Necroptosis-inducible Nanoliposomes for Enhanced Cancer Sonoimmunotherapy in Vitro

Meng-meng Li
Chongqing Medical University

Ya Zhu
Chongqing Medical University

Mi Yang
Chongqing Medical University

Min Zheng
Chongqing Medical University

Haitao Ran (ranhaitao@cqmu.edu.cn)
Chongqing Medical University  https://orcid.org/0000-0002-3820-1970

Wei Zhang
Chongqing Medical University

Research

Keywords: necroptosis, reactive oxygen species, immunogenic cell death, sonodynamic, ovarian cancer

DOI: https://doi.org/10.21203/rs.3.rs-689648/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License
Abstract

**Background:** Necroptosis has emerged as a therapeutic target for stimulating antitumor immune responses in dying tumor cells. However, its suppressed expression of receptor-interacting protein kinase 3 (RIPK3), a key enzyme in necrosis in most cancer cells, limits the clinical translation to exploiting necroptosis.

**Design:** We fabricated a multifunctional phase-transition nanoparticles platform by constructing Lip-ICG-PFP-cRGD, utilizing liposome and indocyanine green (ICG) as the shell and perfluoropentane (PFP) as the core. The platform system represented the combination of sonodynamic therapy (SDT) and immunotherapy for cancer treatment by inducing necroptosis and disrupting the cell membrane through the acoustic cavitation effect mediated by ultrasound. In addition to their inherent contrasting ability under photoacoustic imaging, our liposomes may also be used as an ultrasound imaging probe after being irradiated with low-intensity focused ultrasound (LIFU).

**Results:** We demonstrate that nanoparticles can trigger necroptosis in ovarian cancer cells, which ruptures cell membrane by acoustic cavitation effect. When exposed to LIFU, the nanoparticles effectively facilitate the release of damage-associated molecular patterns by inducing burst-mediated cell-membrane decomposition. Moreover, the PFP phase change caused RIPK3/MLKL-independent necroptosis by acoustic cavitation effect, resulting in the release of biologically active DAMPs (CRT and HMGB1) to facilitate antitumor immunity. Therefore, necroptosis-inducible nanoparticles remarkably enhance antitumor immunity by activating CD8+ cytotoxic T cells and maturing dendritic cells in vitro.

**Conclusion:** We have successfully synthesized Lip-ICG-PFP-cRGD nanoparticles, which can achieve SDT and provoke necroptosis by bubble-mediated cell membrane rupture. The innovative nanoparticle causes immunogenic cell death in cancer cells via RIPK3-independent necroptosis, which is a promising enhancer for cancer immunotherapy.

Introduction

Over the last decade, ovarian cancer has become one of the most lethal gynecological malignancies with high incidence, poor prognosis, mortality rates, and resistance to conventional therapies (Siegel et al. 2021, Torre et al. 2015, Srivastava et al. 2017). According to previous research, up to 30% of ovarian cancer patients die within five years due to relapse and distant metastatic development (Siegel et al. 2021). Consequently, various approaches need to be explored to provide better clinical treatment. Chemotherapy and radiation are two conventional cancer treatments that can reduce cell viability and induce tumor cancer apoptosis by destroying cellular organelles and inhibiting cell division (Erwig and Henson 2008). Apoptosis has been considered as a procedural form of cell death with poor immunogenicity and physiology (Erwig and Henson 2008, Green et al. 2009, Tesniere et al. 2008, Morioka, Maueröder and Ravichandran 2019). Immunogenic apoptosis is unique in that it can elicit an immune response by exposing or releasing molecules, such as cytokines, chemokines, tumor-associated antigens
(TAAs), and damage-associated molecular patterns (DAMPs), including exposure of calreticulin (CRT) at cell surface and release of high mobility group box 1 (HMGB1) and adenosine triphosphate (ATP) (Montico et al. 2018, Galluzzi et al. 2017).

Sensitizers, such as photosensitizers or sonosensitizers, have recently been shown to destroy cancer cells by generating reactive oxygen species (ROS) in response to light or sound stimulation and by eliciting inflammatory responses during cell death (Sang et al. 2019) ICG has been identified as a possible sonosensitizer with minimum toxicity and has been approved by the United States Food and Drug Administration (FDA), exhibiting a low incidence of adverse side effects and can be administered directly for diagnostic purposes (Son et al. 2020). Compared to free ICG, supramolecular systems such as nanoparticles have been demonstrated to significantly increase ROS production when exposed to exogenous energy sources (Lin et al. 2019). A primary immunostimulatory property of immunogenic apoptosis is that it enables the maturation of antigen-presenting cells (APCs), thereby eradicating dispersed or metastatic tumor cells through cytotoxic T lymphocytes (CTLs) (Duan, Chan and Lin 2019). However, because immunogenicity of TAAs and DAMPs can be outstandingly decreased during apoptosis progression, immunogenic apoptosis-inducing approaches have exhibited limited therapeutic responses, followed by proteolysis and intracellular oxidation to prevent autoimmunity (Sachet, Liang and Oehler 2017, Strasser, Jost and Nagata 2009, Kazama et al. 2008).

Necroptosis is a form of controlled and caspase-independent programmed cell death characterized by necrosis-like morphology, combining characters of necrosis and apoptosis, accompanied by cell membrane rupture, leakage of intracellular contents, and cytoplasmic swelling, activating the immune system and resulting in inflammation (Um et al. 2020, Siegel et al. 2021, Zhang and Liu 2013).

Previous studies indicate that receptor-interacting protein kinase 3 (RIPK3) is critical in necroptosis to activate mixed lineage kinase domain-like protein (MLKL), thereby promoting cell membrane disruption (Zhang et al. 2009, Zhao et al. 2012). The immunogenicity of pro-inflammatory molecules (including DAMPs) is not significantly altered during necroptosis, since they are released without being exposed to severe surroundings that trigger oxidation and proteolysis, which is not the case during apoptosis (Kaczmarek, Vandenabeele and Krysko 2013).

Consequently, necroptosis, one of three cell death mechanisms (necroptosis, apoptosis, and type 2 autophagic death), is one of the most effective approaches to boost antitumor immunity, implying its immense promise as a therapeutic target for cancer immunotherapy (Degterev et al. 2005, Aaes et al. 2016, Pasparakis and Vandenabeele 2015, Suntharalingam et al. 2015) However, broad implementations of necroptosis-inducing therapeutics in cancer therapy have been limited in clinics since RIPK3, and MLKL expression levels are downregulated in most cancer cells (Koo et al. 2015).

SDT, which combines low-intensity ultrasound with sonosensitizers, has been investigated as a potential cancer treatment modality (Son et al. 2020). Moreover, SDT demonstrates a high propensity for concentrating ultrasound energy to trigger sonosensitizers in far deeper tissue areas and to induce the
sonosensitizer's local cytotoxicity when adequate sonosensitizer accumulation occurs at the tumor site (Chen et al. 2016, Qian, Zheng and Chen 2016).

Given that, we fabricated a novel multifunctional phase-transition nanoparticles platform system by constructing Lip-ICG-PFP-cRGD using liposome and indocyanine green (ICG) as the shell and perfluoropentane (PFP) as the core. The platform system described herein combined SDT with immunotherapy, inducing necroptosis with RIPK3/MLKL-independent for cancer treatment and eliciting cell membrane disruption through acoustic cavitation effect mediated by ultrasound. Triggered by ultrasound (US), the Lip-ICG-PFP-cRGD elicits ROS-mediated apoptosis. Moreover, the PFP phase change (from droplets to microbubbles) caused RIPK3/MLKL-independent necroptosis by acoustic cavitation effect, resulting in the release of biologically active DAMPs (CRT and HMGB1) to facilitate antitumor immunity (Scheme 1). In addition to their inherent contrasting ability under photoacoustic (PA) imaging, our liposomes may also be used as an imaging probe for US imaging after being irradiated with LIFU.

Such nanoparticles provided us with therapeutic and diagnostic vehicles to destroy ovarian cancer cells and improve antitumor efficacy when combined with SDT and immunotherapy.

**Results And Discussion**

**Characterization**

Previous study developed a robust stealthy phospholipid liposome that demonstrated long circulation and could be stimulated by NIR light to release encapsulated drugs (Ge et al. 2019). Compared to NIR irradiation, US achieves much deeper penetration, and its energy can be regulated in exposed areas.

The liposomes were synthesized using a two-step emulsion method (Zhao et al. 2020). Transmission electron microscopy demonstrated a nearly spherical structure of lip-ICG-PFP and Lip-ICG-PFP-cRGD with smooth surfaces (Figure 1A and B). Light scattering measurements manifested a mean diameter of ~300 nm (PDI 0.135) with PFP incorporation, indicating uniform particle size distributions (Figure 1D). Zeta potentials of lip-ICG-PFP and lip-ICG-PFP-cRGD were -21.22 ± 2.54 mV and -24.49 ± 8.31 mV (Figure 1C) at pH = 7.4, respectively. Furthermore, Lip-ICG-PFP-cRGD could maintain appreciable stability in cell culture medium at 4 °C for 30 days (Figure 1E) without significant diameter changes, suggesting excellent long-term stability.

The UV–vis absorption spectra (Figure 2A) of Lip-ICG-PFP and Lip-ICG-PFP-cRGD display prominent absorption peaks of ICG at 790 nm, indicating that ICG had successful encapsulation into nanoparticles. To calculate the encapsulation efficiency of ICG in liposomes, a standard curve was drawn (Figure 2B), and ICG amount encapsulated in Lip-ICG-PFP-cRGD was determined spectrophotometrically as a 92% loading efficiency. The individual structure of Lip-ICG-PFP-cRGD was discovered to encapsulate vaporable PFP, which generated nanobubbles and fused into microbubbles after triggering by LIFU. The PFP encapsulated inside the liposome was converted into numerous microbubbles within 2 min when treated with LIFU (Figure 2D) and was visualized using optical microscopy. As the power increased,
nanoparticles with PFP cores could be converted into microbubbles. The adjacent microbubbles could fuse with each other to form larger microbubbles until they burst, consistent with previous studies, revealing the excellent ability to function as a US imaging agent and acoustic cavitation eliciting necroptosis.

**In Vitro PA and Ultrasound Imaging of Lip-ICG-PFP-cRGD**

The individual structure of liposome was creatively discovered to encapsulate vaporable PFP, which generated nanobubbles and fused into microbubbles after triggering by LIFU, thus realizing the enhanced US imaging. Thus, it was interesting to investigate the echogenic property of the nanoparticles with US irradiation in vitro. We speculated that phase-changeable liposomes could serve as ultrasound agents to enhance US imaging. US images of liposome demonstrated acoustic reflectivity contrast contributing to the generation of gas bubbling under US irradiation, accord with quantitative production results (Figure 3A, B and C). In this regard, enhanced US imaging showed that Lip-ICG-PFP-cRGD was considered an US contrast agent. The ICG can act as a PA agent. To detect the potential of nanoparticles as a PA probe, PA imaging was acquired using Lip-ICG-PFP-cRGD as the contrast agent in vitro. With the excitation wavelength ranging from 680 nm to 950 nm, PA signal of liposome is strongest at 700 nm to 850 nm (Figure 3E). As demonstrated in Figure 3D, PA images of Lip-ICG-PFP-cRGD dispersed in aqueous solution at various concentrations clearly indicate their contrast-enhancement performances at 800 nm excitation.

**Targeting Efficiency in Vitro**

The efficacy of DiI-labeled liposomes to target ID8 cells was verified by CLSM. As presented in Figure 4A, the fluorescence intensity was almost equally weak in ID8 cancer cells after 1 h of incubation with Lip-ICG-PFP and Lip-ICG-PFP-cRGD, and a higher level of cellular uptake could be observed when the incubation time increased from 2 to 3 h. Considerable fluorescence intensity in ID8 cells incubated with Lip-ICG-PFP-cRGD for 3 h was observed, whereas a much weaker fluorescent signal was exhibited in ID8 cells incubated with Lip-ICG-PFP. This result corresponds to the quantitative analysis (Figure 4B), indicating that cRGD can lead to a higher cell internalization of drugs.

**ROS Generation**

As ICG is one of the promising forms of sonosensitizers, DPBF was used to test ROS production. Since the absorbance intensity of DPBF is reduced at 410 nm in UV-vis spectrum after oxidation by ROS, DPBF consumption assists in calculating ROS production. As displayed in Figure 2C, DPBF consumption in liposome plus LIFU group was notably higher compared to that in control group, and with prolonged irradiation time, the liposomes showed obviously higher ROS production efficacy, suggesting its potential as sonosensitizer for SDT.

To detect the intracellular mechanism of Lip-ICG-PFP-cRGD as sonosensitizers in destroying cancer cells, intracellular ROS levels were tested using DCFH-DA, which could be converted from non-fluorescence status into fluorescent 2,7-dichlorofluorescein (DCF) by ROS. It can be found that Lip-ICG-PFP-cRGD
combined with LIFU irradiation induced a large amount of intracellular ROS production, which demonstrated strong green fluorescence in ID8 cells (Figure 5A). In contrast, neither control cells nor US irradiation induced the intracellular fluorescence. Moreover, similar results were also verified again by the quantitative analysis with flow cytometry (Figure 5 B), indicating that Lip-ICG-PFP-cRGD could efficiently generate ROS for effective SDT. It can be deduced that nanoparticles as sonosensitizers can generate ROS under LIFU irradiation to induce the toxic effect and achieve the therapeutic function afterward.

**Antitumor Effect in Vitro and ICD-induced Immune Priming**

The toxicity of Lip-ICG-PFH-cRGD was measured by CCK-8 assay after co-incubation with ID8 cells for 24 h at different concentrations. As Figure 6 A displays, after 24 h of incubation, Lip-ICG-PFP-cRGD revealed no significant cytotoxicity on ID8 cells at a concentration up to 1000 µg/mL, indicating well in vitro cytocompatibility. No apparent cytotoxicity occurs within the therapeutic concentration range employed in this study. Next, to observe SDT effectiveness of liposomes, ID8 cells treated with various concentration of nanoparticles were irradiated with LIFU for different time range. As expected, the cell viability decreased as the concentration and irradiation time increased, implying a dose-dependent cytotoxic effect. And more than 60% of cells were killed by cytotoxic ROS and acoustic cavitation, eliciting necroptosis in the presence of LIFU (Figure 6B and C).

Necroptosis differs from apoptosis in morphological features such as cytoplasmic swelling and cell membrane rupture. However, cell shrinkage, nuclear fragmentation, and formation of apoptotic bodies are characteristic manifestations of apoptosis (Vanden Berghe et al. 2010). The annexin V/propidium iodide (PI) assay was implemented by treating the samples with ID8 cancer cells (Figure 6D and E) to appraise the cell death mechanism (Yang et al. 2016) When the cells were treated with liposome or LIFU only, no obvious fluorescent signals from annexin V or PI were observed, indicating that there was no apparent morphology change of membrane, further suggesting that LIFU irradiation and nanoparticles have no significant damage to the cells. However, apoptotic features (i.e., chromatin condensation of cells and externalization of phosphatidylserine) were clearly demonstrated in ICG plus LIFU treated cells. Interestingly, cells treated with Lip-ICG-PFP-cRGD plus LIFU exhibited a loss in their cell morphology, indicating that the cell membrane was destroyed by US-mediated intracellular explosion of Lip-ICG-PFP-cRGD. Nanoparticles induced cell membrane disintegration under LIFU irradiation, resulting in membrane fragments, cytosolic components, and leakage of chromatin (Gong et al. 2019). These results suggest that, Lip-ICG-PFP-cRGD could induce cell necrosis by physical disruption of cell membrane under LIFU irradiation. It is worth noting that, regardless of MLKL and RIPK3 expression, the cells showed morphological characteristics of necroptosis when they were treated with Lip-ICG-PFP-cRGD plus LIFU irradiation, suggesting that the occurrence of cell necrosis is independent of RIPK3/MLKL.

Unlike apoptosis, necroptosis stimulates the release of intact DAMPs from the cells, thus effectively mediating the maturation and activation of dendritic cells (DCs). Of the DAMPs released, HMGB1 has often been considered a necroptosis representative marker (Kaczmarek et al. 2013). As depicted in Figure 7A, Lip-ICG-PFP-cRGD plus LIFU could induce a significant extracellular release of HMGB1. Additionally,
immunogenic cell death (ICD) of tumor cells is characterized by eliciting cell surface expression of pro-apoptotic calreticulin (CRT) (Galluzzi et al. 2017). The exposure of CRT on cancer cell surface serves as an “eat me” signal to antigen-presenting cells (e.g., DCs and macrophages) and trigger an anti-tumor immune response. After treatment of Lip-ICG-PFP-cRGD plus LIFU, CRT exposure on ID8 tumor cells was significantly elevated (Figure 7B). To further evaluate the efficacy of turning tumor cells into antigen-presenting cells, DC maturation was investigated. As a result, CD86 expression, a DC maturation marker, remarkably increased via Lip-ICG-PFP-cRGD treatment upon LIFU irradiation compared with other control groups (Figure 7C). In conclusion, it was evident that Lip-ICG-PFP-cRGD effectively provoked LIFU-mediated necroptosis in a RIPK3/MLKL-independent manner, thereby releasing intact DAMPs to enhance the anti-tumor immunity.

Conclusions

In summary, we demonstrate that nanoparticles can trigger necroptosis in ovarian cancer cells expressing low RIPK3, which ruptures cell-membrane by acoustic cavitation effect. The nanoparticles were loaded with PFP and ICG to enhance antitumor efficacy and immunological effects. In addition, the liposome was demonstrated to be a bimodal imaging probe for PA/US imaging. When exposed to low-intensity focused ultrasound, the nanoparticles effectively facilitate the release of damage-associated molecular patterns through burst-mediated cell-membrane decomposition. Therefore, necroptosis-inducible nanoparticles remarkably enhance antitumor immunity by activating CD8+ cytotoxic T cells and maturing dendritic cells in vitro. In summary, the combination of SDT and immunotherapy can significantly improve anti-cancer efficacy and overcome drug resistance through nanoparticles, a promising approach for ovarian cancer therapy.

Materials And Methods

Materials

Liposome includes 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG2000), 1,2-distearoyl-sn-glycero-3-phospho-(1’-rac-glycerol) (DSPG) and cholesterol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Perfluoropentane (PFP), indocyanine green (ICG), propidium iodide (PI) were obtained from Sigma Aldrich (St. Louis, MO, USA).

Reactive Oxygen Species Assay Kit (20,70-dichlorofluorescin diacetate, DCFH-DA), 1,10-dioctadecyl-3,3,30,30-tetramethylindocarbocyanine perchlorate (Dil) were purchased from Santa Cruz Biotechnology (TX, USA). All reagents were of analytical grade and used without further purification.

Preparation of LIP-ICG-PFP-cRGD, LIP-ICG-PFP

The liposomes loaded with ICG and PFP, referred to as Lip-ICG-PFP-cRGD, were synthesized by a two-step emulsion method as described previously (Jian et al. 2014). Briefly, 5 mg of DPPC, 2.0 mg of DSPG, 1.5
mg of DSPE-PEG-cRGD, and 1.5 mg of cholesterol were dissolved in 10 mL of methanol and 10 mL of chloroform. The solution was then transferred to a round bottom flask to form lipid films by rotary evaporation. After that, 2 mL of phosphate buffer saline (PBS) was added to the flask, sonicating the mixture to hydrate the lipid films for 5 min using ultrasound cleaner. Next, 0.05 mL of ICG aqueous solution (10 mg/mL) and 0.2 mL of PFP were added into lipid films and emulsified in an ice bath for 3 min using a sonicator (Heat System Inc, USA). Following that, a solution of LIP-ICG-PFP-cRGD was purified three times by centrifugation (8000 rpm, 5 min), and the supernatant was removed. Finally, 2 mL of PBS were added to the precipitate for further experimentation. The LIP-ICG-PFP liposomes were synthesized by sonicating the hybrid of lipid films without cRGD and PFP in an ice bath for 3 min and then purified three times by centrifugation (8000 rpm, 5 min).

**Characterization of liposome**

Nanoparticles (NPs) carrying PFP and ICG were successfully synthesized using DSPG, DSPE-PEG2000, DPPC, and cholesterol, and were called LIP-ICG-PFP-cRGD, generating a US-mediated cavitation effect. The diameter, zeta potential, and polydispersity index (PDI) of different kinds of NPs or NBs were measured by Zetasizer Nano ZS unit (Malvern Instruments, Malvern, UK) and their morphology and structure were observed by transmission electron microscopy (TEM). Ultraviolet-visible (UV-vis) spectrophotometer with scanning wavelength ranging from 200 to 900 nm was used to determine the absorption spectra of different nanoparticles. UV-vis spectrophotometer (260-Bio, Thermo Fisher Scientific) was used to evaluate the entrapment efficiency and loading of ICG, and entrapment efficiency and loading content were then calculated.

**PA and US Imaging In Vitro**

The agar-gel model (3% agar w/v in double-distilled water) was utilized to evaluate LIP-ICG-PFP-cRGD capacity, which acts as contrast agents for dual-modal imaging. PA imaging of different concentrations of nanoparticles was shown on VEVO LASER PA imaging system (VEVO 2100, Canada).

For US imaging, liposomes were exposed to low-intensity focused ultrasound (2-6 W) for 100-300 s. The contrast-enhanced ultrasound (CEUS) and B-mode images were obtained by an ultrasonic diagnostic instrument (MyLab 90; Esaote, Italy).

**Detection of ROS**

1, 3-Diphenylisobenzofuran (DPBF) was used to determine ROS generation in nanoparticles. A total of 60 µL of DPBF (4 mg/mL), 50 µL of LIP-ICG-PFP-cRGD, and 1 mL of double distilled water were added into Eppendorf tube and exposed to low-intensity ultrasound with 4 W/cm² for 0-10 min in dark. Then, a multimode reader was used to evaluate ROS production by measuring the absorption at 410 nm.

To detect intracellular ROS generation, 2', 7'-dichlorofluorescin diacetate (DCFH-DA) was employed as an indicator. In brief, 1 x 10^5 ID8 cells were seeded in 12-well plates and co-incubated with LIP-ICG-PFP-cRGD
(the concentration of each group was 0.4 mg/mL) for 3 h. All the wells were divided into six groups: control group, NPs group, only LIFU group (4 W/cm² for 60 s), and NPs combined with LIFU group (4 W/cm² for 60 s). Then, each of the wells was washed three times with PBS. Subsequently, the cells were cultured with diluted DCFH-DA for 30 min in dark. The ROS generation was evaluated by flow cytometry.

ID8 cells were seeded in confocal dishes at a density of 1 x 10⁵ for 24 h, and the rest of routine was as described above. Finally, laser scanning confocal microscopy (LSCM, Nikon AJR, Japan) was used to detect ROS generation.

**Determination of Cellular Uptake**

ID8 cells were seeded onto confocal dishes at a density of 1 x 10⁵ for 24 h. Next, the original media was replaced by media containing LIP-ICG-PFP or LIP-ICG-PFP-cRGD labeled with Dil dye for co-incubation with different periods (1.0, 2.0, and 3.0 h). Then, the cells were washed with PBS and cultured with 2-(4-aminophenyl)-6-indolecarbamidine dihydrochloride (DAPI) for 10 min to stain cell nuclei. Subsequently, PBS was used to wash the cells three times, and 4% paraformaldehyde was utilized to fix the cells. Finally, the cellular uptake ability was evaluated by confocal laser scanning microscopy.

**In vitro cell viability and Cell death assay using annexin V/PI staining**

To assess cytotoxicity in vitro, ID8 cells were seeded in a 96-well plate for 24 h, and then, 100 µL of LIP-ICG-PFP-cRGD nanoparticles with various concentrations were added and cultured for another 24 h. Cell viabilities were tested by CCK-8 assay.

To evaluate the therapeutic effects, ID8 cells were seeded in a 96-well plate and co-cultured with LIP-ICG-PFP-cRGD nanoparticles (different concentrations were divided into five groups) for 3 h. PBS was utilized to wash the wells three times, and the media in each well were displaced with fresh media. Next, the groups were exposed to low-intensity ultrasound with various power (4.0 W/cm²) for 200 s in dark, and cell viabilities were determined using CCK-8 method.

ID8 cells were cultured in 6-well dishes for 24 h. Additionally, cells were incubated with liposomes and free ICG for 3 h and washed with PBS. Then, cells were trypsinized and re-suspended with 400 µL of 1% FBS containing annexin V binding buffer. After that, the cells were exposed to LIFU for 200 s (power 4.0 W/cm²). The cell viabilities were determined using CCK-8 method or further incubated for 1 h and stained with FITC annexin V and PI for 10 min. Finally, the cells were observed using a fluorescence microscope and analyzed with flow cytometry.

**The CRT expression and HMGB1 Release Assay**

The ID8 cells were co-cultured with nanoparticles (LIP-ICG-PFP-cRGD 0.8 mg/mL) in 6-well plates for 3 h. All the wells were divided into four groups: control group, only LIFU group, only NPs group, and LIP-ICG-PFP-cRGD plus LIFU group (4 W/cm² for 200 s). The cells were cultured for another 18 h, and the
supernatant was added into another 6-well plate which was cultured with dendritic cells (DC) for 6 h. Subsequently, the cells and supernatants from DC co-cultured were collected to examine the CRT and HMGB1 level by ELISA (LifeSpan BioSciences), according to manufacturer's instructions.

**The expression of CD86 in dendritic cells**

To test the expression of CD86 (a marker for DC maturation), ID8 cells were seeded and co-incubated with different nanoparticles. Following exposure to different treatments, the supernatant was collected separately and added into another 12-well plate cultured with DC for 24 h. After washing with PBS and staining with FITC-conjugated anti-CD86 for 60 min in dark, the samples were analyzed using flow cytometry to quantify CD86 expression in DC.

**Statistical analysis**

The experimental data were analyzed using GraphPad Prism (version 8.0) with the unpaired Student's t-test, or the paired Student's t-test. *P*-values < 0.05 indicated statistically significant difference (*p < 0.05, **p < 0.01).

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Availability of data and material**

All data generated or analyzed during this study are included in this published article.

**Competing interests**

The author reports no conflicts of interest in this work.

**Funding**

This work was supported by the National Natural Science Foundation of China (81630047, 82071926), the Science and Technology Research Program of Chongqing Municipal Education Commission (Grant No. KJZD-K202000402).

**Authors' contributions**
WZ and HTR planned and designed the experiments and drafted the main part of the manuscript. RHT offered the fund to perform this study. MML, YZ, MY, and YC performed the in vitro study and analyzed the data. All authors read and approved the final manuscript.

Acknowledgements

Not applicable.

References

1. Aaes, T. L., A. Kaczmarek, T. Delvaeye, B. De Craene, S. De Koker, L. Heyndrickx, I. Delrue, J. Taminau, B. Wiernicki, P. De Groote, et al. (2016) Vaccination with Necroptotic Cancer Cells Induces Efficient Anti-tumor Immunity. Cell Rep, 15, 274-87.

2. Chen, Y. W., T. Y. Liu, P. H. Chang, P. H. Hsu, H. L. Liu, H. C. Lin & S. Y. Chen (2016) A theranostic nrGO@MSN-ION nanocarrier developed to enhance the combination effect of sonodynamic therapy and ultrasound hyperthermia for treating tumor. Nanoscale, 8, 12648-57.

3. Degterev, A., Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G. D. Cuny, T. J. Mitchison, M. A. Moskowitz & J. Yuan (2005) Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury. Nat Chem Biol, 1, 112-9.

4. Duan, X., C. Chan & W. Lin (2019) Nanoparticle-Mediated Immunogenic Cell Death Enables and Potentiates Cancer Immunotherapy. Angew Chem Int Ed Engl, 58, 670-680.

5. Erwig, L. P. & P. M. Henson (2008) Clearance of apoptotic cells by phagocytes. Cell Death Differ, 15, 243-50.

6. Galluzzi, L., A. Buqué, O. Kepp, L. Zitvogel & G. Kroemer (2017) Immunogenic cell death in cancer and infectious disease. Nat Rev Immunol, 17, 97-111.

7. Ge, R., J. Cao, J. Chi, S. Han, Y. Liang, L. Xu, M. Liang & Y. Sun (2019) NIR-guided dendritic nanoplatform for improving antitumor efficacy by combining chemo-phototherapy. International journal of nanomedicine, 14, 4931-4947.

8. Gong, Y., J. Crawford, B. Heckmann & D. Green (2019) To the edge of cell death and back. The FEBS journal, 286, 430-440.

9. Green, D. R., T. Ferguson, L. Zitvogel & G. Kroemer (2009) Immunogenic and tolerogenic cell death. Nat Rev Immunol, 9, 353-63.

10. Kaczmarek, A., P. Vandenabeele & D. V. Krysko (2013) Necroptosis: the release of damage-associated molecular patterns and its physiological relevance. Immunity, 38, 209-23.

11. Kazama, H., J. E. Ricci, J. M. Herndon, G. Hoppe, D. R. Green & T. A. Ferguson (2008) Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. Immunity, 29, 21-32.

12. Koo, G. B., M. J. Morgan, D. G. Lee, W. J. Kim, J. H. Yoon, J. S. Koo, S. I. Kim, S. J. Kim, M. K. Son, S. S. Hong, et al. (2015) Methylation-dependent loss of RIP3 expression in cancer represses programmed
necrosis in response to chemotherapeutics. Cell Res, 25, 707-25.

13. Lin, H., S. Li, J. Wang, C. Chu, Y. Zhang, X. Pang, P. Lv, X. Wang, Q. Zhao, J. Chen, et al. (2019) A single-step multi-level supramolecular system for cancer sonotheranostics. Nanoscale Horiz, 4, 190-195.

14. Montico, B., A. Nigro, V. Casolaro & J. Dal Col (2018) Immunogenic Apoptosis as a Novel Tool for Anticancer Vaccine Development. Int J Mol Sci, 19.

15. Morioka, S., C. Maueröder & K. S. Ravichandran (2019) Living on the Edge: Efferocytosis at the Interface of Homeostasis and Pathology. Immunity, 50, 1149-1162.

16. Pasparakis, M. & P. Vandenabeele (2015) Necroptosis and its role in inflammation. Nature, 517, 311-20.

17. Qian, X., Y. Zheng & Y. Chen (2016) Micro/Nanoparticle-Augmented Sonodynamic Therapy (SDT): Breaking the Depth Shallow of Photoactivation. Adv Mater, 28, 8097-8129.

18. Sachet, M., Y. Y. Liang & R. Oehler (2017) The immune response to secondary necrotic cells. Apoptosis, 22, 1189-1204.

19. Sang, W., Z. Zhang, Y. Dai & X. Chen (2019) Recent advances in nanomaterial-based synergistic combination cancer immunotherapy. Chem Soc Rev, 48, 3771-3810.

20. Siegel, R. L., K. D. Miller, H. E. Fuchs & A. Jemal (2021) Cancer Statistics, 2021. CA Cancer J Clin, 71, 7-33.

21. Son, S., J. H. Kim, X. Wang, C. Zhang, S. A. Yoon, J. Shin, A. Sharma, M. H. Lee, L. Cheng, J. Wu, et al. (2020) Multifunctional sonosensitizers in sonodynamic cancer therapy. Chem Soc Rev, 49, 3244-3261.

22. Srivastava, S. K., A. Ahmad, O. Miree, G. K. Patel, S. Singh, R. P. Rocconi & A. P. Singh (2017) Racial health disparities in ovarian cancer: not just black and white. J Ovarian Res, 10, 58.

23. Strasser, A., P. J. Jost & S. Nagata (2009) The many roles of FAS receptor signaling in the immune system. Immunity, 30, 180-92.

24. Suntharalingam, K., S. G. Awuah, P. M. Bruno, T. C. Johnstone, F. Wang, W. Lin, Y. R. Zheng, J. E. Page, M. T. Hemann & S. J. Lippard (2015) Necroptosis-inducing rhenium(V) oxo complexes. J Am Chem Soc, 137, 2967-74.

25. Tesniere, A., T. Panaretakis, O. Kepp, L. Apetoh, F. Ghiringhelli, L. Zitvogel & G. Kroemer (2008) Molecular characteristics of immunogenic cancer cell death. Cell Death Differ, 15, 3-12.

26. Torre, L. A., F. Bray, R. L. Siegel, J. Ferlay, J. Lortet-Tieulent & A. Jemal (2015) Global cancer statistics, 2012. CA Cancer J Clin, 65, 87-108.

27. Um, W., H. Ko, D. G. You, S. Lim, G. Kwak, M. K. Shim, S. Yang, J. Lee, Y. Song, K. Kim, et al. (2020) Necroptosis-Inducible Polymeric Nanobubbles for Enhanced Cancer Sonoimmunotherapy. Adv Mater, 32, e1907953.

28. Vanden Berghe, T., N. Vanlangenakker, E. Parthoens, W. Deckers, M. Devos, N. Festjens, C. Guerin, U. Brunk, W. Declercq & P. Vandenabeele (2010) Necroptosis, necrosis and secondary necrosis converge
on similar cellular disintegration features. Cell death and differentiation, 17, 922-30.

29. Yang, H., Y. Ma, G. Chen, H. Zhou, T. Yamazaki, C. Klein, F. Pietrocola, E. Vacchelli, S. Souquere, A. Sauvat, et al. (2016) Contribution of RIP3 and MLKL to immunogenic cell death signaling in cancer chemotherapy. Oncoimmunology, 5, e1149673.

30. Zhang, D. W., J. Shao, J. Lin, N. Zhang, B. J. Lu, S. C. Lin, M. Q. Dong & J. Han (2009) RIP3, an energy metabolism regulator that switches TNF-induced cell death from apoptosis to necrosis. Science, 325, 332-6.

31. Zhang, Y. Y. & H. Liu (2013) Connections between various trigger factors and the RIP1/RIP3 signaling pathway involved in necroptosis. Asian Pac J Cancer Prev, 14, 7069-74.

32. Zhao, J., S. Jitkaew, Z. Cai, S. Choksi, Q. Li, J. Luo & Z. G. Liu (2012) Mixed lineage kinase domain-like is a key receptor interacting protein 3 downstream component of TNF-induced necrosis. Proc Natl Acad Sci U S A, 109, 5322-7.

33. Zhao, X. Z., W. Zhang, Y. Cao, S. S. Huang, Y. Z. Li, D. Guo, X. Y. Wang & H. T. Ran (2020) A Cleverly Designed Novel Lipid Nanosystem: Targeted Retention, Controlled Visual Drug Release, and Cascade Amplification Therapy for Mammary Carcinoma in vitro. Int J Nanomedicine, 15, 3953-3964.

34. Jian, J., C. Liu, Y. Gong, L. Su, B. Zhang, Z. Wang, D. Wang, Y. Zhou, F. Xu, P. Li, et al. (2014) India ink incorporated multifunctional phase-transition nanodroplets for photoacoustic/ultrasound dual-modality imaging and photoacoustic effect based tumor therapy. Theranostics, 4, 1026-38.

Figures
Figure 1

Characterization of nanoparticles. (A, B) TEM images of Lip-ICG-PFP and Lip-ICG-PFP-cRGD. (C) Zeta potential of the Lip-ICG-PFP and Lip-ICG-PFP-cRGD. (D) Size distribution of Lip-ICG-PFP-cRGD. (E) Size changes of Lip-ICG-PFP-cRGD at 4 °C for 30 days (n=3).
**Figure 2**

(A) Identification of liposomes by UV-vis spectroscopy. (B) The concentration-absorbance standard curve of ICG. (C) DPBF consumption of liposomes under LIFU irradiation. (D) Optical images of Lip-ICG-PFP-cRGD after LIFU irradiation (2 min, 2-6 W).

**Figure 3**

(A) Comparison of grayscale values for different concentrations and LIFU intensities. (B) Bar graph showing grayscale values in US. (C) Bar graph showing grayscale values in CEUS. (D) PA average and intensity graphs for different concentrations.
(A) B-mode and CEUS images of Lip-ICG-PFP-cRGD after LIFU irradiation under different conditions (2-6 W/cm², 1 min). (B, C) The grayscale value in B-mode and CEUS after irradiated with LIFU (the data are shown as mean ± SD, n=3). (D) PA images of Lip-ICG-PFP-cRGD with different concentration gradient. (E) Quantitative PA intensities of liposome in vitro. (F) Plot of PA values of Lip-ICG-PFP-cRGD with various concentration gradient.

Figure 4

Targeting efficiency of liposomes in vitro. (A) CLSM images of ID8 cells after incubation with Lip-ICG-PFP-cRGD and Lip-ICG-PFP nanoparticles for elevated time. (B) Flow cytometry results of ID8 cells after incubation with Lip-ICG-PFP-cRGD and Lip-ICG-PFP nanoparticles for 2 h.
Figure 5

Intracellular ROS generation capacity of Lip-ICG-PFP-cRGD. (A) CLSM images and (B) flow cytometry analysis of ROS generation in ID8 cells treatment with various treatments, as detected with DCFH-DA.
Figure 6

(A) Relative cell viability of ID8 cells after incubation with different concentrations of Lip-ICG-PFP-cRGD. (B, C) Relative cell viability of ID8 cells after various treatments. (The error bars represent standard deviation, n=3). (D) Fluorescence microscope image of annexin V/PI-stained cells. (E) Flow analysis of apoptosis of ID8 cells after various treatments.
Figure 7

(A, B) Quantitative assay of CRT exposure and release of HMGB1 into the medium using ELISA. (The error bars represent standard deviation, n=3). (C) The amount of CD86+ DCs through flow cytometry after various treatment in vitro.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Scheme1.jpg
- GraphicalAbstract.jpg