Research article

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Fe₂P nanorods based photothermal therapy combined with immune checkpoint inhibitors for pancreatic cancer

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Abstract: Treatment of pancreatic cancer is faced with great difficulties and challenges due to high lethality and metastasis. Synergism of targeted therapy and immunotherapy has been considered as ideal strategy to both eliminate primary tumors and control metastases. For the treatment of advanced pancreatic cancer, we demonstrated a local photothermal therapy (PTT) following administration of monoclonal antibody of programmed death ligand 1 (αPD-L1). Fe₂P nanorods were employed as a Fenton agent and photothermal agent, which modified with DSPE-PEG₂₀₀₀-Mal for improved biocompatibility and Mal mediated-antigen presentation. Under a low dose laser irradiation at 980 nm, Fe₂P-PEG-Mal nanorods (NRs) mediated PTT could induce immunogenic tumor cell death that can cause dendritic cells (DCs) infiltration and maturation. In a bilateral pancreatic tumor model, the local treatment of NRs-PTT on primary tumor could cause the increased infiltration of cytotoxic T lymphocytes (CTLs)

and decreased residential of M2 macrophages in untreated distal tumors. Furthermore, subsequently intervened αPD-L1 could enhance cell death triggered by CTLs in distal tumors through reversing immunosuppression. An orthotopic pancreatic tumor model was used to further confirm the therapeutic outcome. Finally, the combination of NRs based PTT and αPD-L1 based immunotherapy was able to significantly eliminate orthotopic pancreatic tumors and reduce mesentery metastases. Thus, the strategy may provide a more effective treatment for pancreatic cancer.

Keywords: combination therapy; immunotherapy; metastasis; pancreatic cancer; photothermal therapy.

1 Introduction

Pancreatic cancer is one of the most lethal malignancies with high fatality rate, poor curative effect, and unsatisfactory prognosis [1]. Nearly 80% of patients diagnosed with pancreatic cancer have unresectable primary tumor, and even more than 50% of patients have distal metastasis, who achieved a merely 3% five-year survival rate [2, 3]. As traditional cancer treatments in clinical, chemotherapy and radiotherapy have made some progress, such as overcoming drug resistance by tumor targeting and reducing nonselective collateral damage to normal tissue by interstitial brachytherapy [4, 5]. Nevertheless, the efficacy of chemotherapy and radiotherapy could be restricted by dense tumor stroma and uneven dose distribution in pancreatic tumor, respectively [6, 7]. In addition, these two traditional therapies have limited efficacy on metastases [8, 9]. For these reasons, a better treatment option for advanced pancreatic tumor is highly demanded.

Near-infrared (NIR) light triggered photothermal therapy (PTT) was known as less invasive but higher specificity, which can destroy targeted-tumor through converting light energy into heat energy. Nanomaterials have been widely explored in PTT [10, 11]. Especially, gold nanoshells-based PTT has been studied in clinical trial on

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the treatment of prostate cancer, exhibiting 94% (15/16) of male patients without tumor markers in 12 months [12]. For pancreatic cancer, gold nanoparticles, silica/gold nanoplateform, graphene oxide and iron oxide nanoparticles, have been employed as photothermal agents for primary tumor ablation [5, 13–15]. Moreover, gold nanoshells, coupled with indocyanine green and uPAR, were designed as imaging agents and photothermal agents, which can effectively eliminate orthotopic pancreatic tumor with imaging guidance [16]. Taken together, local-selective PTT has shown outstanding antitumor performance, but remains less effective in controlling metastatic tumor.

Immune therapeutic strategies have been rapidly developed for cancer in recent years [17]. For instance, T cells expressing CD19 chimeric antigen receptors (CARs) have been approved by U.S. Food and Drug Administration (FDA) to treat B cell malignancies [18, 19]. Two immune checkpoint inhibitors, PD-1/PD-L1 and CTLA-4 antibody, were already on the market with capability to relieve malignant tumor [20, 21]. The unsatisfied therapy responses aforementioned may attribute to limited efficacy of immunotherapy alone in solid tumors. Therefore, the combination of photo-immunotherapy has attracted more attention. CAR T cell therapy and PD-L1 antibody have enhanced anti-melanoma efficacy when combined with PTT [22, 23]. Ipilimumab, a CTLA-4 inhibitor, combined with PTT exhibited the remarkable therapeutic outcomes to completely clear subcutaneous and distal lung metastases on a stage IV melanoma patient [24]. Inspired by such properties, combination between PTT and immunotherapy will be a promising therapy modality for pancreatic cancer to cause not only ablation of primary tumor, but also activation of immunity to control tumor metastasis.

Magnetic iron oxide Fe₃O₄ has been approved by FDA for T₂-weighted magnetic resonance imaging (MRI) agent. Recently, iron materials were figured out to suppress cancer progress by inducing a pro-inflammatory immune response with M1 macrophages polarization [25]. Iron materials can also be utilized as Fenton agents to convert endogenous H₂O₂ into hydroxyl radicals (•OH) under acidic environment, which are generally considered as the strongest reactive oxygen species (ROS). Lately, ferrous phosphide (Fe₂P) has gained intensive interests, because it comprises not only excellent ferromagnetism and higher transverse relaxation (T₂) value, but also outstanding photothermal conversion efficiency [26].

Herein, Fe₂P nanorods were employed as Fenton agents and photothermal agents for the treatment of pancreatic cancer. In addition, an immune checkpoint inhibitor, αPD-L1, was introduced into treatment strategies (Scheme 1), aiming to achieve enhanced antitumor

effect. Antitumor activity and immune response were studied *in vitro* and *in vivo*.

2 Results

2.1 Synthesis and characterization of NRs

Fe₂P nanorods were achieved according the previously described method [27]. DSPE-PEG₂₀₀₀-Mal was capped on prepared Fe₂P nanorods to improve hydrophilicity and biocompatibility, in addition to enhance antigens capture because of the presence of Mal modifier [28]. The morphologies and composition of resulting nanorods (Fe₂P-PEG-Mal NRs, NRs) were confirmed by transmission electron microscopy (TEM) and X-ray photoelectron spectroscopy (XPS). The TEM images displayed well-defined rod-phased structure (Figure 1A). Such structure is conducive to cellular uptake [29]. The XPS spectra indicated the presence of Fe, P, and O (Figure 1B). The binding energies (BEs) were attributed to Fe²⁺ at 710.8 and 724.5 eV, to P 2p_{1/2} and P 2p_{3/2} at 130.1 and 129.1 eV, respectively. Meanwhile, the BE at 132.9 eV was caused by P–O species. The absorption spectrum of NRs dispersions was shown in Figure 1C, illustrating a wide band from 400 to 1000 nm. The standard absorption curve of NRs at 980 nm showed a good linear relationship.

It was found that NRs could convert NIR laser energy into heat energy for PTT. We further confirmed the photothermal properties of NRs by detecting the temperature elevation during laser irradiation. As shown in Figure 1D and E, the higher concentration of NRs solution, the higher temperature elevation during irradiation with 980 nm laser. In addition, with increasing power densities of laser, the temperature rises of NRs solution became more intense (Figure 1G and H). In particular, the final temperature of solution at 200 µg/mL increased to 70.6 °C under laser irradiation at 0.5 W/cm², revealing excellent photothermal conversion performance of NRs under laser irradiation. Afterwards, NRs solution (200 µg/mL) was irradiated with a 980 nm laser at 0.5 W/cm² last 10 min and then cooled for another 10 min, finally carrying three thermal cycles in total. The temperature curves exhibited that photothermal property of NRs was stable after repeated laser irradiation (Figure 1F). To examine the MR imaging property of NRs, T₂-weighted MRI was performed *in vitro* by a clinical 3.0 T MRI scanner at room temperature. As presented in Figure 1I, imaging region became darker with the increasing content of Fe, accompanied by the decrease of MR signal. Such a good performance

Scheme1: Strategic overview of antitumor immunity induced by α PD-L1 potentiated NRs based PTT.

offered the prepared nanorods a great potential as MR contrast agents.

2.2 *In vitro* immunogenic immune response induced by NRs based PTT

To determine intracellular localization of NRs in tumor cells, FITC functionalized Fe₂P NRs were incubated with Panc02-H7 cells for 12 h and stained with LysoTracker (indicating lysosomes) and Hoechst 33342 (indicating nucleus). As shown in Figure 2A, Fe₂P-FITC NRs were accumulated in lysosomes. Then, the intracellular oxidative stress triggered by ferrous ion of NRs in Panc02-H7 tumor cells was studied by fluorescence staining with DCFH-DA as the ROS probe. The cells treated with NRs displayed green fluorescence that indicating the production of ROS, depicting the presence of Fenton reaction. Surprisingly, compared with other groups, NRs + L group displayed stronger fluorescence, showing that laser illumination could amplify the intrinsic oxidative stress of NRs (Figure 2B). A standard cell counting kit-8 (CCK-8) assay was then employed to determine the cytotoxic effect of NRs and NRs + L. Panc02-H7 tumor cells were incubated with NRs at various concentrations (0, 5, 10, 20 μ g/mL) for 12 h

followed with or without laser irradiation. As shown in Figure 2C, it had no obvious inhibitory effect on cell viability when treated with NRs-only, even at a high concentration of 20 μ g/mL. However, NRs showed obvious photo-cytotoxicity at the concentration of 20 μ g/mL, because the cell viability after laser irradiation was less than 40%. Afterwards, two double stain apoptosis detection kits, annexin V-FITC/PI and calcein-AM/PI, were accomplished to further evaluate the phototoxicity on cells. As shown in Figure 2D, the cell apoptosis in NRs + L group reached 71.4%, which was much higher than the other groups. Most of cells in the group of NRs + L were dead, indicated by red fluorescence (Figure 2E). In contrast, most of cells in other groups were still viable, indicated by green fluorescence.

Calreticulin (CRT) is belonging to damage-associated molecular patterns (DAMPs). The exposure of CRT on cell surface is associated with enhanced immunogenic cell death (ICD), releasing phagocytic signal to activate dendritic cells (DCs) [30]. The treatment of NRs + L was evidenced to induce more CRT translocation to cell surface (Figure 2F). However, other groups had no obvious effect on CRT translocation. CD86 expression and cytokines release were acted as typical maturation markers of DCs. We next used flow cytometry to measure

Figure 1: Characterization of Fe₂P-PEG-Mal NRs.

(A) TEM image of NRs. (B) XPS spectra of NRs. Left: Fe 2p. Right: P 2p. (C) UV-Vis-NIR absorbance spectrum of NRs. Insert: Standard curve of NRs absorption at 980 nm. (D) Near infrared thermal images of NRs solution at different concentrations (0, 50, 100 and 200 µg/mL) under 980 nm laser irradiation at 0.5 W/cm². (E) Temperature elevations of solution during laser irradiation based on infrared thermal imaging data in (D) ($n = 3$). (F) Temperature curves of NRs (200 µg/mL) during three times "on/off" irradiation with 980 nm laser at 0.5 W/cm². (G) Near infrared thermal images of NRs (200 µg/mL) under 980 nm laser irradiation at different power densities (0.1, 0.25, 0.5 and 0.75 W/cm²). (H) Temperature elevations of solution during laser irradiation based on infrared thermal imaging data in (G) ($n = 3$). (I) MR images of solution (insert) and linear relationship between MR signal and concentrations of Fe.

the upregulation of CD86 on DCs that stimulated by treated tumor cells. Compared to control group, tumor cells treated with NRs + L could greatly promote DCs maturation (Figure 2G). As shown in Figure 2H, tumor cells treated with NRs + L could stimulate the secretion of TNF- α from DCs, which was significantly higher than that in other groups. In addition, the secretion of TNF- α from DCs co-cultured with NRs-only or tumor cells treated with NRs was also increased, which may be related to the Fenton effect of NRs. Overall, these results validated that the treatment of NRs + L induced ICD *in vitro*, with high level tumor cell death and enhanced maturation of DCs.

2.3 *In vivo* antitumor effect on pancreatic tumors

To demonstrate antitumor effect on primary tumors, a pancreatic tumor model was established. Tumor-bearing mice were randomly assigned to different groups, including control, L, NRs, and NRs + L groups ($n = 3$). During laser irradiation, a rapid elevation of temperature on tumor surface was monitored by an infrared thermal camera *in vivo*. As shown in Figure 3A and B, the surface temperature increased to 52 °C for sufficient tumor ablation in NRs + L group, while the temperature remained 41.6 °C in L-only group.

Figure 2: Immunogenic tumor cell death induced by Fe₂P-PEG-Mal NRs based PTT.

(A) Fluorescence images of intracellular localization of NRs in Panc02-H7 cells. Scale bar: 20 μ m. (B) Fluorescence images of ROS production in Panc02-H7 cells stained with DCFH DA (a ROS probe, green). Scale bar: 100 μ m. Bar graph demonstrated percentage of ROS cells ($n = 3$, $*P < 0.05$, $**P < 0.01$). (C) Cell viability with or without laser irradiation (980 nm, 0.5 W/cm², 2 min) at different concentrations of NRs ($n = 4$, $*P < 0.05$, $**P < 0.01$). (D) Flow cytometric analysis of cell apoptosis induced by different treatments staining with Annexin V-FITC/PI. (E) Fluorescence images of cell death induced by different treatments staining with calcein-AM/PI Live cells green, calcein-AM Dead cells red, PI. Scale bar: 100 μ m. Bar graph demonstrated percentage of cell death rate ($n = 3$, $*P < 0.05$, $**P < 0.01$). (F) Fluorescence images of CRT on the surface of Panc02-H7 tumor cells. Scale bar 20 μ m. Bar graph demonstrated percentage of CRT cells ($n = 3$, $*P < 0.05$, $**P < 0.01$). (G) Flow cytometric analysis of CD 86 expression on the surface of DCs stimulated by treated Panc02-H7 cells (gated on CD11c⁺ cells). (H) ELISA analysis of TNF α released from DCs stimulated by NRs or treated Panc02-H7 cells ($n = 3$, $*P < 0.05$, $**P < 0.01$ and # $P < 0.05$ vs. indicated groups).

Figure 3: *In vivo* antitumor effect of Fe₂P-PEG-Mal NRs based PTT on primary tumors.

(A) Infrared thermal images of Panc02-H7 tumor-bearing mice under laser irradiation. (B) Temperature elevations on tumor surface during laser irradiation based on infrared thermal imaging data in (A) ($n = 3$). (C) H&E staining images of excised tumors after different treatments (Upper: 4×, scale bar: 200 μm. Lower: 40×, scale bar: 20 μm). (D) Immunofluorescence images of excised tumors stained with anti-CD11c. Scale bar: 100 μm.

To further investigate killing effect on the treated tumors, hematoxylin and eosin (H&E) staining assay was carried out on harvested tumor section. The results showed that NRs + L treatment could significantly increase the amount of necrosis and apoptotic cells than other groups (Figure 3C). In addition, to evaluate the infiltration of DCs into treated tumors, excised tumors after treatments from mice were stained with anti-CD11c for assessment through immunofluorescence analysis. Compared to L or NRs treatment, NRs + L treatment induced a larger number of DCs infiltration (marked by CD11c) into treated tumors (Figure 3D).

Inspired by the immune responses induced by NRs based PTT, we wondered whether the therapeutic effects could be valuable for metastasis controlling. The abscopal effect of NRs based PTT was evaluated in accordance with the treatment schedule illustrating in Figure 4A. Panc02-H7 cells were subcutaneously injected into both flanks to build the bilateral pancreatic tumor model. Tumors on left flanks were considered as primary tumors, while those on right flanks were considered as distal tumors. When

primary tumors reached 200 cm³, tumor-bearing mice ($n = 3$) were assigned to three different groups, including control, NRs + L and NRs + L + αPD-L1 groups. Mice were sacrificed at day 7 after treatments and then the corresponding distal tumors were resected for following immunofluorescence staining, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay and H&E staining.

Cytotoxic T lymphocytes (CTL, marked by CD8) can release various cytotoxins to eliminate tumor cells, while M2 macrophages (marked by CD206) lead to tumor tolerance. As shown in Figure 4B, the distal tumors in NRs + L group demonstrated great increases in CD8 infiltration, indicating the enhanced systemic antitumor effects. Meanwhile, M2-related CD206 exhibited a remarkable loss in distal tumors after NRs + L treatment, which indicated the decreased immunosuppression in tumor microenvironment (Figure 4C). Particularly, when injected αPD-L1 following NRs + L treatment, the infiltration of CD8 and loss of CD206 in distal tumors were further improved. The increased tumor cell death in distal tumors in

Figure 4: Abscopal effect of Fe₂P-PEG-Mal NRs based PTT.

(A) Schematic illustration of the treatment protocol for research of the abscopal effect induced by NRs based PTT in the presentation of α PD-L1. B–D) Representative images of excised distant tumors stained with anti-CD8a (B), scale bar: 100 μ m), anti-CD206 (C), scale bar: 100 μ m), TUNEL and H&E (D), scale bar: 200 μ m). Bar graphs demonstrated the number softumor-infiltrating CD8⁺, CD206⁺ and TUNEL⁺ cells indistant tumors ($n = 3$, * $P < 0.05$, ** $P < 0.01$ and # $P < 0.05$ vs. indicated groups).

NRs + L + α PD-L1 group was confirmed by TUNEL assay and H&E staining (Figure 4D). These results revealed that the treatment of NRs + L could trigger the production and infiltration of CTL, and the killing effect of CTL was further potentiated by introduction of α PD-L1 with improved microenvironment, evidencing the superior therapeutic efficacy of combination between PTT and immunotherapy.

2.4 Therapeutic efficacy on orthotopic pancreatic tumors

In our previously study, glycosylated chitosan (GC), an immunoadjuvant, had been explored to combine with interventional PTT for the treatment of orthotopic pancreatic tumors, showing significant inhibition on tumor

Figure 5: Biodistribution of Fe₂P-PEG-Mal NRs.

(A) T₂-weighted MR image (left, white arrows refer to tumor regions) and photographic image (middle) of orthotopic pancreatic tumor-bearing mice. H&E staining (lower right) of orthotopic pancreatic tumor. Scale bar: 200 μ m. (B) Fluorescence distribution of NRs 24 h after intravenous injection. (a) Fluorescence images of injected mice *in vivo*. (b) Biological distribution of NRs in heart, liver, spleen, lung and kidney. (c) Bar graph based on the intensity of fluorescent signals in (b). (C) H&E staining images of major organs collected from mice treated with or without NRs. Scale bar: 200 μ m.

metastases [31]. Based on this, we wondered whether NRs could also contribute to the treatment of orthotopic pancreatic cancer under the help of α PD-L1. To test the hypothesis, we established an orthotopic pancreatic tumor model ($n = 4$) [32, 33]. After intravenous injection of NRs for 12 h, the T₂-weighted MR images of orthotopic pancreatic tumor were recorded (a white arrow refer to tumor regions). Orthotopic tumor lesions were further confirmed by operation and H&E staining (Figure 5A). Through these results, the remarkable imaging capability of NRs *in vivo* was confirmed, which helps to accurately diagnose the lesion areas and guide visual treatment. Cy5-labeled Fe₂P NRs were utilized to validate tumor-targeting and bio-distribution *in vivo*. After intravenous injection of Cy5-labeled Fe₂P NRs, images of mice in supine position, dissected orthotopic tumor and major organs (heart, liver, spleen, lung and kidney) were conducted by a fluorescent

imager (Figure 5B). As revealed by *ex vivo* imaging, the red fluorescence was abundantly accumulated in tumor even at 24 h post injection, representing the efficient passive tumor targeting ability of the prepared nanorods. H&E was employed to stain major organs collected from mice treated with or without NRs. As shown in Figure 5C, no obvious damage was observed, supporting the biosafety of NRs.

In order to check the therapeutic effects, the orthotopic pancreatic tumors were surgically exposed and irradiated directly with a 980 nm laser (Figure 6A). As shown in Figure 6B, the temperature on orthotopic pancreatic tumors increased to 62.6 $^{\circ}$ C for sufficient tumor ablation. Then, all tested mice were sacrificed for anatomical analysis after the mice of sham group died (dotted circles and white arrows refer to tumor regions) (Figure 6C). Within 11 days of therapeutic period, the body weights of each group were observed without measurable variations (Figure 6D), further indicting the excellent biosecurity of our therapeutic strategies. Mice in sham group showed noticeable distended abdomen, ascribed to massive ascites accumulated. In comparison, there was no obvious ascites in other treatment groups (Figure 6C and E). There was no significant difference in mean tumor weight between NRs + L group and NRs + L + α PD-L1 group (Figure 6F), which may be due to the fact that pancreas and tumor are tightly bound and not easy to separate from orthotopic pancreatic tumors. Notably, NRs based PTT showed enhanced therapeutic effect when combined with α PD-L1, in terms of the decreased number of mesenteric metastases (Figure 6C and G). These results revealed the NRs based PTT, in combination with α PD-L1, exerting antitumor effect by clearing primary tumors under laser irradiation and inducing antitumor immunity to control distant metastasis.

3 Conclusion

In summary, we reported an effective combined therapy of NRs based PTT with α PD-L1 based immunotherapy for pancreatic cancer. The presence of ferrous iron-mediated Fenton reaction led to increased immunogenic tumor cell death under a low dose laser irradiation of 980 nm. Meanwhile, enhanced antigens capture was caused by Mal modifier of NRs. Such excellent properties offered great potentials to accelerate activation and infiltration of antigens presentation cells to trigger CTL response. The introduced immune checkpoint inhibitor, α PD-L1, further potentiated the relief of immunosuppression and improved antimetastatic effect, as shown by increased cell death in distal tumors, and reduced amounts of mesenteric

Figure 6: Therapeutic efficacy of combination therapy on orthotopic pancreatic tumors.

(A) Surgical exposure of orthotopic pancreatic tumor for laser irradiation. (B) Temperature elevations and near infrared thermal image (insert) on tumor surface during laser irradiation ($n = 4$). (C) Images of tumor-bearing mice. Left column: Comparison of the amount of ascites. Middle column: Comparison of the size of orthotopic pancreatic tumors. Right column: Comparison of the number of mesenteric metastases. (D) The changes of body weights of mice after different treatments ($n = 4$). (E–G) Volume of ascites (E), weight of orthotopic pancreatic tumors (F), insert: representative images of the tumors collected from the mice, and number of mesenteric metastases (G) from treated mice. ($n = 4$, * $P < 0.05$, ** $P < 0.01$).

metastases. Our work suggested a promising strategy for the treatment of pancreatic cancer to inhibit the growth of primary tumors, as well as attack diffuse metastases, which will open up future applications on synergistic combination of PTT and immunotherapy.

4 Experimental section

4.1 Preparation of Fe₂P-PEG nanorods

Fe₂P nanorods were synthesized according to the previously described method [27]. Briefly, Fe(acac)₃ (141.2 mg,

Aladdin, Shanghai, China) and oleylamine (20 mL, Aladdin) were introduced to a 100 mL three-necked flask with a magneton under nitrogen. Then, the mixture was heated to 260 °C. Subsequently, another 20 mL of oleylamine was rapidly injected into the mixture. After heating continuously for an hour, 4 mL of trioctylphosphine (Aladdin) was quickly injected into the mixture, and then the temperature of solution was increased to 320 °C for 2 h. The products were obtained by centrifugation and washed several times with cyclohexane (Aladdin). After volatilization of cyclohexane, Fe₂P NRs (5 mg) and DSPE-PEG₂₀₀₀-Mal (25 mg, Ponsure Biotechnology, Shanghai, China) or DSPE-PEG₂₀₀₀-NH₂-FITC (25 mg, Ponsure Biotechnology)

were dispersed in 5 mL dichloromethane (Aladdin), followed by sonication for 2 h at 25 °C. Dichloromethane was then evaporated, and the PEGylated Fe₂P nanorods (NRs) were re-dispersed in 1.5 mL deionized water under ultrasound for 2 h. Finally, 3 kDa filters (Millipore, MA, USA) were used to remove excess DSPE-PEG₂₀₀₀-Mal to obtain pure NRs.

4.2 Characterization of nanorods

The morphologies of NRs were confirmed by a transmission electron microscope (TEM, Tecnai G2, FEI, OR, USA). The chemical composition of NRs was measured by X-ray photoelectron spectroscopy (XPS, Axis Supra, Shimadzu, Kyoto, Japan). The absorbance of NRs was analyzed via a UV-Vis-NIR spectrophotometer (Evolution 220, Thermo Fisher, MA, USA). The photothermal properties of NRs were evaluated during laser irradiation by an infrared thermal camera (220S, FOTRIC, Shanghai, China). The magnetic resonance signals of NRs were measured by a clinical MRI scanner (MAGNETOM Skyra 3.0T, Siemens, Erlangen, Germany).

4.3 Cell culture

Panc02-H7 cell line was authenticated based on viability, recovery, growth, morphology, and isoenzymology. Bone marrow DCs (BMDCs) were harvested from C57BL/6 mice using standard methods. Panc02-H7 cells were cultured with Dulbecco's Modified Eagle Medium (DMEM, Gibco, NY, USA), BMDCs were culture with RPMI 1640 medium (Gibco). All cell culture mediums were supplied with 10% FBS (Gibco), penicillin (50 U/mL, Gibco) and streptomycin (50 mg/mL, Sigma-Aldrich, MO, USA). Additional granulocyte-macrophage colony-stimulating factor (GM-CSF, PeproTech, NJ, USA) was added into the culture medium for BMDCs. Cells were cultured in a humidified incubator supplemented with 5% CO₂ at 37 °C.

4.4 Cell staining assays

Panc02-H7 cells were cultured on cell slide in 24-well plates for 10 h, and then incubated with or without NRs for another 12 h, following with or without 980 nm laser irradiation (0.5 W/cm² at 2 min). For subcellular localization analysis, cells were stained with Lyso-Tracker Red (lyso-some indicator, Invitrogen, NY, USA) and Hoechst 33342 (nucleus indicator, Invitrogen). For ROS production

analysis, cells were stained with 2',7'-dichlorofluorescein diacetate (DCFH-DA, Invitrogen) immediately after laser irradiation. For calreticulin (CRT) expression analysis, cells were stained with anti-rabbit CRT polyclonal antibody (Immunoway, Beijing, China) and Alexa Fluor Plus 488-Goat anti-rabbit IgG (Invitrogen) at 1 h after laser irradiation. For cell death assay, cells were stained with annexin V-FITC/propidium iodide (PI, Sigma-Aldrich) and calcein acetoxymethyl ester (calcein-AM, Invitrogen)/PI after viroous treatments, and then detected by flow cytometry (CytoFLEX, Beckman Coulter, CA, USA) and fluorescence microscope (MD43-N, Mshot, Guangzhou, China), respectively. For evaluation of DCs maturation, the treated DCs were stained with FITC anti-mouse CD11c antibody and PE anti-mouse CD86 antibody (Biolegend) before measurement by flow cytometry (CytoFLEX).

4.5 Cell viability assays

CCK-8 (Dojindo, Kumamoto, Japan) assay was carried out to analyze cell viability. To detect cytotoxicity of NRs, tumor cells were cultured in 96-well plates for 10 h, and incubated with NRs for another 24 h. For photo-cytotoxicity of NRs, tumor cells were incubated with NRs for 12 h, then irradiated by 980 nm laser at 0.5 W/cm² for 2 min, and cultured for another 12 h. CCK-8 was incubated with treated cells for 1 h, and the absorbance at 450 nm was read by a 96-well plates reader (AMR-100, Allsheng, Hangzhou, China).

4.6 Animal models and *in vivo* treatment

Female C57BL/6 mice (6–8 weeks) were purchased from Guangdong Medical Laboratory Animal Center. The feeding and treatment of the mice were conducted in compliance with the Guide for the Care and Use of Laboratory Animals by Hainan University Institutional Animal Care and Use Committee.

To establish a primary tumor model, C57BL/6 mice were subcutaneously injected with Panc02-H7 cells (1×10^5) on left flanks. When primary tumors reached 200 mm³, mice were randomly assigned into four groups ($n = 3$), including control, L, NRs and NRs + L groups. The mice in NRs and NRs + L groups were intratumorally injected 100 µL of NRs (2 mg/kg). Then the Mice were anaesthetized by inhaling 2% v/v isoflurane, and primary tumors were irradiated with a 980 nm laser for 10 min (0.5 W/cm²). A thermal imaging camera was used to record the temperature on the tumor surface during laser

irradiation. Tumors were collected one day later for H&E staining and CD11c immunofluorescence staining (FITC anti-mouse CD11c antibody, Biolegend).

For abscopal effect assay, C57BL/6 mice were subcutaneously injected with Panc02-H7 cells (1.2×10^5) into left flanks (act as primary tumors) and injected with Panc02-H7 cells (0.8×10^5) into right flanks (act as distal tumors). After primary tumors reached 200 mm³, bilateral pancreatic tumor-bearing mice were divided into four groups ($n = 3$), including control, L, NRs + L and NRs + L + α PD-L1 groups. The primary tumors were intratumorally injected with 100 μ L of NRs (2 mg/kg), followed by irradiation with a 980 nm laser for 10 min (0.5 W/cm^2). Anti-mouse PD-L1 (α PD-L1, BioCell, CA, USA) (200 μ g per mouse) were intraperitoneally injected three days after treatment.

4.7 Orthotopic pancreatic tumor model and *in vivo* treatment

Panc02-H7 cells (5×10^4) were injected into the pancreatic tail of C57BL/6 mice to obtain pancreatic tumors, which were then cut into $1 \times 1 \text{ mm}^3$ pieces and surgically implanted into pancreas to establish the orthotopic pancreatic tumor model. Tumors were observed by a clinical MRI scanner (MAGNETOM Skyra 3.0T) *in vivo*. To validate passive tumor-targeting and biodistribution, Cy5-labeled Fe₂P NRs were prepared and intravenously injected into orthotopic pancreatic tumor-bearing mice, then the distribution of red fluorescence was observed by a fluorescent imager of Caliper IVIS Lumina XR (PerkinElmer, MA, USA). For tumor treatment, when tumors reached 200 mm³, mice were randomly assigned to three groups ($n = 4$), including sham controls (sham), NRs + L and NRs + L + α PD-L1 groups. The mice were weighed every two days. For laser irradiation, the orthotopic pancreatic tumors were surgically exposed. When the mice in sham group died, mice in each aforementioned group were sacrificed to measure the amount of ascites and metastases. In the meantime, tumors of each group were taken out, weighted and photographed.

4.8 Immunofluorescence assay

Tumors were collected and fixed in 4% paraformaldehyde, and cut into 5- μ m thick sections. These sections were stained with H&E, terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL, Sigma-Aldrich), FITC anti-mouse CD8a antibody and FITC anti-mouse CD206 (MMR)

antibody (Biolegend). Samples were analyzed by fluorescence microscopy (Mshot).

4.9 Statistical analysis

Values are expressed as mean $\pm$ standard error of the mean (SEM). All the data were analyzed with a one-way analysis of variance (ANOVA) followed by Bonferroni posttest. A P value of <0.05 was considered statistically significant, which all significant values were performed as follows: $^{\#}P < 0.05$, $^*P < 0.05$ and $^{**}P < 0.01$. All error bars were indicated as $\pm$ SD.

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