**Sonodynamical reversion of immunosuppressive microenvironment in prostate cancer via engineered exosomes**

Dingyi Wang\(^a\)*, Zhuo Wan\(^b\)*, Qian Yang\(^a\)*, Jianmei Chen\(^c\), Yunnan Liu\(^d\), Fan Lu\(^e\) and Jie Tang\(^a\) **

\(^a\)Department of Ultrasound, The First Medical Center of Chinese PLA General Hospital, Beijing, China; \(^b\)Department of Hematology, Tangdu Hospital, Fourth Military Medical University, Xi’an, People’s Republic of China; \(^c\)Department of Health Medicine, The Fourth Medical Center of PLA General Hospital, Beijing, China; \(^d\)Department of Ultrasound, Tangdu Hospital, Fourth Military Medical University, Xi’an, People’s Republic of China; \(^e\)Department of Biochemistry and Molecular Biology, State Key Laboratory of Cancer Biology, Fourth Military Medical University, Xi’an, People’s Republic of China

**ABSTRACT**

Prostate cancer (PCa) responds poorly to routine immunotherapy due to the tumor immunosuppressive microenvironment. Here, we describe an ultrasound-based drug delivery strategy to stimulate potent anti-tumor immunity via exosomes encapsulated with sonosensitizers Chlorin e6 (Ce6) and immune adjuvant R848, namely Exo\(^{\text{Ce6} + \text{R848}}\). Exo\(^{\text{Ce6} + \text{R848}}\) was constructed by simple co-incubation of Ce6 and R848 with HEK 293T cell-derived exosomes. The properties of exosomes were not affected after loading Ce6 and R848, and the exosomes were accumulated in the tumor site after intratumoral injection. In vitro and in vivo assays showed that ultrasonic irradiation enhanced R848-mediated DCs maturation when Exo\(^{\text{Ce6} + \text{R848}}\) was engulfed by DCs, as demonstrated by the upregulated expression of CD80 and CD86. Furthermore, these engineered exosomes together with ultrasound irradiation could synergistically reprogram macrophages from an immunosuppressive M2-like phenotype to an anti-tumor M1-like phenotype, further activating effector T cells and reverting the immunosuppressive microenvironment. The exosome delivery strategy not only supplies a paradigm for overcoming side effects of systemic delivery of Ce6 and R848, but also offers an effective combination regimen of cancer immunotherapy.

**ARTICLE HISTORY**

Received 10 January 2022
Revised 14 February 2022
Accepted 14 February 2022

**KEYWORDS**

Prostate cancer; exosomes; sonodynamic therapy; immunoadjuvant; synergistic effect

**CONTACT** Fan Lu lufan@fmmu.edu.cn

© 2022 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
exosomes have the advantage of inherent stability, biocompatibility, biological barrier permeability, low toxicity, and low immunogenicity (You et al., 2018; Pullan et al., 2019; Nezhadi et al., 2021).

Here, we proposed an exosome-based strategy to locally deliver Ce6 and R848 at tumor sites. Our study revealed that locally delivered Ce6 and R848 activated DCs and reverted the tumor suppressive microenvironment in the xenograft model upon ultrasound irradiation.

Materials and methods

Cell lines

HEK293T cells, DC2.4 and RAW264.7 cells were originally purchased from ATCC (Manassas, VA). The mouse PCa cell line RM-1 cells provided by Procell Life Science & Technology Co., Ltd. (Wuhan, China), DC2.4 and RAW264.7 cells were carefully expanded and maintained in RPMI 1640 Medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (FBS) (Exocell Bio, Shanghai, China) and 1% penicillin–streptomycin solution (Hyclone, Logan, UT). HEK293T cells were cultured in DMEM medium (Hyclone, Logan, UT) with 10% FBS and 1% antibiotics. The cells were incubated under a saturating humidity atmosphere at 37 °C containing 5% CO₂.

Animal tumor model

The male C57BL/6 mice (8–12 weeks old, 21–24 g) from the experimental animal center of the Air Force Military Medical University (Xi’an, China) were housed and processed in accordance with the Institutional Animal Care and Use Committee in the Air Force Military Medical University. 1 × 10⁶ RM-1 cells suspended in 100 μL of PBS were subcutaneously implanted into the mice at the right hind leg to establish the animal tumor model.

Exosome isolation

Exosomes from the donor HEK293T cells were isolated by ultracentrifugation in the study. For ultracentrifugation, cell culture supernatants were collected after 48 h of serum-free culture, followed by centrifugation at 300×g for 5 min to remove dead cells and then at 3000×g for 25 min to eliminate residual cellular debris. The resulting supernatants were then filtered through 0.22 μm filters, followed by additional centrifugation at 100,000×g for 3 h. The sediment was resuspended in PBS, and the mixture was additionally centrifuged at 100,000×g for 1 h to obtain relatively pure exosomes. The exosomes were resuspended in PBS and stored at −80 °C till use.

Preparation and characterization of Exo³⁸⁴⁸ + Ce6

Exo³⁸⁴⁸ + Ce6 was synthesized using co-incubation technique. Chlorin e6 was purchased from Cayman Chemical (Ann Arbor, MI). Resiquimod (R848) was purchased from MedChemExpress (Monmouth Junction, NJ). Briefly, 30 μL of R848 (2.5 μg/μL) and 30 μL of Ce6 (5 μg/μL) were gently incubated with 300 μL purified exosomes (10⁹ particles/μL) for 2 h at 37 °C. Free R848 and Ce6 were removed by another round of exosome isolation.

To verify the characterization of ExoCtrl and Exo³⁸⁴⁸ + Ce6, the exosomes were added onto the grid and stained with 2% uranyl acetate, followed by imaging with the transmission electron microscope (JEM200EX TEM, JEOL Ltd., Tokyo, Japan). Isolated exosomes were diluted to 500 ng/mL and subjected for size distribution analysis by Nanoplas.

Loading capacity of exosomes

The concentrations and loading capacity of exosomes were quantified using characteristic UV–vis absorptions by the Nanodrop-2000 spectrophotometer (Thermo Scientific, Waltham, MA). Briefly, Ce6 and R848 dry products were dissolved in DMSO with concentration of 10 mM and stored at −20 °C. To determine the drug concentration at maximum loading rate, different doses of Ce6 and R848 (30 μL) were added to 300 μL purified exosomes (10⁹ particles/μL) for incubating 2 h at 37 °C and then centrifuged at 100,000×g for 60 min to remove the excess free drug. The supernatant containing the free drug was collected and absorption intensity at the maximum absorption peak (A₂) was measured. The same dose of drug was added to DMSO and absorption intensity (A₁) was measured. The loading rate was calculated by the following equation: loading rate=(A₁ − A₂)/A₁×100%.

Western blotting

Total protein from HEK293T cells, exosomes, and DC2.4 was homogenized in RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentrations were measured using a BCA Protein Assay Kit (Thermo, Waltham, MA). Total protein from HEK293T cells, exosomes, and DC2.4 was homogenized in RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentrations were measured using a BCA Protein Assay Kit (Thermo, Waltham, MA). Total protein from HEK293T cells, exosomes, and DC2.4 was homogenized in RIPA Lysis Buffer (Beyotime Biotechnology, Shanghai, China), and the protein concentrations were measured using a BCA Protein Assay Kit (Thermo, Waltham, MA). Equal amounts of protein samples (15 μg) were electrophoresed in 5% and 12% SDS-PAGE (120 V for stacking gel and 160 V for separation gel) and then transferred onto polyvinylidene fluoride (PVDF) membranes. Subsequent to blocking by 5% nonfat milk for 1 h, the membranes were incubated with primary antibodies, anti-GM130 (11308-1-AP, Proteintech, Rosemont, IL), anti-TSG101 (ab83, Abcam, Cambridge, UK), anti-GAPDH (D110016-0100, BBI Life Sciences, Shanghai, China), or anti-Hsp70 (ab2787, Abcam, Cambridge, UK) overnight at 4 °C. After washing three times in TBST, the membranes were cultured with anti-rabbit (7074, CST, Boston, MA) and anti-mouse (7076, CST, Boston, MA) secondary antibodies corresponding to the primary antibodies at room temperature for 1 h.

Exosomes tracking in vivo

Exosomes were labeled with Dil/DIR (at the final concentration of 10 μM, Invitrogen, Carlsbad, CA) by incubating with the dye at the ratio of 500:1 in volume for 30 min. Unlabeled free dyes were then removed by centrifugation after washed with PBS. Mice were then injected with 200 μL labeled...
exosomes via tail vein or intratumor. For fluorescence imaging in vivo, different tissues from the mice that injected with DiR-labeled exosomes were harvested. IVIS® Lumina II in vivo imaging system was used for exosome localization in the whole body and individual organs. For test of the sliced section, Dil-labeled exosomes were prepared similarly before tail vein or intratumor injection. Four hours after injection, mice were sacrificed and the organs and tumors were fixed with 4% parafomaldehyde for 15 min and then washed with PBS twice. The cell nuclei were stained with Hoechst 33342 (1:1000, Beyotime Biotechnology, Shanghai, China) for 10 min. The whole process was kept from light. The fluorescence signal for the labeled exosomes and the blue nuclei were visualized by Nikon A1 Spectral Confocal Microscope (Nikon, Tokyo, Japan).

To further verify the distribution of exosomes in vivo, exosomes were electroporated with one OD miR54 at 700 V/150 mF in 4 mm wide electroporation cuvettes. Relative expression of miR54 in different issues was analyzed by qPCR as described below.

**Optimal parameters for SDT**

To investigate the efficacy of SDT based on different concentrations of ExoCe6 and intensities of ultrasound, the cell viability of RM-1, DC2.4, and RAW264.7 cells was determined with a Cell Counting Kit-8 (CCK-8) assay (SK2060, Coolaber, Shanghai, China). The cells cultured in 96-well plates were incubated with different concentrations of ExoCe6 (5 × 10⁸, 10⁹ or 2 × 10⁹ particles/mL) for four hours, then exposed to ultrasound of various intensities (0.1, 0.5, or 1.0 W/cm²) at a frequency of 1 MHz, using a 20% duty cycle, for 5 min. Notably, exosomes became obviously accumulated at the tumor site since the beginning of intratumor injection. For therapeutic reason, ultrasound was conducted and repeated three times. The therapeutic effectiveness in each studied group was evaluated by measuring the size of the primary tumors over time. Besides, some of the tumor tissues were collected from different groups of mice on the second day after the last administration for further analysis. At the end of the experiment, the tumors were dissected and weighed from the sacrificed mice.

**ROS detection**

ROS generation in DC2.4 was measured using the fluorescent probe dihydroethidium (DHE, Beyotime Biotechnology, Shanghai, China). DC2.4 with density of 10⁶ cells per mL was seeded on glass bottom cell culture dish and subjected to PBS, ExoCtrl, ExoR848, ExoCe6+US, and ExoR848+Ce6+US. The exosome concentration was 10⁹ particles/mL, the groups treated with Ce6 were exposed to ultrasound at a frequency of 1 MHz, a power density of 2.0 W cm⁻² using a 20% duty cycle, for 5 min. Notably, exosomes became obviously accumulated at the tumor site since the beginning of intratumor injection. For therapeutic reason, ultrasound was conducted and repeated three times. The therapeutic effectiveness in each studied group was evaluated by measuring the size of the primary tumors over time. Besides, some of the tumor tissues were collected from different groups of mice on the second day after the last administration for further analysis. At the end of the experiment, the tumors were dissected and weighed from the sacrificed mice.

**In vivo tumor treatment**

Tumor development over time was estimated using the equation: tumour volume = length × width × height × π/6. When the tumors had reached an average size of 30 mm³, animals were randomly distributed into five groups (the PBS group, the ExoCtrl group, the ExoR848 group, the ExoCe6+US group, and the ExoR848+Ce6+US group, n = 5 in each group). On the 10th, 12th, and 14th day of subcutaneous tumor bearing in mice, 20 × 10⁶ particles/kg body weight of exosomes (10⁹ particles/mL), loaded with 0.2 mL Ce6 (5 μg/μL, loading rate 70.08%) or/and 0.2 mL R848 (2.5 μg/μL, loading rate 58.56%) were injected into tumors in the different groups. Following induction of anesthesia (intraperitoneal injection of 1% pentobarbital sodium), the groups treated with Ce6 were exposed to ultrasound 2 h later since injection at a frequency of 1 MHz, a power density of 2.0 W cm⁻², using a 20% duty cycle, for 5 min. Notably, exosomes became obviously accumulated at the tumor site since the beginning of intratumor injection. For therapeutic reason, ultrasound was conducted and repeated three times. The therapeutic effectiveness in each studied group was evaluated by measuring the size of the primary tumors over time. Besides, some of the tumor tissues were collected from different groups of mice on the second day after the last administration for further analysis. At the end of the experiment, the tumors were dissected and weighed from the sacrificed mice.

**Real-time qPCR for gene expression analysis**

Total RNA of the cells or tumor tissues was extracted with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Reverse-transcription was conducted using PrimeScript First-Strand cDNA Synthesis Kit (Takara, Beijing, China) for mRNA and miRcute First-Strand cDNA Synthesis Kit (Tiangen Biotech, Beijing, China) for miRNA. Gene expression was analyzed using PrimeScript RT Master Mix (Roche, Basel, Switzerland) or miRcute miRNA qPCR Kit (Roche, Basel, Switzerland). GAPDH and U6 were served as an internal control in mice for standardization between samples and relative RNA levels of target genes. The fold change of target gene was calculated using the 2⁻ΔΔCt method. The specific primers for individual genes are shown in the Supplementary Table 1.

**H&E staining and immunofluorescence**

Mice were sacrificed and tumor tissues were dissected. Tissues were fixed in 4% paraformaldehyde, dehydrated with sucrose solutions, embedded with paraffin, and then cut into slides. The sections were then stained with hematoxylin and eosin.

After incubation with 5% bovine serum albumin (BSA) for 1 h, the sections were incubated with primary antibody obtained from Servicebio (Woburn, MA): anti-CD11c rabbit (GB11059), anti-CD3 rabbit (GB130140), anti-CD4 rabbit (GB130141), anti-CD8 rabbit (GB130142), anti-Foxp3 Rat
In this study, we used HEK 293 T cell-derived exosomes and constructed ExoCe6 by co-incubation technique (Figure 1(A)), which displayed the typical exosome morphology as revealed by transmission electron microscopy (Figure 1(B)). Nanoparticle tracking analysis showed that the size distribution of ExoCtrl and ExoCe6 ranged from 30 to 150 nm and the size of ExoR848 was slightly larger than the parental Exo. As shown in Figure 1(C), there were two peaks for the engineered exosomes. However, they remain in the range of exosomes. Moreover, western blot analysis revealed the exosomal inclusive marker TSG101 and CD9 were found to be expressed in both ExoCtrl and ExoCe6, while the exclusive marker GM130 was absent (Figure 1(D)). These data demonstrated that exosomes were successfully extracted and their properties were not affected after loading Ce6 and R848.

Ce6 and R848 concentrations were determined by the absorption measurement (Figure S1A). The UV–vis spectrum revealed that Ce6 and R848 had the maximum absorption peak at 400 nm and 250 nm (Figure S1B,D), respectively. There was a good linear relationship between the absorbance and concentration of Ce6 and R848 (Figure S1C,E). When the concentration of R848 was 2.5 µg/µL and the concentration of Ce6 was 5 µg/µL, loading efficiency was 58.56% and 70.08% in 10^5/mL exosome solution, respectively (Figure 1(E,F)).

Optimization of exosome injection routes

To track the efficient distribution of exosomes to major organs (heart, liver, spleen, lung, and kidney) and the tumor issue, we injected DiR/DiI-labeled exosomes into the tumor directly or via tail vein (Figure 2(A)). As expected, both ex vivo fluorescent imaging and confocal microscope analysis showed that the liver, spleen, and lung were the dominant targets of exosomes with the strongest fluorescence intensity both when injecting into the tumor directly and via tail vein. However, compared with tail vein injection, the distribution of exosomes in tumor issues increased significantly via intratumoral injection (Figure 2(B–D), Figure S2). To further confirm the results, mice were injected with cel-miR54-loaded exosomes as cel-miR54 has no homolog in mice (Figure 2(E)). qPCR analysis of cel-miR54 abundance showed the enrichment of exosomes in tumor tissues can be significantly increased by intratumoral injection compared with tail vein injection (Figure 2(F)).

ExoR848+Ce6-mediated enhancement of antigen presentation and macrophages repolarization in vitro

To determine the optimal SDT parameters of in vitro experiment to make it have certain killing activity on tumor cells, while not affecting the viability of immune cells, we incubated RM-1, DC2.4, and RAW264.7 cells with three different concentrations of ExoCe6 and ultrasonic intensities. The results showed that no obvious cytotoxicity of ultrasound treatment was observed for all the cell types. Furthermore, stimulation with 10^6 particles/mL ExoCe6 and the intensity of 0.1 W/cm² resulted in the highest relatively viability in DC2.4 (90.22%) and RAW264.7 cells (84.96%) compared with tumor cells (70.59%) (Figure S3). Confocal images in vitro revealed efficient endocytosis of exosomes both by DCs and RM-1 cells (Figure S4).

The mechanism of ExoR848+Ce6+US enhancement of DCs maturation was first determined by detecting intracellular ROS levels using DHE. As shown in Figure 3(C), after ExoR848
or ExoCe6+US treatment, abundance of ROS was generated in these groups, as verified by the strong red fluorescence in cells. The ROS intensity of ExoR848+Ce6+US group was the strongest of all (Figure 3(D)). ROS generation in cells was also quantified by flow cytometric analysis, further suggesting the significantly highest ROS generation of ExoR848+Ce6+US group in all the groups (Figure S5). We also evaluated the expression of Hsp70 on the surface of DCs after different treatment groups to further enhance its antigen presentation ability. As shown in Figure 3(B), ExoR848 and ExoCe6+US could increase Hsp70 expression. Moreover, the combination treatment exhibited an obvious effect in comparison with other groups. Based on the above results, the synergistic effect which induced the DCs maturation to express the co-stimulatory molecules CD80/CD86 was verified by flow cytometry. As shown in Figure 3(E–H), the ExoR848+Ce6+US group resulted in a much higher level of CD80 and CD86 expression than other treatment groups.

To study the effect of ExoR848+Ce6+US on the macrophage polarization, interleukin-4 was first added to induce M2 macrophage polarization, followed by incubation with various treatments. Compared with the PBS and ExoCtrl group, ExoR848, ExoCe6+US, and ExoR848+Ce6+US could decrease the expression of M2-related genes (Mrc-1, Fizz-1, and II-10) and increase the expression of M1-related genes (iNos, Il-6, and II-1β), indicating a polarization from the M2 phenotype to M1 phenotype (Figure S6).

Enhanced antitumor efficacy and inflammatory response of ExoCe6+R848+US on PCa tumor-bearing mice

Animal studies were performed in mice bearing PCa to evaluate the synergistic anticancer effect of R848 and SDT in vivo (Figure 4(A)). As shown in Figure 4(B–D), all exosomes loading Ce6 or/and R848 showed obvious therapeutic effects, and tumor growth was most inhibited in mice treated with ExoCe6+R848+US. On the 8th day after the last treatment, tumor volume in the ExoCe6+R848+US was 86 ± 32 mm³. In comparison, tumors grew to 1224 ± 179 mm³, 1079 ± 188 mm³, 434 ± 132 mm³, and 362 ± 132 mm³ in the PBS group, ExoCtrl group, ExoCe6+US group, and ExoR848 group,
respectively (Figure 4(B,C)). Regardless of different treatments, the body weights of mice in all groups remained stable (Figure 4(E)).

The synergistic immunologic adjuvant and SDT based on exosomes were further explored on tumor tissue sections. As shown in Figure S7, tumor tissues from animals treated with PBS and ExoCtrl had fewer blood vessels and less inflammatory infiltration. In contrast, the blood vessels and inflammatory cells in tumor tissues from animals receiving ExoCe6 + US, ExoR848, or ExoCe6 + R848 + US increased obviously. Moreover, tumor tissue from animal treated with ExoCe6 + R848 + US showed the most significant increase in blood vessels and inflammatory cells.

To further investigate the immune cytokine storm induced by systemic immunity, inflammatory cytokines in tumor tissues from mice receiving different treatments were analyzed by qPCR (Figure 5(A)). The ExoCe6 + R848 + US therapy could obviously downregulate the secretion of the anti-inflammatory cytokine Il-10 and Tgf-β in the tumor microenvironment (Figure 5(B,C)). At the same time, the
combination therapy exhibited a pleasing pro-inflammatory effect, leading to 6.48-, 12.67-, 640.98-, 23.45-, and 18.56-fold upregulation of IFN-γ, TNF-α, IL-1β, IL-6, and IL-12 in comparison with the control group (Figure 5(D–H)).

Accordingly, ExoCe6+R848+US treatment resulted in a highest number of mature DCs (49.41±3.35%) among all groups, which are 23.03%±3.01% in the Exo R848 group, 15.51%±1.65% in the Exo Ce6+US group, 5.83%±1.20% in the Exo Ctrl group, and 3.84%±0.47% in the PBS group, respectively (Figure 6(B,C,F,G)). To further investigate whether the ExoCe6+R848+US therapy induced effective anti-tumor T cell responses, the numbers of tumor-infiltrating T cells in various treatment groups were measured. In comparison with the rare tumor infiltration of CD3+CD8+ T cells in the PBS group (2.93±0.81%) and Exo Ctrl group (2.79±0.97%), the effector T cells were obviously increased via treatments of other exosomes. In particular, ExoCe6+R848+US treatment showed the best effect to promote the tumor infiltration of CD3+CD8+ T cells (27.27±2.74%) (Figure 6(D,E,H,I)). In contrast, the number of CD3+CD4+ T cells did not change significantly after different treatment groups (Figure 6(D), Figure S8).

In contrast, ExoCe6+R848+US and ultrasonic radiation synergistically reduced the populations of Tregs (CD4+Foxp3+) and increased M1/M2 ratio (F4/80+CD86+/F4/80+CD206-) as determined by flow cytometry and immunohistochemistry (Figure 7). Compared with the PBS group, the number of Tregs was 0.87, 0.50, 0.35, and 0.11-fold lower in the Exo Ctrl, ExoCe6+US, ExoR848+, and ExoCe6+R848+US group, respectively (Figure 7(A,B,E,F)). The combination therapy showed a greater decline than the ExoR848+ group. Besides, as shown in Figure 7(C,D,G–J), the M1/M2 ratio of ExoCe6+US and/or
**ExoR848** groups exhibited a significant increase compared with the PBS and Exo Ctrl group. Notably, the ratio of ExoCe6+R848+US group was 0.81 ± 0.04 while the value of the ExoR848 group was 0.53 ± 0.12, which indicated an enhanced M1 polarization effect of the combination therapy.

**Discussion**

In this study, we demonstrated that engineered ExoCe6+R848 can inhibit the progression of PCa upon ultrasound irradiation in tumor-bearing mouse model. Mechanistically, ExoCe6+R848 could stimulate robust maturation of tumor-infiltrating DCs, promote M1 macrophages polarization and pro-inflammatory cytokines production, and decrease anti-inflammatory factors, further inhibiting Tregs and activating effector T cells in TME. As professional APCs, DCs play a central role in initiating and regulating tumor immune responses. Only mature DCs can migrate to tumor-draining lymph nodes to present antigenic peptides to CD8+ T cells and subsequently recognize and destroy cancer cells. Instead of allowing maturation of DCs and a DC-mediated antitumor response, DCs in tumors can be redirected toward a dysfunctional, tolerogenic, or even immunosuppressive phenotype (Pfirschke et al., 2017; Wculek et al., 2020; Jhunjhunwala et al., 2021). Therefore, conversion of tumor-infiltrating DCs from tolerogenic to immunogenic by the effective strategies is of great importance.

In this study, we efficiently incorporated Ce6 and R848 into the exosome membrane by co-incubation technique. Since both Ce6 and R848 are lipophilic, they can adhere to lipid bilayer of exosomes via co-incubation technique. Due to the lipophilic characteristics, R848 and Ce6 themselves could be rarely loaded inside the exosomes (which is similar as the cytosol) even if they penetrate into the exosomes. This passive drug loading method, which is relatively simple and straightforward, can maintain the membrane integrity of exosomes. HEK293T cells-derived exosomes were chosen for the reason that they were easy for manipulation, free of immunity, and widely used in animal models. Compared with liposomes and other polymeric nanoparticles, which have intricate shortcomings such as activation of immune system, low circulating capability, stability, and high toxicity (Elsharkasy et al., 2020; Herrmann et al., 2021), exosome-based delivery strategy can overcome these shortcomings with minimal toxicity, excellent biocompatibility, and an immune privileged status, which allows for decreased drug clearance according to their characteristics and natural origin (Batrakova & Kim, 2015; Pullan et al., 2019; Patil et al., 2020).

In this study, exosomes were administrated via intratumor injection. The injection route has characteristics that ensure a long retention time at tumor site to increase the chance for extravasation due to the high permeability and retention effect (EPR). Besides, compared with vein injection, intratumor injection enhances the local concentration by reducing...
exosomes phagocytized by the mononuclear phagocyte system (MPS, e.g. liver and spleen).

Previous studies found that R848 increased the expression of immune genes including TLR7, IL6, Myd88, and IRF3, related to the TLR7/8 signaling pathway. These signaling pathways lead to activation of the transcriptional factor NF-kB, which, upon translocating to the nucleus, promoted the transcription of genes characteristic of DCs maturation, including genes for cytokines (e.g. IL-12, IL-1β, and TNF-α), co-stimulatory molecules (CD80, CD86), and MHC molecules (Zhou & Sun, 2015; Alam et al., 2018). However, prolonged exposure to R848 causes significant cytotoxicity and limits its continued use (Schmid et al., 2017; Michaelis et al., 2019). The proposed exosome delivery systems can limit dissemination of the drug into the systemic circulation following intratumoral injection.

Exosomes containing Ce6 engulfed by DCs can promote translocation of Hsp70 on to the cell surface and produce ROS under ultrasound. Previous studies have demonstrated that membrane-bound Hsp70 could promote the release of IL-6 and IL-1β as well as DCs maturation by the evaluation of CD80 and CD86 expression (Kupnner et al., 2001; Zhu et al., 2016; Zininga et al., 2018). Notably, excessive ROS production may result in premature death, while moderate ROS production is essential for DC activation and function (Grassi et al., 2007; Paardekooper et al., 2019). A recent study has demonstrated that ROS-mediated DNA oxidation enhanced immune recognition by DCs and DC-derived ROS was responsible for STING-initiated antitumor immune responses (Hu et al., 2021). Nonetheless, future researches need to explore the optimal intensity and frequency of ultrasound and dose of exosomes to induce DC maturation without killing immune cells at the tumor site.

Besides the direct impact on DCs, remodeling of the tumor microenvironment by the engineered exosomes could also be responsible for the observed therapeutic effects. In the tumor microenvironment, TAMs and Tregs are the most abundant immunosuppressive cells that inhibit T cells activation and proliferation. TAMs exhibit either an immunosuppressive M2-like phenotype, releasing pro-inflammatory cytokines IL-12, IL-1, IL-6, and TNF-α (Poh & Ernst, 2018; Chen et al., 2019; Pathria et al., 2019). The proposed strategy here repolarized TAMs from M2 to M1 phenotype to enhance cancer immunotherapy. The synergistic effect of TAM polarization

Figure 5. Effects of engineered exosomes on inflammatory cytokine. (A) Schematic diagram of the experimental procedure. $2 \times 10^5$ particles/kg body weight of exosomes were intratumorally injected three times at an interval of two days. Insonation (1 MHz, 2.0 W cm$^{-2}$, 20% duty cycle, 5 min) was applied two hours after administration. Tumors were excised for inflammatory cytokine analysis on day 16 after inoculation RM-1 cells. (B–H) Relative expression level of IL-10, Tgf-β, Ifn-γ, Tnf-α, IL-1, IL-6, and IL-12 in tumor tissues with different treatments ($n = 5$). Data are expressed as mean ± SEM, one-way ANOVA, *p < .05, ExoCe6-US versus PBS, ExoR848, ExoCe6-R848-US versus ExoCtrl, **p < .01, ExoCe6-US versus ExoCtrl.
and DCs maturation could unleash the suppressed T cells caused by the tumor microenvironment, activating effector T cells and improving their infiltration into tumors. Besides, it has been reported that tumor cells secreted the immunosuppressive molecules, such as IL-10 and TGF-β, inhibited the maturation of DCs and increased the production of Tregs (Tormoen et al., 2018; Hu et al., 2020). We found that tumor tissues of ExoR848⁺Ce6⁺US group displayed

Figure 6. Exosomes in each treatment group for activating DCs and anti-tumor T-cell immune response by flow cytometric analysis and immunofluorescence. (A) Schematic diagram of the experimental procedure. Exosomes (10⁹ particles/mL) were injected three times at an interval of two days, and their dose was 2 × 10⁹ particles/kg body weight per injection. Insolation (1 MHz, 2.0 W cm⁻², 20% duty cycle, 5 min) was applied two hours after administration. Tumors were excised for immunological analysis on day 16 after inoculation RM-1 cells. Flow cytometric analysis of (B) proportion of mature DCs (CD80⁺CD86⁺) over CD11c⁺ DCs and (D) CD8⁺ T cells over CD3⁺ T cells in the tumor tissues with different treatments. (C, E) Statistical results of (B) and (D), respectively (n = 5). (F) Immunofluorescence staining of CD11c⁺CD86⁺ in tumor sections after different treatments. (G, I) Statistical results of (F) and (H), respectively (n = 5). Data are expressed as mean ± SEM, one-way ANOVA, *p<.05, ExoR848⁺US versus ExoCtrl; #p<.05, ExoR848⁺Ce6⁺US versus ExoR848.
lower levels of these inhibitive factors, relieving the local tumor-mediated immunosuppression and generating an effective systemic immunity.

**Conclusions**

In summary, we here confirmed that SDT and immunoadjuvant delivered via exosome-based delivery strategy not only evoked the DCs but also re-modulated the immunosuppressive tumor microenvironment in PCa tumor-bearing mouse model. The exosome delivery strategy not only supplies a paradigm for overcoming the systemic side effects of Ce6 and R848, but also offers a rational design of an effective combination regimen, which is promising for clinical translation.

**Acknowledgements**

We are very grateful to the technical help of Jing Zhang from the Department of Ultrasound in Tangdu Hospital, Fourth Military Medical University.

*Ethics approval and consent to participate:* All of the animal experiments were performed in accordance with the protocols of Animal Experimentation and Ethics Committee of Fourth Military Medical University.

*Consent for publication:* Not applicable.
Acknowledgments

We thank... (2014). The role of heat shock protein (hsp70) in dendritic cell maturation: hsp70 induces the maturation of immature dendritic cells but reduces DC differentiation from monocyte precursors. Eur J Immunol 31:1602–9.

Kwon ED, Drake CG, Scher HI, et al. (2014). Ipilimumab versus placebo after radiotherapy in patients with metastatic castration-resistant prostate cancer that had progressed after docetaxel chemotherapy (CA184-043): a multicentre, randomised, double-blind, phase 3 trial. Lancet Oncol 15:700–12.

Li Q, Zhao Z, Qin X, et al. (2021). A checkpoint-regulatable immune niche created by injectable hydrogel for tumor therapy. Adv Funct Mater 31:2104630.

McHale AP, Callan JF, Nomikou N, et al. (2016). Sonodynamic therapy: concept, mechanism and application to cancer treatment. Adv Exp Med Biol 880:429–50.

Michaelis KA, Norgard MA, Zhu X, et al. (2019). The TLR7/8 agonist R848 remodels tumor and host responses to promote survival in pancreatic cancer. Nat Commun 10:4682.

Nezhadi S, Saadat E, Handali S, et al. (2021). Nanomedicine and chemo-therapeutics drug delivery: challenges and opportunities. J Drug Target 29:185–98.

Paardekooper LM, Vos W, van den Bogaart G. (2019). Oxygen in the tumor microenvironment: effects on DC function. Oncotarget 10:883–96.

Pathria P, Louis TL, Varner JA. (2019). Targeting tumor-associated macrophages in cancer. Trends Immunol 40:310–27.

Patil SM, Sawant SS, Kunda NK. (2020). Exosomes as drug delivery systems: a brief overview and progress update. Eur J Pharm Biopharm 154:259–69.

Peng Y, Jia L, Wang S, et al. (2018). Sonodynamic therapy improves anti-tumor immune effect by increasing the infiltration of CD8+ T cells and altering tumor blood vessels in murine B16F10 melanoma xenograft. Oncol Rep 40:2163–70.

Pfirschke C, Siwicki M, Liao H-W, et al. (2017). Tumor microenvironment: no effector T cells without dendritic cells. Cancer Cell 31:614–5.

Poh AR, Ernst M. (2018). Targeting macrophages in cancer: from bench to bedside. Front Oncol 8:49.

Pullan JE, Confiel MJ, Osborn JK, et al. (2019). Exosomes as drug carriers for cancer therapy. Mol Pharm 16:1789–98.

Runcie KD, Dallos MC. (2021). Prostate cancer immunotherapy—finally in from the cold? Curr Oncol Rep 23:88.

Schmid D, Park CG, Hartl CA, et al. (2017). T cell-targeting nanoparticles focus delivery of immunochemistry to improve antitumor immunity. Nat Commun 8:1747.

Siegel RL, Miller KD, Fuchs HE, et al. (2021). Cancer statistics, 2021. CA Cancer J Clin 71:7–33.

Stultz J, Fong L. (2021). How to turn up the heat on the cold immune microenvironment of metastatic prostate cancer. Prostate Cancer Prostatic Dis 24:697–717.

Tormoen GW, Crittenden MR, Gough MJ. (2018). Role of the immunosuppressive microenvironment in immunotherapy. Adv Radiat Oncol 3:520–6.

Wculek SK, Cueto FJ, Mujal AM, et al. (2020). Dendritic cells in cancer immunology and immunotherapy. Nat Rev Immunol 20:27–9.

You B, Xu W, Zhang B. (2018). Engineering exosomes: a new direction for anticancer treatment. Am J Cancer Res 8:1332–42.

Zhou SX, Sun L. (2015). Immune effects of R848: evidences that suggest an essential role of TLR7/8-induced, Mydd88- and NF-kB-dependent signaling in the antiviral immunity of Japanese flounder (Paralichthys olivaceus). Dev Comp Immunol 49:113–20.

Zhu H, Fang X, Zhang D, et al. (2016). Membrane-bound heat shock proteins facilitate the uptake of dying cells and cross-presentation of cellular antigen. Apoptosis 21:96–109.

Zininga T, Ramatsui L, Shonhai A. (2018). Heat shock proteins as immunomodulators. Molecules 23:2846.