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In situ rewiring TAMs as annihilators by “Spark-relay” nanoinitiator with flexible reactant ratio for amplifying solid tumor immunotherapy

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Abstract Tailoring tumor-associated macrophages (TAMs) into the tumoricidal phenotype represents a high-profile strategy for tumor immunotherapy. However, the existing TAMs repolarization strategies are restricted by hostile microenvironmental stress and metabolic compensation, leading to limited anti-tumor phenotype sustainability. Herein, a “Spark-Relay” nanoinitiator (SRN) with flexible S/R reactant ratio was meticulously designed for rewiring TAMs as tumoricidal bioreactor and precisely overcome metabolic compensation. Briefly, SRN reshaped TAMs *in situ* relying on tissue tropism of macrophage membrane, firstly upregulating their reactive oxygen species (ROS) production *via* burst release of Spark element and initially shifting the TAMs into the anti-tumoral phenotype, subsequently elevating NO production by releasing Relay element, which is converted to NO *via* reaction with ROS and iNOS, leading to the generation of additional ROS and creating a positive feedback loop, thereby strengthening the metabolic rewiring of TAMs from passively defensive oxidative phosphorylation to positively offensive glycolysis, which significantly enhanced the antitumoral activity of TAMs and shrunk tumor immunosuppressive microenvironment, emerging as 5.5-fold increase of tumor-suppressive/supportive ratio (TSSR) of TAMs, 4.0-fold elevation of CD8⁺ T cell infiltration, contributing to a satisfactory tumoricidal efficacy and providing a cascade amplification mode for TAMs-based cancer therapeutics.

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1. Introduction

Nowadays, tumor immunotherapies represented by immune checkpoint therapy have made significant progress, but only 10%–30% patients suffering from solid tumors show a positive response due to immunosuppressive tumor microenvironment (ITME)^{1–4}. Tumor-associated macrophages (TAMs), which account for nearly 50% of immune cells in malignant solid tumors, play a pivotal role in shaping the ITME⁵. Characterized by high plasticity and heterogeneity, TAMs can be broadly classified into two main types: proinflammatory macrophages which primarily rely on glycolysis for rapid energy production and tumoricidal decisive engagement; anti-inflammatory macrophages that turn to oxidative phosphorylation (OXPHOS) that conducted passive attrition war and tumor support⁶. In most malignant solid tumors, the predominant TAMs are tumor-supportive, which tremendously facilitate tumor proliferation, metastasis and postoperative recurrence, and even worse restrict the activity and infiltration of T cells by secreting immunosuppressive factors such as IL-10 and PF4^{7,8}. It's even reported that the response rate of T cell-based immunotherapy critically depends on not only the presence of T cells but also the phenotype of TAMs⁹. Given these vital effects TAMs exert in the tumor progression and immunotherapy efficiency, TAMs intervention strategies have garnered significant attention as potential in solid tumor therapies^{10,11}.

Recently, various macrophage regulating tactics have been explored in preclinical and clinical studies for enhancing their tumor fighting ability, such as phagocytosis potentiators, surface receptor loading strategies and TAMs repolarizing agents represented by CSF1R inhibitors and Toll-like receptor agonists^{12–14}. However, it's intractable to keep up tumor repelling capacity of TAMs under continuous negative factors from tumor cells. The reversibility of TAMs is a double-edged sword, drug-mediating polarization is easily skewed back to tumor-supportive phenotype in tumor cell-dominant TME after drug removal when they had no time to reverse the ITME. So far, some multiple-pathway TAMs amendment routes from metabolic function modulation view have gradually developed for durable tumor attack potential^{15,16}, especially metabolic reprogramming TAMs through regulating their endogenous free radicals and signal pathways¹⁷.

Among numerous signal molecules in TAMs orienteering, reactive oxygen species (ROS) were demonstrated an effective initiator of tumor-suppressive phenotype polarization *via* ROS–NF- κ B proinflammatory signal pathway activation^{18,19}. Many ROS-generating agents such as photosensitizers and ferric tetroxide nanoparticles have been proven the efficacy in disrupting mitochondrial function and tightening the raw materials pyruvate of OXPHOS, increasing the glycolytic activity of macrophages and promoting the TAMs repolarization²⁰, which could be defined as Spark agent. However, the generation and elimination of ROS are both instantaneous and a single signaling molecule is insufficient to sustain pro-inflammatory signals long-term and tends to elicit metabolic compensation, so a powerful downstream cascade enzymatic reaction which called Relay agent is vital for effective

and enduring TAMs rewiring. Inspiring by this, connecting a ROS stimulator (Spark agent) with a ROS-responsive downstream reaction (Relay agent) in series is a promising route for prolonging the anti-tumor phenotype sustainability of TAMs.

A thorough exploration of ROS catalytic biochemical reactions identified the production of nitric oxide (NO) from L-arginine as a process relying on ROS generation and suitable strategy for prolonging the TAMs-polarization effect of ROS^{6,19,21}. NO could decrease OXPHOS metabolic activity by nitrifying with mitochondrial electron transport chain, redirecting macrophage metabolism toward glycolysis pathway and thereby influencing the phenotype of TAMs^{22,23}. The endogenous NO could be generated from arginine, catalyzed by ROS plus inducible nitric oxide synthase (iNOS), which is highly expressed in tumor-suppressive TAMs²⁴. Moreover, NO could subsequently accelerate ROS production, leading to a positive feedback loop of proinflammatory pathways. However, the presence of multiple immunosuppressive signals such as IL-4 in TME impedes TAMs repolarization and NO synthesis²⁵. Therefore, it is necessary to put a primary initiator for NO production. In summary, the generation of ROS and NO radicals could be sequentially connected and effectively prolong the persistence of TAMs with the proinflammatory (anti-tumoral) phenotype.

Nevertheless, the ROS production in TAMs suffers from elimination by reduction systems, especially GSH, which provides a solid braking for proinflammatory stage sustainability^{6,26}. Fortunately, we have previously developed a library of GSH depletion nano-adjuvants which could effectively elevate ROS content and activate immune response^{27,28}. Especially, cinnamaldehyde-grafting polyethyleneimine (PEI-Cin, PC) neoadjuvant which derives from a natural food additive approved by FDA could elevate intracellular ROS levels by depleting glutathione (GSH). Therefore, PC could be used as a model Spark agent.

Herein, based on ROS generation capacity of Spark agent and pro-inflammatory sustained capacity of Relay agent, we developed a novel “Spark-Relay” nanoinitiator SRN with sequential radical release and cascade proinflammatory responses in TAMs. In this initiator, PC was chosen as a ROS trigger (Spark agent) and L-arginine-grafting polyethyleneimine (PEI-Arg, PA) was picked as a NO donor (Relay agent), as it can be catalyzed into NO *via* the reaction between active guanidine group and ROS/iNOS, propagating the pro-inflammatory function of ROS. After entering TAMs, SRN released Spark agent quickly, upregulating intracellular ROS levels, and then activated ROS–NF- κ B pro-inflammatory signal pathway, which promoted its tendency to tumoricidal phenotypic and related protein expression. Furthermore, ROS and the upregulated iNOS expression are utilized to enzymatically degrade the additional supplemented arginine to produce NO, which synergistically impaired the mitochondrial function along with ROS. Consequently, the TAMs shifted from their mitochondrial-dependent OXPHOS metabolism to glycolysis metabolic pathway, leading to a cascading repolarization towards the anti-tumor phenotype and assisting tumor immunotherapy.

2. Materials and methods

2.1. Materials

PEI (MW = 1.8 k), cinnamaldehyde and Fmoc-Arg-OH were purchased from Aladdin (Shanghai, China). Fetal bovine serum (FBS) was purchased from TransGen Biotech Company (Beijing, China). The ROS reactive oxygen species detection kit was purchased from Beyotime Biotechnology Company (Shanghai, China). iNOS antibodies were purchased from ZEN-biotechnology Company (Chengdu, China). The apoptosis kit was purchased from Yisheng Company (Shanghai, China). Tumor necrosis factor (TNF- α) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Biolegend (San Diego, CA, USA). The flow antibodies were mostly purchased from Elabscience (Wuhan, China).

2.2. Cell culture and animals

RAW 264.7 cells and 4T1 mouse breast cancer cells were obtained from PerkinElmer and cultured in DMEM supplemented with 10% FBS at 37 °C incubator with 5% CO₂. Female BALB/c mice (18–22 g) were bought from Yangzhou University. All the animal experiments were performed in compliance with the Guide for Care and Use of Laboratory Animals and were approved by China Pharmaceutical University (Approval No. SYXK2021-0011).

2.3. Synthesis and characterization of S-PC and R-PA

Cinnamaldehyde grafted polyethyleneimine (PEI-Cin, S-PC) was synthesized by Schiff base reaction (Supporting Information Fig. S1). Based on our previous research²⁷, 100 mg PEI (MW = 1.8 k) and 100 mg Cin were dissolved in 2 mL ethanol, respectively, then the Cin ethanol solution was dropwise added to PEI and stirred for 12 h at room temperature. After that, organic solvents and free Cin were removed using spin drying and vacuum drying to produce a pale yellow viscous solid (S-PC).

Fmoc-Arg grafted polyethyleneimine (PEI-Fmoc-Arg, R-PA) was synthesized by amide reaction (Supporting Information Fig. S2). Briefly, Fmoc-Arg-OH (0.56 mmol, 22.2 mg), EDC (1.12 mmol, 214.7 mg), and HOBT (1.12 mmol, 151.35 mg) were dissolved in 4 mL of DMF and stirred for 1 h for the carboxyl activation. The reaction solution was then mixed with 200 mg of PEI (MW = 1.8 k) and 145 mg of DIPEA (1.12 mmol) and allowed to react for another 24 h. Finally, the mixture was dialyzed (MWCO: 1.5 kDa) for 48 h and then lyophilized to obtain the R-PA.

The structures of S-PC and R-PA were characterized by nuclear magnetic resonance hydrogen spectroscopy (¹H NMR, 500 MHz) and the grafting ratio was calculated.

2.4. Preparation and characterization of PCA(SR) and MPCA (SRN)

PCA(SR) nanomicelles were self-assembled by emulsification. 5 mg S-PC and 5 mg R-PA were dissolved in 50 μ L of ethanol, respectively. Then SR nanomicelles were formed by preparing a mixture of S-PC and R-PA in different ratios, which was then slowly added drop by drop into 1 mL of distilled water. The particle size and PDI were investigated by particle size analyzer to determine the optimized proportion of S-PC and R-PA. Subsequently, HSR was prepared by adding HA (MW = 10,000) and co-incubating with SR to neutralize the positive charge of SR.

Macrophage membranes (MM) were obtained by ultrasonic fragmentation and centrifugation. The obtained MM and SR nanoparticle solutions were mixed and prepared SRN nanomicelles by ultrasonic assay. The particle size and zeta potential of SRN were measured by dynamic light scattering (DLS) and the morphology of SRN was characterized by TEM. Furthermore, the surface proteins of the nanomicelles were characterized using SDS-PAGE electrophoresis.

The GSH depletion capacity of SRN nanomicelles was investigated by Ellman's method. To begin, 0.1982 g of GSH assay reagent 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) powder was weighed and dissolved in 50 mL of 50 mmol/L disodium hydrogen phosphate solution (pH = 7.0) to prepare a 10 mmol/L DTNB storage solution, which was stored in a refrigerator at 4 °C protected from light. Next, the DTNB working solution was prepared by diluting the DTNB storage solution to 0.1 mmol/L with 0.25 mol/L Tris-HCl buffer (pH = 8.3) before assay and stored away from light. After reacting 10 mmol/L GSH with PBS, 10 mmol/L S-PC, R-PA, SR and SRN, respectively, for 10 min at room temperature, 20 μ L of each of the above reaction mixtures was taken and combined with 100 μ L of 0.1 mmol/L DTNB working solution. Finally, the absorbance was measured at 412 nm by an enzyme meter.

2.5. In vitro release of cinnamaldehyde and NO

In vitro release of cinnamaldehyde by SRN was conducted in PBS at different pH (5.0, 6.5 and 7.4). Typically, SRN solution was put in a dialysis bag (MWCO 3500) and immersed in a tube with 30 mL of PBS at different pH under stirring. 1 mL medium was collected and replaced with fresh PBS at different time. The release profile of cinnamaldehyde was determined by UV–vis absorbance at 280 nm according to the calibration curves.

The release of NO from SRN was performed in PBS with different H₂O₂ concentrations (100 μ mol/L, 500 μ mol/L and 1 mmol/L). The acquisition of samples was the same as above. NO was determined by quantifying nitrite through the Griess reaction. In detail, 50 μ L of released solution, 50 μ L of Griess reagent A and 50 μ L of Griess reagent B were added to 96-well plates, and the absorbance at 550 nm was measured using a microplate reader. The release of NO was determined according to the calibration curves.

2.6. Intracellular distribution of SRN

The intracellular distribution of SRN was evaluated on RAW264.7 cells. Ce6-SRN was prepared by adding hydrophobic dye Ce6 in the ethanol at the above SRN preparation process. RAW264.7 cells were inoculated in confocal dishes at a density of 5×10^4 /dish, and then treated with tumor-conditioned medium (TCM) for 24 h to induce TAMs. Then TAMs were treated with Ce6-SRN. After incubation for 4 h, the cells were washed with PBS for three times. Subsequently, the nuclei were stained with DAPI for 15 min and washed twice, after which the fluorescence co-localization was observed by confocal microscope.

2.7. Intracellular ROS level

Intracellular ROS level was detected by 2',7'-dichlorofluorescein diacetate (DCFH-DA) fluorescent probe. RAW264.7 cells (1×10^5 per well) were inoculated in a 12-well plate and cultured for 6 h. After adhering, the cells treated with TCM for

24 h to induce TAMs. The medium was discarded and the TAMs were incubated with DMEM (Control), S-PC (7.5 $\mu\text{g/mL}$), R-PA (7.5 $\mu\text{g/mL}$), SR (7.5 $\mu\text{g/mL}$) and SRN (7.5 $\mu\text{g/mL}$) for 24 h. Then, each group was incubated with DCFH-DA (8 $\mu\text{mol/L}$) for 20 min in the dark. After washed three times with PBS, the intracellular DCFH-DA fluorescence intensity was measured by flow cytometry to evaluate the intracellular ROS level.

2.8. *NF- κ B, iNOS, NO and TNF- α detection*

The protein expressions such as NF- κ B and iNOS were verified by Western blot. RAW264.7 cells (2×10^5 per well) were seeded in 12-well plates and cultured for 6 h. Following cell adherence, TAMs were induced as before. One group was left untreated with TCM as M0 control, while the other groups were incubated with DMEM (Control), S-PC (7.5 $\mu\text{g/mL}$), R-PA (7.5 $\mu\text{g/mL}$), SR (7.5 $\mu\text{g/mL}$) and SRN (7.5 $\mu\text{g/mL}$) for 24 h, and then the cells were collected for Western blot analysis. The supernatant was collected for extracellular NO release detection by Griess reagent. Briefly, 50 μL of supernatant, 50 μL of Griess reagent A and 50 μL Griess reagent B were added to the 96-well plate sequentially, and the absorbance at 540 nm was measured by microplate reader. Finally, the release amount of NO in the supernatant was calculated according to the standard curve. In addition, the content of TNF- α in the supernatant was detected by ELISA kit according to manufacturer's instructions.

2.9. *Metabolic investigation of macrophages*

The OCR and ECAR of macrophages were measured by cell energy metabolism analyzer Seahorse XFe96. RAW 264.7 cells (2×10^5 per well) were seeded on XFe96 FluxPax microplate and incubated with TCM for 24 h. Subsequently, the cells were treated with DMEM, S-PC, R-PA, SR and SRN for 24 h. In addition, to investigate the metabolic difference between extreme M1-like TAMs (tumor suppressive) and M2-like TAMs (tumor supportive), RAW 264.7 cells were incubated with LPS (1 $\mu\text{g/mL}$) and TCM for 48 h separately. Seahorse XF Cell Mito Stress kit (1.5 $\mu\text{mol/L}$ oligomycin, 2 $\mu\text{mol/L}$ FCCP, 0.5 $\mu\text{mol/L}$ rotenone/antimycin A) and Seahorse XF glycolysis (10 mmol/L glucose, 1.5 mmol/L oligomycin, and 50 mmol/L 2-DG) were used to determine OCR and ECAR values, respectively, and the OCR curve and ECAR curve were obtained after statistical analysis.

2.10. *Examination of macrophage repolarization in vitro*

The phenotypic transformation of macrophages was analyzed by flow cytometry. RAW264.7 cells (1×10^5 per well) were inoculated in 12-well plates for 6 h and were stimulated by TCM for another 24 h, followed by incubation with DMEM (Control), S-PC (7.5 $\mu\text{g/mL}$), R-PA (7.5 $\mu\text{g/mL}$), SR (7.5 $\mu\text{g/mL}$) and SRN (7.5 $\mu\text{g/mL}$) for 24 h. After treatment, the cells were washed twice with PBS and then incubated with CD86-PE (5 $\mu\text{g/mL}$) for 30 min at 4 $^{\circ}\text{C}$ in the dark. Then, the cells were fixed with 100 μL of 4% paraformaldehyde for 10 min and the cell membrane was disrupted with 0.1% Triton for another 10 min. Subsequently, the cells were incubated with CD206-APC (5 $\mu\text{g/mL}$) for 30 min in a light-free environment. Finally, the expression of CD86 and CD206 in macrophages was detected by flow cytometry.

2.11. *Transwell co-culture experiments*

The killing ability of macrophages on tumor cells was investigated by Transwell co-culture experiments. TAMs (1×10^5 per well) were inoculated in the upper chamber of the Transwell (0.4 μm), while 4T1 cells (5×10^4 per well) were inoculated in the lower chamber. After incubating for 12 h, DMEM (Control), S-PC (7.5 $\mu\text{g/mL}$), R-PA (7.5 $\mu\text{g/mL}$), SR (7.5 $\mu\text{g/mL}$) and SRN (7.5 $\mu\text{g/mL}$) were added for 24 h. Subsequently, 4T1 cells in the lower chamber were digested with trypsin without EDTA and then stained with Annexin V (5 μL) and PI (5 μL) for 15 min. The apoptosis of 4T1 cells was detected by flow cytometry.

2.12. *In vivo distribution experiments*

To investigate the distribution of nanomicelles *in vivo*, Ce6-SRN was prepared as above. 4T1 tumor-bearing mice were established by subcutaneously injecting 4T1 cells (5×10^6 per mouse) into the right side of BALB/c female mice. When the tumor size reached 75 mm^3 , the mice were randomly divided into three groups ($n = 3$) and administered with free Ce6, Ce6-SR, and Ce6-SRN (0.5 mg/kg Ce6) through tail vein injection. Fluorescence images of biodistribution in mice were observed at various time points (2, 6, 12, 24, 36 h) using the IVIS system. In addition, the mice were euthanized at 36 h post-injection, and both tumors and other organs were collected for imaging and fluorescence quantification with IVIS system.

2.13. *In vivo anti-tumor activity*

To establish a model of 4T1 tumor-bearing mice and evaluate the anti-tumor effects of different preparations *in vivo*, 4T1 cells (5×10^6 per mouse) were injected subcutaneously into the right side of the mice. When the tumor size reached 75 mm^3 , the mice were randomly divided into five groups ($n = 6$). Mice in each group received injections of Saline, S-PC, R-PA, SR, and SRN (PC at 5 mg/kg) every three days *via* tail vein starting from Day 0. Tumor size and mouse body weight of each mouse were measured and recorded every two days. The tumor volume was calculated according to Eq. (1):

$$\text{Tumor volume (mm}^3\text{)} = 0.5 \times \text{Length} \times \text{Width}^2 \quad (1)$$

On the 14th day, the mice were executed and the tumor tissues were collected for photography and weighing. The collected tumor tissues underwent various analyses, including H&E staining and immunofluorescence analysis for CD206 and TUNEL. In addition, tumor tissues were collected from 3 mice randomly selected from each group for tumor immune activation analysis. Meanwhile, the same model was established to investigate the survival of 4T1 mice with breast cancer. After four times of tail intravenous administration every three days, the survival of mice was monitored until the 60th day.

2.14. *In vivo immune activation studies*

The collected tumor tissues were placed in a 6-well plate and sheared to the size of 1 mm^3 after added into 3 mL of collagenase IV (0.5 mg/mL), then shaken at constant temperature for 45 min until digested completely. Finally, the single cell suspension was obtained through 100 μm cell sieve after grinding and

homogenization. Then, the supernatant was collected for the detection of cytokines at $300\times g$ while the collected cells were washed with PBS containing of 1% FBS and centrifuged again. Subsequently, CD45-PerCP/cy5.5, CD11b-PE/cy7, F4/80-FITC and CD86-PE mixed antibodies were added and incubated for 30 min at 4 °C. Then, the cells were centrifuged at $300\times g$ for 5 min, followed by fixation with 100 μ L of 4% paraformaldehyde for 15 min. To perforate the membrane, 0.1% Triton X-100 was added and incubate for 10 min. CD206-APC antibody was then added to incubate for 30 min. Finally, the proportion of TSSR in tumor tissues was detected by flow cytometry after PBS washing. In addition, the content of TNF- α in the tumor microenvironment was detected by ELISA kit. Simultaneously, immunohistochemical analysis was carried out on CD8⁺ T cells within the tumor tissues.

2.15. Biosafety assessment

Healthy BALB/c mice were randomly divided into five groups ($n = 5$) for the biosafety assessment. Then the mice in each group were injected with Saline, S-PC, R-PA, SR, and SRN (PC at a dose of 5 mg/kg) through tail vein every three days, with the first administration counted as Day 0. On the 14th day, blood samples were collected from the orbits of the mice for routine blood analysis (PLT and WBC). Additionally, major organs including the heart, liver, spleen, lungs, and kidneys were collected for hematoxylin and eosin (H&E) staining.

2.16. Statistical analysis

Student's *t*-test was conducted to analyze the significant difference between two sample groups by GraphPad Prism version 8 software, with all experimental data performed three or more times. And One-way ANOVA was carried out to compare the significance of multiple groups. The results are shown as mean $\pm$ standard deviation (SD). *P* value < 0.05 was considered statistically significant between the data sets, with all significant values listed: **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

3. Results and discussion

3.1. Preparation and characterization of SRN initiators

Polyethyleneimine rich in modifiable amino groups was selected as the main frame and hydrophilic end of the TAMs-intervening platform. In order to flexibly adjust the ratio of Spark agent-PC (S-PC) and Relay agent PA (R-PA), diverse functionalized polyethyleneimines formed hybrid micelles for multi-node TAMs repolarization. Firstly, S-PC was synthesized by Schiff base reactions with a 35% grafting percentage based on the peak area of ¹H NMR (Supporting Information Figs. S1 and S3). Besides, Arg-modified polyethyleneimine (R-PA) was synthesized by amide reaction with 23.5% grafting percentage (Supporting Information Figs. S2 and S4). Furthermore, the FTIR spectrum of S-PC and R-PA also confirmed their structures (Supporting Information Fig. S5). Then, PCA(SR) mixed nanomicelles were prepared through the self-assembly of S-PC and R-PA mixed in various mass ratios, allowing flexibility in their preparation. A S-PC to R-PA ratio of 2.5:1 was preferred based on particle size, PDI and optimized TAMs repolarization TSSR proportion (Fig. 1A, Supporting Information Fig. S6 and Fig. 1G). As shown in

Fig. 1B–D, SR had spherical morphology with particle size $\sim$ 216.6 nm and zeta potential $\sim$ 13 mV.

For enhancing the circulating stability and tumor chemotaxis of SR, macrophage membrane (MM) was picked as the coating layer of SR, due to the universally reported solid tumor taxis of macrophages. In order to modify electronegative MM on the surface of highly positive SR, biocompatible hyaluronic acid was primarily covered on SR for electron shelter and then MM was decorated through ultrasonic method, leading to the complete construction of biomimetic nanoreactor SRN. Compared to PCA (SR), the particle size of HPCA(HSR) increased a bit and the potential changed from positive to negative (Fig. 1C and D). Next, the consistent protein bands of SRN with fresh MM by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1E) proved the successful encapsulation of MM on SRN. Besides, the changes in particle size and zeta potential of SRN compared with HSR also demonstrated the successful modification of the film components on HSR (Fig. 1C and D). The hydration size and Zeta potential of SRN were 210 ± 1.8 nm and -31.6 ± 0.4 mV, which could maintain colloidal stability on the whole in physiological environment for at least one week (Fig. 1F). Additionally, cytotoxicity results of SRN on macrophages in Supporting Information Fig. S7 revealed that the cell viability of macrophages remains over 90% after SRN treatment, demonstrating its biosecurity.

3.2. SRN sequentially released free radicals in TAMs

The major dilemma of TAMs regulation therapies in tumor treatment is the anti-tumor durability, which is limited by that intervened TAMs tend to easily transform back to tumor-supportive phenotype in ITME. Hence, reliable regulator platform is crucial for TAMs repolarization. Herein, SRN was designed to address this issue, sequentially releasing endogenous radicals inside TAMs and acting as a cascade-proinflammatory nanoreactor (Fig. 2A). At first, the cellular uptake of SRN by TAMs was detected. For tracking the cellular distribution, hydrophobic dye Chlorin e6 (Ce6) was covalently linked on the amines of PEI to label the nanoreactor SRN. The particle size of Ce6-SRN was detected, which has uniform and similar size with SRN (Supporting Information Fig. S8). As shown in Fig. 2B, Ce6-SRN mainly distributed in the cytoplasm of TAMs, colocalizing with DiO-labelled MM on SRN. Then, the endocytosis route of SRN by macrophages was verified with several endocytosis inhibitors, which represented membrane fusion and giant pinocytosis mediated endocytosis mode were the main entry patterns (Supporting Information Fig. S9).

After entering TAMs, the Schiff base of S-PC in SRN breaks in the acidic tumoral environment to rapidly release cinnamaldehyde, inducing a large amount of ROS production via GSH depletion of the aldehyde group in the cinnamaldehyde, as schemed in Fig. 2C. Firstly, the burst release of cinnamaldehyde from SRN in an hour in Fig. 2D leads to the remarkably declining GSH in Fig. 2E and elevated ROS in Fig. 2F, detected by Ellman's assay and DCFH-DA probe, respectively. ROS have been widely reported to promote TAMs repolarization to the anti-tumor phenotype possibly by activating pro-inflammatory signaling pathways such as ROS–NF- κ B signal path^{18,29–32}. Despite this, the production of ROS is instantaneous and explosive in most cases and the whole macrophage polarization is a continuum, proper subsequent downstream stimulation is vital for more permanent anti-tumor orienteering progress¹¹.

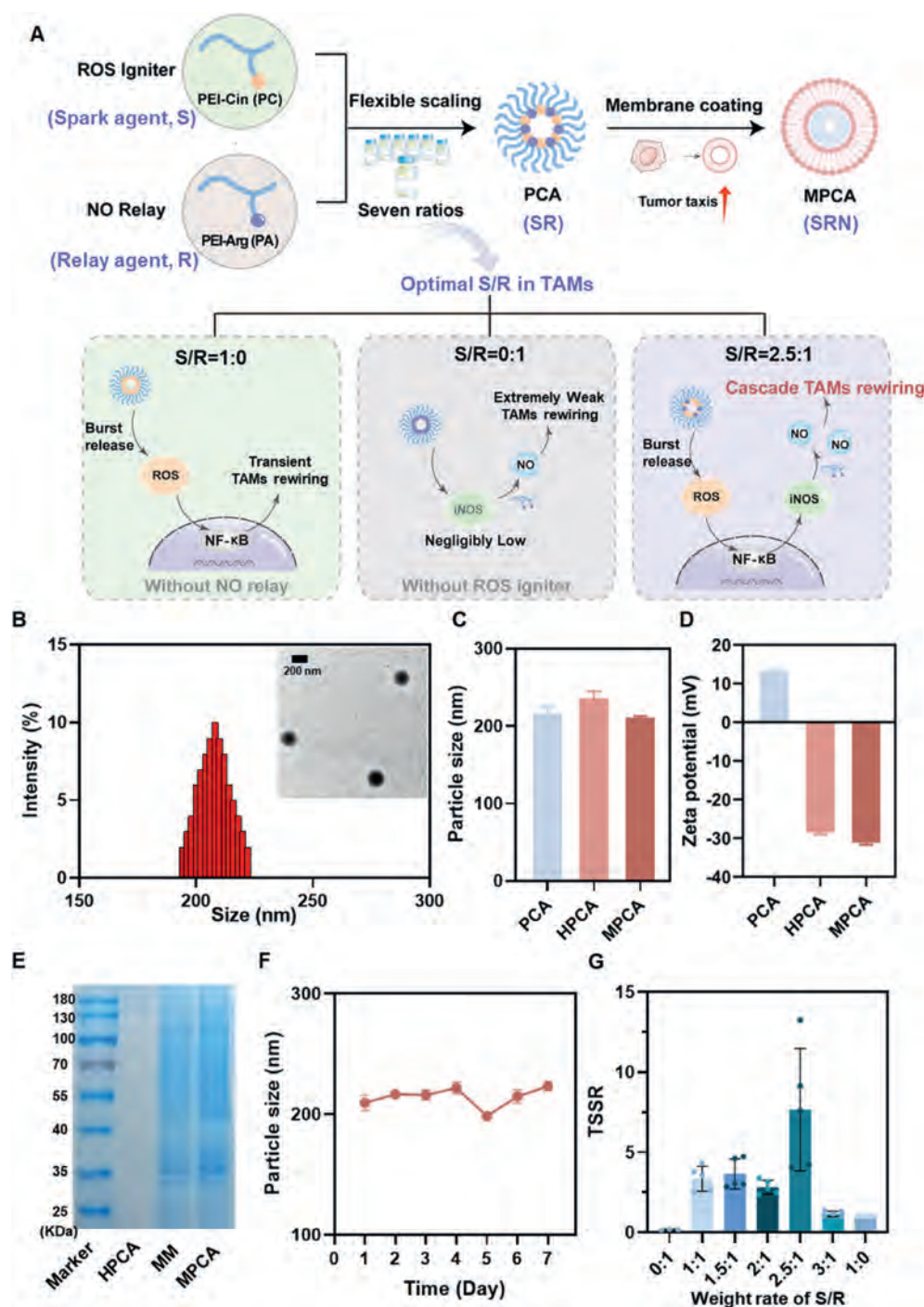


Figure 1 Construction and characterization of biomimetic nanoreactors SRN. (A) The preparation of nanomicelles PCA (SR) by self-assembly method and the ratio optimization of PC:PA (S/R). (B) Size distribution of SR. Inset: representative TEM image of SR. Scale bar, 200 nm. (C) The hydrodynamic size of PCA (SR), HPCA (HSR) and MPCA (SRN). (D) The Zeta potential of PCA (SR), HPCA (HSR) and MPCA (SRN). (E) Representative SDS-PAGE protein analysis of PCA (SR), macrophage membrane (MM) and MPCA (SRN). (F) Stability assessment of SRN in medium containing serum during 7 days. (G) Relative tumor suppressive/supportive ratio (TSSR) of TAMs phenotype after 24 h-treatment of different weight rate of S/R. The total PEI concentration of different groups was fixed as 6 $\mu\text{g/mL}$. Data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and $P > 0.05$ is not significant.

As observed in Fig. 2G, ROS was rapidly generated in just 2 h after SRN treatment, 2–3-fold compared with control group, yet without obvious elevation between 4 and 32 h except for a moderate increase at the 8th hour, confirming the explosiveness and instantaneity of ROS. Contributed by the ROS stimulus, the

downstream pro-inflammatory path NF- κ B and iNOS (specific anti-tumor phenotype marker) significantly increased (Fig. 2H and Supporting Information Fig. S10), predicted the primary anti-tumor-skew. Soon after, R-PA in SRN took over the relay baton of further TAMs rewiring as portrayed in Fig. 2I, decomposed into

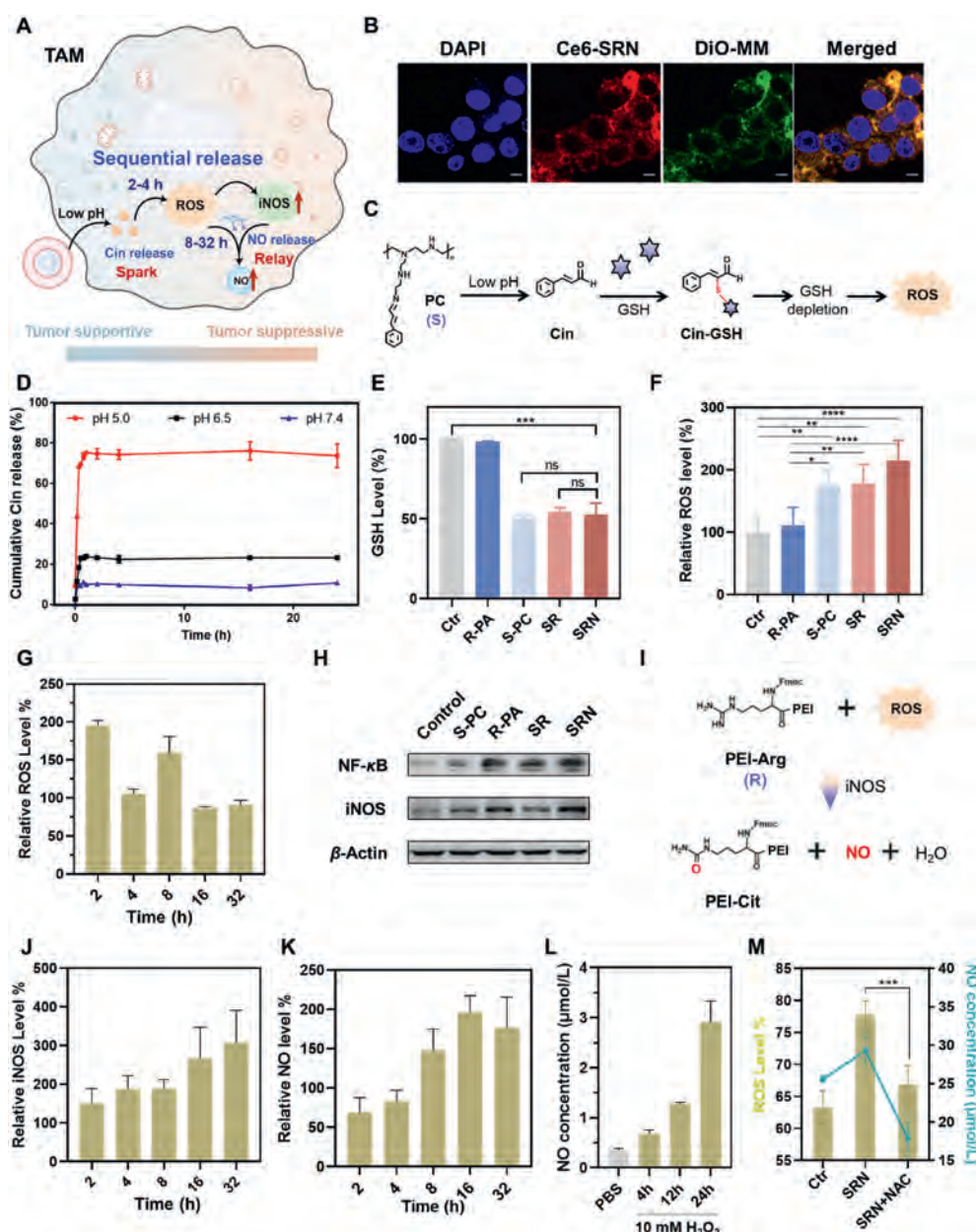


Figure 2 Free radicals sequential release in TAMs induced by SRN nanoreactor. (A) The illustration of the cellular progress of SRN in TAMs. (B) Representative fluorescent images of TAMs incubated with different preparations for 4 h. The total PEI concentration in SRN is 6 $\mu\text{g/mL}$ and the Ce6 concentration in SRN is 2 $\mu\text{g/mL}$. Blue: cell nuclei; Red: Ce6; Green: DiO. Scale bars: 5 μm . (C) The production process of ROS induced by SRN nanoreactor in TAMs. (D) The release profile of cinnamaldehyde from SRN at different pH. (E) The GSH level after various treatments. (F) ROS generation in TAMs after different treatments for 8 h. The total PEI concentration in SRN is 6 $\mu\text{g/mL}$. (G) ROS generation in TAMs comparing with untreated group as the control after different treatments for different time. (H) The NF- κB and iNOS expression of TAMs after different treatments for 24 h. (I) The production process of NO induced by SRN nanoreactor in TAMs. (J) The change of iNOS expression after SRN treatment at different time. (K) NO generation in TAMs comparing with untreated group as the control after different treatments for different time. (L) The *in vitro* NO generation without or with 10 mmol/L H_2O_2 at different time. (M) NO generation in TAMs after SRN treatment without or with ROS inhibitor NAC (5 mmol/L) at 8 h. Data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and $P > 0.05$ is not significant.

NO relying on foregoing ROS and iNOS catalyzation. Unlike ROS, the generation of iNOS and NO is time-dependent (Fig. 2J and K, Supporting Information Figs. S11–S13). Specifically, NO revealed distinct elevation until the 8th hour, implying a follow-up process after ROS stimulus and explaining the ROS elevation at

the 8th hour by ROS regeneration from NO effect, highlighting the positive feedback loop of ROS and NO. Additionally, inhibitor studies were carried out to further clarify this feedback. Treating TAMs with an iNOS inhibitor SMT (100 $\mu\text{mol/L}$, *S*-methylisothiourea sulfate, a potent and relatively selective

inhibitor of iNOS and would reduce NO production), which decreased ROS levels by ~40% (Supporting Information Fig. S14), supporting NO's role in sustaining ROS generation.

Thereafter, *in vitro* verification and cellular competitive inhibition experiments were conducted to elucidate the ROS-dependent and responsive character of NO production. As shown in Fig. 2L of *in vitro* verification, nearly 3 $\mu\text{mol/L}$ NO was released *via* a ROS-responsive manner. Besides, the competitive inhibition of classic antioxidant *N*-acetyl cysteine (NAC) was utilized for scavenging ROS produced by SRN, provoking immediately declined NO production in Fig. 2M, consistently illustrating the ROS-responsive release of NO and time sequence of the release of ROS and NO free radical. As a result of sequential and synergetic release and effect of these two crucial radicals, the tumor-supportive phenotype specific marker CD206 exhibited a time-dependent attenuation in Supporting Information Fig. S15, implying TAMs far away from tumor-supportive direction and a cascade-proinflammatory effect of SRN by ROS–NF- κB –iNOS–NO signaling pathway.

3.3. SRN-induced metabolic reprogramming of macrophages

The two main products of SRN, ROS and NO, have been reported to inhibit OXPHOS metabolism in macrophages, thereby altering their metabolic pattern and promoting polarization toward the tumoricidal phenotype^{25,33,34}. To understand metabolic changes and their effects on phenotypic polarization of macrophages during SRN treatment, the changes of mitochondrial OXPHOS and glycolysis were investigated in RAW264.7 cells. Regarding the control groups, lipopolysaccharide (LPS)-stimulated RAW264.7 cells—regarded as an extreme proinflammatory (M1) phenotype—were used as the positive control group, tumor-conditioned medium (TCM)—stimulated RAW264.7 cells—regarded as an extreme anti-inflammatory (M2) phenotype—were used as the model group, and unstimulated RAW264.7 cells were considered M0 macrophages. Afterwards, the OXPHOS in all macrophage groups was evaluated by measuring the oxygen consumption rate (OCR) of RAW264.7 with Seahorse XF, while glycolysis was evaluated by measuring the extracellular acidification rate (ECAR).

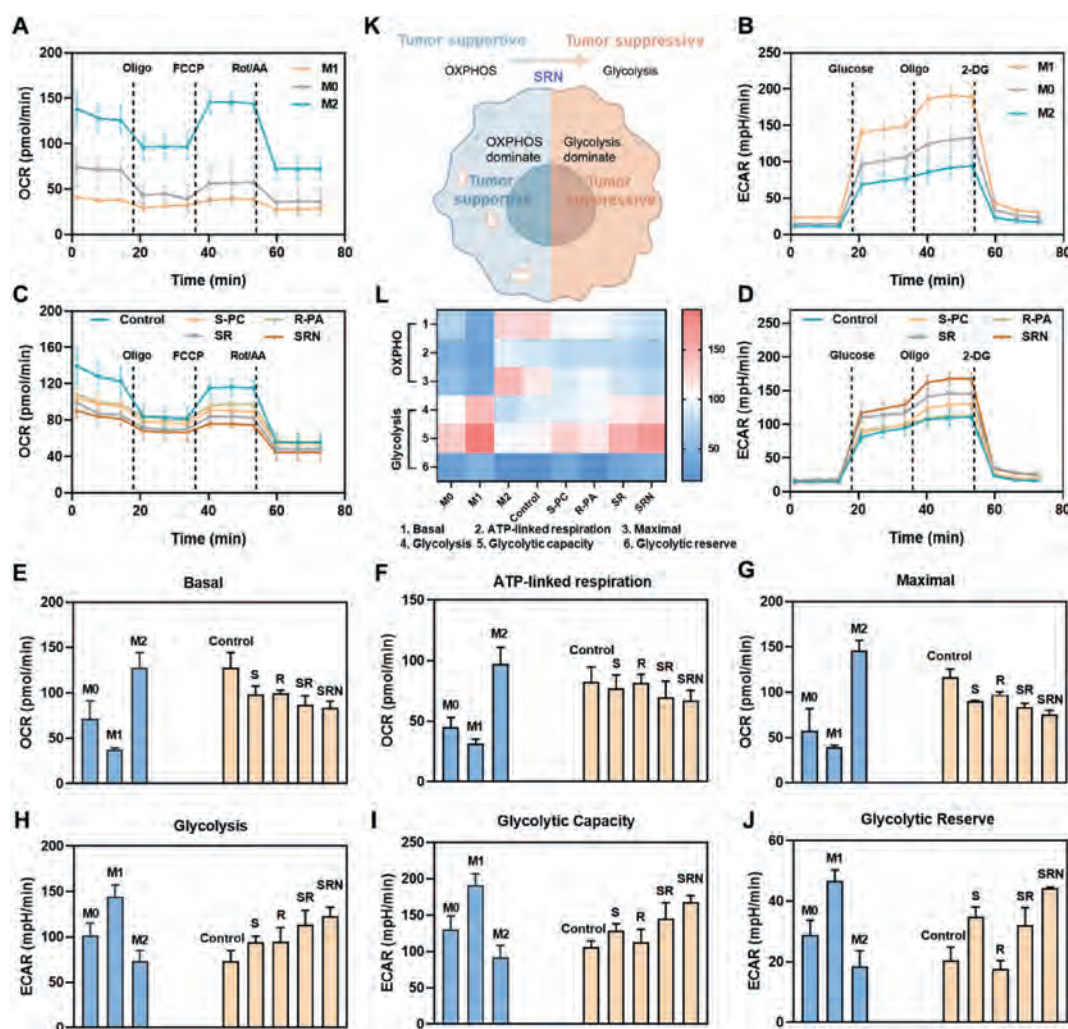


Figure 3 SRN induced metabolic polarization in RAW264.7 cells. (A) OCR and (B) ECAR of RAW 264.7 cells after DMEM, LPS and IL-4 stimulation. (C) OCR and (D) ECAR of RAW 264.7 cells after treatment with different preparations. (E–G) Quantitative analysis of (E) basal respiration, (F) ATP-related respiration and (G) maximum respiration provided by the OCR curves. (H–J) Quantitative analysis of (H) glycolysis, (I) glycolytic ability and (J) glycolytic reserve provided by the ECAR curves. (K) The schematic diagram of the SRN twisting metabolic mode of TAMs, from OXPHOS to glycolysis-dominated energy supply pattern. (L) Metabolic heatmap of glycolysis and OXPHOS after different treatments. Data are presented as the mean $\pm$ SD ($n = 3$).

As shown in Fig. 3A, M0 and M2 groups exhibited the classical OCR toothed curves and M2 group exhibited high levels of OXPHOS. In contrast, M1 group had a flatter curve and exhibited low levels of OXPHOS. On the contrary, M1 group presented high levels of ECAR compared to M0 and M2 groups (Fig. 3B), demonstrating that it relied mainly on glycolysis for energy supply. These results suggest that different environmental stimuli would make macrophages tend to favor different metabolic modes. Notably, the SRN treatment achieved the reprogramming of metabolic pattern of macrophage, transforming the original high levels of OXPHOS in M2 into high levels of glycolysis (M1) (Fig. 3C and D). Similar metabolic reprogramming trends were observed with the metabolic parameters of basal respiration: ATP-related respiration and maximum respiration provided by the OCR curves (Fig. 3E–G) and glycolysis, glycolytic ability and glycolytic reserve provided by the ECAR curves (Fig. 3H–J), as well as aggregate heat map (Fig. 3K and L). Comparing to SRN group, single S-PC or R-PA groups only brought out moderate metabolic rewiring capacity on TAMs. These results support that the ROS production leads to mitochondrial dysfunction, and the subsequent NO generation further inhibits OXPHOS through nitrosylation of the mitochondrial electron transport chain, which in turn drives metabolic shifts toward glycolysis in TAMs. Therefore, SRN effectively regulated the metabolic shift of macrophages from OXPHOS to glycolysis *via* mitochondrial function impairment, which led to much quicker energy supply and further promoted their polarization towards the tumoricidal phenotype.

3.4. SRN exerts antitumor effect in TNBC tumor cells

The above findings mechanistically verified that SRN could sequentially release pharmacophores, activate the ROS–NF- κ B–iNOS–NO signaling pathway, promote the metabolic reprogramming of TAMs and ultimately promote their polarization to the anti-tumor phenotype. The core issue of TAMs-based cancer therapies is to reduce anti-inflammatory macrophage and increase pro-inflammatory (anti-tumor) macrophages, in other words, to elevate the tumor suppressive/supportive ratio (TSSR). Thus, we examined whether SRN could successfully reprogram TAMs from immunosuppressive phenotype to immune-supportive phenotype by flow cytometry. First, TAMs were obtained by pretreatment with TCM for 24 h, followed by co-incubated with S-PC, R-PA, SR and SRN for 24 h. The results showed that SRN treatment significantly increased the proportion of antitumor TAMs (CD86⁺, 39.7%) and decreased the proportion of tumor-promoting TAMs (CD206⁺, 12.2%) compared with control group (Fig. 4A). In addition, the ratio of TSSR (CD86⁺/CD206⁺) was 3.25, which is 16-fold higher than that of control group (0.2) (Fig. 4B). Remarkably, the R-PA group exhibited the worst TAMs rewiring capacity, inducing only a negligible increase of TSSR, mainly due to the lack of a ROS-initiator and cannot inspire effective NO generation, which particularly highlighted the superiority of sequential and cascade effect of SRN nanoreactor on TAMs. Additionally, as can be seen in Supporting Information Fig. S16, SRN also elevated the expression of MHC-II and

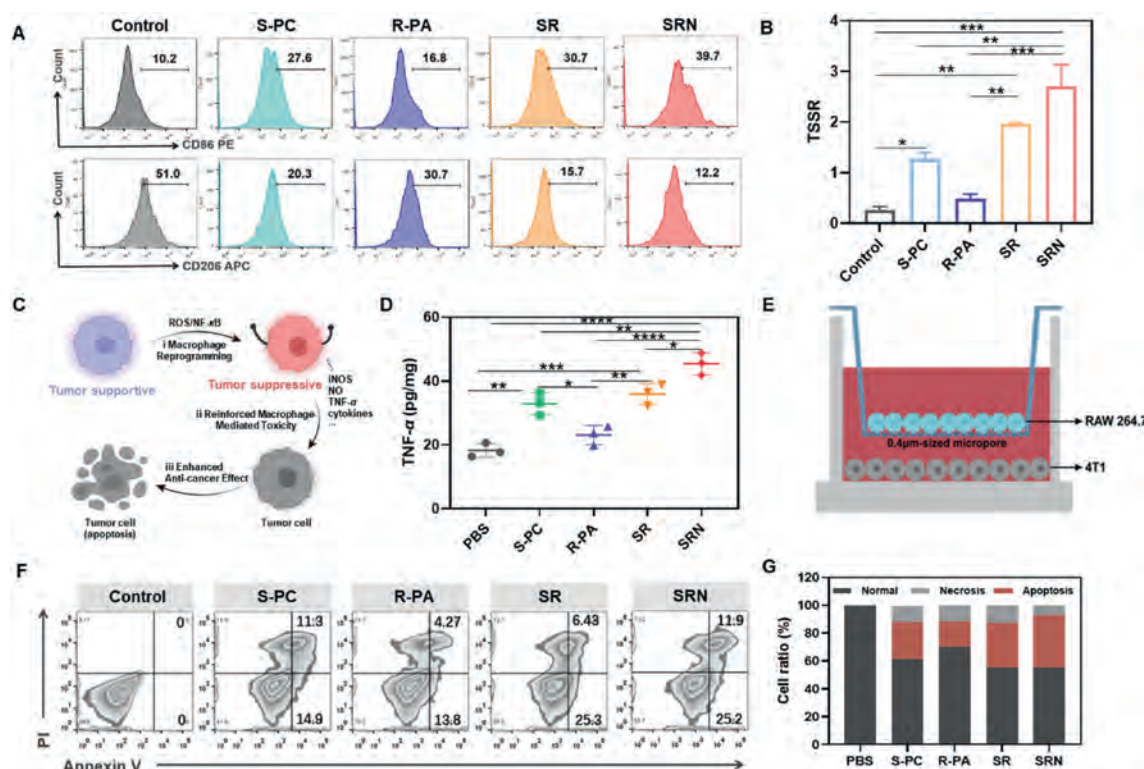


Figure 4 Macrophage repolarization and its antitumor effect. (A) Flow cytometry analysis of tumor-suppressive macrophages (CD86⁺) and tumor supportive macrophages (CD206⁺) after different treatments for 24h. (B) Quantification of the tumor-suppressive and tumor supportive macrophages ratio (TSSR) ($n = 3$). (C) Schematic of SRN-mediated macrophage repolarization and the tumor cells killing effect. (D) Secretion of TNF- α by repolarized macrophages after various treatments for 24 h by ELISA kit ($n = 3$). (E) Schematic of the co-culture system of RAW264.7 and 4T1 tumor cells in transwell model. (F) Apoptosis analysis of 4T1 cells co-cultured with RAW264.7 macrophages after different treatments for 24 h. (G) Quantitative analysis of (F). The total PEI concentration in SRN is 6 μ g/mL. Data are presented as the mean $\pm$ SD ($n = 3$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$, and $P > 0.05$ is not significant.

secretion of IL-12 (another proinflammatory markers of tumor-suppressive macrophages) while decreasing the expression of Arg-1 and CD163 (anti-inflammatory markers of tumor-supportive macrophages), 2.53-fold MHC-II expression and 9.08-fold IL-12 secretion in SRN group comparing with control group, further verifying shift of TAMs toward a pro-inflammatory phenotype. What's more, human-derived cell line THP-1 was also utilized for TAMs rewiring evaluation, showing similar TAMs repolarization effect of SRN as on RAW264.7 (Supporting Information Fig. S17) and enhancing clinical relevance of this research. Moreover, the comparison with “empty MM carrier” control group was exploring for investigating the immunomodulatory level of MM, presenting similar MHC-II expression and TSSR with control group (Supporting Information Fig. S18) and so excluding the immunostimulatory effect of MM itself. The above results demonstrated that SRN successfully promoted the repolarization TAMs *via* an ingenious cascade mode, which would restore and enhance the anti-tumor ability of macrophages.

Other than the TAMs repolarization and maintaining capacity, effective tumoricidal activity is a key characteristic in TAMs-based therapies, which is the ultimate goal in these treatments. NO release is a vital route in the antitumoral activity of TAMs^{23,35}, and it has been fully demonstrated above that SRN could efficaciously prompt

the NO generation in TAMs. Furthermore, in addition to NO production, tumor-suppressive TAMs would secrete inflammatory factors such as TNF- α to enhance the killing of tumor cells (Fig. 4C). Consequently, we detected the secretion of TNF- α of repolarized macrophages by ELISA. The content of TNF- α in the supernatant increased significantly after SRN treatment, indicating that SRN could mediate the secretion of TNF- α from TAMs (Fig. 4D). To determine whether repolarized TAMs have antitumor effect, we investigated the apoptosis-inducing ability of TAMs treated with different preparations on 4T1 cells by transwell co-culture system (Fig. 4E). The results showed that S-PC and SR treatment induced apoptosis in 26.2% and 31.78% of 4T1 cells after 24 h, respectively. However, R-PA treatment only induced 18.07% apoptosis because of its poor repolarization ability to TAMs. Notably, SRN treatment resulted in the highest apoptosis rate of 37.1% in 4T1 cells, indicating its superior ability to kill tumor cells (Fig. 4F and G).

The above results indicate that SRN promotes the cascade repolarization of TAMs by activating the ROS–NF- κ B–iNOS–NO pro-inflammatory signaling pathway and reprogramming its metabolism, and repolarized TAMs exert an effective tumor-killing effect through the secretion of NO and TNF- α , which demonstrates the great potential of SRN in anti-tumor immunotherapy.

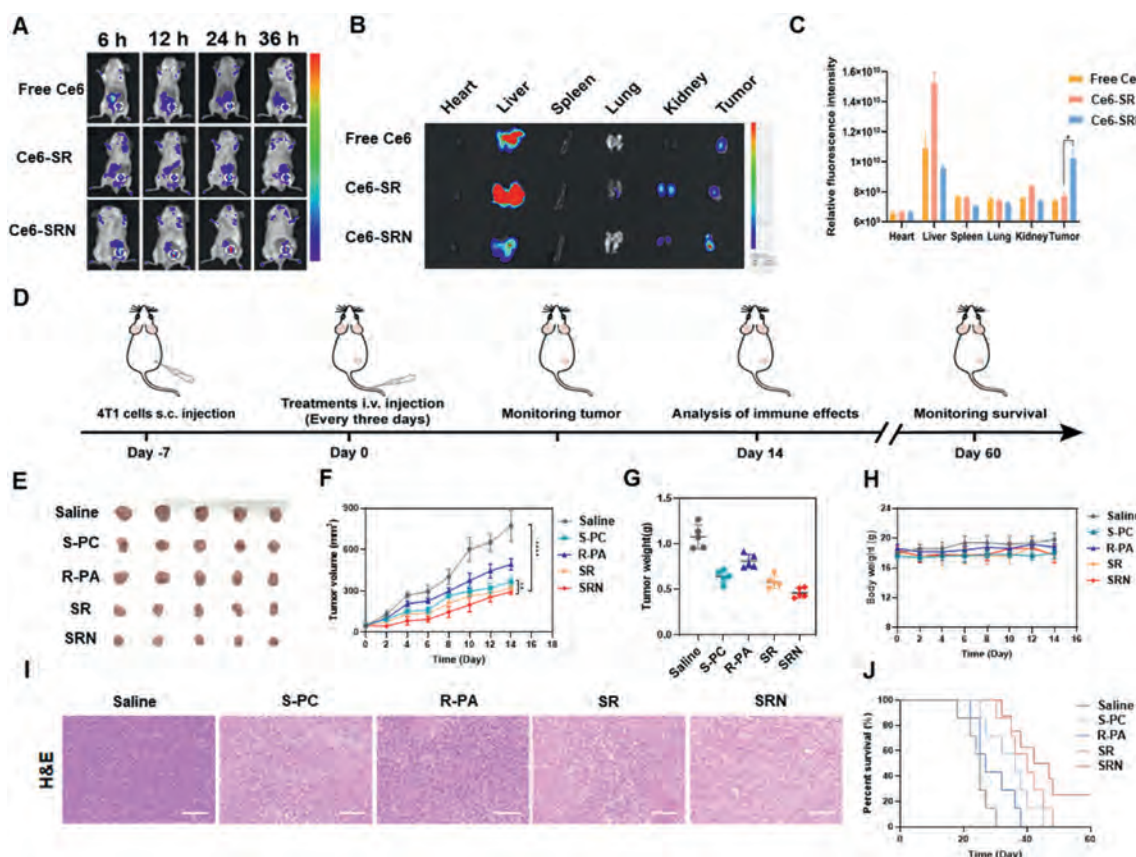


Figure 5 Tumor accumulation and antitumor effects of SRN *in vivo*. (A) *In vivo* fluorescence images after intravenous injection of free Ce6, Ce6-SR, and Ce6-SRN at 6, 12, 24 and 36 h. (B) *Ex vivo* fluorescence imaging of tumors and major organs at 36 h. (C) Semiquantitative analysis of (B). (D) Establishment of the 4T1 orthotopic tumor model and treatment strategy. (E) Representative images of tumor tissues after various treatments ($n = 5$). (F) Tumor growth curves of mice recorded during treatment ($n = 5$). (G) The weight of tumors in mice at the end of treatment. (H) Average body weight curves of 4T1 tumor-bearing mice during treatment. (I) H&E staining images of tumor tissue from mice. Scan bar = 50 μ m. (J) Survival curves of 4T1 tumor-bearing mice during 60-day observation period ($n = 7$). Data are presented as the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and $P > 0.05$ is not significant.

3.5. SRN accumulated in tumor regions and exerted antitumor effects *in vivo*

Afterwards, the *in vivo* anti-tumor performance of SRN was monitored with TNBC mice model. At first, the advantage of MM coating on tumor tropism was explored by *in vivo* imaging. The Ce6-labelled SRN was utilized for visualizing the tumor accumulation of the nanoreactor. As shown in Fig. 5A, after injection of Ce6-SRN *via* tail vein, Ce6 fluorescence signal appeared at the tumor site at 6 h and gradually enhanced until 24 h. Besides, the fluorescence signal of Ce6-SRN group was much stronger than Ce6-SR group, effectively proving the tumor taxis capacity of SRN coated by MM. The tumors and main organs of mice were collected at 36 h for *ex vivo* fluorescence imaging and semi-quantitative analysis. The results (Fig. 5B and C) showed that free Ce6 and Ce6-SR had strong fluorescence signals in the liver, but did not have obvious fluorescence in the tumor, while Ce6-SRN still had strong fluorescence in the tumor and relatively less liver accumulation, indicating that their tumor accumulation was stronger than that of free Ce6 and Ce6-SR. The results demonstrated the good tumor accumulation and retention capacity of macrophage membrane-mimicking nanoreactors.

After determining their adequate tumor accumulation, the antitumor effect of SRN in BALB/c mice was investigated at the administration procedures in Fig. 5D. When the tumor size reached approximately 75 mm³, Saline, S-PC, R-PA, SR-PCA and SRN were injected intravenously every three days, and the tumor volume was monitored every two days. The tumor volume (Fig. 5E and F, Supporting Information Fig. S19) showed that

both S-PC (48% of saline group) and SR (41% of saline group) exhibited moderate therapeutic effects compared to saline treatment, and R-PA (64% of saline group) had a poorer antitumor effect due to poorer macrophage repolarization without ROS-initiator. Notably, the SRN group (38% of saline group) showed the best tumor suppression effect, with a significantly smaller tumor volume than the saline group, and the SRN group had the lowest tumor weight among all groups (Fig. 5G). Hematoxylin and Eosin (H&E) staining revealed that the nucleus of SRN group was dissolved and the cytoplasm was loose, which demonstrated a strong tumor-killing effect (Fig. 5I). In addition, there was no significant decrease in the body weight of the mice in each group during the treatment period, indicating that SRN has good biosafety (Fig. 5H). All the tumor-bearing mice in the saline groups died within 25 days. Importantly, SRN treatment prolonged the survival time from 25 to 42 days, indicating strong antitumor efficiency (Fig. 5J). Additionally, tumor-bearing mice were administered SRNs with varying S/R ratios to evaluate the *in vivo* advantages of flexible ratio control. As could be seen in Supporting Information Fig. S20, S/R ratio (2.5:1) group revealed the best tumor-inhibitory effect (nearly 65%) in several ratios, which is consistent with the findings in above cellular level experiments, and low content S groups (S/R = 1:1 or 0:1) presented the worst tumor-inhibitory effect (only 30%–40%), highlighted the importance of a slightly higher S ratio once more. And this phenomenon prompts that flexible and precise drug ratio modulation would be a counteraction for the complex heterogeneity of solid tumors and compensatory paths of TAMs.

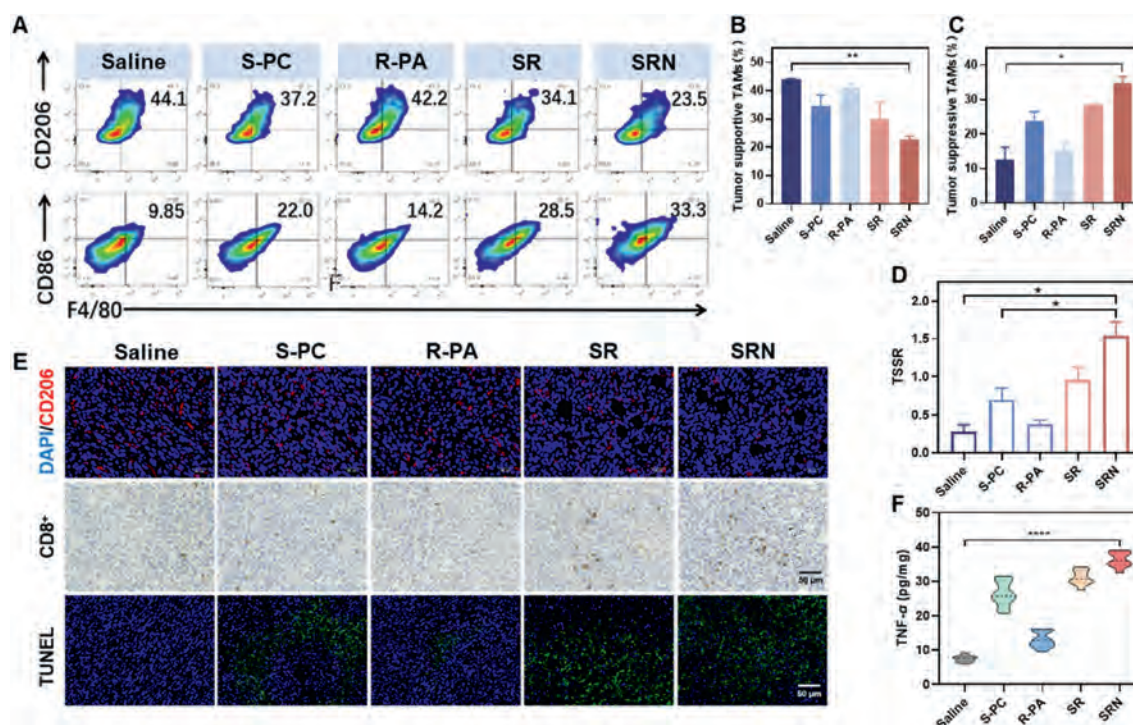


Figure 6 Macrophage-polarizing efficacy and immunosuppressive TME remission of SRN. (A) Flow cytometry analysis of the proportion of F4/80⁺ CD86⁺ macrophage and F4/80⁺ CD206⁺ macrophage in tumor tissues. (B) Quantification of F4/80⁺ CD206⁺ macrophages. (C) Quantification of F4/80⁺ CD86⁺ macrophages. (D) Quantification of the proportion of TSSR (CD86⁺/CD206⁺). (*n* = 3). (E) Immunofluorescence staining images for CD206⁺ (red); Immunohistochemical images for cytotoxic CD8⁺ of tumor sections after treatments; Immunofluorescence staining images for TUNEL (green). (F) Contents of TNF-α in plasma on Day 14 after various treatments by ELISA kit (*n* = 3). Data are presented as the mean ± SD. **P* < 0.05, ***P* < 0.01, and ****P* < 0.001.

While PEI-Cin/PEI-Arg shows promise in solid tumor therapies, its clinical translation faces challenges, primarily due to long-term toxicity of cationic polymers PEI (*e.g.*, cellular damage from non-degradable cationic residues). To address this, replacing PEI with biodegradable polycations like polyamidoamines (PAAs) or poly(β -amino ester)s (PBAEs) could mitigate toxicity while maintaining advantages of cationic polymers. Future work will focus on optimizing these alternatives for improved biocompatibility and therapeutic performance.

3.6. SRN induces potent antitumor immune responses *in vivo* and reprograms the ITME

TAMs are the primary contributors to the ITME³⁶. The rewiring of TAMs to anti-tumor TAMs can enhance the phagocytosis of tumors by macrophages and the secretion of inflammatory factors, thus reversing the transformation from “cold” tumor to “hot” tumor^{37,38}. Therefore, to verify the ability of SRN-induced macrophages to participate in antitumor immune response *in vivo*, we first evaluated the repolarization of intratumor TAMs by flow cytometry. The results indicated a significant increase in CD86-expressing macrophages (by 33.3%) and a decrease in CD206-expressing macrophages (by 23.5%) in the SRN group compared to the control group (CD86: 9.85% and CD206: 44.1%) (Fig. 6A–C). In addition, the TSSR increased from 0.28 ± 0.06 to 1.54 ± 0.13 , which was 5.5-fold higher than that of the control

group (Fig. 6D). Immunofluorescence results also demonstrated that CD206 fluorescence decreased significantly after SRN treatment (Fig. 6E), which was consistent with the result of flow cytometry. The immunohistochemistry results demonstrated an increased infiltration of intratumoral CD8⁺ T cells following SRN treatment (Fig. 6E). Subsequently, the level of TNF- α in the tumor tissue was detected using ELISA kit. The results revealed a significant increase in TNF- α secretion in tumor tissue after SRN treatment compared to control group (Fig. 6F). In addition, TUNEL staining showed a pronounced green fluorescence in tumor cells following SRN treatment, demonstrating its effective induction of apoptosis in tumor cells (Fig. 6E). The above results further demonstrated that SRN could effectively promote the repolarization of TAMs in tumor tissues, reversing immunosuppressive TME, and significantly enhancing the anti-tumor immune efficacy.

3.7. SRN effectively alleviates tumor recurrence in TNBC tumor resection models

Generally speaking, standard therapy for TNBC now is surgery excision along with subsequent adjuvant chemotherapy such as Taxanes. In the tumor postoperative process, TAMs secreted a large number of cytokines, thereby assisting the residual tumor cells in escaping immune surveillance and accelerating their recurrence^{39,40}. Therefore, based on the above results, we

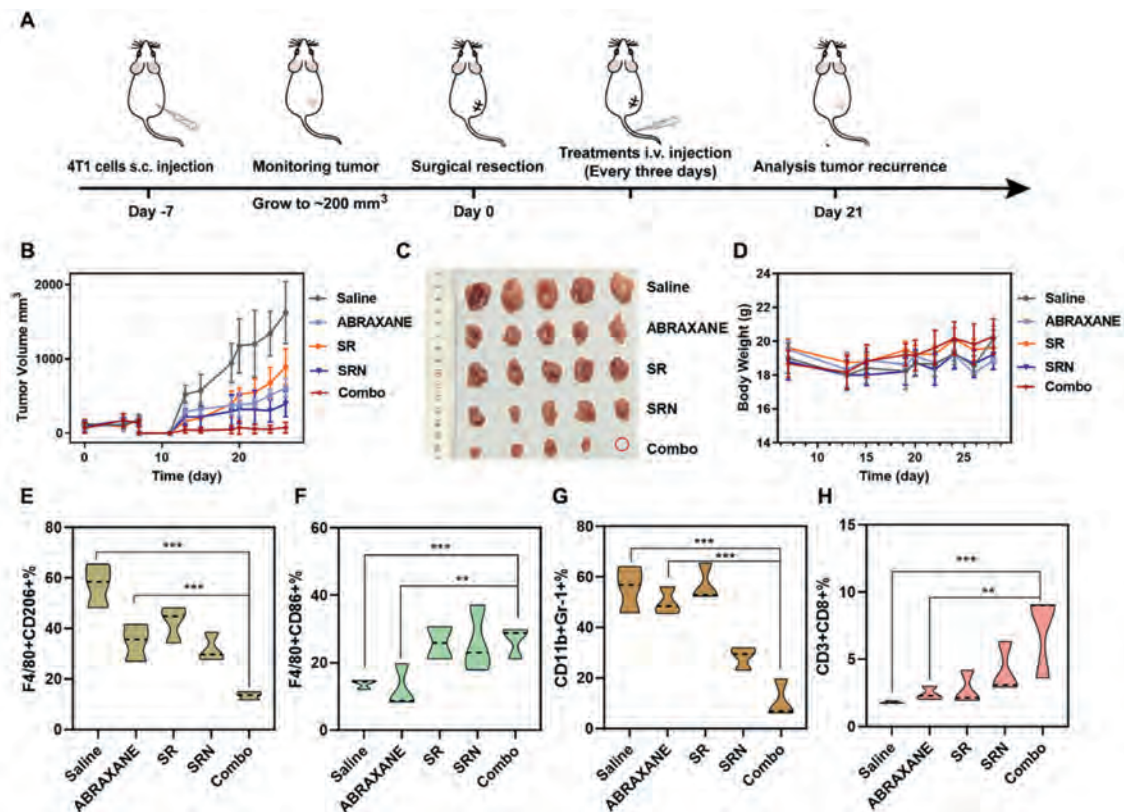


Figure 7 Evaluation of *in vivo* treatment efficacy after different treatments in the 4T1 breast cancer post-surgery model. (A) Establishment of the 4T1 post-surgery model and treatment strategy. (B) Tumor growth curve ($n = 6$). (C) Representative images of tumors collected in different groups ($n = 5$). (D) Body weight of mice in different groups ($n = 6$). (E–H) Statistical analysis of tumor infiltrated (E) F4/80⁺CD206⁺ macrophages (gated on CD45⁺CD11b⁺ cells), (F) F4/80⁺CD86⁺ macrophages (gated on CD45⁺CD11b⁺ cells), (G) CD11b⁺Gr-1⁺ cells (MDSCs, gated on CD45⁺ cells), (H) CD3⁺CD8⁺ cells (cytotoxic T cells, gated on CD45⁺CD3⁺ cells) ($n = 3$). Data are presented as mean $\pm$ SD; * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

hypothesized that the SRN could potentially alleviate tumor recurrence. In this part, the surgical procedure and treatment regimen are illustrated in Fig. 7A. The 4T1 breast cancer model was established by injecting 4T1 cells subcutaneously near the breast of female mouse. After tumors grew to $\sim 200 \text{ mm}^3$, the tumor tissues were resected. After resection, different formulations were injected through vein once every three days for 3 times, including Saline, Albumin-bound paclitaxel (ABRAXANE), SR, SRN, SRN + ABRAXANE (Combo). Among these groups, combo group represented the lowest recurrence rate and the highest tumor inhibitory rate (93.1%, Fig. 7B and C), much higher than ABRAXANE alone (62.7%), revealing the combination effect of cytotoxic chemotherapeutics and TAMs regulating agents. And combo group represented steady body weight growth in Fig. 7D. Besides, the immunosuppression relief of combination therapy based on SRN was verified by flow cytometry analysis. It was shown that the proportion of tumor-supportive TAMs and MDSCs of combo group was lowest, which decreased about 77.1% and 89.4% in comparison to that of the saline group, respectively, indicating the effective TAMs repolarization in the surgical site (Fig. 7E–G and Supporting Information Fig. S21). Correspondingly, the proportion of tumoricidal TAMs elevated by 2.5-fold than that of saline group. All these conversions of ITME contributed to the elevated infiltration of cytotoxic T cells (5.2-fold, Fig. 7H and Fig. S21), indicating the immune activation capacity of SRN combo group. Therefore, SRN has a good adjuvant activity on tumor post-surgery therapies.

3.8. Biological safety *in vivo*

Biosafety is a key indicator for the successful application of antitumor drugs *in vivo*. The biosafety of SRN was verified by H&E staining and blood routine analysis. After the same procedure as tumor therapy process, the levels of red blood cells (RBC) and white blood cells (WBC) remained within the normal range (Supporting Information Fig. S22). In addition, the H&E staining revealed no significant physiological changes in the major organs such as heart, liver, spleen, lung and kidney of mice in SRN group compared with the saline group (Supporting Information Fig. S23), indicating that the macrophage membrane-modified nanomicelles SRN had a favorable biosafety profile. Moreover, high dose tolerance was carried out for further safety assessment. After SRN treatment of 10-fold of therapeutic dose, there's no significant difference in complete blood routine test, blood biochemistry tests and H&E staining images of the main organs between SRN group and healthy mice (Supporting Information Figs. S24 and S25), suggesting the absence of acute inflammation and liver and kidney injury.

4. Conclusions

In this study, a TAMs metabolic intervention platform SRN was constructed for cascading proinflammatory effects and potentiating antitumor immunotherapy efficacy. Focusing on the dilemmas of TAMs-based anti-tumor therapies, limitations of TAMs rewiring and anti-tumor sustainability, the nanoinitiator SRN collaborated explosive ROS release (Spark effect) and slow degradation of NO donor (Relay effect) *via* ROS–NF- κ B–iNOS–NO signaling pathway, sequentially releasing metabolic regulators in TAMs and contributing to more permanent anti-tumor potential stimulation. Notably, the weak TAMs

rewiring effect of Relay agent alone emphasized the crucial position of ROS-initiator and the value of sequence strategy. Furthermore, the upregulation of ROS and NO regulated the metabolic paradigm shift from OXPHOS to glycolysis in TAMs, which further promoted its repolarization towards the antitumoral phenotype. Finally, TAMs *in situ* engineering agent SRN effectively increased the activation and infiltration of CD8⁺ T cells in solid tumors, which improved the immunosuppressive TME and exerted potent antitumor and anti-recurrence effects. In summary, the biomimetic nanoreactor SRN designed in this study facilitates the anti-tumor immunotherapy effect of TAMs by regulating their metabolic exhaustion and pathway compensation, showing a promising application prospect in solid tumor immunotherapy. Furthermore, this research highlighted the significance of temporal release of ROS and NO generation on macrophage polarization efficiency and inspired subsequent research to counteract the complex heterogeneity and compensatory processes of TAMs through flexible and precise drug ratio modulation. By optimizing the dose, release time sequence of ROS initiator and NO donor, more macrophage-related diseases such as other tumor types with a dense immunosuppressive microenvironment (*e.g.*, pancreatic cancer), infection and inflammatory diseases would attain better treatment outcomes.

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Author contributions

Xinping Luo: Conceptualization design, Writing original draft, Methodology, Data Curation. Yingmeng Jiang: Writing original draft, Methodology, Data Curation. Qing Zhang: Methodology, Project administration. Jie Yu: Data Collection. Chenxi Zhou: Methodology, Data Curation. Zhanwei Zhou: Conceptualization, Writing - original draft, Writing - review & editing. Minjie Sun: Conceptualization, Writing - review & editing. All of the authors have read and approved the final manuscript.

Conflicts of interest

The authors declare no conflict of interest.

Appendix A. Supporting information

Supporting information to this article can be found online at <https://doi.org/10.1016/j.apsb.2025.10.036>.

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