Supporting information

The Conjugate of Rhein and Artesunate for Inducing Immunogenic Cell Death to Prepare Cancer Vaccine and Suppress Tumor Growth

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1. Material and Methods

Chemicals and reagents

Rhein (98%) , artesunate (99%) , and ethylene glycol were all purchased from Meryer (Shanghai) Chemical Technology Co., Ltd. 4-Dimethylaminopyridine (DMAP, 99%) and N,N'-Dicyclohexylcarbodiimide (DCC, 95%) were acquired from TCI Development Co. Ltd. Sulfuric acid, some inorganic salts, and organic solvents were procured from Sinopharm Chemical Reagent Co. Ltd. Fluorescent antibody dyes were mainly sourced from Biolegend and BD Biosciences.

4T1 cells were obtained from the China Center for Type Culture Collection at Wuhan University and were cultured in RPMI 1640 medium containing 1% double antibody and 10% FBS.

Female BALB/c mice (18±2 g, 4-6 weeks old) were purchased from Liaoning Changsheng Biotechnology Co., Ltd. All mice were cared for in accordance with the guidelines for the Care and Use of Laboratory Animals, and the procedures were approved by the China Animal Care and Use Committee of Wuhan University.

Preparation and characterization of Rhe-EE

Add ethylene glycol (80 mL) to a single-neck flask and heat to approximately 95°C. then add Rhein powder (1 g, 3.52 mM) to the flask under stirring conditions. Slowly add 2 mL of H_2SO_4 (98%). Allow the reaction to proceed for 4 hours until the solution clears. Extract the organic phase three times with 50 mL of dichloromethane, followed by washing with water three times. Subsequently, wash with a saturated solution of $NaHCO_3$ until the aqueous layer becomes colorless, then collect the organic phase. Add anhydrous Na_2SO_4 and allow to stand overnight. After filtration, rotary evaporation yields powder of Rhein ethylene glycol ester (yield: 81%). ^{1}H NMR (400 MHz, $CDCl_3$，δ ppm): 11.96 (s, 1H), 11.90 (s, 1H), 8.35 (d, J = 1.2 Hz, 1H), 7.89 (s, 1H), 7.81 (d, J = 7.4 Hz, 1H), 7.69 (s, 1H), 7.28 (d, J = 8.4 Hz, 1H), 4.53 – 4.46 (m, 2H), 4.02–3.94 (m, 2H).

Preparation and characterization of Rhe-EE-ARS

Rhe-EE-ARS was synthesized under a nitrogen atmosphere. Rhein ethyl ester (0.336 g, 1.0 mM), DMAP (0.062 g, 0.5 mM), and Artesunate (0.385 g, 1.0 mM) were sequentially added to 10 mL of anhydrous dichloromethane (DCM). Subsequently, DCC (0.206 g, 0.1 mM) dissolved in 5 mL of anhydrous DCM was slowly dripped into the above solution, and the resulting mixture was allowed to react in an ice bath for 1 hour. After further reaction at room temperature for 48 hours, deionized water was added to the reaction mixture and stirred, followed by filtration to remove N, N'-dicyclohexylurea. The organic phase was then extracted from the mixture using DCM, dried overnight with anhydrous Na_2SO_4, and subsequently separated by column chromatography to yield yellow crystals (yield: 40%). ^{1}H NMR (400 MHz, $CDCl_3$，δ ppm): 12.00 (s, 1H), 11.94 (s, 1H), 8.39 (s, 1H), 7.92 (s, 1H), 7.85 (d, J=7.4, 1H), 7.70

(t, J=8.0, 1H), 7.30 (d, J=8.4, 1H), 5.73 (d, J=9.7, 1H), 5.34 (s, 1H), 4.55 (s, 2H), 4.43 (s, 2H), 2.71 (s, 3H), 2.67 – 2.61 (m, 1H), 2.47 (s, 1H), 2.28 (d, J=10.5, 1H), 1.96 (d, J=15.2, 1H), 1.83 (s, 1H), 1.71 (d, J=11.9, 1H), 1.65 (d, J=10.7, 1H), 1.53 (s, 3H), 1.40 (d, J=15.1, 1H), 1.35 (s, 3H), 1.21 (s, 2H), 0.90 (d, J=5.9, 3H), 0.79 (d, J=7.0, 3H).

Cellular uptake and intracellular distribution

A confocal laser dish was used to seed fresh RPMI 1640 medium with 5×10^4 4T1 cells, which were subsequently cultured for 24 hours. Following this, the cells were incubated in medium containing Rhe-EE-ARS or Rhe at a concentration of 2 μM for 12 hours. Post-incubation, the original medium was aspirated, and the cells were washed twice with PBS. Subsequently, 500 μL of fresh medium containing Mito-Tracker Red (200 nmol/L) was added for staining and incubated for 15 minutes. Following two washes with PBS, the cells were stained with Hoechst 33342 at a concentration of 10 μg/mL for 10 minutes. After two additional PBS washes, the cells were observed using confocal laser scanning microscopy (CLSM).

Detection of surface-exposed CRT

Exploring cell surface CRT under different concentrations of Rhe-EE-ARS: Fresh RPMI 1640 medium containing 1×10^5 4T1 cells was added to a 6-well plate, and the cells were cultured for 24 hours. Subsequently, the cells were incubated in medium containing different concentrations of Rhe-EE-ARS (0.25 μM, 0.5 μM, 1 μM, 2 μM, 4 μM) for 24 hours. The original medium was then removed, and the cells were washed twice with PBS. Cells were collected, stained with fluorescent anti-CRT antibody, and analyzed by flow cytometry.

Quantitative detection of cell surface CRT under different drug conditions: Fresh RPMI 1640 medium containing 1×10^5 4T1 cells was added to a 6-well plate, and the cells were cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, ARS, or the mixture of Rhe and ARS (Rhe/ARS) (all at a concentration of 2 μM) for 24 hours. The original medium was then removed, and the cells were washed twice with PBS. Cells were collected, stained with fluorescent anti-CRT antibody, and analyzed by flow cytometry.

Observation of cell surface CRT under different drug conditions: Cells (5×10^4 cells/dish) were seeded into a confocal laser dish and cultured for 24 hours. Subsequent processing steps were the same as described above. The cells were then stained with a fluorescent anti-CRT antibody for 30 minutes. After fixation, the cells were washed twice with PBS and stained with Hoechst 33342 (at a concentration of 10 μg/mL) for 10 minutes before rinsing. The cells were subsequently observed under confocal laser scanning microscopy (CLSM).

Detection of HMGB1 and ATP

Fresh RPMI 1640 medium containing 5×10^4 4T1 cells was added to a confocal laser dish, and the cells were cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, and ARS (at a concentration of 2 μM) for another 24 hours. After the incubation period, the original medium was

removed, and the cells were washed with PBS. The cells were fixed with paraformaldehyde, followed by treatment with a permeabilization agent for 30 minutes. Subsequently, the cells were stained with a fluorescent anti-HMGB1 antibody for 30 minutes, followed by staining with Hoechst 33342 for 10 minutes. After washing with PBS, the cells were observed using confocal laser scanning microscopy (CLSM).

For ATP detection, fresh RPMI 1640 medium containing 1×10^5 4T1 cells was added to a 6-well plate, and the cells were cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, ARS, and a mixture of Rhe and ARS (Rhe/ARS) (each at a concentration of 2 μM) for another 24 hours. The supernatant was collected and centrifuged, followed by ATP measurement according to the instructions provided in the ATP ELISA kit. Each group was set up with three replicates.

Intracellular ROS detection

Observation of intracellular ROS distribution by CLSM: Fresh RPMI 1640 medium containing 5×10^4 4T1 cells was added to a confocal laser dish and cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, and ARS (each at a concentration of 2 μM) for 12 hours. After incubation, the original medium was removed, and the cells were washed with PBS. Then, 1 mL of serum-free medium containing DCFH-DA (10 μM) was added, and the cells were incubated in the dark for 30 minutes. Afterward, the cells were washed twice with PBS, followed by addition of fresh RPMI 1640 medium for further incubation of 12 hours. Subsequently, the medium was discarded, and the cells were washed twice with PBS. Then the cells were stained with Hoechst 33342 for 10 minutes, washed with PBS, and observed by CLSM.

Quantification of intracellular ROS: Fresh RPMI 1640 medium containing 1×10^5 4T1 cells was added to a 6-well plate and cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, ARS, and Rhe/ARS (each at a concentration of 2 μM) for 12 hours. After incubation, the original medium was removed, and the cells were washed with PBS twice. Following this, 1 mL of serum-free medium supplemented with DCFH-DA (10 μM) was administered, and the cells were incubated in darkness for 30 minutes. Subsequently, the cells underwent two washes with PBS before fresh RPMI 1640 medium was added for an additional 12-hours incubation period. Later, the medium was aspirated, and the cells were subjected to two additional washes with PBS. Finally, the cells were resuspended in PBS and subjected to flow cytometry analysis.

Cell death

Cell cytotoxicity was evaluated using the standard MTT assay. In brief, 100 μL of fresh RPMI 1640 medium containing 2×10^3 4T1 cells was added to each well of a 96-well plate. The cells were cultured for 24 hours. Subsequently, 100 μL of fresh medium containing different concentrations of drugs was added, and the cells were cultured for 48 hours. Then 20 μL of MTT solution (5 mg/mL in PBS) was added to each well and incubated for 4 hours. Then, the medium was removed, and 150 μL of DMSO was

added to dissolve the formazan crystals. The absorbance of the DMSO solution at 570 nm in each well was recorded using a microplate reader.

Apoptosis of 4T1 cells was also assessed using the annexin-APC/PI apoptosis detection kit. Briefly, 2 mL of fresh RPMI 1640 medium containing 1×10^5 4T1 cells was added to each well of a 6-well plate, and the cells were cultured for 24 hours. The cells were then incubated in medium containing different drugs (at a concentration of 2 μM) for 24 hours. Subsequently, cells were collected for annexin-APC/PI apoptosis detection.

Mitochondria membrane potential assay

Fresh RPMI 1640 medium containing 5×10^4 4T1 cells was added to a confocal laser dish, and the cells were cultured for 24 hours. Subsequently, the cells were incubated in medium containing Rhe-EE-ARS, Rhe, and ARS (each at a concentration of 2 μM) for 24 hours. After the incubation period, 1mL of fresh medium containing JC-1 (10 μg/mL) was added, and the cells were cultured in the dark for 15 minutes. The cells were then washed twice with PBS and observed using confocal laser scanning microscopy (CLSM).

4T1 cells (1×10^5 cells per well) were seeded in a 6-well plate and cultured in medium containing Rhe-EE-ARS, Rhe, ARS and Rhe/ARS (each at a concentration of 2 μM) for 24 hours. After incubation, the original medium was removed, and the cells were washed twice with PBS. Subsequently, 1 mL of serum-free medium containing JC-1 (10 μg/mL) was added, and the cells were cultured in the dark for 15 minutes. The cells were then washed twice with PBS and resuspended for analysis by flow cytometry.

In vitro preparation of tumor vaccines

A 6-well plate was initially seeded with 1×10^5 4T1 cells in fresh RPMI 1640 medium, followed by incubation for 24 hours. Subsequently, the cells were treated with Rhe-EE-ARS (at a concentration of 2 μM) in the medium for an additional 24 hours. Following the incubation period, the cells were washed with PBS to remove the original medium. Subsequently, the cells were harvested and exposed to X-rays (60 Gy) for the generation of prophylactic tumor vaccines, which were designated as the R-A group. Conversely, if the cells were treated with PBS (in the same volume as the Rhe-EE-ARS solution), the resulting vaccines were categorized as the PBS group.

In vivo prophylactic tumor vaccination study

Mice were randomly divided into three groups: Control, PBS, and Rhe-EE-ARS (R-A) groups. The Control group remained unvaccinated, while the PBS group received immunization with inactivated cell vaccines that did not induce ICD effects. On days 0 and 7, cancer vaccines (2×10^6 cells per mouse) were subcutaneously injected into the left dorsal area of the mice. The day when the mice completed the two immunizations was recorded as day 0.

Concurrently, 4T1 cells were subcutaneously injected into the right dorsal area of the mice (n=8) at a density of 1×10^7 cells/mL (100 μL) on day 0 to investigate the in vivo tumor inhibitory effect. Mouse body weights and tumor volumes were monitored

at scheduled time points. The in vivo anti-tumor study was terminated when the mean tumor volume reached 2000 mm^3, and three mice were then sacrificed. Blood, organs, tumors, and lymph nodes were collected for further analysis. Blood samples underwent routine blood tests, while tumors and organs were subjected to hematoxylin and eosin (H&E) staining for observation.

Cell suspensions of lymph nodes were prepared for flow cytometry analysis as follows: Lymph nodes were mechanically crushed to obtain cell suspensions. For DC analysis, cell suspensions from lymph nodes were co-stained with anti-CD11c-APC, anti-CD80-FITC, and anti-CD86-PE fluorescent antibodies at 4°C for 30 minutes, followed by washing and collection for flow cytometry analysis.

Tumors were digested in RPMI 1640 medium containing hyaluronidase (0.1 mg/mL), DNase (0.1 mg/mL), collagenase (0.1 mg/mL), and FBS (2%) for 1.5 hours to obtain single-cell suspensions of T cells in tumors for evaluation. The staining procedure was as follows: Cells were treated with Leoko Act Cktl and Golgipiug for 4-6 hours at 37°C, incubated with CD16/32 for 5 minutes at 4°C, stained with Fixable Viability Stain 700 for 5-7 minutes, and then labeled with anti-CD44-BV510 antibody, anti-CD45-APC-CY7 antibody, anti-CD3-FITC antibody, anti-CD4-PE antibody, anti-CD8-APC antibody, and anti-CD62L-BV650 antibody for 30 minutes. Subsequently, cells were incubated in a fix/perm mixture for 50 minutes, stained with IFN-γ-PE-CY7 for 40 minutes, washed, and collected for flow cytometry.

Mice were defined as dead when the tumor size reached 2000 mm^3, and the survival of the remaining mice was monitored throughout the study period.

Statistical analysis

Statistical analyses were carried out using Student's t-test. $P < 0.05$ implies significant. Data are presented as the mean ± SD.

2. Supporting Figures

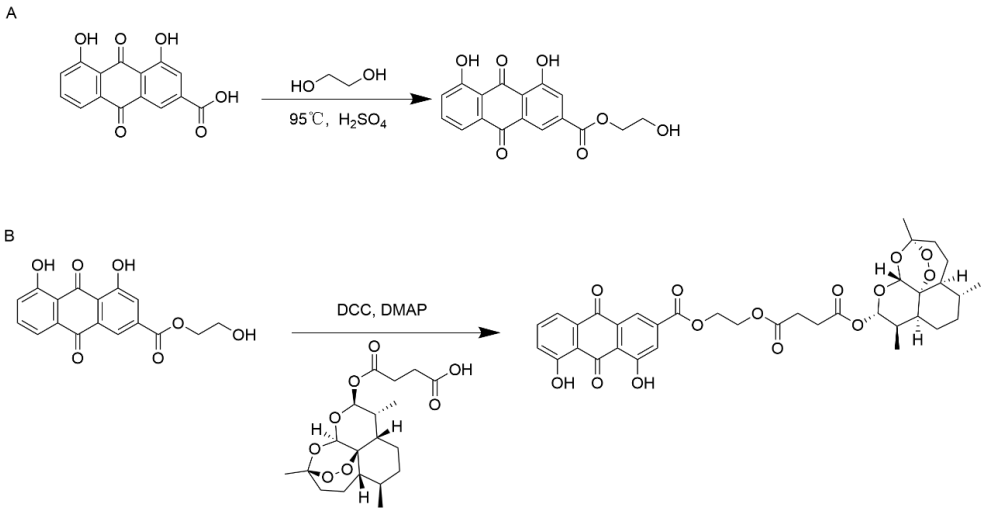

Figure S1 Synthesis routes of Rhe-EE (A) and Rhe-EE-ARS (B) .

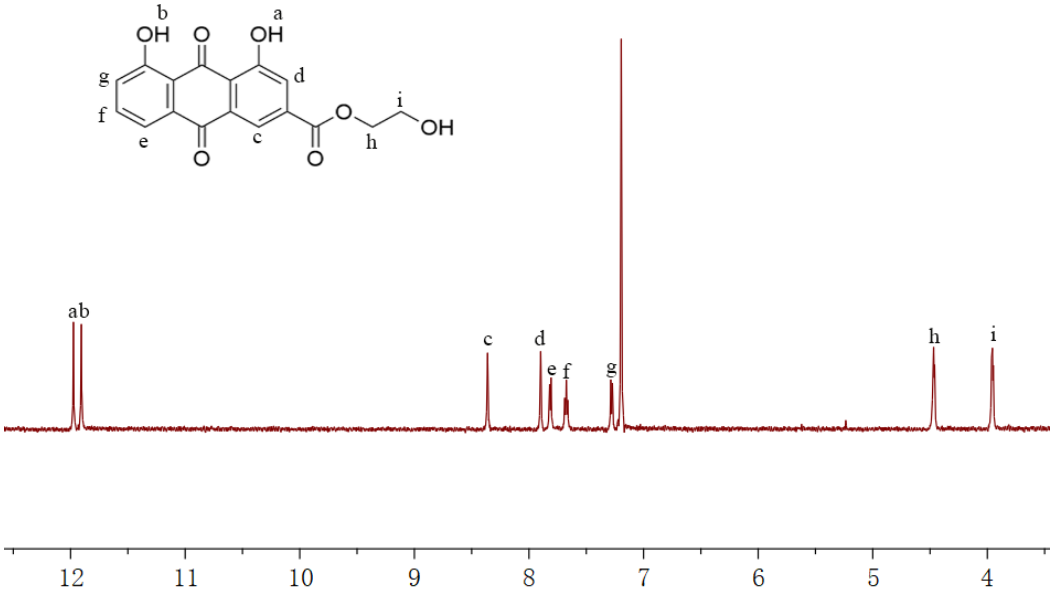

Figure S2 ^{1}H NMR spectrum (400 MHz) of Rhe-EE in $CDCl_3$ at 25℃.

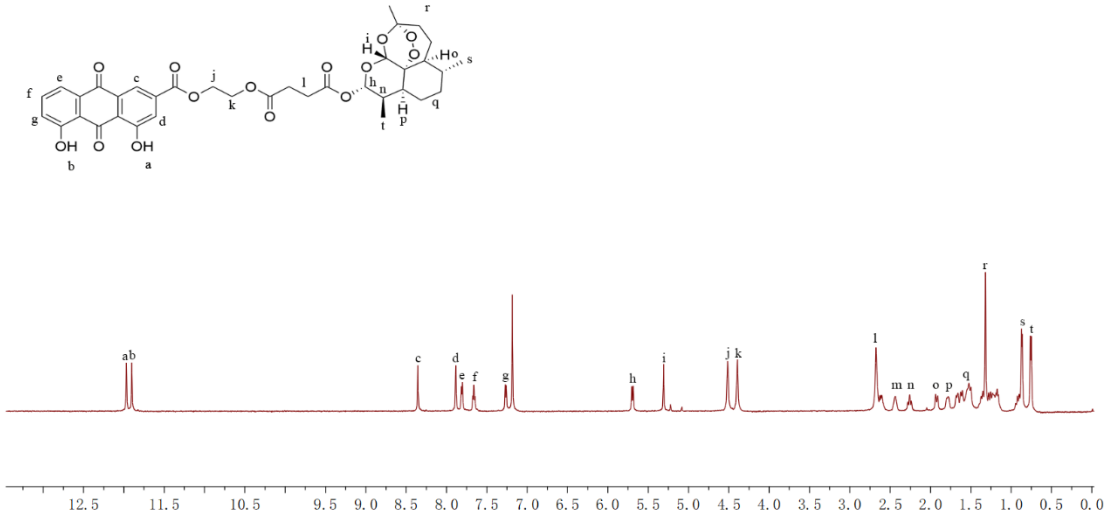

Figure S3 ^{1}H NMR spectrum (400 MHz) of Rhe-EE-ARS in $CDCl_3$ at 25℃.

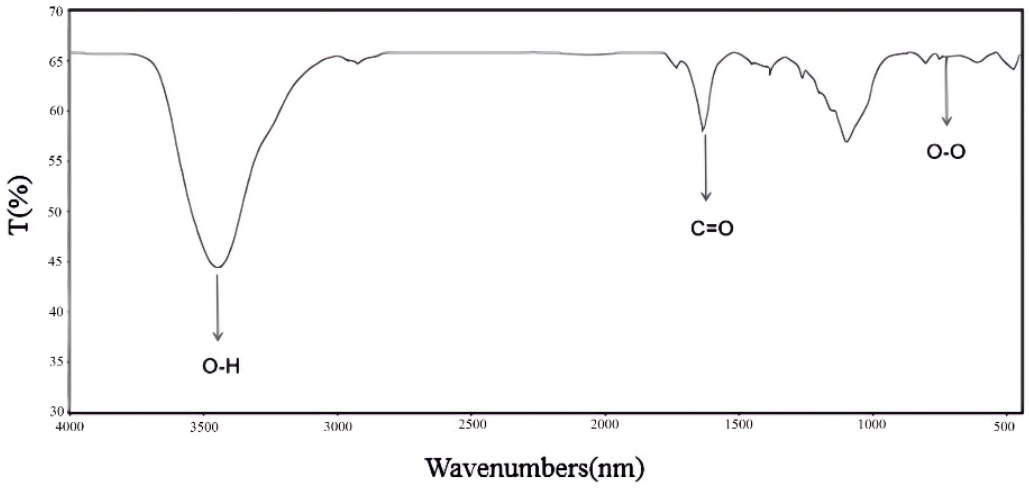

Figure S4 The FT-IR of Rhe-EE-ARS.

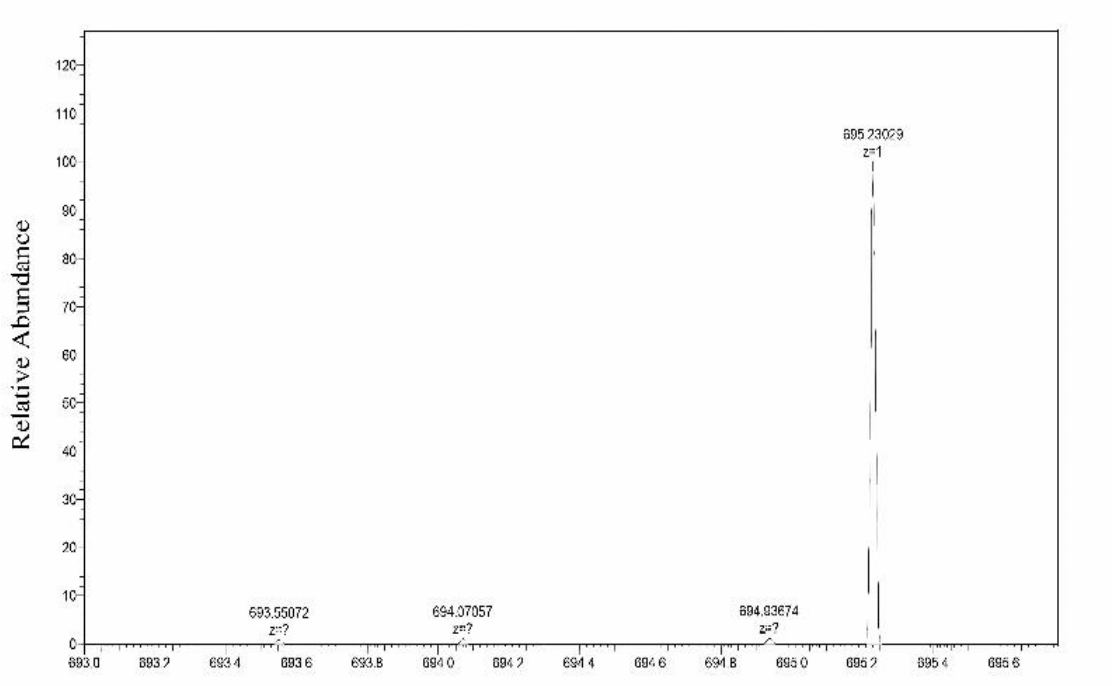

Figure S5 The mass spectrometry of Rhe-EE-ARS.

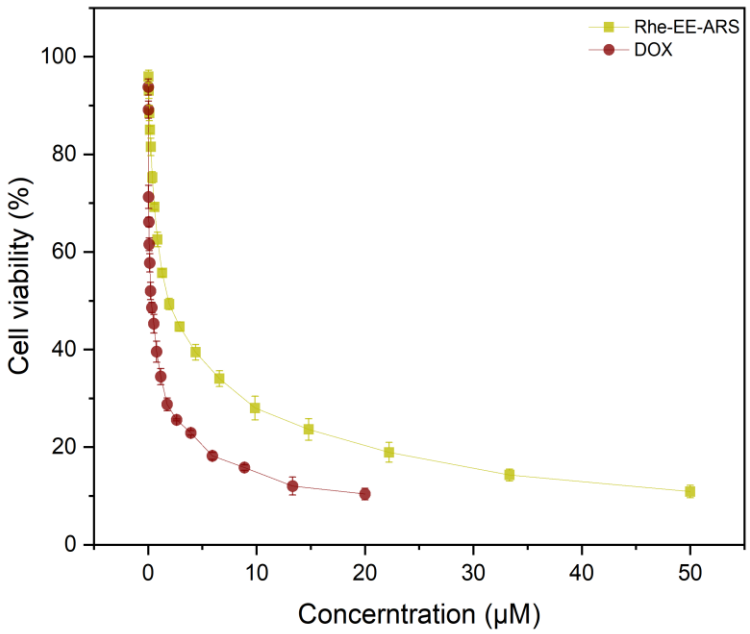

Figure S6 In vitro cell viability of 4T1 cells after treated with Rhe-EE-ARS and free DOX for 48 hours.

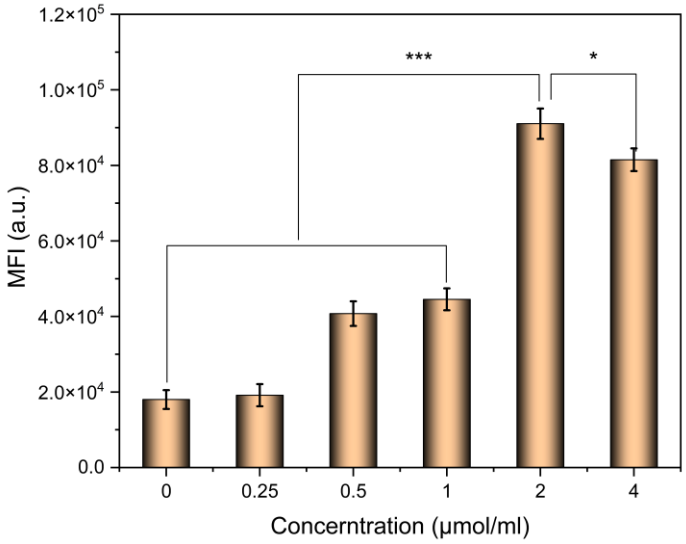

Figure S7 The MFI value of CRT was measured by flow cytometry. Data are presented as mean ±S.D. (n = 3), ***$p < 0.001$ and *$p < 0.05$.

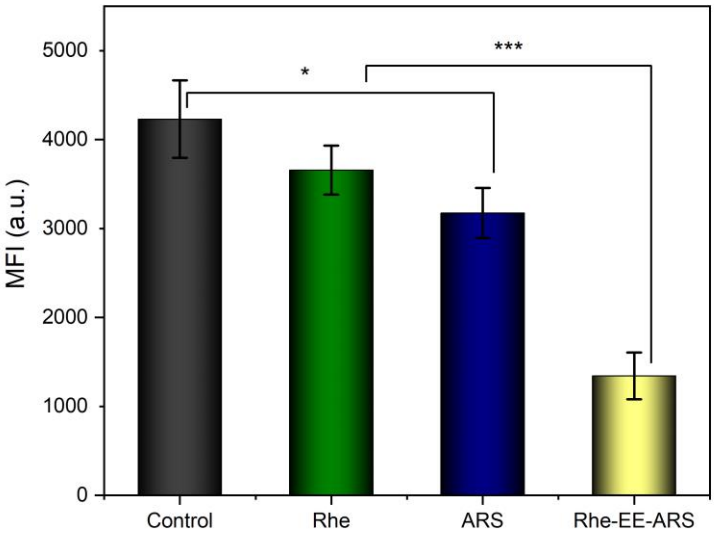

Figure S8 The MFI value of HMGB1 was measured in CLSM images. Data are presented as mean ±S.D. (n = 3), ***p < 0.001 and *p < 0.05.

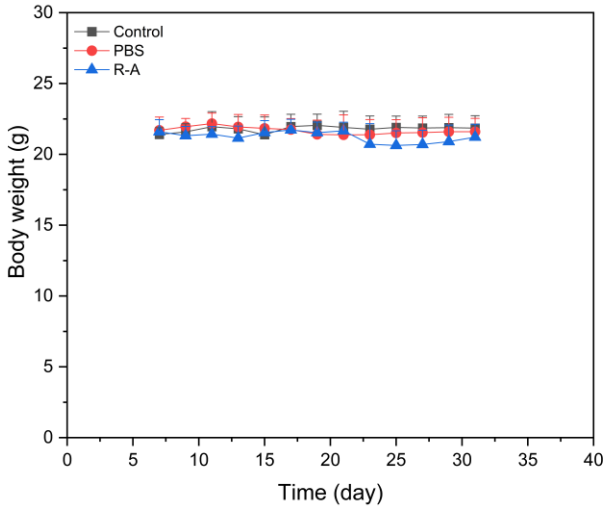

Figure S9 Body weight changes during different treatments, (n =8).

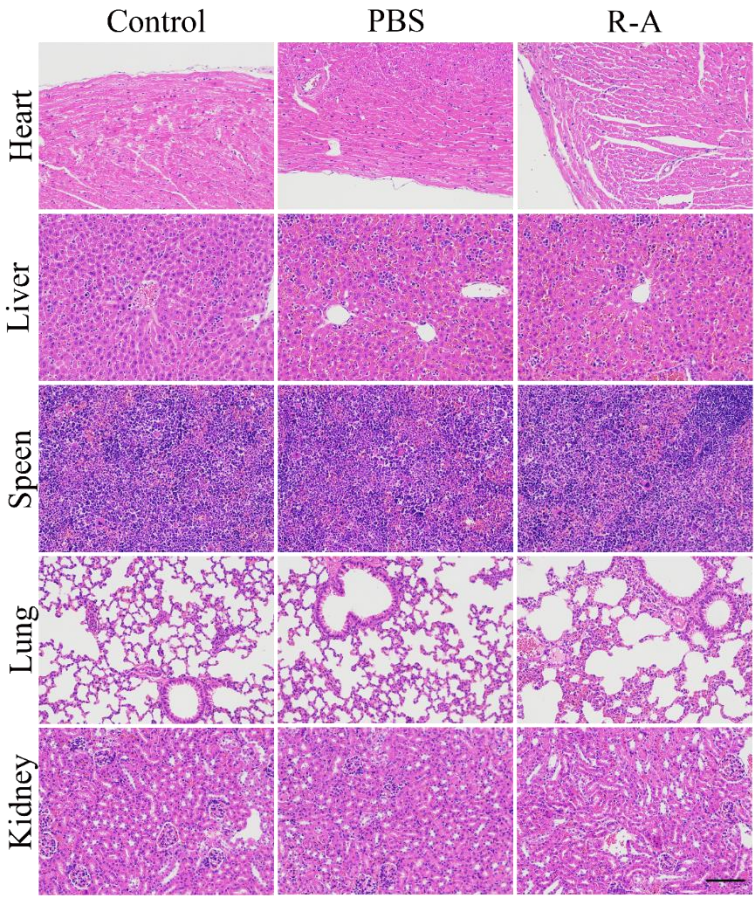

Figure S10 H&E staining images of heart, liver, spleen, lung and kidney, scale bar=100 μm.

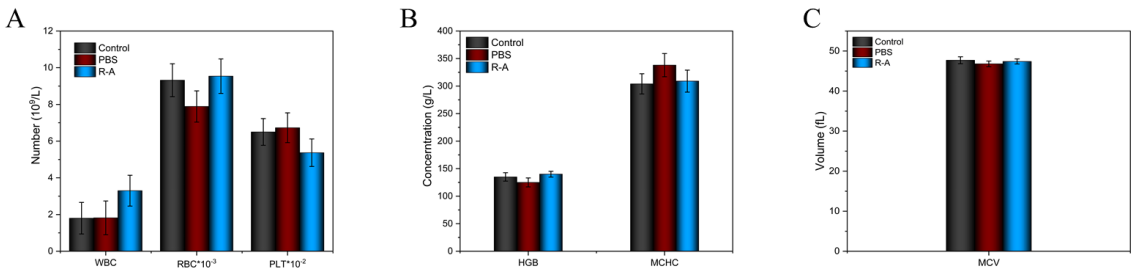

Figure S11 Blood biochemistry analysis of mice immunized by various vaccine formulations. (A) WBC, RBC, PLT. (B) HGB, MCHC. (C) FL, (n = 3).