In situ injectable hydrogel-loaded drugs induce anti-tumor immune responses in melanoma immunochemotherapy

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ABSTRACT

Melanoma is a highly aggressive tumor located in the skin, with limited traditional therapies. In order to reduce the side effects caused by traditional administration method and amplify the killing effect of immune system against tumor cells, an in situ injectable hydrogel drug delivery system is developed for the first time which co-delivers doxorubicin (Dox) and imiquimod (R837) for the synergistic therapy of melanoma. The mechanical properties and stability of the hydrogel are characterized and the optimal doses of hydrogel and drugs are also identified. As a result, the co-delivery system effectively suppresses melanoma growth and metastatic progression both in vitro and in vivo. Further studies show that the co-delivery system causes immunogenic cell death, activation of antigen presenting cells, comprising dendritic cells and M1 macrophages, and secretion of related cytokines consisted of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), subsequently with the activation of T lymphocytes and natural killer cells in spleen and tumor area. The co-delivery system also decreases the suppressive immune responses, including infiltration of M2 macrophages and secretion of interleukin-10 (IL-10), in vivo. Besides, other death modes are induced by the co-delivery system, including apoptosis and non-apoptotic cell death. In a word, this co-delivery system induces melanoma cell death directly and activates immune system for further tumor killing simultaneously, which shows probability for precise targeted tumor therapy.

1. Introduction

Melanoma, a malignant tumor derived from melanocytes, is called "cancer that rises with the sun" [1]. Among various skin tumors, melanoma is the most aggressive form, accounting for more than 80% of the deaths related to skin cancers [2]. According to the statistics collected from the American Cancer Society, there were approximately 91,270 new diagnosed melanoma cases and 9,320 new death melanoma cases in 2018 [3]. What's more, latest reports from the World Health Organization showed that there were 288,000 new melanoma cases and 61,000 melanoma deaths happening every year all over the world [4].

In the early stage of melanoma, the success of surgery is the major reason for the high survival rate, while the survival rate is significantly reduced after metastasis. And for the patients with unresectable advanced or metastatic melanoma, current treatment mainly focused on the chemotherapy, systemic immunotherapy, and molecular targeted therapy [5]. However, systemic chemotherapy had very limited efficacy for the treatment of melanoma because of the serious side effects [6].

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Fortunately, studies have found that certain chemotherapy drugs, including doxorubicin (Dox), cyclophosphamide, epirubicin, idarubicin, mitoxantrone, and oxaliplatin, induced immunogenic death of cancer cells in the therapy for various tumors [7]. In our study, an in situ injectable hydrogel drug delivery system was applied, which delivered Dox to explore the anti-cancer effect.

Immunogenic cell death (ICD) refers to the activation of the immune system through the induction of damage-associated molecular patterns (DAMPs) [8]. The ICD-inducing DAMPs, which were comprised of calreticulin (CRT), high mobility group box 1 (HMGB1), adenosine S'-triphosphate (ATP), heat shock protein (HSP)-70 and HSP-90, stimulated the maturation of antigen presenting cells, including dendritic cells and macrophages, to promote their phagocytosis of dying cancer cells [9]. Tumor specific CD8+ cytotoxic T lymphocytes (CTL) were then activated through the antigen presentation from the antigen presenting cells, which led to the infiltration of CTLs into the tumor site [10]. The functionality of NK cells is enhanced by cytokines produced by activated DCs or other innate immune cells [11]. Moreover, studies confirmed that chemotherapeutic drugs, which could induce the ICD of cancer cells, have already become a novel type of treatment for melanoma [12]. In order to further amplify the immune response induced by the chemotherapeutic drug, we added Imiquimod (R837), which is an immune adjuvant activating toll-like receptors (TLR) and nuclear factor-kappa B (NF-kB), in the co-delivery system with Dox [13].

Compared with the traditional chemotherapy, local drug delivery could not only reduce the side effects caused by systemic chemotherapy, but continuously release the chemotherapeutic drugs at the tumor site [14]. As an emerging polymer material, hydrogel, whose cross-linked hydrophilic polymer chains formed three-dimensional networks, is an extremely versatile drug delivery vehicle for small molecules and biomacromolecules [15]. The hydrogel has been widely used in pharmaceutical applications to control the release of drugs and provide stability for the loaded protein drugs [16]. Furthermore, the in situ injectable hydrogel has a wide range of biomedical applications such as cell encapsulation, drug delivery, cancer treatment and wound dressing [17].

Two kinds of hydrogels have been used for the sustained release of Dox and R837: Zhang et al. developed a thermal-sensitive hydrogel co-delivering Dox and R837 as an therapeutic tumor vaccine [18] and Yan et al. found one matrix metalloproteinase sensitive hydrogel delivering Dox and R837 as an therapeutic tumor vaccine [18]. For the in situ hydrogel injection, we prepared them in the same way as noted above.

2. Materials and methods

2.1. Materials and cell culture

The four-arm polyethylene glycol thiol (PEGSH, ≥95%, MW = 5000) was purchased from Huateng Pharmaceutical (Changsha, China) and stored at −20 °C. And poly (ethylene glycol) diacyrlate (PEGDA) and possessed an uncomplicated preparation procedure. After in situ gelation, Dox and R837 were continuously released to trigger the immunogenic cell death in vitro and in vivo thus increasing the anti-tumor immune responses, which has great application potential in the tumor treatment.

2.2. Antibodies and reagents

Primary antibodies against LC3, Poly-ADP-ribose polymerase-1 (PARP-1), CD161c (NK1.1) were purchased from Cell Signaling Technology (Boston, Massachusetts, USA); N-cadherin, E-cadherin, Vimentin, P62, CRT, HSP-70, HSP-90, GPX-4, Ki67, PD-L1, Bax and β-actin were purchased from Proteintech (Wuhan, China); poly-ADP ribose (PAR) and Anti-cleaved N-terminal Gasdermin-D (N-GSDMD) were purchased from Abcam (Cambridge, UK); CD8a was purchased from Thermofisher (Waltham, Massachusetts, USA); CD4, CD11c, F4/80, CD86 and CD206 were purchased from CUSABIO (Houston, USA). Horseradish peroxidase (HRP) conjugated anti-rabbit/mouse secondary antibodies were purchased from Abbkine (Wuhan, China) and the ECL-plus kit was from Advansa in USA. Cell Counting Kit-8 (CCK-8) was from 7 Sea Biotech (Shanghai, China).

2.3. Preparation of injectable hydrogel and drug delivery system

The injectable hydrogel was obtained by a Michael addition reaction between PEGSH and PEGDA. For the preparation of 100 μL hydrogel (20 wt%), 15.625 mg PEGSH and 4.375 mg PEGDA (the mass ratio of PEGDA to PEGSH is 0.28 in the hydrogel) were first dissolved in 50 μL PBS (phosphate buffered saline, PH = 8.0, 10 min of nitrogen ventilation before use to remove the dissolved oxygen), respectively. Then, the two solutions were mixed thoroughly and we confirmed the gelation by the tube inversion method after 5 min.

To establish the in vitro drug delivery system, sterilized drug-coated hydrogel, which had been placed at 37 °C for 24 h after gelation, was immersed in the culture medium at 37 °C for 24 h and then the immersed extract was acquired and directly used to culture the melanoma cells. The concentrations of hydrogel indicate the ratio of immersed hydrogel volume versus culture medium volume. For the preparation of drug-coated hydrogel, we first dissolved 5.7998 mg Dox with 1 mL PBS to get a 10 mM Dox solution and dissolved 3 mg R837 with 1 mL DMSO (dimethyl sulfoxide) to get a 3 mg/mL R837 solution. To get 2 mL 5% hydrogel extract containing 1μM Dox and 10 μg/mL R837, 0.2 μL 10 mM Dox solution and 6.67 μL 3 mg/mL R837 solution were added in 50 μL PEGDA solution (without solid precipitation). After mixing the solution thoroughly, 50 μL PEGSH solution was added in the solution to make the hydrogel. For the different concentrations of hydrogel and coated drugs, we prepared them in the same way as noted above.

For the in situ hydrogel injection in vivo, we first mixed the drug-coated PEGDA solution and PEGSH solution in an EP tube thoroughly and then rapidly suctioned the mixture into the syringe for the quick subcutaneous injection nearby the tumor site in 1 min to avoid gelation.

2.4. Rheological properties

A rotational rheometer (Anton Paar, MCR-92, Austria) was used for rheological performance testing, and 5 wt%, 10 wt%, 20 wt%, 30 wt% and 40 wt% hydrogel was used for the testing. The storage modulus (G’) and loss modulus (G″) of the hydrogel were observed by changing the angular frequency. In addition, the results of time sweep (0–10 min, 1 Hz) and frequency sweep (1–100 rad/s, strain of 1%) were collected at 37 °C.
2.6. Release test of PEGDA and PEGSH after gelation in vitro

After gelation for 24 h, 1 mL hydrogel was immersed in the EP tube with 10 mL PBS. Then the swelling ratios (Q) at the indicated time points were determined gravimetrically as the following:

\[ Q = \frac{(m_w - m_d)}{m_d} \times 100 \]

where \( m_w \) is the weight of the swollen hydrogel, and \( m_d \) is the weight of the hydrogel immediately after gelation. Measurements of the swelling characterization were performed in triplicate.

2.6. Release test of PEGDA and PEGSH after gelation in vitro

Different concentrations of the hydrogel (1%, 3%, 5%, 7%, 9%) were immersed in the EP tube with 2 mL PBS. Then they were incubated at 37 °C and we obtained the efflux (10 μL) at different time points (1 h, 2 h, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h, 48 h). Equal volume of fresh PBS was added into the EP tube at the same time. The release of PEGDA and PEGSH was then detected using an ultraviolet–visible spectrophotometer (Thermo-Fisher Scientific, Carlsbad, CA, USA) at 250 nm and 380 nm, respectively.

2.7. Release test of Dox and R837 in vitro

The hydrogel of different doses (1%, 3%, 5%, 7%, 9%) coated with Dox (0.5 μM, 1 μM, 2 μM, 4 μM) and R837 (1 μg/mL, 10 μg/mL, 20 μg/mL, 40 μg/mL) was immersed in EP tubes containing 2 mL PBS. During continuous incubation at 37 °C, the efflux (10 μL) was obtained at the indicated time points (1 h, 2 h, 3 h, 6 h, 12 h, 18 h, 24 h, 36 h, 48 h, 3 d, 4 d and 8 d) and we added the equal volume of fresh PBS into the EP tube at the same time. The release of Dox and R837 was then detected using the ultraviolet–visible spectrophotometer at 480 nm and 235.5 nm, respectively.

2.8. In vitro cytotoxicity assay of hydrogel, Dox and R837

The cytotoxicity of the hydrogel, Dox and R837 was evaluated by the CCK-8 assay on B16F10 cells. The B16F10 cells were seeded on a 96-well cell culture plate at a density of 5 × 10^4 cells per well and incubated overnight at 37 °C. To analyze the cytotoxicity of the hydrogel, culture medium in the 96-well cell culture plate was replaced by different concentrations of the hydrogel extract (1%–10%) and the wells only replaced by the culture medium were served as the control group. Cell viability was assessed by CCK-8 assay at 24 h and 48 h according to the manufacturer’s instructions. And for Dox and R837, the medium was replaced by the medium with different concentrations of Dox and R837. Then, CCK-8 assay was performed to assess the cytotoxicity at 24 h. To estimate the viability of the cells, the absorbance of 450 nm (OD450) was measured with a 96-well plate reader (DG5032, Hua Dong, Nanjing, China).

2.9. Cell viability and apoptosis assays of different groups

B16F10 cells and HaCaT cells were cultured in 96-well cell culture plates at a density of 1 × 10^4 cells/well for 24 h. Then, the medium in the 96-well plates was replaced by the culture medium with different treatments. Cell viability was assessed by CCK-8 assay at 1 d, 2 d, 3 d and 4 d post-treatment according to the manufacturer’s instructions. The 96-well plate reader (DG5032, Hua Dong, Nanjing, China) was used to measure the viability of the cells at the absorbance of 450 nm (OD450).

For colony formation assays, B16F10 cells at a density of 1 × 10^3 cells/well were plated into 6-well plates after different treatments. And 14 days after plating, colonies were visualized by crystal violet staining.

And for the analysis of apoptosis by Hoechst33258 (Invitrogen), different groups of B16F10 cells were cultured on the coverslip of a chamber. The chamber was then rinsed with PBS for 3 times, and we added 500 μL of RPMI-1640 culture media containing 5 μg Hoechst33258 in the chamber. After incubating at 37 °C for 15 min, the apoptosis of different groups was detected through microscopic visualization of condensed chromatin and micronucleation.

2.10. Western blotting

Protein samples from cells and tissues were extracted by RIPA buffer (10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140 mM NaCl). Equivalent protein samples (30 μg protein extract was loaded on each lane) were subjected to SDS-PAGE gels. The proteins were then transferred onto PVDF membranes (Millipore, Massachusetts, USA) and blocked with TBST containing 5% BSA for 1 h at room temperature. The membranes, probed with the indicated primary antibodies, were incubated at 4 °C overnight. Secondary antibodies were bound with the primary antibodies for 1 h at room temperature and we detected the lanes by ECL plus kit (NCM biotech, Suzhou, China). Detection was performed using the Odyssey infrared imaging system (LI-COR, Lincoln, USA). The gray values of the Western blotting lanes were measured by Image J (http://rsb.info.nih.gov/ij/). The relative protein expression levels were calculated by the gray values of the indicated lanes versus the gray values of β-actin lanes.

2.11. Cell metastasis assays of different groups

For the cell migration assay, B16F10 cells with different treatments were suspended and seeded in the transwell chambers (Corning, NY, USA) with 8-μm pore membranes at the density of 2 × 10^5 cells/chamber. And after culturing at 37 °C for 24 h, the migrated cells were fixed by 4% paraformaldehyde and the cells on the upper side of the membrane were removed by wiping. Then we washed the membrane by PBS before it was stained by crystal violet. Images of the cells were obtained from the optical microscope.

For the wound healing assay, B16F10 cells were suspended and seeded in a 6-well plate at the cell density of 1 × 10^5 cells/well. We then scratched the wound by a 10 μL plastic pipette tip after the cells growing more than 90% confluence. The width of the wound was recorded and photographed at 0 h and 24 h. The wound area was measured using Image J and the migration rate (%) was calculated by the ratio between the wound area difference of the two time and the wound area of the initial time.

2.12. Extracellular ATP and HMGB1 detections

After different treatment for 24 h, the culture medium was collected for the detection of ATP and HMGB1 release. Extracellular ATP release following the indicated treatments was measured by an ATP Detection Kit (Solarbio, Beijing, China) according to the manufacturer’s instruction. The levels of HMGB1 (high mobility box 1, 4 A Biotech, Beijing, China) in the culture medium were analyzed by the enzyme-linked immune sorbent assay (ELISA) kits according to the manufacturer's protocols.

2.13. Co-culturing between dendritic cells and melanoma cell extract and analysis of dendritic cells maturation by flow cytometry

The B16F10 cells with different treatments were suspended at the density of 1 × 10^5 cells/mL and washed by PBS for 2 times. Then, we extracted the proteins and cell fragments of B16F10 cells by using the Ultrasonic Cell Disrupter System. The doses of these extracts were measured by Bradford method. We added the extract (100 μg/mL) into the culture medium of DC2.4 cells to induce their mature. And 18 h after inducing, we suspended the DC2.4 cells in PBS containing 0.1% BSA at the density of 1 × 10^5 cells/mL. 1 μL anti-CD86-APC (Biolegend, San Diego, CA, USA) was added in 100 μL of the cell suspension to stain the B16F10 cells after being blocked with 1 μL CD16/32 antibody.
(BioLegend, San Diego, CA, USA). We then measured the maturation of dendritic cells by detecting the CD86 positive cell ratio through flow cytometry following the manufacturer's protocols.

2.14. Annexin V-FITC/PI apoptosis staining and detection

The B16F10 cells treated with PBS, Dox (1 μM) and Dox-coated hydrogel with or without R837 (10 μg/mL) were suspended in 1 × 10⁵ cells/mL, and 5 μL Annexin V (Vazyme, Nanjing, China) and 5 μL propidium iodide staining solution (Vazyme, Nanjing, China) were added to 100 μL of the cell suspension. 400 μL binding buffer was next added to the cell suspension. After incubated at room temperature for 10 min in the dark, the stained cells were assayed and quantified using a FACSort Flow Cytometer (Beckman Coulter, Brea, CA, USA). Cell debris was excluded from the analysis by an appropriate forward light scatter threshold setting. What's more, compensation was used wherever necessary.

2.15. In vivo experiments design

All animal experiments were carried out followed the protocols approved by Hunan University Animal Use and Care Committee (Changsha, Hunan, China; SYXK2018-0006) and the animal procedures were conducted under the Guidance for the Operation of Animals (Scientific Procedures) Act (1986). Female C57BL/6 mice (Hunan SJA Laboratory Animal, Changsha, China), 6-8 weeks old, were housed in a sterile environment with micro isolator cages and allowed free access to water and food. B16F10 cells (5 × 10⁵ cells in 0.1 mL PBS solution) were subcutaneously injected into the left flank (the primary tumor) and right flank (the distant tumor) of each mouse to build bilateral melanoma tumor model. Then the mice were randomly divided into 4 groups (n = 6 for each group) when the tumor volumes reached 50-100 mm³. Volume of the tumors was calculated as follows: volume = (tumor length × tumor width)²/2. Each mouse in the different groups were treated with PBS (100 μL) for the control group, PBS (100 μL) and Dox (5 mg/kg) for the Dox group, Dox (5 mg/kg)-coated hydrogel (100 μL) for the Gel + Dox group and Dox (5 mg/kg) with R837 (50 μg)-coated hydrogel (100 μL) for the Gel + Dox + R837 group by subcutaneous injection nearby the primary tumor sites at the first day of the treatment. What's more, for the further validation of the advantages of the sustained-released hydrogel on anti-tumor therapies, we performed another animal experiment with the treatment of PBS (100 μL) for the control group, Dox (5 mg/kg) and R837 (50 μg) for Dox + R837 group and Dox (5 mg/kg) with R837 (50 μg)-coated hydrogel (100 μL) for Gel + Dox + R837 group. After 7 and 14 days of the different treatments, the mice were sacrificed by CO₂ inhalation followed by cervical dislocation. The splenocytes for the co-culture with B16F10 cells and the extraction of the splenocytes, blood cells and tumor cells from mice for the flow cytometry were collected 7 days after the treatments; the tumors and tissues for the holistic analysis of the tumor size, tumor weight and tissue analysis for Western blotting assay and IHC analysis were collected 14 days after the treatments. Most immune responses and immune cells activation get to the highest level 1 week after the treatments in vivo [20-24], so we harvested the spleens and tumors and collected blood from mice 7 days after different treatments in order to analyze the authentic changes of the immune cells for the co-culture and flow cytometry analysis. And for the other analysis based on the tumor tissues, we harvested the tumors, hearts, livers and lungs after 14 days, when the differences of the tumors were significant, to maximize the effects of the treatments and immune responses in the tumor sites. Additionally, after obtaining and measuring the fresh tumors, one part of the tumor was used for paraffin slides, and the other part was quickly immersed in RPMI buffer for the later protein isolation for Western blotting analysis.

2.16. Release test of dox in vivo by HPLC

Blood at the time point of 1 and 14 days after the treatments were collected. After centrifuging the blood at 3000 rpm/minute for 10 min, the serum was drawn from the supernatants. 50 μL serum, 50 μL methanol and 0.1 g anhydrous sodium sulfate were mixed by vortex meter for 3 min and then placed in 4 °C for 1 h. The samples for loading in the High Performance Liquid Chromatography (HPLC) system were drawn from the supernatants of the mixture after centrifuging at 12,000 rpm/minute for 15 min. P320p High Pressure Constant Flow Pump (EliteHPLC, Dalian, China), UV230II UV-vis detector (EliteHPLC, Dalian, China) and Sino-Chrom ODS-BP column (250 mm × 4.6 mm, 5 μm, EliteHPLC, Dalian, China) were used for the HPLC system. The mobile phase was comprised of (A) water/(B) acetonitrile (organic and water phase both contained 0.1% formic acid), and the gradient elution program was as follows: 90% A (initial), 90-80% A (5-6 min), 80-20% A (6-30 min), 20-0% A (30-31 min), 0-90% A (38-39 min) and 90%A (46min). During the program, the constant flow rate was 1 mL/min and the injection volume was 40 μL. The wavelength for the detecting was 233 nm.

2.17. Cytokine detection

The blood samples of the mice were collected after 1, 3, 7 and 14 days of treatment. Then, the blood samples were centrifugated at 3000 rpm/minute after incubating at 37 °C for 1 h. The levels of IFN-γ (interferon-γ, 4 A Biotech, Beijing, China), TNF-α (tumor necrosis factor-α, 4 A Biotech, Beijing, China) and IL-10 (interleukin-10, 4 A Biotech, Beijing, China) in the serum were analyzed by the ELISA kits according to the manufacturer’s protocols.

2.18. Splenocytes co-culturing analysis

We harvested the spleens 7 days after the different treatments. After the spleens were isolated, we prepared single-cell suspensions by passing the suspensions through a cell strainer. B16F10 cells were seeded in 24-well plates and 96-well plates at cell densities of 1 × 10⁵ cells/well and 1 × 10⁴/well for 24 h, respectively. Then, splenocytes were added in the culture medium of B16F10 cells at the indicated ratio (5:1, 10:1 and 20:1) for the co-culturing. We measured the cell viability of different groups by CCK-8 assay after 24 h. And 14 days after the co-culturing, colonies were visualized by crystal violet staining and the culture medium with splenocytes in the culture plates were refreshed with new complete RPMI-1640 culture medium on the day 7 in order to avoid the nutrient depletion.

2.19. Analysis of different groups of T cells and dendritic cells

For the analysis of T cells and dendritic cells, spleens and tumors were harvested and blood was collected after 7 days of different treatments. We used the cell strainers to prepare the single-cell suspensions of spleens and tumors and centrifuged the blood after dilution with the equivalent volume of PBS and adding the Lymphocyte Separation Medium (Mitobio, Shanghai, China) to get suspensions of blood cells. These suspensions were diluted by PBS containing 0.1%BSA at the density of 1 × 10⁶ cells/mL and then 100 μL of the dilution was taken and blocked by 1 μL CD16/32 antibody. After drying with Zombie Aqua Fixable Viability kit (Biolegend, San Diego, CA, USA) for 15 min, 1 μL anti-CD45-FITC (Biolegend, San Diego, CA, USA), anti-CD11b-BV421 (Biolegend, San Diego, CA, USA), anti-CD11c-PE (Biolegend, San Diego, CA, USA), anti-CD86-BV605 (Biolegend, San Diego, CA, USA), anti-CD3-APC (Biolegend, San Diego, CA, USA), anti-CD4-PECy7 (Biolegend, San Diego, CA, USA) and anti-IFN-γ-APC (Biolegend, San Diego, CA, USA) were added in the suspensions. The CD4 positive, CD8a positive and CD11c positive cell ratio of the blood cells, splenocytes and tumor cells, IFN-γ positive cell ratio of CD8a positive cells from tumors, and CD86 positive cell ratio of dendritic cells from blood, spleens and tumors were detected by flow cytometry according to the manufacturer's protocols.
2.2. H&E staining and immunohistochemistry

For immunohistochemistry (IHC), the organs and tumors were fastened in 4% paraformaldehyde for 24 h. After dehydrated in a graded series of ethanol and embedded in paraffin, the tumor tissues were sectioned by LEICA system according to standard protocols. Tumor sections (3 μm) were deparaffinized and stained with Hematoxylin and Eosin (H&E). For IHC, slides were retrieved by EDTA-Citrate Antigen Retrieval Solution (Beyotime Technology, China). Briefly, 1% hydrogen peroxide was used to block the endogenous peroxidase activity. The slides were incubated with primary antibodies anti-Ki67 (1:5000), anti-N-cadherin (1:1000), anti-C8a (1:200), anti-CD4 (1:500), anti-CD11c (1:200), anti-CD64 (anti-NK1.1, 1:150), anti-F4/80 (1:200), anti-CD86 (1:200) and anti-CD206 (1:100) overnight at 4 °C and then incubated with secondary antibodies conjugated to peroxidase-labelled dextran polymer according to the manufacturer’s instructions. A light microscope was used to observe the slides. All IHC results were read blindly by two independent researchers.

2.2.1. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad, San Diego, CA, USA). All assays were repeated independently more than three times. Data were represented as means ± standard error of mean (SEM) in the figures. P values were calculated using the Student’s paired t-test.

3. Results

3.1. Hydrogel design, material characteristics and drug release property

Herein, by mixing the solution of PEGDA and PEGSH, we prepared an injectable hydrogel (PEGDA-PEGSH) to serve as a carrier for the targeted therapy. Fig. 1A shows the three-dimensional network structure of the hydrogel. Within the hydrogel, one PEGSH molecule has 4 sulfhydryl groups (Fig. 1B) and one PEGDA molecule contains two carbon-carbon double bonds (Fig. 1C), between which a Michael addition reaction occurs. The conditions of the gelation reaction are mild and rapid, gelling quickly in situ and will be degraded slowly in mice (Fig. S1A-F). The hydrogel could maintain its existence for about 2 weeks subcutaneously in vivo (Fig. S1E-F). And in vitro, when the two solutions were mixed together at room temperature, they changed from liquid to solid within a few minutes (Fig. 1D).

In addition, the rheological properties during gel formation were also measured. The rheological properties of 5 wt%, 10 wt%, 20 wt%, 30 wt% and 40 wt% hydrogels were measured in the time sweep mode by the rotational rheometer (Fig. 1E-I). We found that when the two solutions were mixed together for 1 min, the elastic modulus (G\textsuperscript{′}) was equal to the viscous modulus (G\textsuperscript{″}), which proved the formation of the 20 wt% hydrogels (Fig. 1G). And 9 min after hydrogel formation, the elastic modulus increased constantly to a high level (~2600 Pa) which meant the hydrogel became extremely stable (Fig. 1G). We also found that 5 wt% and 10 wt% hydrogels did not gel in 10 min (Fig. 1E-F), while 30 wt% and 40 wt% hydrogels gelled so fast that the gelation had been finished at the beginning of the measurement (Fig. 1H-I). These results indicated that 20 wt% hydrogels with gelation time of 1 min was appropriate, which not only possessed a fast gelation speed but also provided suitable time for the in situ hydrogel injection. By the contrast, 5 wt% and 10 wt% hydrogels had slow gelation speed, which was detrimental to the in situ gelation in vivo. 30 wt% and 40 wt% hydrogels gelled too fast to perform the subcutaneous injection. Therefore, considering the injection operation, cost and potential unknown toxicity of PEGSH, we chose 20 wt% hydrogel for subsequent experiments. What’s more, we also tested the variation of storage modulus (G\textsuperscript{′}) and loss modulus (G\textsuperscript{″}) of hydrogel with frequency. As the frequency changed, the storage modulus (G\textsuperscript{′}) changed very little, indicating that the gel changed...
from the liquid status to the solid status and had good toughness (Fig. 1J). Swelling characterization is also of great significance for the further use of the hydrogel. Thus, we measured the swelling behavior of the hydrogel to learn about the physical properties. 6 days after the measurement began, swelling ratios got to ~230% and kept the level until ~12 days (Fig. 1K), which began to decrease at ~14 days, meaning that the degradation of the hydrogels started at that time (Fig. 1K).

Moreover, the release profile of PEGDA and PEGSH after gelation was examined by the UV–vis spectrometry and the result showed that there were very low doses of PEGDA and PEGSH released from the hydrogel they composed (Fig.S2A-B). Next, we examined the release profile of different concentrations of Dox (Fig.1L, Fig.S2C-F, K) and R837 (Fig.1M, Fig.S2G-J) in different dose of hydrogels. As the concentrations of Dox and R837 increased, the release rate also increased. About 60% of Dox and R837 were released in 2 days. The release rate decreased afterwards, and almost all Dox and R837 in the hydrogels were released in about 4 days. What’s more, different doses of drugs or hydrogels showed no significant difference for the release profiles (Fig. 1L-M, Fig.S2C-F, K). The results indicated that the hydrogel possessed a good drug release rate and a capability of sustained release. And for assessing the biodistribution of the drugs released from the hydrogel in vivo, HPLC was used to measuring the Dox content in blood serum, which was collected at 1 and 14 days after the treatments. Chromatograms of the sample made of methanol and 20 μg Dox (Fig.S3A), the blank serum sample (Fig.S3B) and the blank serum sample with 20 μg Dox (Fig.S3C) showed the chromatographic peak of Dox, which served as the reference chromatographic peak of Dox for the further analyzing of the different groups. The results showed that Dox was not detected in mice blood of all the groups (Fig.S3D-K), which might be caused by the functioning of Dox in the tumor sites, metabolism by tumor cells and short terminal half-life in blood [25]. However, the low concentration of Dox in the blood circulation means the less side effects caused by Dox compared with that of the oral or intravenous administration, which will be conductive to reducing the side effects caused by systemic chemotherapy.

3.2. The co-delivery system suppressed melanoma cell growth and induced cell death

In order to determine the optimal concentration of the chemotherapeutic drug Dox loaded by the hydrogel in this study, cell viability of B16F10 cells was detected by CCK-8 assay after administration of 0.25–4 μM Dox for 1 day (Fig. 2A). As a result, treated cells showed a decrease in viability with increasing Dox doses. When the dose of Dox got to and exceeded 1 μM, the cell viability reached the minimum level. Thus, we chose 1 μM as the optimal concentration for the further study, which has been verified by other studies of melanomas [26,27]. And for Imiquimod, cell viability was measured at 1 day after 1.25–20 μg/mL R837 treatment (Fig. 2B). The results showed that cell viability did not change obviously after treatment of multiple doses of R837, compared with that of the control group (P > 0.05, Fig. 2B). Based on our results and other studies about R837, 10 μg/mL was chosen as the optimal dose [28,29]. For the hydrogel, CCK-8 assay was performed to analyze the cytotoxicity of different hydrogel doses (1%–10%) to evaluate its cytocompatibility (Fig. 2C). And the results indicated that cell viability had a little decrease when 6% hydrogel was used, and this decrease would be continuous when higher concentrations (more than 6%) of hydrogel were used (Fig. 2C). However, when the concentration of hydrogel was lower than 6%, there was no toxicity to cells at all. Considering our demands of enough volume to coat the drugs and good biocompatibility, we eventually chose 5% hydrogel to perform the further experiments in vitro.

To explore the effect of co-delivery system on tumor cell growth, colony formation experiments were performed, the results showed that co-delivery of Dox and R837 significantly inhibited the colony formation compared with other groups (Fig. 2D). Additionally, B16F10 cells were stained with Hoechst33258 after different treatments, and micronucleation was observed (Fig. 2E). Moreover, proliferation of B16F10 cells and HaCaT cells was detected by CCK-8 assay after the same treatments (Fig. 2F-G). It is noteworthy that the cell viability of R837 or hydrogel treated group showed no obvious difference compared with that of the control group, while the cell viability of Dox, Dox/hydrogel and Dox/R837/hydrogel treated groups was significantly inhibited (Fig. 2F). The Dox treatment group showed more effective inhibitory functions than that of the hydrogel groups at the beginning, which indicated that drugs in the hydrogels were released continuously and the drug doses were lower in the culture medium than that of the Dox group at that time (Fig. 2F). What’s more, the results revealed that there was no significant difference among the groups in HaCaT cells (P > 0.05), which illustrated that the co-delivery system and drug treatment had no toxicity on the normal healthy cells (Fig. 2G).

3.3. The co-delivery system suppressed the metastatic progression in vitro

To explore whether the co-delivery system inhibited the metastatic progression in vitro, the wound healing assay was firstly performed (Fig. 3A). The results revealed that all the treatment groups showed reduced migration areas after 24 h compared to the control group (Fig. 3A) and the Dox/R837/hydrogel group had the lowest migration rate (Fig. 3A and B). In addition, a transwell assay was also applied to analyze the migration of B16F10 cells with different treatments, which showed that the Dox/R837/hydrogel co-delivering system markedly reduced the number of migrated cells (Fig. 3C and D). Moreover, Western blotting analysis of the metastasis and epithelial-mesenchymal transition (EMT) markers was performed after different treatments (Fig. 3E). The epithelial cadherin (E-cadherin) is a growth and invasion suppressor and blocks the tumorigenesis and tumor dissemination by stimulating tissue organization, promoting the biophysical adhesion processes and inhibiting apoptosis [30]. Expression of E-cadherin was found to increase after treatments and the Dox/R837/hydrogel co-delivering treated group showed the highest expression level among them (Fig. 3E and F), which indicated that the Dox/R837/hydrogel co-delivering system had the strongest invasion inhibiting effect. And neuronal cadherin (N-cadherin) is a key adhesion protein in synaptogenesis and replaces E-cadherin during the EMT process thus contributing to tumor progression and metastasis [31]. The expression level of N-cadherin significantly decreased after different treatments especially in the Dox/hydrogel and Dox/R837/hydrogel co-delivering groups (Fig. 3E and G). Vimentin, one intermediate filament protein in normal mesenchymal cells, is overexpressed in multiple epithelial tumors like melanomas and is served as a marker for tumor growth and metastasis [32]. Our results revealed that the expression level of Vimentin markedly decreased after the treatments (Fig. 3E and H), which represented that the metastasis progression of B16F10 cells was repressed. Taken together, these results proved that Dox/R837/hydrogel co-delivering system effectively suppressed the EMT and metastatic progression in vitro.

3.4. The co-delivery system induced tumor immunogenic cell death and activated anti-tumor immune responses in vitro

Chemotherapeutic drugs induce tumor cells to release DAMPs, which exert a powerful immune effect when being combined with pattern recognition receptors expressed by immune cells, thus inducing the ICD [8]. Therefore, the release of DAMPs is closely relevant to the anti-tumor immune response. Among the various DAMPs, cell surface exposure of CRT, HSP-70 and HSP-90 and extracellular release of ATP and HMGB1 promote the tumor cells uptake by antigen presenting cells (APCs), thus starting the ICD pathways [33]. In the present study, after Dox/R837/hydrogel co-delivering treatment, the protein expression levels of HSP-70, HSP-90 and CRT increased significantly (Fig. 4A-D) and the extracellular release of ATP and HMGB1 was also the highest level (Fig. 4F-G), which revealed that the co-delivering treatment released multiple DAMPs to induce ICD. And for PD-L1, which is the hot-spot...
Fig. 2. Dox/R837/PEGDA-PEGSH suppressed melanoma cells growth and induced apoptosis.

(A) Cell viability was detected by CCK-8 assay after administration of 0.25–4 μM Dox for 24 h to determine the optimal concentration of Dox. (B) Cell viability was measured by CCK-8 assay after administration of 1.25–20 μg/mL R837 for 24 h to determine the optimal concentration of R837. (C) CCK-8 assay analyzed the cytotoxicity of the different concentrations of the hydrogel (1%–10%) after treatment for 24 h and 48 h to evaluate its cytocompatibility. (D) Colony formation experiment showed Dox/R837/PEGDA-PEGSH co-delivering treatment significantly inhibited colony formation compared with other groups. (E) B16F10 cells were stained with Hoechst33258 after grouping for different treatments. Scale bar = 100 μm. (F-G) The proliferation of B16F10 cells (F) and HaCaT cells (G) was detected by CCK-8 assay after different treatments at the time points of 1 day, 2 days, 3 days and 4 days. Data are present as means ± SEM (n = 5). ****P < 0.0001.
immunoinhibitory molecule for tumor immunotherapy nowadays [34], our results showed that the Dox/R837/hydrogel co-delivering suppressed the expression of PD-L1 thus stimulating the activation of T cells (Fig. 4A and E), which would be conducive to the studies of immunotherapy in the further research.

Maturation of dendritic cells after the antigen stimulation is also a fundamental segment in the ICD process [35]. CD86, whose enhanced expression marked the cell surface maturation of dendritic cells [36], was detected in dendritic cells by flow cytometry after the co-culture between B16F10 cells and DC2.4 cells. The results showed that the percentage of CD86 positive dendritic cells gradually increased in the groups with treatment, which proved the activation of antigen presenting cells (Fig. 4H–I). In a word, these results suggested that the co-delivering system could induce the DAMPs releasing from melanoma cells and activate dendritic cells thus stimulating the ICD progression.
3.5. The co-delivery system triggered multiple cell death modes including apoptosis and non-apoptotic death

BAX, a protein of the BCL-2 family, is a key executioner protein of the apoptotic pathway [37]. The results of Western blotting analysis showed that the expression level of BAX increased after treatments, especially in the Dox/R837/hydrogel co-delivering, which triggered apoptosis.

Considering whether the co-delivering system induces cell death only through apoptosis, non-apoptotic cell death status was also studied. As the golden markers for ferroptosis, parthanatos and pyroptosis respectively [38–40], the expression level of GPX-4, PAR/PARP-1 and N-GSDMD were detected in our present study. As shown in Fig. 5A and C–H, GPX-4 decreased evidently while PAR/PARP-1 and N-GSDMD increased markedly after treatments, and the most significant effect was observed in the Dox/R837/hydrogel co-delivering group. These results indicated that ferroptosis, parthanatos and pyroptosis were induced by...
Dox/R837/PEGDA-PEGSH not only triggered apoptosis, but also triggered non-apoptotic cell death methods.

(A) Western blotting analysis of BAX, GPX-4, PAR, PARP-1, N-GSDMD, P62, and LC-3 expression in B16F10 cells after the different treatment for 24 h. (B-H) Relative protein expression levels of BAX (B), GPX-4 (C), PAR (D), PARP-1 (E), N-GSDMD (F) and P62 (G) and LC-3II/I protein expression fold (H) in B16F10 cells after different treatment analyzed from Western blotting were shown in each bar. (I) Annexin V and PI positive cells rates of B16F10 cells after the different treatment were shown in each bar. (J) Flow cytometry analysis of Annexin V-FITC/PI staining of the B16F10 cells after different treatment. Data are present as means \( \pm SEM \) (n ≥ 3). Statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs the control group.
the co-delivering system, which might play a minor role in tumor growth suppression. In addition, LC3II/I and p62 were investigated simultaneously to estimate the status of autophagy. As a result, only the Dox/R837/hydrogel co-delivering treatment caused the decline of LC3II/I ratio and the rise of p62 expression (Fig.5A,5G-H), suggesting the inhibition of autophagy.

Moreover, the flow cytometry results of Annexin V-FITC/PI staining further proved the occurrence of apoptosis (Fig. 5J and 1). In the flow cytometry analysis, PI-positive is the marker for the regulated necrotic cells [41], including cells with necrosis, ferroptosis and parthanatos [42]. The rate of PI-positive only cells increased after the Dox/R837/hydrogel treatment (Fig. 5J,SSA), which also verified the results of Western blotting analysis (Fig.5A,5C-E): ferroptosis and parthanatos were induced by the Dox/R837/hydrogel co-delivering system. Taken together, the co-delivery system triggered not only apoptosis, but also non-apoptotic cell death modes.

3.6. Anti-tumor immune responses induced by the co-delivery system in spleen, blood and tumor tissues

In order to verify the systemic immune responses and therapeutic effects induced by injectable hydrogel-encapsulated drug and immune adjuvant on mouse melanoma, a bilateral melanoma tumor model was constructed and the hydrogel was injected in situ around the primary tumors. When the tumor volume reached 50–100 mm³, the mice were randomly divided into four groups followed by different treatments, blood cells, spleen cells and tumor cells were extracted 7 days after treatment, and the mice were euthanized after 14 days for the analysis of tumors (Fig. 6A). The extracted splenocytes were co-cultured with melanoma cells in a corresponding proportion, then stained with the crystal violet. The results showed that the splenocytes from the Dox/R837/hydrogel treated mice significantly inhibited the growth of melanoma cells, compared with that of the other groups at all proportions, indicating the activation of the immune system (Fig. 6B). Fig.6C and Fig.S1 showed a schematic diagram of the co-culture between splenocytes and melanoma cells in different ratios. Similarly, the CCK-8 assay revealed that the cell viability was the lowest in the co-delivering group, which proved the strengthened suppressing effect of splenocytes on melanoma cell growth (Fig. 6D–F).

What’s more, the flow cytometry analysis proved that after co-delivering treatment, CD8a positive cells in blood, spleens and tumors increased compared with that of other groups, which indicated that the infiltration of CTLs had higher proportions in blood, spleens and tumor tissues respectively (Fig. 6G,SSC-E). And through detecting the expression of IFN-γ, we found that the proportion of IFN-γ positive cells in CD8a positive cells also increased after different treatments, which confirmed the activation of CTLs in tumor tissues (Fig. 6H,SSB). The infiltration and activation of dendritic cells in blood, spleens and tumors tissues were also analyzed. The results showed that the ratios of CD11c positive cells were the highest among all the groups in blood, spleens and tumors (Fig. 6I), which indicated that the co-delivering system effectively increased the infiltration of dendritic cells and activated the ICD process in vivo. And for the analysis of activated dendritic cells, CD86 was used to serve as the maturation marker [36]. The results revealed that the ratio of CD86⁺ cells increased in the Dox/hydrogel and Dox/R837/hydrogel groups (Fig. 6J), which further demonstrated the activation of dendritic cells in vivo. The activation of immune cells in spleen, blood and tumor tissues showed the initiation of ICD induced by the co-delivery system in vivo.

3.7. Anti-tumor effects and systemic immune responses induced by the local chemoinmunotherapy in vivo

To demonstrate the abscopal therapeutic effects induced by the co-delivering treatment, a bilateral melanoma tumor model was constructed to evaluate the systemic anti-tumor immune responses. The results showed that comparing with Dox treated alone, Dox/hydrogel and Dox/R837/hydrogel treatments significantly inhibited the tumor growth (Fig. 7A), tumor weight (Fig. 7B) and tumor volume (Fig. 7C) of the primary tumors. Additionally, immune adjuvant R837 further strengthened the therapeutic effect, which confirmed that the immune system played a role in this process. And the same trends appeared in the distant tumors (Fig. 7D–F), which further demonstrated the therapeutic efficacy of the systemic anti-tumor immune responses induced by the co-delivering system. For the validation of the benefits for the sustained-released hydrogel on anti-tumor therapies, another animal experiment was performed with the treatment of control, Dox/R837 and Dox/R837/hydrogel. We found that Dox/R837/hydrogel co-delivering treatment significantly inhibited the tumor growth, tumor weight and tumor volume of the primary tumors (Fig.5A-C) and distant tumors (Fig.5D-F) than that of the Dox/R837 group, which indicated the advantages of the sustained-released hydrogel. Besides, there was no obvious weight loss or tissue injury observed in the mice after all the treatments (Fig.7H, Fig.5A-C and Fig.S10), indicating that the treatments based on the hydrogels were highly biocompatible in vivo.

Inflammation-related cytokines played essential roles in the anti-tumor immune responses by modulating cellular functions of the immune cells [43]. In this study, the concentrations of the cytokines, including TNF-α, IFN-γ, and IL-10, in the serum were quantitatively evaluated by ELISA at different time points after different treatments (Fig. 7I–K). TNF-α and IFN-γ are important proinflammatory cytokines in the anti-tumor immune responses, which induce the commitment, development and mature of multiple immune cells [44]. Compared with the control group, the concentrations of TNF-α (Fig. 7I) and IFN-γ (Fig. 7J) in serum had a significant increase in the treated groups, indicating the optimal efficiency of the treatments in triggering the anti-tumor immune responses. Additionally, R837 was found to elevate the anti-inflammatory cytokine IL-10, which would impair the priming of anti-tumor immune responses [45]. Hence, in this study, the level of IL-10 in serum was also measured. The results showed that the concentration of IL-10 decreased significantly in the serum of the Dox/hydrogel group (Fig. 7K), indicating the inhibitory effect of Dox on IL-10. However, the co-delivering treated group possessed the higher IL-10 levels in serum than that of the Dox treated only group, which were still lower than that of the control group (Fig. 7K). The result indicated that R837 redeemed the intense inhibitory effect of Dox on the anti-inflammatory cytokines, but this redeemed effect was not enough to recover the levels of anti-inflammatory cytokines to that of the control group, which could be applied to reduce the cardiotoxic effects caused by Dox [46,47]. To sum up, the present study found that the proinflammatory cytokines in serum were up-regulated by the co-delivering system, while the anti-inflammatory cytokine was down-regulated, which helped to learn about the systemic immune responses induced by the local chemoimmunotherapy in vivo.

To further explore the inhibitory effects of the co-delivering treatment, the immunohistochemical assays were performed. As a marker for tumor cell proliferation and growth broadly used in routine pathological investigation [48], the expression of Ki67 was found to be the minimum in the tissues of the co-delivering treated group (Fig. 7G, L), which indicated the maximum suppression of tumor growth. What’s more, N-cadherin expression showed a decreased tendency in the treated groups and reduced to the lowest level in the co-delivering treated group which represented the inhibition of metastatic progression by the co-delivering system in vivo.

3.8. Immune cells infiltration and immunogenic cell death activation in the tumor sites

To evaluate the types of immune cells infiltrated in the tumor sites induced by the co-delivering treatment, the markers of multiple immune cells were analyzed by immunohistochemistry assays. CD8a, the cell surface marker of the tumor-infiltrating CTLs, showed higher expression after co-delivering treatment, which confirmed the increased infiltration...
of CD8⁺ CTLs in the tumor tissues (Fig. 8A and G). CD4⁺ T lymphocytes relayed signals to activate the CD8⁺ CTLs in the priming of anti-tumor immune responses [49]. In the present study, the rate of CD4⁺ cells increased in the tumor areas after the co-delivering treatment, which indicated the increased infiltration of CD4⁺ T lymphocytes. As the marker of dendritic cells, CD11c exhibited enhanced expression levels in the groups with treatments and the co-delivering treated group possessed the highest rate of CD11c⁺ cells (Fig. 8A, L). Similar results were obtained for the CD161c⁺ cells, suggesting the activation of natural killer cells (Fig. 8A, M). Moreover, macrophages also play essential roles in the anti-tumor immune responses [50], and in melanoma, tumor sites are enriched with immune suppressive cells like M2 macrophages [51]. In the present study, to further explore the infiltration of M1 macrophages and M2 macrophages in the tumor sites, F4/80 (marker for all the mouse macrophages), CD86 (marker for the M1 macrophages) [52] and CD206 (marker for the M2 macrophages) [53] were analyzed. The results showed that the rates of F4/80⁺ cells and CD86⁺ cells raised in the co-delivering treated groups, while the rate of CD206⁺ cells decreased, especially in the Dox/hydrogel group (Fig. 8A and 8I-K), which indicated that M1 macrophages were activated and the infiltration of M2 macrophages decreased in the tumor sites after the Dox/hydrogel treatment. We also found that the rate of CD206⁺ cells in the co-delivering treated group showed no significant difference compared with that of the control group, which might due to the stimulation of R837 on M2 macrophages [54]. In a word, the analysis of infiltrated immune cells revealed that multiple types of immune promoted cells have been activated and...
infiltrated in the tumor sites after the co-delivering treatment, while the infiltration of immune suppressive M2 macrophages decreased. The same infiltration tendency of these immune cells was shown in the distant tumors (Fig. S11A-H), which demonstrated that the resident immune cells and effector immune cells in the distant tumors were activated by the local chemoimmunotherapy from the primary tumor site. Additionally, the expression levels of the DAMPs (HSP-70, HSP-90 and CRT) and PD-L1 were detected by Western blotting in the tumor tissues. And the results showed the occurrence of immunogenic cell death in vivo (Fig. 8B–F), which were consistent with that of the in vitro results. All these results indicated that the local chemoimmunotherapy we proposed could be used to treat melanoma and was a potential therapeutic strategy for future clinical application.

4. Discussion

Melanoma is a malignant tumor originating from melanocytes in the basal layer of the epidermis. It has the characteristics of high malignancy, early metastasis, poor prognosis, and high mortality [47]. Traditional methods of treating melanoma include surgery, radiotherapy and chemotherapy, but due to the toxicity of most chemotherapy drugs, many patients would have serious adverse reactions after the chemotherapy...
Fortunately, increasing studies have proved that the combination of chemotherapy and immunotherapy could be served as a new strategy for the treatment of solid tumors [56,57]. In addition, considering the systemic toxicity of chemotherapy, previous studies have used nanoparticles [58] and liposome formulations [56] to target the delivery of ICD-inducing drugs thus lessening the adverse reaction and improving the immunogenicity of ICD-inducing agents, aiming to achieve long-term clinical benefits. In our study, we used a biocompatible and sustained-release hydrogel based on PEGDA and PEGSH to deliver the chemotherapy drug Dox and the immune adjuvant R837 in situ in the treatment of melanoma, which could induce the ICD and inhibit the tumor growth in vitro and in vivo.

The use of injectable hydrogel for local treatment could overcome most disadvantages of the low soluble chemotherapeutic drugs, thereby reducing the amount of drugs required and increasing the amount reaching to the tumor site [17]. The properties for the hydrogel to function in melanomas require: 1) the hydrogel have sufficient mechanical strength to go through the capsule of melanomas [59]; 2) the hydrogel is biocompatible and does not cause adverse reactions or immune reactions when using; 3) the hydrogel can be dissolved or bio-degraded when releasing the coated drugs [60]; 4) the hydrogel should provide a release profile of slow and sustained release for the indicated period of administration time [61]. The PEGDA-PEGSH hydrogel developed by Yom-Tov et al. has been found to have a simple preparation procedure and stable physical properties, which might be used in drug delivery and regenerative medicine [62]. And to explore whether this hydrogel was suitable for biomedical applications, we performed a few experiments to verify its characterizations. In our study, the gel formation does not require light irradiation, organic solvents or cross-linking catalysts, it gels quickly at 37 °C in about 1 min (Fig. 1G) and the storage modulus (G’) got to and stabilized at ~2600Pa in 10 min after mixing with a stable loss modulus (G’’) at ~27Pa (Fig. 1G). These results showed that the hydrogel we made was a soft material and possessed the matched mechanical properties with the tissues [63,64] and hydrogel with the similar stiffness could be served as a brilliant embedding carrier for the local delivery of chemotherapy drugs [65,66]. The hydrogel also possessed stable properties after being gelled (Fig. 1J), long stable period with suitable swelling properties (Fig. 1K), the ability of sustained releasing (Fig. 1L–M, S2C–K) and a good biocompatibility (Fig. 2C). The Michael-type addition reaction between the PEGDA and PEGSH is a rapid, simple and high efficient reaction [67]. We performed a release experiment, which made hydrogels by only mixing PEGDA and PEGSH together, and tested whether unreacted PEGDA or PEGSH existed in the PBS solution after gelation by using the UV–vis spectrophotometer.

Fig. 8. Immune cells infiltration and immunogenic cell death activation in the primary tumor sites. (A) IHC staining analyzed of CD8a (marker for CTLs), CD4 (marker for helper T cells), CD11c (marker for dendritic cells), CD161c (marker for NK cells), F4/80 (marker for macrophages), CD86 (marker for M1 macrophages) and CD206 (marker for M2 macrophages) from paraffin-embedded sections of the tumor tissues. Scale bar = 200 μm. (B) Western blotting analysis of HSP-70, HSP-90, CRT and PD-L1 expression in tumor tissues after the different treatment in vivo. (C-F) The protein levels of HSP-70 (C), HSP-90 (D), PD-L1 (E) and CRT (F) of the tumor tissues after different treatments were shown in each bar. (G-M) CD8a (G), CD4 (H), F4/80 (I), CD86 (J), CD206 (K), CD11c (L) and CD161c (M) positive cells rates of the tumor tissues after different treatments were shown in each bar. Data are present as means ± SEM (n ≥ 3). Statistical significance: *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 vs the control group.

[55]
And the results showed that PEGDA and PEGSH were detected in the PBS solution with small quantities essentially zero (Fig.S2A-B). Furthermore, the hydrogel degraded slowly in mice (Fig.S1). The factors which promote the degradation of the hydrogel are complex, including the hydrolysis of hydrogel [68], surface erosion [69] and the surrounding tissue environment [70]. A three-stage degradation mechanism proposed by Chen et al. could be used to satisfactorily explain the complicated and multifactorial degradation process of hydrogel in vivo [71]: in the first stage, surface erosion played the pivotal role; subsequently, the hydrolysis of the hydrogel appeared and began to participate in the degradation process together with surface erosion; in the last stage, the hydrolysis prevailed in the degradation process. And the dissolved polymers and their degradable wastes were mainly eliminated through the circulatory system and urinary system. Therefore, the hydrogel based on PEGDA and PEGSH is suitable as a carrier for sustained release of drugs in vivo.

Dox is a first-line chemotherapeutic agent for the therapy of melanoma [72] and also an effective ICD inducer [56]. There is a lot of evidence showing that Dox could cause ICD and the use of Dox to initiate immunotherapy seems to be a gratifying treatment [73]. ICD is characterized by DAMPs expressed on the surface of dying tumor cells, such as HMGB1, ATP, CRT, HSP-70 and HSP-90. The extracellular release of HMGB1 and ATP can attract and activate the APCs. Then, CRT, HSP-70 and HSP-90 on the surface of dying cells activate the “eat-me” of APCs [74]. As a topical immune response modifier against malignant skin tumors in clinical practice [75], Imiquimod (R837) is incorporated in various types of hydrogels to control the scarring process [76], treat skin diseases [77] and cure malignant tumors [78]. Therefore, to strengthen the immune response and ICD induced by the Dox/hydrogel delivery system, we added the immune adjuvant R837 into the delivery system. The co-delivery of Dox and Imiquimod was also investigated in other studies for the cancer therapies. For local administration, Zhang et al. developed a thermal-sensitive hydrogel co-delivering Dox and R837 as an in situ therapeutic tumor vaccine [18] and Yan et al. found one matrix metalloproteinase sensitive hydrogel co-delivering Dox and R837 for the therapy of metastatic breast cancer in situ [19]. And for the systematic administration, there was only one study focused on the polymer micelles, which delivered R837 and Dox to tumor-associated macrophages and tumor cells through intratumoral injection and intravenous injection, respectively [79]. However, our research differed from these studies in the following points: 1) we used a more efficient and biocompatible type of hydrogel; 2) we have explored more specific mechanisms of the immune activation and responses compared with them; 3) we are the first to detect the changes of cell death and autophagy status after the co-delivery, including the immune genetic cell death, apoptosis, ferroptosis and so on. In the present study, we found that after the co-delivering treatment, the expression levels of HSP-70, HSP-90 and CRT were the highest (Fig. 4A-D, 8 B-D, 8 F), with increased extracellular release of ATP and HMGB1 (Fig. 4F-G), which meant the highest ICD induction.

Additionally, various studies have reported that Dox could repress the development of metastasis in melanoma therapies [80,81]. And in the present study, we also found that the co-delivering treatment could effectively repress the metastatic progression both in vitro and in vivo (Fig. 3A-H, 7G, 7 M). It is traditionally believed that Dox can cause cell death by inducing apoptosis in melanoma [82]. In recent years, non-apoptotic cell death modes were found to be induced by Dox. Typical ferroptosis was found to appear in Dox-treated cardiomyocytes and target genes failed to prevent to rescue the ferroptosis caused by Dox [83]. Additionally, pyroptosis occurred in Dox-treated heart tissues. Through increasing the expressions of NOX1 (NADPH oxidases 1) and NOX4 and inducing mitochondrial fission by Drp1 (dynamin-related protein 1) activation, Dox induced pyroptosis in cardiomyocytes via caspase-1-dependent manner [84]. Moreover, Dox activated necroptosis together with apoptosis in doxorubicin induced cardiotoxicity [85]. In our study, we also found that Dox could induce non-apoptotic cell death modes while causing ICD, including pyroptosis, ferroptosis and parthanatos (Fig.5A, 5C-H). Among these non-apoptotic cell death modes induced by Dox in the present study, parthanatos was first found to be induced by Dox and the underlying mechanism will be explored in our future study. What’s more, based on our results, adding imiquimod to the Dox/hydrogel treated group produced some benefits. As shown in Fig. 5A-H, the expression levels of BAX, PAR and PARP-1 obviously further increased while GPX-4 markedly further decreased in the Dox/R837/hydrogel co-delivering group, compared with that in Dox/hydrogel group, which indicated that imiquimod further enhanced Dox/hydrogel-induced cell death, including apoptosis, parthanatos and ferroptosis. In parallel, the expression level of P62 further increased while the LC3II/I ratio further decreased in the Dox/R837/hydrogel co-delivering group, suggesting that Imiquimod further inhibited autophagy, which might also further promote cell death in the other way. In addition, these benefits further suppressed tumor cell growth in the Dox/R837/hydrogel co-delivering group in vitro (Fig. 2D). Furthermore, it was reported that Imiquimod, as a toll-like receptor (TLR)-7 and TLR-8 agonist, induced the secretion of pro-inflammatory cytokines by activating the transcription factor kappa-B (NF-kB) pathway [13], which might be speculated as the cause of higher cell death modes expression in the Dox/R837/hydrogel co-delivering group. The specific mechanisms of R837 in the cell death modes need further exploration.

The ICD-inducing DAMPs stimulated the maturation of antigen presenting cells, like dendritic cells and macrophages, which presented antigens to CTLs and secreted related cytokines thus strengthening the activation of NK cells [9,10]. Therefore, CTLs and NK cells bound to the targeted tumor cells and released granyme and perforin to cause the death of tumor cells, thus achieving the purpose of killing tumor cells [86]. And in our study, the splenocytes extracted after 7 days of treatments were co-cultured with tumor cells and the result showed a significant inhibitory effect on the growth of tumor cells (Fig. 6B-F), which proved the activation of the immune cells. Additionally, in the analysis of dendritic cells maturation induced by melanoma cells in vitro and in vivo, the dendritic cells were found to possess the highest level of maturation (Fig. 4H and -I, 6J, 5I-K) and tumor sites infiltration (Fig. 6L, 5H, 8A) after the Dox/R837/hydrogel co-delivering treatment. Moreover, the infiltration of M1 macrophages was also increased after the treatments (Fig. 8A and J). These results revealed the participation of antigen presenting cells in the ICD process after the co-delivering treatment. And through the immunohistochemical analysis and flow cytometry of tumor and spleen tissues, we found that a large amount of CTLs infiltrated in blood, tumor tissues and spleens and activated in tumor tissues after the co-delivering treatment (Fig. 6G-H, 8A, 8G, 5A-B, E). The immunohistochemical analysis also showed the enhanced infiltration of NK cells in the tumor sites (Fig. 8A, M). Besides, the immune suppressive cells also play essential regulatory roles in ICD [87] and in the present study, we found that the Dox/hydrogel treatment significantly repressed the activation of M2 macrophages, which are enriched in melanomas [88]. Furthermore, the release of cytokines from activated immune cells plays pivotal roles in the whole process of ICD [11]. Thus, to further support the ICD induced by the co-delivering treatment, we measured the levels of the pro-inflammatory cytokines, TNF-α and IFN-γ, and the level of the anti-inflammatory cytokine, IL-10. The results showed increased levels of TNF-α and IFN-γ and decreased level of IL-10 in the anti-tumor immune response (Fig. 7I-K). In a word, ICD could be effectively engendered by the Dox/R837/hydrogel treatment in the immunchemotherapy for melanoma.

Immunotherapy has become an effective clinical strategy for the treatment of cancer. PD-L1 is highly expressed on the surface of the tumor cells and is critical to the damage of T cells [89]. According to the former researches, blocking the interaction between PD-1 and its ligand PD-L1 could produce an impressive anti-tumor response [89]. Currently, immune-checkpoint inhibitors related to PD-1, PD-L1 and CTL-associated antigen 4 (CTLA4) have been approved for clinical use [90]. The relationships of PD-L1 and Dox, the commonly used chemotherapeutic drug which induces tumor cell apoptosis and promotes tumor antigen
release thus increasing the immunogenicity of tumor cells, have attract full concerns recently. On the one hand, some studies found that Dox could promote the expression of PD-L1 in tumor cells [91,92]; on the other hand, there are some studies showing the inhibitory effect of Dox on PD-L1 [93,94]. The relationships between them seem unclear. And in the present study, we found that the expression of PD-L1 on the surface of melanoma cells was reduced after the co-delivering system (Fig. 4A,E,8B, 8E). The present study focused on ICD and immune activation induced by the drug delivery system and further exploration of how Dox regulating PD-L1 will be carried out in our future studies.

In short, the drug delivery system based on hydrogel could effectively activate the immune system to repress tumor growth and metastasis. The combined therapy of chemotherapy drugs and immunity has shown great potential for the treatment of melanoma.

5. Conclusion

In this study, we used a biocompatible and sustained-releasing in situ injectable hydrogel for the local delivery of the chemotherapy drug Dox and the immune adjuvant R837 to the tumor site. The stability and safety of the co-delivery system composed by the injectable hydrogel, Dox and R837 were proved. Additionally, the co-deliver system effectively inhibited the growth and metastatic progression of B16F10 cells. We found that the co-delivery system directly induced the immunogenic cell death of melanoma cells and increased the mature of dendritic cells to activate the immune system thus inhibiting the tumor growth in vitro. Furthermore, the co-delivery system could bring about various death modes, thereby leading to more deaths of melanoma cells. And in vivo, the co-delivery system also induced immunogenic cell death effectively, which killed the tumor cells and restricted the tumor growth and metastasis. In summary, the in situ co-delivery system effectively inhibited the growth and metastatic progression of melanoma in vitro and in vivo and activated the responses of the immune system to induce the cell death, which exhibited a bright future in the targeted therapy of tumors.

Credit author statement

Jiehan Li: Investigation, Methodology, Formal analysis, Writing – review & editing. Guang Luo: Investigation, Methodology. Chuchu Zhang: Writing – original draft, Validation. Shuaiyu Long: Data curation.
Leiming Guo: Data curation, Methodology. Ge Yang: Validation. Feng Wang: Writing – review & editing. Lingling Zhang: Supervision, Writing – review & editing. Liyang Shi: Conceptualization, Methodology. Yang Fu: Supervision, Writing – review & editing. Yingjie Zhang: Project administration, Methodology, Writing – review & editing.

Data availability statement

The data sets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of competing interest

The authors declare that they have no conflict of interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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