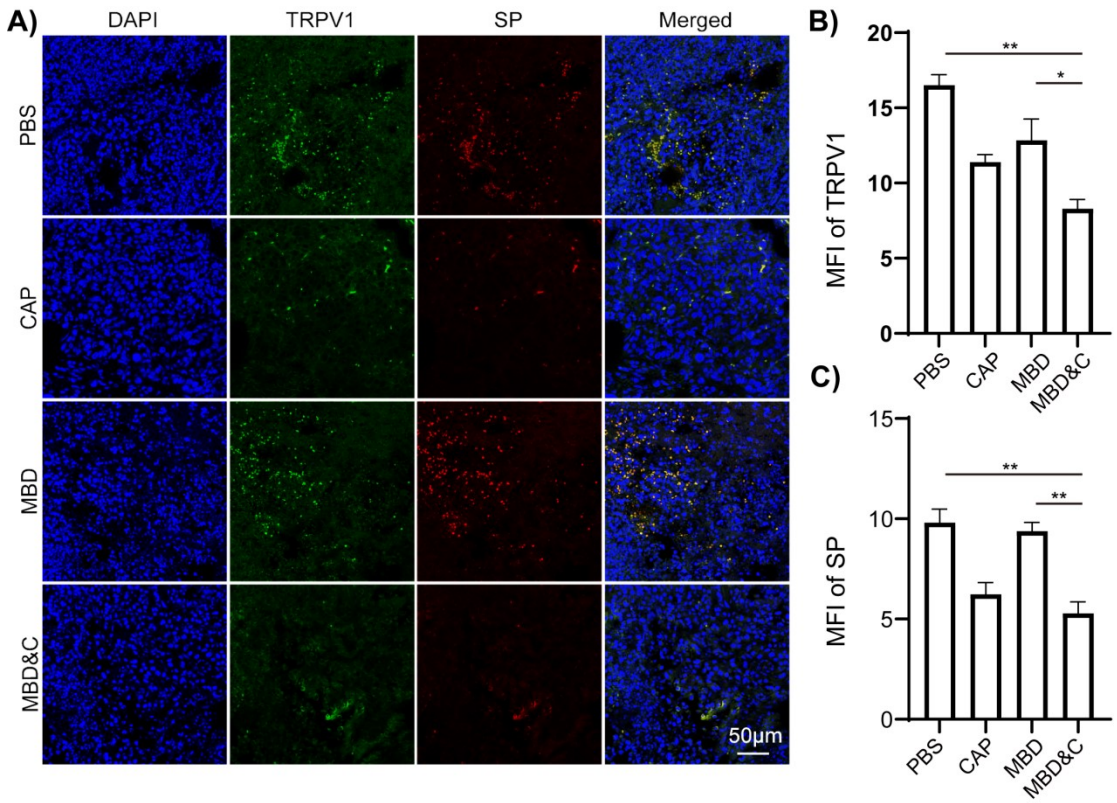

Fig. S9. (A) Immunofluorescence staining for TRPV1 and SP. Scale bar = 50 μm. (B) Quantification of the mean fluorescence intensity of TRPV1 and (C) SP. (*p<0.0 and 5**p < 0.01).

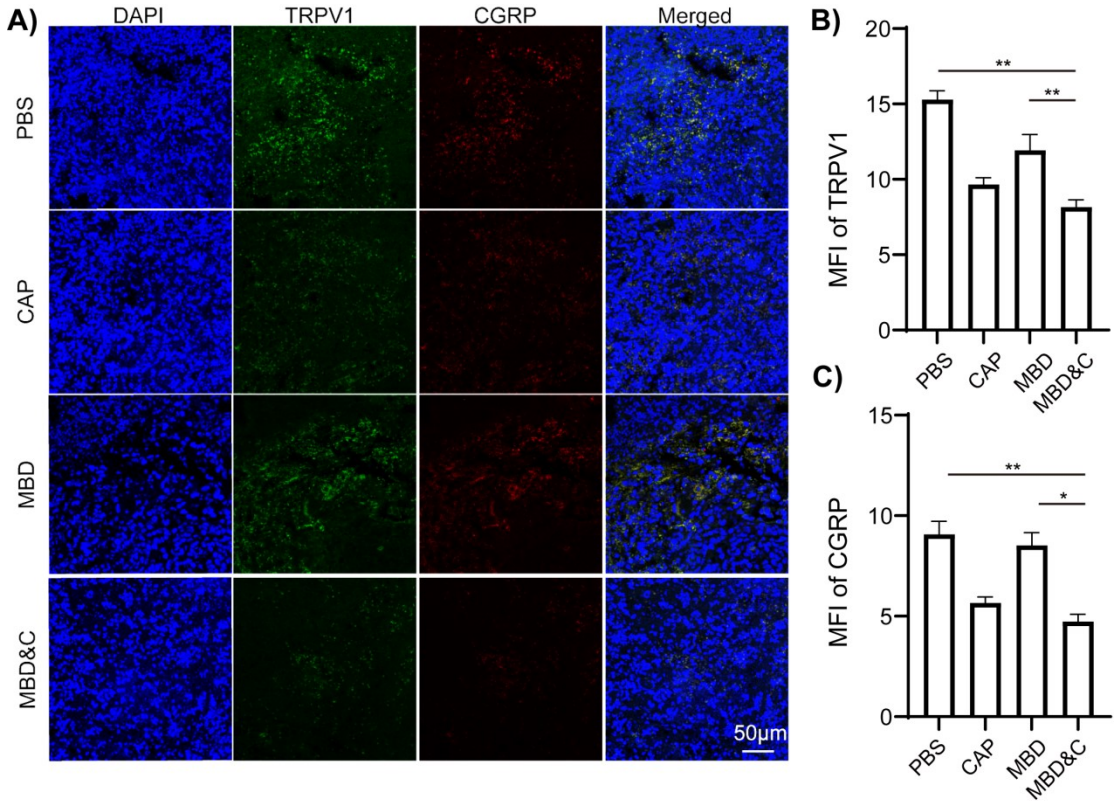

Fig. S10. (A) Immunofluorescence staining for TRPV1 and CGRP. Scale bar = 50 μm. (B) Quantification of the mean fluorescence intensity of TRPV1 and (C) CGRP. (*p < 0.05 and **p < 0.01).

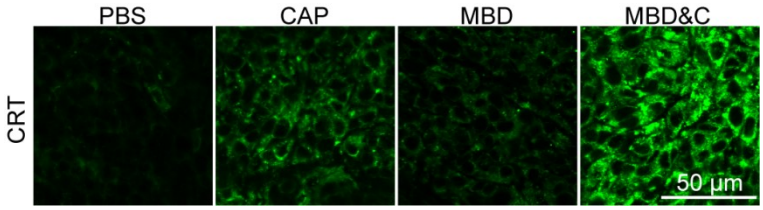

Fig. S11. Immunofluorescence detection of tissue CRT. Scale bar = 50 μm.

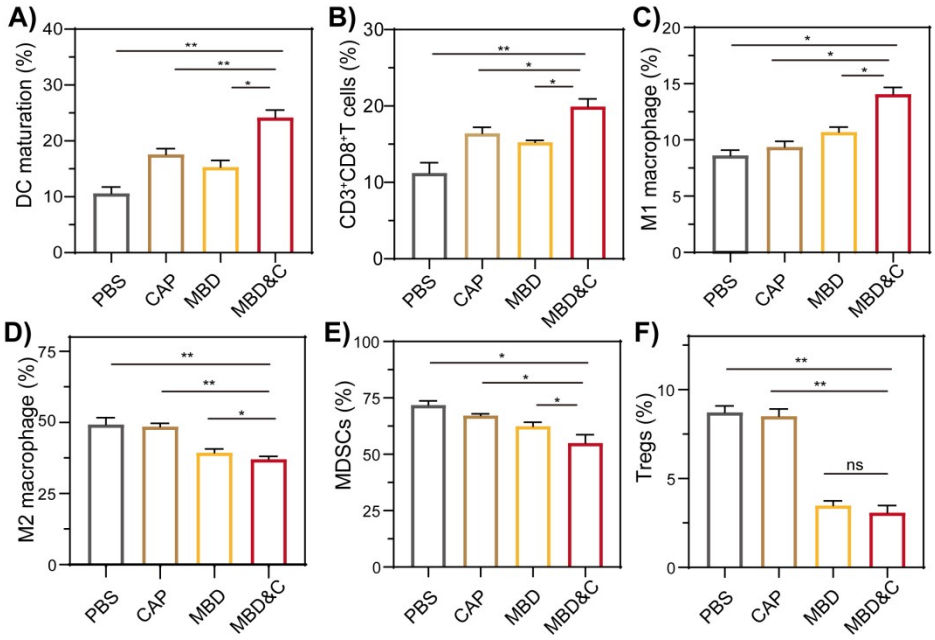

Fig. S12. Quantification of (A) DC cells maturation, (B) $CD3^{+}CD8^{+}$ T cells, (C) M1 macrophage, (D) M2 macrophage, (E) MDSCs and (F) Tregs in mouse after different treatments. (*p < 0.05 and **p < 0.01).

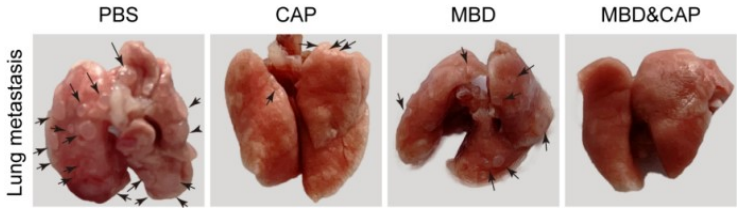

Fig. S13. Lung metastases at the end of the last treatment.

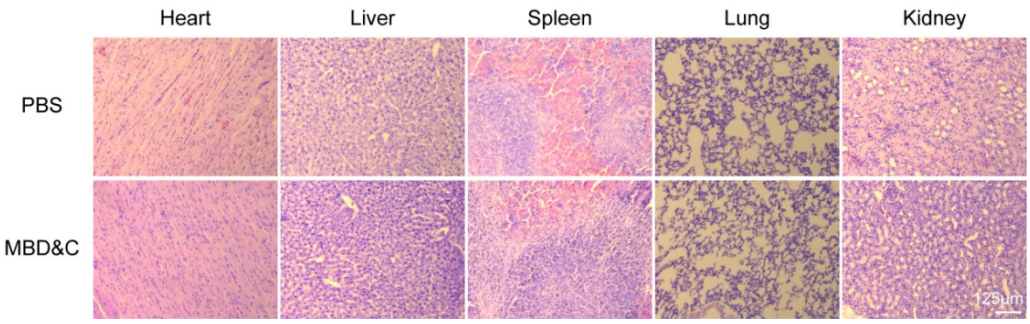

Fig. S14. H&E staining of the major organs at the end of treatment. Scale bar = 125 μm.

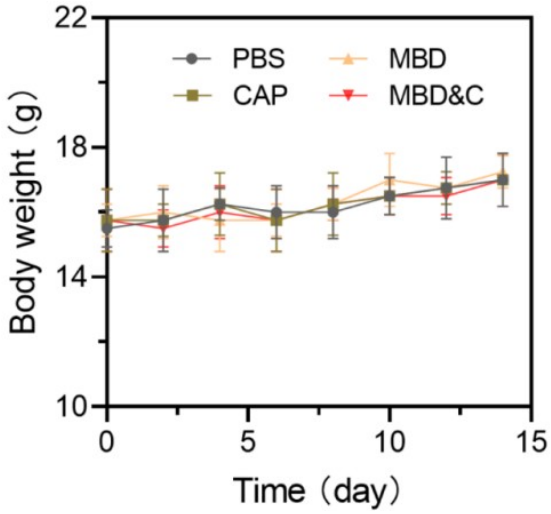

Fig. S15. Body weight of mice during treatment.