Electronic Supplementary Material (ESI) for Materials Advances.
This journal is © The Royal Society of Chemistry 2023

Supplementary Information

Modulating tumor-associated macrophages with natural nanomodulators by neutralizing acidic tumor microenvironment for tumor treatment

Lei Peng[a], Chenxu Zhang[a], Guanlun Zhou[a], Ao Yu*[b,c] and Yongjian Wang*[a]

a. Key Laboratory of Bioactive Materials, Ministry of Education, College of Life Sciences, Nankai University, Tianjin 300071, China.

b. Haihe Laboratory of Sustainable Chemical Transformations, Tianjin 300192, China.

c. Tianjin Key Laboratory of Molecular Recognition and Biosensing, College of Chemistry, Nankai University, Tianjin 300071, China.

Supporting Figures

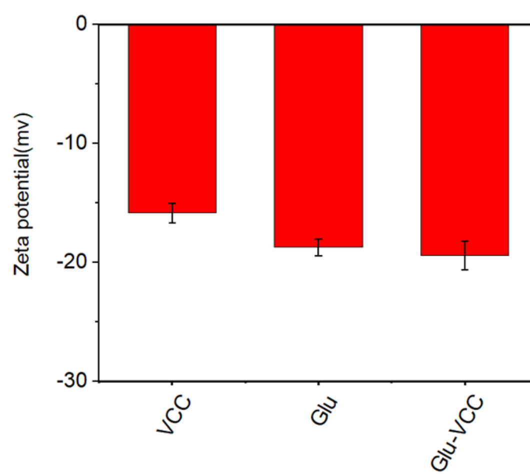

Fig. S1 The zeta potential of VCC, Glu and Glu-VCC.

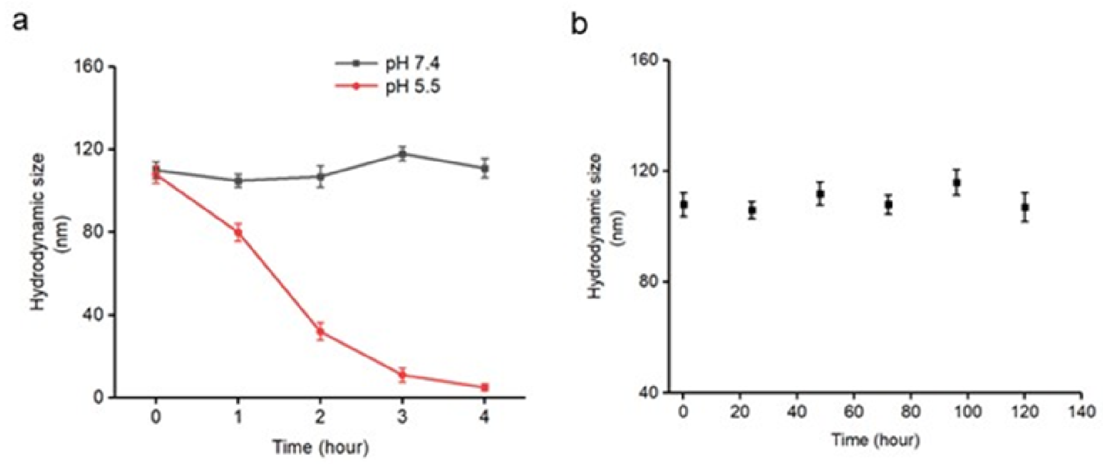

Fig. S2 pH-Dependent hydrodynamic size variation of Glu-VCC (a) in different pH and (b) in DMEM containing 10% fetal bovine serum.

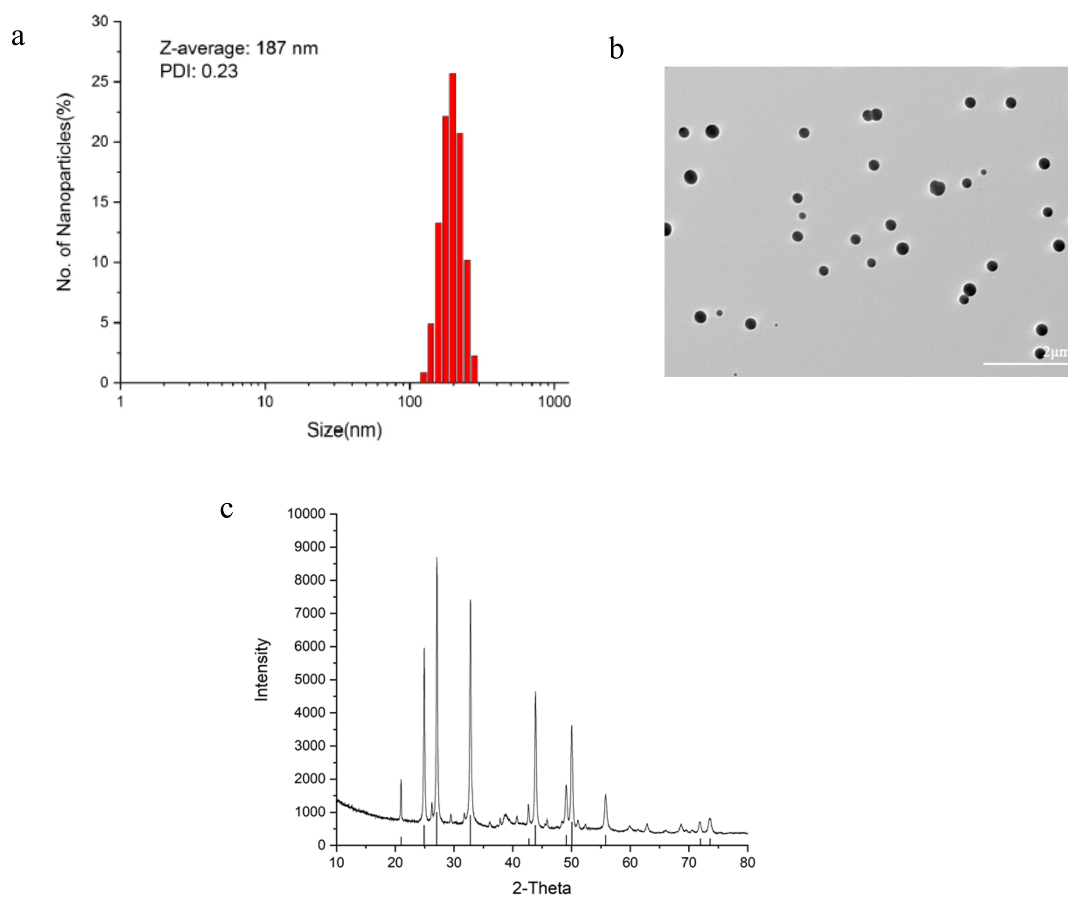

Fig. S3 Characterization of $CaCO_3$ nanoparticles. a) size distribution; b) transmission electron microscopy, scale bar: 2 μm; c) XRD pattern.

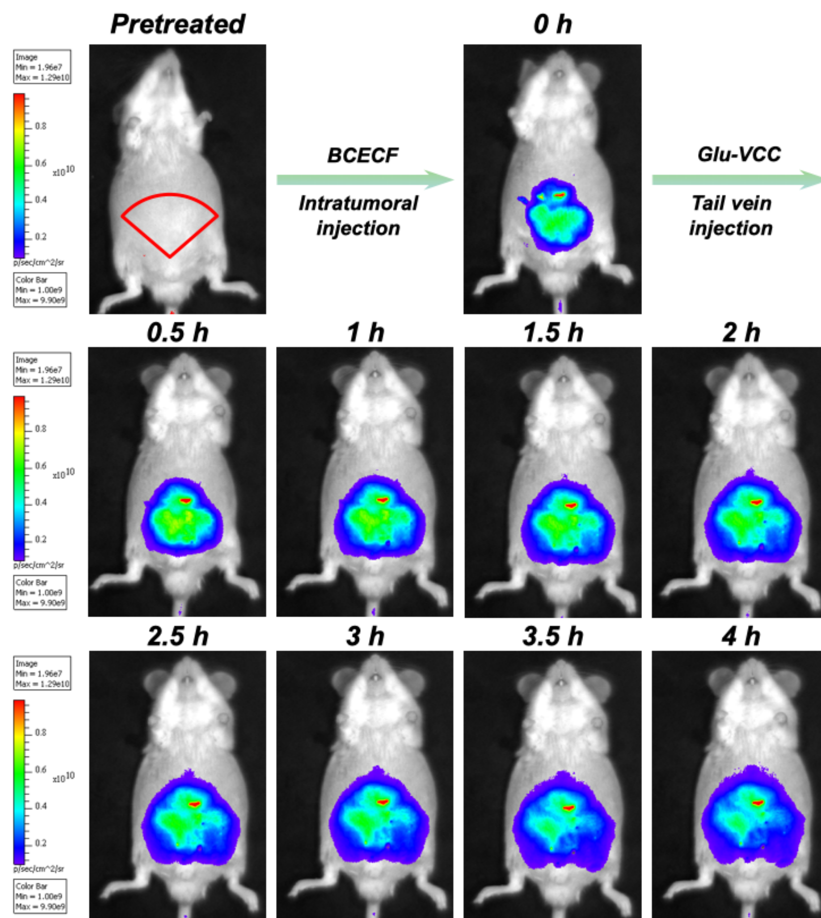

Fig. S4 The imaging map of the pH of the tumor region of ICR mice in the living imaging system, the red sector frame area is the H22 cell inoculation area.

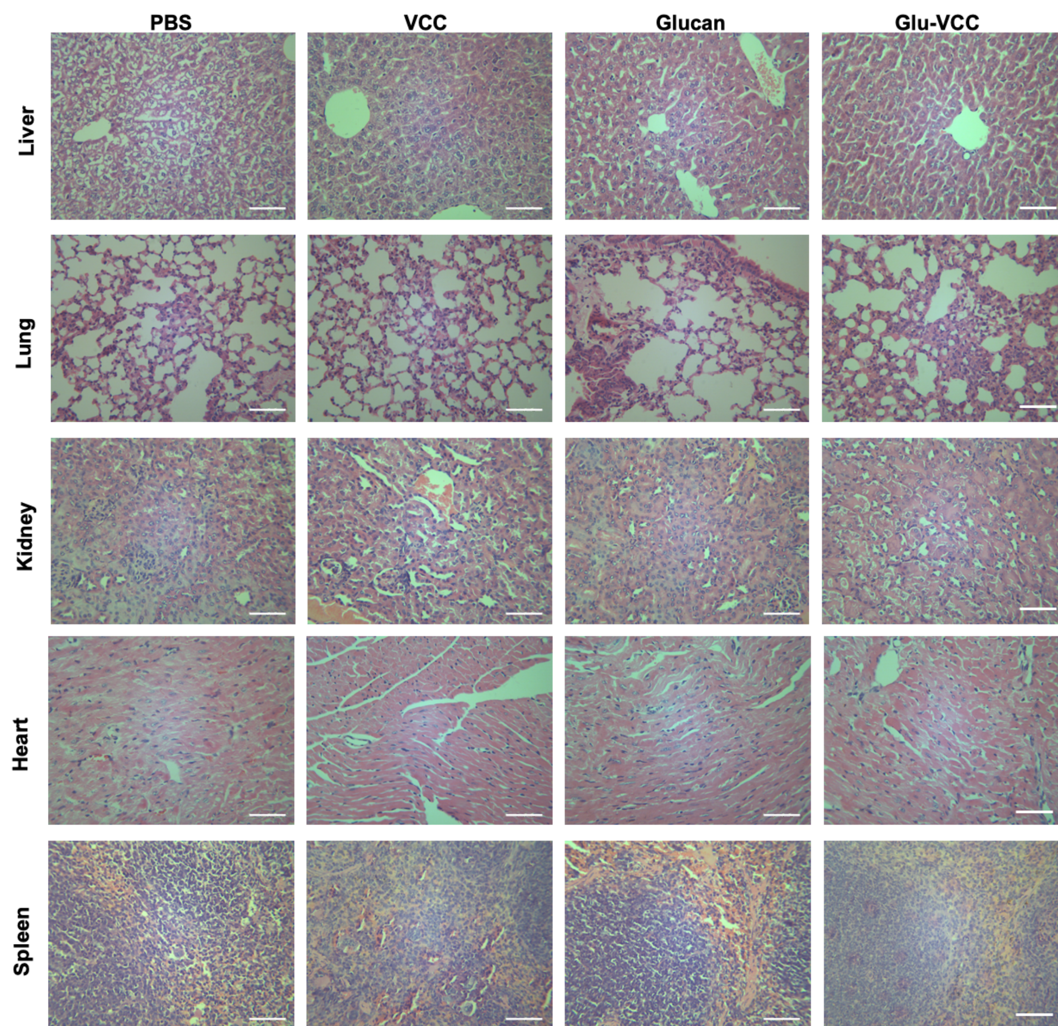

Fig. S5 4T1 bearing Balb/c mice treated with PBS, VCC, Glu, Glu-VCC. H&E staining of liver, lung, kidney, heart and spleen. Scale bar: 100 μm.

Materials and Characterization

All chemical reagents are purchased from Aladdin (Shanghai, China). Yeast-derived β-Glucan (S24487, Molecular weight 20000, Yuanye, China, water soluble). The F4/80 Antibody-APC was supported by eBioscience™ and the IL-12 (p40/p70) Antibody was obtained from BD Pharmingen™. Besides, the IL-10 Antibody, CD86 Antibody, CD206 Antibody, and all of the second antibodies were provided by Biosynthesis Biotechnology Inc. (Bioss, Beijing, China). The EDTA Antigen Retrieval Solution was from Beijing Solarbio Science & Technology Co., Ltd. Annexin V-FITC/PI staining kit was purchased from Dalian Meilun Biotechnology Co., Ltd. Mouse tumor tissue macrophage cell isolate kit (TBD1092MAC) was from Tianjin Yanyang Biological. The Mouse IL-12 p70 Uncoated ELISA KIT and Mouse IL-10 Uncoated ELISA KIT were from Invitrogen™.

The morphology and dry size of the nanoparticles were observed by Transmission electron microscopy (JEM-2800) at a working voltage of 100KV. The DLS and zeta potential measurements were performed by dynamic light scattering (Nano-ZS90 instrument, Malvern). The crystal ratio of the materials was obtained by X-ray diffraction (XRD) (D/max 2550, Rigaku) with Cu-Kα (1.5406 Å) radiation. The Infrared spectroscopy was detected by Fourier transform infrared spectrometer (TENSOR II, Bruker)

Methods

Preparation of Glu- VCC nanoparticles

1.98 g of $CaCl_2$ was dissolved in 20 mL of deionized water, and 0.4 g of β-Glucan was added to the $CaCl_2$ solution and stirred at 200 rpm for one minute until β-Glucan was completely dissolved. 3.816 g of Na_2CO_3 was dissolved in 20 mL of deionized water for backup. 20 mL of $CaCl_2$ (with β-Glucan) solution was preheated to 40°C, and the rotation speed was adjusted to 600 rpm, then 10 mL of Na_2CO_3 solution was slowly dropped into the $CaCl_2$ solution. After the addition was completed, the reaction liquid was ta centrifuged at 8000 rpm for 5 minutes, then discarded the supernatant, added

8 mL of deionized water to wash the pellet, and centrifuge again. After repeating centrifugation three times, the precipitate was washed once with absolute ethanol, centrifuged at 8000 rpm for 5 minutes, the supernatant was discarded and the pellet was re-suspended in 5 mL of deionized water, then freeze-dried to obtain a solid powder. The product was stored at 4 °C. The preparation process of the VCC nanoparticles was similar to that of the Glu-VCC nanoparticles. It was necessary to add 0.08 g of sodium tripolyphosphate to the Na_2CO_3 solution, and the reaction temperature was controlled at 0°C.

Determination of β-Glucan loading in Glu- VCC by Congo red method

2 mg β-Glucan was dissolved in 2 mL of deionized water, then diluted to 20 mL to obtain 100 μg mL^{-1} standard solution, and then gradually diluted to 50, 25, 12.5, 6.25, 3.125 and 1.56 μg mL^{-1} as standard solutions. 3 mg of Congo red was dissolved in PBS and diluted to 30 mL. Put 1 mL of sample solution into 2 mL Congo red stain solution, immediately vortexed for 10 seconds, let stand for 30 minutes at room temperature, and then tested the absorbance at 550 nm. 1 mg Glu-VCC sample was dissolved in 0.5 mL hydrochloric acid solution of pH 5.0 until the calcium carbonate was completely decomposed, then adjust the pH to 7.0 with NaOH solution, and made up to 5 mL with deionized water. 1 mL of the sample solution was added to 2 mL of Congo red stain solution, vortexed for 10 seconds, and then allowed to stand for 30 minutes. The absorbance was measured at 550 nm, and the loading of β-Glucan in Glu-VCC was converted according to the standard curve.

pH-responsive capacity of nanoparticles in the acidic environment

Glu-VCC nanoparticles (4 mg) was dispersed in 4 mL of solution (buffer solution with pH 7.4 or 5.5) at room temperature. At set time intervals, the size of the nanoparticle was measured by a Nano-ZS90 instrument (Malvern).

Determination of lactic acid content in the medium

After 4 days of continuous culture of cancer cells, the medium was taken for the detection of the lactic acid content, and the detection process referred to the instructions

for the lactic acid content detection kit. Briefly, after mixing 100 μL of the medium with 1 mL of the extract, 0.8 mL of the supernatant was taken by centrifugation at 12,000 g for 10 minutes, mixed with 0.15 mL of the extract, and centrifuged again to remove the supernatant. At the same time, the standard contained in the kit was diluted by dilution. Added reagents one, two, and five to the sample tube to be tested and the standard tube, and set a control tube (with distilled water instead of reagent 2) and a blank tube (with distilled water instead of the sample), incubated at 37°C for 20 minutes, then added reagent 6 and The color developing solution was incubated at 37°C for 20 minutes in the dark, centrifuged at 12000 g for 10 minutes, the supernatant was discarded, and 1 mL of ethanol was added to fully dissolve the precipitate. Finally, the absorbance of each tube at 570 nm was measured. The standard curve was prepared by taking the standard concentration as the abscissa and the ΔA standard (equal to A standard-A blank) as the ordinate. The lactic acid content in the solution was calculated based on the ΔA measurement (equal to the A-assay control).

pH detection of the medium

After the cells were continuously cultured for 4 days, the medium was aspirated, and centrifuged at 200 g for 5 minutes, the supernatant was taken out, and the pH was measured with a pH meter.

Induction of RAW264.7 polarization

RAW264.7 cells were inoculated at 2.4×10^5 cells per well in six-well plates and continued to be cultured for 24 h. After that, the old medium was aspirated, and the cells were further cultured with fresh medium containing 25 mM lactic acid or 100 ng mL^{-1} Lipopolysaccharide (LPS) for 24 h for bright-field observation.

Flow cell preparation

RAW264.7 cells were inoculated into a six-well plate, and after 24 hours the old medium was aspirated and discarded. 25 mM lactic acid, 25 mM lactic acid + 1 mg mL^{-1} VCC, 25 mM lactic acid + 100 μg mL^{-1} β-Glucan, 25 mM lactic acid+1 mg mL^{-1} Glu-VCC and continue to incubate for 24 h. Afterwards, the supernatant was discarded

and the cells were washed three times with PBS. The cells were counted and prepared for further testing.

Cellular immunofluorescence staining

The cells were seeded in a six-well plate. After 12 hours, the drugs were added to each treatment group and cultured for 24 hours. Then, the supernatant was discarded and washed with PBS three times. Each well was fixed with 1 mL of 4% paraformaldehyde solution at room temperature for 20 minutes. Then washed with PBS three times, added antigen repair solution to cover the entire bottom of the dish, and let stand for 5 minutes at room temperature. The cells were washed three times with PBS, then immunostaining blocking solution was added, and the cells were blocked for 1 hour, and slowly shaken on a shaker. After washed three times with PBS, the primary antibody was diluted to the antibody dilution in a reference ratio, and then incubated for 6 hours at room temperature in a six-well plate. Washed three times with PBS for 10 minutes each time. added a proportionally diluted fluorescent secondary antibody, and continued to incubate at room temperature for 1 hour in the dark. The cells were washed with PBS for 5 minutes, repeated three times, and placed under a microscope to observe photographs.

Flow cytometry staining

The cells to be tested were packed in 1×10^6 cells per 100 μL, and 2 mL of cell fixing solution was added and fixed at room temperature for 20 minutes. Washed with 2 mL PBS, centrifuged at 300 g for 5 minutes, discarded the supernatant and repeated the washing process. 2 mL of 0.1% Triton X-100 was added to perforate the cell membrane for 3 minutes (for intracellular labeling, the cell membrane indicates that the labeling can be omitted). Washed with 2 mL PBS, centrifuged at 300 g for 5 minutes, discarded the supernatant and repeated. The primary antibody was diluted in the flow-through antibody labeling solution according to the reference ratio,and 100 μL of antibody was added to the cells, and incubated at 4°C for 30 minutes in the dark. The cells were washed by adding 2 mL of antibody labeling solution, centrifuged at 300 g

for 5 minutes, the supernatant was discarded and the washing process was repeated. 200 μL of immunostaining blocking solution was added to each well and blocked at 4°C for 30 minutes. Next, incubated directly with the fluorescent secondary antibody and incubated at 4°C for 40 minutes in dark. The cells were washed by adding 2 mL of antibody labeling solution, centrifuged at 300 g for 5 minutes, the supernatant was discarded, and the washing process was repeated once, and then detected by Flow cytometry. An isotype control group was set up to eliminate the background of non-specific fluorescent signals.

Animals ethics statement:

All animal experiments were performed in strict accordance with the institutional guidelines for animal experimentation and were approved by the Animal Ethics Committee of Nankai University (2021-SYDWLL-000209).

Construction of H22 subcutaneous tumor model and detection of pHe in the *tumor area*

To increase the effective observation area of the pH-sensitive BCECF fluorescent probe, planted tumor on the back of the mouse according to Robert A. Gatenby et al. in Chapter 1.1.1.1. We inserted a 1 mL syringe needle into the abdominal cortex of ICR mice, and then 200 μL of H22 cell suspension (3×10^6 cells per mL) was squeezed into the abdominal cortex of ICR mice maintained with a uniform fan-shaped. After 6 days of the inoculation, the abdominal tumor of ICR mice was approximately 2 cm × 2 cm × 0.2 cm flat on the abdomen of ICR mice and then used for further pH detection.

When detecting the pH of the tumor area, the mice were first anesthetized, and 100 μL of 1 mM BCECF solution was injected into the subcutaneous tumor area of the abdomen. The fluorescence signal of the tumor area was observed using a small animal living imaging system, and a map was taken as the initial signal intensity map. Then, 200 μL of 20 mg mL^{-1} Glu-VCC nanoparticles were injected into the tail vein, and photographs were taken at intervals of 0.5 hours. It should be emphasized that the BCECF dye itself cannot penetrate the cell membrane, and the modified BCECF AM can penetrate the cell membrane. Therefore, the BCECF dye is used to avoid the

interference of the pH environment in the tumor cells, thereby selectively detecting pHe outside the tumor cell membrane.

Construction of 4T1 tumor-bearing Balb/c mouse model

Mouse breast cancer cells 4T1 were cultured in the laboratory, centrifuged to collect cells to prepare cell suspension, and 200 μL of 4T1 cells (1×10^7 mL^{-1}) were inoculated into the right flanks of 6-week-old BALB/c female mice. Until the tumor volume reached 100 mm^3, experiments were started. To order to avoid the interference of the hair in the experiment, the hair of the mouse tumor area was removed by using a razor and a hair removal solution.

Determination of intratumoral pHe of 4T tumor-bearing Balb/c mice by micro pH composite electrode method

To continuously monitor the change of pHe in the tumor area, a small pH composite electrode was used to directly inject into the tumor of 4T1 tumor-bearing Balb/c mice. Before measurement, the electrodes were first calibrated using standard buffers of pH 4.0, pH 7.0, and pH 9.0. The fur of the right forelimb subcarinal area of Balb/c mice was removed, and then a hole was punched into the tumor with a needle of 10 mL syringe (about 5-7 mm deep), and the micro pH composite electrode was inserted along the puncture point. In the mouse tumor, the electrode was fixed. Subsequently, 200 μL of 20 mg mL^{-1} Glu-VCC nanoparticles were injected into the Balb/c mice from the tail vein, and then the pH of the tumor was monitored, and data was read and recorded every 5 minutes.

Separation of TAMs from Balb/c mice

The separation of TAMs was mainly carried out according to the instructions in the kit. Briefly, the Balb/c mice in each treatment group were sacrificed, immersed in 75% alcohol, sterilized, and the tumor was dissected. After weighing the tumor, the tumor was cut into pieces of about 1~2 mm^3 and transferred to the 70 μm cell screen. The tumor fragments were finely ground with a piston in a 10 mL syringe, and the sieve was continuously rinsed with a

homogenate rinse until all cells were collected through a sieve in a 50 mL centrifuge tube. After centrifugation at 450 g for 10 minutes, the supernatant was discarded, and the sample dilution was added to adjust the cell density to about 1×10^9 cells per milliliter. Took a sterile siliconized tube, and added the separation solution 1 and the separation solution 2 (3:1 by volume) slowly. The total volume of the separation solution was about twice that of the cell suspension. The cell suspension was then slowly added dropwise to the separation solution and centrifuged at 500 g for 25 minutes. After careful removal, six layers of stratification could be seen.

The second layer of macrophage was taken into a new centrifuge tube, and 10 mL of washing solution was added for pipetting. After centrifugation at 400 g for 10 minutes, the supernatant was discarded and 10 mL of washing solution was added. After centrifugation at 300 g for 10 minutes, the supernatant was discarded and the washing process was repeated. Finally, the resulting cells were resuspended in 1 mL PBS for flow cytometry.

Tissue section immunofluorescence staining

The tumor of the removed 4T1 tumor-bearing Balb/c mice were washed twice with PBS, and then transferred to a 20% sucrose solution for dehydration, and then transferred to a 30% sucrose solution to continue dehydration. Stored in the cassette at - 20°C for later use. After dehydration, the tumor is frozen and sliced.

The frozen sections stored at -20°C were taken out and allowed to stand at room temperature for 5 minutes. After the OCT was melted, washed with PBS three times, and the immunostaining blocking solution (containing Triton X-100) was added and blocked at 4°C for 2 hours. Discarded the immunostaining blocking solution, added the primary antibody diluted with 1:200, and incubate in a humid box at 4°C overnight. Then, the primary antibody dilution was discarded and washed the slices with PBS three times, then the fluorescently labeled secondary antibody (1:400) was added and incubated in a humid box at 4°C for 2 hours in the dark. The secondary antibody dilution was discarded, washed three times with PBS, and mounted with

Fluoroshield Mounting Medium With DAPI, and the coverslips were fixed with nail polish and then observed with a fluorescence microscope.

Antitumor Effect on 4T1 Tumor Xenografts

To confirm the anticancer effect in vivo, mice were randomly divided into four groups (n=5): PBS, VCC, Glu, and Glu-VCC. Two hundred microliters of 4T1 cells (3×10^5 cells) were inoculated into the right flanks of mice. Six days later, anticancer treatments were performed. β-Glucan and its formulations were administered by tail vein injection with a volume of 200 μL per injection. The injection concentrations of β-glucan, Glu-VCC and $CaCO_3$ nanoparticles were 5 mg mL^{-1}, 25 mg mL^{-1}, and 22.5 mg mL^{-1}, respectively. PBS and glucan were administered every three days for a total of 6 times, respectively. Glu-VCC and VCC were injected for two times at every injection day for a total of 12 times, respectively. The tumor size and body weight of the mice were recorded every three days. The tumor volume was calculated according to the following formula: tumor volume (mm^3) = 0.5 × length × $width^2$. Three days after the final injection, the mice were sacrificed. Tumors and major organs (heart, liver, spleen, lung, and kidney) were collected for H&E staining.