Photoacoustic-immune therapy with a multi-purpose black phosphorus-based nanoparticle

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ABSTRACT

Effective therapeutic strategies to precisely eradicate primary tumors with minimal side effects on normal tissue, inhibit metastases, and prevent tumor relapses, are the ultimate goals in the battle against cancer. We report a novel therapeutic strategy that combines adjuvant black phosphorus nanoparticle-based photoacoustic (PA) therapy with checkpoint-blockade immunotherapy. With the mitochondria targeting nanoparticle, PA therapy can achieve localized mechanical damage of mitochondria via PA cavitation and thus achieve precise eradication of the primary tumor. More importantly, PA therapy can generate tumor-associated antigens via the presence of the R848-containing nanoparticles as an adjuvant to promote strong antitumor immune responses. When combined with the checkpoint-blockade using anti-cytotoxic T-lymphocyte antigen-4, the generated immunological responses will further promote the infiltrating CD8 and CD4 T-cells to increase the CD8/Foxp3 T-cell ratio to inhibit the growth of distant tumors beyond the direct impact range of the PA therapy. Furthermore, the number of memory T cells detected in the spleen is increased, and these cells inhibit tumor recurrence. This proposed strategy offers precise eradication of the primary tumor and can induce long-term tumor-specific immunity.

KEYWORDS

photoacoustic therapy, mitochondrial targeting, black phosphorus, immunotherapy, checkpoint blockade

1 Introduction

Developing effective therapeutic strategies to precisely eradicate primary tumors, inhibit metastases, and prevent tumor relapses, without side effects are the ultimate objectives in the battle against cancer. The current gold-standard cancer treatment approaches are surgery, chemotherapy, and radiotherapy, but none can completely achieve this goal [1]. Cancer immunotherapy is a very popular approach that stimulates the innate and adaptive immune systems against tumors and has been progressing rapidly, showing tremendous promise as a next generation tool for cancer treatment strategy [1, 2]. An important immune resistance mechanism involves immune inhibition (immune checkpoints) that normally mediates immune tolerance and tumor escape [2–6]. Checkpoint blockades are a corresponding monotherapy that will only succeed in the setting of a pre-existing antitumor immune response [1, 4]. Therefore, to expand the efficacy of immunotherapy, a checkpoint blockade [4, 5, 7, 8] was combined with other traditional clinical therapies to induce de novo antitumor immune responses [9].

Chemotherapy and radiation therapy can be combined with immunotherapy, for immune stimulation. This leads to tumor-derived immunogenic cell death (ICD) and phagocytosis via released tumor antigens, promoting an antitumor immune

response [10–17]. This can be further combined with anti-cytotoxic T-lymphocyte antigen-4 (CTLA4) checkpoint blockades to suppress regulatory T-cells (T_{reg} s) for a greater therapeutic effect. Chemotherapy or radiation therapy can be combined with immunotherapy, for significant antitumor efficacy and even a long-term tumor-specific immunity [18–21]. However, the severe side effects of chemotherapy and radiation therapy still have not been overcome.

Synergistic photo-immunotherapy combination with CTLA4 checkpoint blockade is a promising treatment for cancer because it is non-invasive, with low systemic toxicity, good laser controllability, and excellent photoradiation-dependent tumor selectivity [22–26]. However, the photodynamic therapy (PDT) effect was confined via low singlet oxygen (1O_2) to yield efficiency in the tumor site due to hypoxia in the tumor and aggregation-caused quenching (ACQ) of traditional photosensitizers (PSs) [27–33]. Another common phototherapy strategy (photothermal therapy (PTT)) includes a light-absorbing agent that accumulates in a tumor with near-infrared light. PTT converts light energy into heat and thermally ablates tumor cells. This indicates the ability to generate antitumor immunological effects by producing tumor-specific antigens from ablated tumor cell residues [6, 34–37]. However, PTT may overheat in the general targeting area, thus causing

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unnecessary damage to normal tissues.

Photoacoustic therapy has recently emerged by targeting nanoparticles to tumor cells using photoacoustic (PA) shockwave to specifically destroy tumor cells [38–40]. The PA shockwave is created by the cavitation (growth and collapse) of nano- and micro-bubbles around nanoparticles heated by laser pulses. This produces a strong and localized mechanical disruption of the nearby tissue [38, 41–43]. The impact range of the PA shockwave is well defined due to the rapid decay of the sound wave intensity from the target tissue (approximately tens of micrometers) [44]. Versus other available therapies, PA therapy provides a distinct potential advantage: It can precisely eradicate primary tumors with minimal side effects on normal tissue. Despite these encouraging results, whether and how PA therapy can trigger any immunological response and exert any effect in inhibiting tumor metastasis remain largely unknown.

Recently, black phosphorus (BP) has emerged as an inorganic two dimensional nanomaterial with many novel properties, such as a large near-infrared extinction coefficient, a high photothermal conversion efficient, and an ultra-high surface to volume ratio that may allow for high drug-loading ability, as well as excellent biocompatibility and biodegradation, i.e., the final degradation products are nontoxic phosphate and phosphonate [45, 46]. Thus, BP nanoparticles are an important bio-material being used for bioimaging, phototherapy, and drug delivery [47, 48].

Herein, we report a therapeutic strategy that combines adjuvant BP nanosheets-based PA therapy with a checkpoint-blockade immunotherapy. Biodegradable BP nanosheets with large near-infrared extinction coefficients were chosen as a PA therapy agent for converting laser energy to a PA shockwave. BP nanosheets were modified with tumor-targeting cyclic Arg-Gly-Asp (RGD) peptide, mitochondrion-targeting triphenylphosphine (TPP), and Resiquimod (R848) an immune-adjuvant with a Toll-like receptor 7 and 8 (TLR7/TLR8) agonist, for producing an adjuvant effect. The nanoparticle termed here is BP/TPP/

RGD/R848. The mitochondria are mechanically destroyed by PA shockwaves when the BP/TPP/RGD/R848 specifically target mitochondria, with pulsed laser irradiation. This leads to cell apoptosis and thus precise PA therapy for eradication of a primary tumor with minimal side effects. More importantly, the PA therapy can generate tumor-associated antigens in the presence of the R848-containing nanoparticles as the adjuvant. This adjuvant can in turn promote strong antitumor immune responses. In combination with the checkpoint-blockade using CTLA4, the generated immunological responses will be further promoted to inhibit the growth of distant tumors beyond the direct impact range of PA therapy. This leads to a long-term immune effect to confine tumor cell regrowth. Figure 1 illustrates our overall treatment strategy with BP/TPP/RGD/R848.

2 Materials and methods

2.1 Materials

BP was purchased from XF Nano Materials Tech Co., Ltd (Nanjing, China). N-Methyl pyrrolidone (NMP) (99.5%, anhydrous), N-hydroxysuccinimide (NHS), 1-hydroxypyrrolidine-2,5-dione, and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) were purchased from Aladdin Reagents. The C[RGDyK] was purchased from TOP (Shanghai, China). Resiquimod (R848) was procured from InvivoGen (CA, USA). The (3-carboxypropyl) triphenylphosphonium bromide (TPP) and NMP were obtained from J&K Chemical CO.

2.2 Preparation of BP nanosheets

The BP was prepared by a simple liquid phase stripping technique. Briefly, BP (30 mg) powder was added to NMP (30 mL) in a 100 mL sealed conical tube and sonicated with a sonic tip for 4 h at 1,200 W. The ultrasonic frequency was from 19 to 25 kHz and the ultrasound probe worked every 2 s with an interval of 4 s. The dispersion was then sonicated in an ultrasonic bath continuously for another 1 h at 300 W. The temperature of the

Figure 1 (a) Nanoparticle synthesis. (b) Mechanisms of combining PA therapy with anti-CTLA4 therapy.

sample solution was kept below 277 K by an ice bath. The resulting dispersion was centrifuged for 20 min at 7,000 rpm, and the supernatant containing BP nanosheets was gently decanted. The BP nanosheet solution was centrifuged for 20 min at 12,000 rpm, and then the precipitate was repeatedly rinsed with water and re-suspended in an aqueous solution.

2.3 Synthesis of BP/R848

To produce the immuno-adjuvant R848 coating, BP (0.4 mg), was mixed in tri-distilled water (0.2 mL) and sonicated for 30 min to obtain BP dispersion ($2 \text{ mg}\cdot\text{mL}^{-1}$). The R848 ($0.5 \text{ mg}\cdot\text{mL}^{-1}$, 0.2 mL) and bovine serum albumin (BSA) ($0.5 \text{ mg}\cdot\text{mL}^{-1}$, 0.2 mL) were added to the BP dispersion, and the mixture was uniformly mixed, sonicated in a water bath for 1 h, and stirred at room temperature for 6 h. An immunoadjuvant-modified BP (BP/R848) was obtained.

2.4 Synthesis of mitochondrial targeting molecule-modified BP/R848/HS-PEG^{2k}-NH₂/TPP nanoparticle

TPP (8 mg) dissolved in dimethyl sulfoxide (DMSO) (0.2 mL). The EDC (9 mg) and NHS (6 mg) were added and stirred for 4 h. The solution was then treated with HS-PEG^{2k}-NH₂ ($20 \text{ mg}\cdot\text{mL}^{-1}$, 0.5 mL). The reaction was carried out for 12 h at room temperature to a final concentration of HS-PEG^{2k}-NH₂/TPP (0.2 mmol). Next, BP/R848 ($1 \text{ mg}\cdot\text{mL}^{-1}$) and HS-PEG^{2k}-NH₂/TPP (0.2 mmol) were mixed and stirred at room temperature for 12 h, and distilled water was used to wash three times. The final BP/R848/HS-PEG^{2k}-NH₂/TPP ($1 \text{ mg}\cdot\text{mL}^{-1}$) complex was obtained by centrifugation at 7,000 rpm for 10 min.

2.5 Synthesis of dual targeting potency of molecule modified BP/R848/HS-PEG^{2k}-NH₂/TPP/RGD nanoparticle

TPP (8 mg) was dissolved in DMSO (0.2 mL). Then, EDC (9 mg) and NHS (6 mg) were added and stirred for 4 h. The solution was treated with HS-PEG^{2k}-NH₂ ($20 \text{ mg}\cdot\text{mL}^{-1}$, 2 mL) and C[RGDyK] peptide (10 μg). The reaction was carried out for 12 h at room temperature to reach a final concentration of HS-PEG^{2k}-NH₂/TPP/RGD (0.2 mmol). Then BP/R848 ($1 \text{ mg}\cdot\text{mL}^{-1}$) and HS-PEG^{2k}-NH₂/TPP/RGD (0.2 mmol) were mixed and stirred at room temperature for 12 h, and distilled water was washed three times. The final HS-PEG^{2k}-NH₂/TPP/RGD complex ($1 \text{ mg}\cdot\text{mL}^{-1}$) was obtained by centrifugation at 7,000 rpm for 10 min.

2.6 Characterization of the nanoparticle

Transmission electron microscopy (TEM) images were collected on a field emission high-resolution 2100F transmission electron microscope (JEOL, Japan) operating at an acceleration voltage of 200 kV. The atomic force microscopy (AFM) was performed on drop-cast flakes on Si/SiO₂ substrates using the Series 5500 atomic force microscopy (Agilent, USA) under AC mode (tapping mode) in air. The size distribution of the nanoparticles was measured via a Malvern Zetasizer Nano ZS90 (Malvern, UK) instrument. The samples were recorded from 4,000 to 500 using a Nicolet Model 759 Fourier transform infrared spectrometer (FT-IR), and the samples were compressed with KBr crystals to detect infrared spectra. The optical characteristics of Cy5.5, BP, R848, TPP and BP/R848/TPP/Cy5.5 were investigated by UV–vis absorption (Lambda-35 UV–vis spectrophotometer, PerkinElmer, MA, USA).

2.7 Cell culture

4T1 cells and U87-MG cells were incubated in Dulbecco's modification of Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin,

$10,000 \text{ U}\cdot\text{mL}^{-1}$) in a 37 °C humidified atmosphere containing 5% CO₂. The DC2.4 cells, 4T1 cells, and MCF-7 cells were incubated in RPMI-1640 medium with 10% FBS and 1% antibiotics (penicillin-streptomycin, $10,000 \text{ U}\cdot\text{mL}^{-1}$) at 37 °C in a humidified atmosphere containing 5% CO₂. Murine breast cancer 4T1 cells were cultured under standard medium and conditions. A cell counting assay (CCK-8, Dojindo Laboratories, Kumamoto, Japan) was used to measure the cell viabilities.

2.8 For confocal fluorescence imaging

BP/TPP/RGD/R848 was labeled with Cy5.5-NHS. The 4T1 cells were cultured with BP/TPP/RGD/R848/Cy5.5 and staining with MitoTracker to visualize mitochondria, under a confocal fluorescence microscope. Mitochondria were stained by MitoTracker (Beyotime Biotechnology, Catalog number: C1048) strictly following the standard protocol.

Mitochondrial regulation of apoptosis by JC-1 assay: The 4T1 cells were cultured with Laser, BP/TPP/RGD/R848 or BP/TPP/RGD/R848+Laser in a cell culture dish. After 12 h incubation at 37 °C, the mitochondria were stained with JC-1 ($10 \text{ mg}\cdot\text{mL}^{-1}$) in DMEM for another 30 min. After washing with phosphate buffer saline (PBS) for 3 times, the cells were observed under a laser scanning confocal microscopy.

2.9 To assess the changes in nuclear morphology of apoptosis by PA therapy

4T1 cells were cultured with Laser, BP/TPP/RGD/R848 or BP/TPP/RGD/R848+Laser in 35 mm glass-bottomed dishes. After 12 h of incubation at 37 °C, tumor cells were irradiated for 1 min by the pulsed laser (808 nm, at a fluence of $20 \text{ mJ}\cdot\text{cm}^{-2}$, 20 Hz repetition rate, and 4 ns pulse duration). These samples were co-stained with calcein-acetylmethoxylate (calcein AM) and propidium iodide (PI) for 30 min at 37 °C and washed twice with PBS. Images were acquired with a confocal laser scanning microscope (ZEISS LSM 510 META, Germany). Calcein-AM and PI were purchased from Sigma-Aldrich Corporation (MO, USA).

2.10 Apoptosis experiments

The 4T1 cells were cultured with Laser, BP/TPP/RGD/R848 or BP/TPP/RGD/R848+Laser in cell culture dish. After 12 h of incubation at 37 °C, tumor cells were irradiated for 1 min by the pulsed laser (808 nm, at a fluence of $20 \text{ mJ}\cdot\text{cm}^{-2}$, 20 Hz repetition rate, 4 ns pulse duration). Samples containing 10,000 cells each were run on a FACS Canto II flow cytometer with excitation at 488 nm. Fluorescein isothiocyanate (FITC) and PI were obtained from Sigma-Aldrich Corporation (MO, USA). The fluorescence emission of FITC was measured at 515–545 nm and the PI complexes were 564–606 nm.

2.11 In vitro transwell system experiment

To evaluate the activation of DCs by tumor-associated antibodies produced by PA therapy, the 4T1 cells were first seeded onto the abluminal side of the transwell filter (0.4 μm pore size) at a density of 1.0×10^5 cells per filter (1.12 cm² surface area, Corning Incorporated Life Sciences, USA). These samples were then cultured with BP/TPP/RGD, BP/TPP/RGD+Laser, R848, BP/TPP/RGD/R848 or BP/TPP/RGD/R848+Laser. The 4T1 cells were irradiated for 1 min by the pulsed laser (808 nm, at a fluence of $20 \text{ mJ}\cdot\text{cm}^{-2}$, 20 Hz repetition rate, 4 ns pulse duration). The DC2.4 cells were cultured in the lower chamber of the transwell system. Both cells were incubated under normal culture conditions for 12 h. The DCs was collected and stained with anti CD80-PE and anti CD86-APC, and then sorted by flow cytometry.

2.12 Animal models

Female Balb/c mice (10–20 g, 5–6 weeks) were obtained from Southern Medical University (Guangzhou, China). The animal study protocol was approved by the Institutional Animal Care and Use Committee at Southern Medical University (Guangzhou, China). To study the immune cells in secondary tumors, tumors were harvested from mice in different groups and homogenized into single cell suspension following the well-established protocol. Those antibodies were anti-CD8a-APC (Biolegend, Clone: 53–6.7, Catalog: 100712), anti-CD4-PerCP (Biolegend, Clone: GK1.5, Catalog: 100434) and used according to the manufacturer's protocols.

Cytotoxic T lymphocytes (CTLs) and helper T-cells were stained with CD4⁺CD8⁺ and CD4⁺CD8[−]. To analyze CD4⁺ helper T-cells, cells in the secondary tumor were further stained with anti-CD3-FITC (Biolegend, Clone: 145-2C11, Catalog: 100203), anti-CD4-PerCP (Biolegend, Clone: GK1.5, Catalog: 100434), and anti-Foxp3-PE (Biolegend, Clone: NRRF-30, Catalog: 126404) antibodies according to the standard protocol. CD4⁺ helper T-cells were classified into T_{eff} (CD4⁺Foxp3[−]) and T_{regs} (CD4⁺Foxp3⁺). For analysis of memory T-cells, lymph nodes harvested from mice after various treatments were homogenized into single-cell suspensions and stained with anti-CD3-FITC (Biolegend, Clone: 145-2C11, Catalog: 100203), anti-CD8-APC (Biolegend, Clone: 53-6.7, Catalog: 100712), anti-CD62L-PE (Biolegend, Clone: MEL-14, Catalog: 104407), and anti-CD44-PerCP (Biolegend, Clone: IM7, Catalog: 103031) antibodies. Central memory T-cells (T_{CM}) and effector memory T-cells (T_{EM}) were CD3⁺CD8⁺CD62L⁺CD44[−] and CD3⁺CD8⁺CD62L[−]CD44⁺, respectively.

2.13 Cytokine detection

Serum samples and cell culture supernatants were isolated from mice after various treatments and diluted for analysis. TNF- α (BioLegend), IL-12 (BioLegend), and IFN- γ (BioLegend) were analyzed with ELISA kits according to vendor's instructions.

3 Results and discussion

3.1 Characterization of the nanoparticle

We first characterized the BP/TPP/RGD/R848 nanoparticles.

TEM revealed that the BP/TPP/RGD/R848 nanoparticle exhibited uniform spherical morphology with diameters of ~ 55 nm (Fig. 2(a)). The AFM result was in accordance with the TEM analyses (Fig. 2(b)). BP nanosheets exhibited a uniform morphology with diameters of ~ 5 nm (Figs. S1 and S2 in the Electronic Supplementary Material (ESM)), which is consistent with previous studies [45, 46, 49]. The hydrodynamic diameter of BP/TPP/RGD/R848 was determined to be ~ 65 nm (Fig. 2(c)), which is 57 nm larger than BP nanosheets (~ 6 nm) (Fig. S3 in the ESM). The presence of TPP, RGD, and R848 on the BP nanosheets surface was confirmed by Fig. 2(d). The absorption bands at $3,350\text{ cm}^{-1}$ were associated with $-\text{NH}_2$ groups in R848; bands at $1,633\text{ cm}^{-1}$ were associated with the $-\text{C}=\text{C}-$ groups in RGD; and bands at 790 and 770 cm^{-1} were attributed to the three substituted benzene vibration, which was observed in TPP, RGD and R848. These results confirmed that TPP, RGD, and R848 were modified on the BP nanosheets through the formation of amide bonds between the $-\text{COOH}$ of TPP and the $-\text{NH}_2$ of polyethylene glycol (PEG). The optical absorbance spectrum of BP/TPP/RGD/R848 exhibits typical features of TPP and R848 with peak absorbance at 262 and 365 nm (Fig. 2(e)). Due to the good extinction coefficient of BP/TPP/RGD/R848 (Fig. S4 in the ESM), we observed a strong PA signal when the nanoparticles were irradiated with a single laser pulse (808 nm , $20\text{ mJ}\cdot\text{cm}^{-2}$) with an ultrasonic transducer (Fig. 2(f)), indicating the generation of a mechanical wave.

3.2 The damage mechanism and the physical damage range of PA therapy

Integrin $\alpha_v\beta_3$ is overexpressed by tumor cells and is a receptor for extracellular matrix proteins after exposure to RGD [21]. To investigate the cancer-targeting properties of BP/TPP/RGD/R848 based on the decoration of RGD moieties, two tumor cells were studied for cellular uptake of Cy5.5-labelled BP/TPP/RGD/R848 by flow cytometry: MCF-7 cells with lower expressed $\alpha_v\beta_3$ integrin and 4T1 cells with overexpressed $\alpha_v\beta_3$ integrin. The fluorescence intensity of 4T1 cells was stronger 38% than that of MCF-7 cells (Figs. 3(a)–3(c)). These results indicated that the BP/TPP/RGD/R848 nanoparticle was specific to 4T1 breast tumor cells, these targeting are mainly mediated by the interaction between RGD and integrin. Next, the subcellular localization of BP/TPP/RGD/R848 was

Figure 2 Characterization of nanoparticles. (a) TEM imaging of BP/TPP/RGD/R848. (b) AFM image of BP/TPP/RGD/R848. (c) Hydrodynamic diameter distribution of BP/TPP/RGD/R848 obtained by dynamic light scattering (DLS) analysis. (d) FTIR spectra of BP, TPP, RGD, R848, and BP/TPP/RGD/R848. (e) Absorption spectra of BP, TPP, RGD, R848, and BP/TPP/RGD/R848. (f) PA signals of BP/TPP/RGD/R848 at $0.4\text{ mg}\cdot\text{mL}^{-1}$ upon pulsed laser (808 nm , $20\text{ mJ}\cdot\text{cm}^{-2}$) irradiation.

Figure 3 The dual targeting effect of nanoparticles was verified *in vitro*. (a) Flow cytometry analyses of 4T1 cells upon incubation with Control (black line), BP/TPP/R848/Cy5.5 (red line), and BP/TPP/RGD/R848/Cy5.5 (blue line). The “Control” condition received no nanoparticle. (b) Flow cytometry analyses of MCF-7 cells upon incubation with Control (green line) and BP/TPP/R848/Cy5.5 (cyan line) and BP/TPP/RGD/R848/Cy5.5 (purple line). (c) The detected mean fluorescence intensities of all groups in (a) and (b). Error bars represent the standard deviation of separate measurements ($n = 3$). (d) Confocal images show subcellular localization of BP/TPP/RGD/R848/Cy5.5. (e) Comparison of mitochondrial membrane potentials (JC-1 assay) in 4T1 cells by confocal laser scanning microscopy (CLSM) after different treatments. P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

studied in cultured tumor cells. 4T1 cells stained with MitoTracker (to label mitochondria) were incubated with BP/TPP/RGD/R848. Confocal fluorescence images showed the co-localization of the two color channels, indicating that BP/TPP/RGD/R848 was in the mitochondria. Further, the Pearson correlation coefficients were higher than 0.956 (Fig. 3(d)). Together, these results indicated that BP/TPP/RGD/R848 localized in the mitochondria.

Mitochondrion is a subcellular organelle that is the respiratory center of the cells [43]; its damage can directly cause cellular apoptosis. The PA shockwave generated in PA cavitation could have destructive effects [50]. Whether the PA shockwave could damage mitochondria was investigated after BP/TPP/RGD/R848 localized in the mitochondria. JC-1 is a cationic dye used to evaluate mitochondrial function [51]. The green monomer of JC-1 can enter the cytoplasm and aggregate in normal mitochondria, forming a large number of red J-aggregates, the fluorescence transition from red to green indicates the mitochondrial dysfunction. The results demonstrate normal mitochondrial function (red fluorescence) in 4T1 cells treated with PBS, Laser, and BP/TPP/RGD/R848. Green fluorescence was observed for BP/TPP/RGD/R848+Laser, indicating mitochondrial dysfunction (Fig. 3(e)). The results demonstrate that with an irradiated pulsed laser, the PA shockwave generated in mitochondria targeting nanoparticle-mediated PA cavitation

could destroy cellular mitochondria.

We next used CCK-8 to measure the viability of 4T1 cells under different treatment conditions to investigate the *in vitro* therapeutic efficacy of mitochondria-targeting nanoparticle-mediated PA therapy. Our results show that the effect of mitochondria targeting nanoparticle-mediated PA therapy depends on the concentration of nanoparticles at the same pulsed laser energy (808 nm, 20 mJ·cm⁻²). The cell viability decreases significantly as the concentration of nanoparticles increases. The activity was reduced to 32.4% when the concentration of the nanoparticles was 50 μg·mL⁻¹ (Fig. S5 in the ESM). Therefore, we selected this optimal nanoparticle dose of 50 μg·mL⁻¹ as well as a pulsed laser fluence of 20 mJ·cm⁻² for PA therapy in all the following experiments.

To visually display the therapeutic efficiency of BP/TPP/RGD/R848, cells were pretreated with the presence or absence of pulsed laser irradiation and then stained with Calcein-AM and PI to identify live and dead cells, respectively. Versus other groups, more red fluorescent cells were found for the BP/TPP/RGD/R848+Laser group; this was more lethal to tumor cells upon pulsed laser irradiation (Fig. 4(a)).

Furthermore, the ratio of cell apoptosis and necrosis after different treatments was analyzed by flow cytometry with Annexin V-FITC/PI double staining (Fig. 4(b)). As expected, Control, Laser and BP/TPP/RGD/R848 treatment alone induced remarkable cell survival. In contrast, BP/TPP/RGD/R848+Laser treatment induced obvious cell apoptosis. Statistical analysis

Figure 4 *In vitro* models validate the effectiveness and accuracy of PA therapy. (a) Fluorescent confocal images showing the effect of the nanoparticle and/or pulsed laser irradiation on cell death. The “Control” condition received no nanoparticle and laser treatment. Calcein AM staining (live cells) is labeled in green and PI staining (dead cells) is labeled in red. (b) Cell apoptosis and necrosis analyzed by flow cytometer with Annexin V-FITC/PI double staining after different treatments. (c) Optical images of the co-plated 4T1 cells and MCF-7 cells before and after PA therapy. The 4T1 cells are marked with the arrows. (d) 4T1 cells specifically targeted by the BP/TPP/RGD/R848 nanoparticle and the control MCF-7 cells were mixed and co-plated in a single layer (4T1 & MCF-7). The viabilities of 4T1, MCF-7&4T1, and MCF-7 cells are shown after the identical dose of PA therapy (50 μg·mL⁻¹, 20 mJ·cm⁻²). Data are shown as mean ± SD ($n = 3$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

showed that the percentage of cells undergoing apoptosis significantly increased from 2.36% (Control) to 71.98% for the group of BP/TPP/RGD/R848+Laser, which confirmed PA cavitation to tumor cells. These results indicated that cell apoptosis was a major cell death modality for BP/TPP/RGD/R848-based PA therapy.

To determine the physical range of damage generated by the PA shockwave, we assessed the cell viability of a mixed cell culture preparation. We detected the co-plated 4T1 cells and MCF-7 cell morphology before and after PA therapy with an optical microscope. The 4T1 cells with BP/TPP/RGD/R848+Laser were destroyed (Fig. 4(c), first column, marked with arrows), and the adjacent MCF-7 cell morphology remained intact due to the specificity of the nanoparticles.

As a control, we plated 4T1 cells and MCF-7 cells alone with the nanoparticle. In these control groups, a shockwave could be generated within every cell depending on the efficacy of the PA therapy for the different cell types, and cell viability would be reflected accordingly. After subjecting all three groups to identical doses of PA treatment ($50 \mu\text{g}\cdot\text{mL}^{-1}$, $20 \text{ mJ}\cdot\text{cm}^{-2}$), the cytotoxic efficacy of the therapy was tested with a CCK-8 cell viability assay. We found that the viability of the co-plated cells (63.7%) was statistically higher than that of the 4T1 cells alone (31.4%) and lower than the MCF-7 cells alone (82.3%) (Fig. 4(d)). These results suggest that PA therapy could be a precise method to kill tumor cells with minimal damage to the

adjacent tissue with a proper dosimetry.

3.3 PA therapy triggers an immunological response *in vitro*

Dendritic cells (DCs) are the most potent antigen presenting cells (APCs). DCs efficiently capture antigens in peripheral tissues and process these antigens to form major histocompatibility complex (MHC-peptide) complexes [52]. These immature DCs acquire a unique ability to migrate from the periphery of the secondary lymphoid organ to the T cell region following antigen uptake [53]. As cells move, they mature and alter the distribution of cell surface molecules, attracting resting T-cells and presenting their antigenic load [54]. The up-regulation of co-stimulatory molecules, CD80 and CD86, as the typical markers on the surface of DCs may indicate the level of DC maturation [22]. Whether and how PA therapy triggers an immunological response and inhibits tumor remains largely unknown. Thus, we selected a universally recognized immature DC cell line, DC 2.4 cells [36], to investigate the immune activation characteristics after PA therapy.

A transwell system was then used to study such effects at the *in vitro* level [22]. In our experiment, the upper chamber containing 4T1 tumor cells after various treatments was placed into the transwell system. The lower chamber was seeded with DC2.4 (Fig. 5(a)). The DC maturation and cytokine

Figure 5 *In vitro* transwell system experiment. (a) Scheme showing the design of the transwell system experiment. 4T1 tumor cells and their residues were placed in the upper chamber, and DC2.4 were cultured in the lower chamber. (b) Quantification of CD86 and CD80 expression by flow cytometry for various samples in the *in vitro* transwell system experiment. (c) Statistical analysis of flow cytometry data in (b). Error bars represent the standard deviation of separate measurements ($n = 3$) and (d) the secretion of IL-12p40, (e) TNF- α , and (f) IFN- γ in DC2.4 suspensions after treatment for 20 h in the transwell system experiment. Data are shown as mean $\pm$ SD ($n = 3$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

secretion from DCs were then evaluated by flow cytometry and enzyme-linked immunosorbent assay (ELISA), respectively. Versus the DC maturation level after culturing DC2.4 without 4T1 cells (Fig. S6 in the ESM), the addition of BP/TPP/RGD/R848 nanoparticles into this system resulted in a slight increase of DC maturation (Figs. 5(b) and 5(c)). In contrast, if those 4T1 cells were treated by PA therapy using BP/TPP/RGD+Laser or BP/TPP/RGD/R848+Laser, their residues could remarkably promote DC maturation, particularly if BP/TPP/RGD/R848+Laser was used (Figs. 5(b) and 5(c)).

Consistent with DC maturation data, we also found that 4T1 tumor cell residues post-PA therapy treatment with BP/TPP/RGD/R848+Laser triggered the highest levels of interleukin-12 (IL-12), tumor necrosis factor- α (TNF- α), and interferon- γ (IFN- γ) secretion by DC2.4 (Figs. 5(d)–5(f)). Our results suggest that tumor-associated antigens from tumor cell residues post-PA

therapy could trigger effective DC maturation, especially with R848-loaded nanoparticles as the adjuvant.

3.4 Distribution of nanoparticles *in vivo*

The preferential accumulation of BP/TPP/RGD/R848 in tumor regions was investigated on a systemic level. The biodistribution of BP/TPP/RGD/R848/Cy5.5 was examined using an *in vivo* fluorescence imaging system at multiple time points post-injection. BP/TPP/RGD/R848/Cy5.5-treated mice have much higher fluorescence intensities in their tumors, indicating accumulation of BP/TPP/RGD/R848 in tumors (Figs. 6(a) and 6(b)). The fluorescence intensity reaches a maximum for BP/TPP/RGD/R848/Cy5.5 twelve hours post-injection. The PA images of solid tumors at various time points result was in accordance with the fluorescence intensities analyses (Fig. S7 in the ESM). Considering the biosafety of nanoparticles *in*

Figure 6 The pharmacokinetics of BP/TPP/RGD/R848/Cy5.5 and activation effect of different treatments on DC cells *in vivo*. (a) *In vivo* fluorescence imaging of mice with 4T1 cancer upon treatment with BP/TPP/RGD/R848/Cy5.5 at different time intervals. (b) Fluorescence intensity statistics for (a). (c) Quantification of CD86 and CD80 expression by flow cytometry for various treatments *in vivo*. (d) Statistical analysis of CD86 and CD80 on the surface of DCs in (c) and (e) the secretion of IL-12p40, (f) TNF- α and (g) IFN- γ levels in sera from mice detected at 72 h after various treatments. Data are shown as mean $\pm$ SD ($n = 3$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

vivo, we sacrificed the mice after 12, 24, 36, and 48 h of injection of nanoparticles and harvested their main organs. The main organs and tumor tissues were spectrally imaged by an ODYSSEY Infrared Imaging System (Fig. S8(a) in the ESM). The averaged fluorescent intensity of each imaged organ was calculated for a semiquantitative biodistribution analysis (Fig. S8(b) in the ESM). A strong fluorescent signal was observed in tumors and reticuloendothelial systems including the liver, spleen and lung. In addition, the absorption of BP gradually decrease with extend incubation time (Fig. S9(a) in the ESM), and it was almost colorless and transparent by the eighth day (Fig. S9(b) in the ESM). These results implied the biodegradability of BP. Finally, histological analysis of major organs was examined for potential side effects exhibiting undetectable pathological abnormalities and adverse reactions (Fig. S10 in the ESM), which suggested negligible systemic toxicity of the BP/TPP/RGD/R848-based PA therapy.

3.5 PA therapy triggers immune system activation *in vivo*

We next determined whether PA therapy with our BP/TPP/RGD/R848 could trigger immunological responses *in vivo*. BALB/c mice bearing 4T1 breast tumor were intravenously injected with BP/TPP/RGD or BP/TPP/RGD/R848 and then irradiated by 808 nm laser at 20 mJ·cm⁻² for 20 min to ablate tumors via the PA effect from our previous studies [39–41, 55]. At 72 h after PA therapy, mice were killed, and their spleens were collected for flow cytometry after co-staining with various markers. The results suggested that CD80 and CD86 were significantly upregulated *in vivo*, and PA tumor ablation with adjuvant nanoparticles (BP/TPP/RGD/R848+Laser) induced a high level of DC maturation (Figs. 6(c) and 6(d)), which appeared to be much higher than that observed for adjuvant nanoparticles alone (BP/TPP/RGD/R848 without laser) or PA therapy with BP/TPP/RGD in the absence of immune-adjuvant. Therefore, after the tumor is destroyed via PA therapy, DCs may be recruited to the ablated tumor site as antigen-presenting cells to trigger immune responses.

Tumor-associated antigens in tumor debris post PA therapy can be transported into nearby lymph nodes and then processed by DCs to stimulate DC maturation via the adjuvant nanoparticles. Cytokine secretion is also important in immune responses. In a parallel experiment, the sera of mice bearing 4T1 tumors after different treatments were collected at 72 h to analyze the changes of various cytokines including IL-12, TNF- α , and IFN- γ (Figs. 6(e)–6(g)). Similarly, although BP/TPP/RGD/R848 injection alone or PA therapy with BP/TPP/RGD could increase the secretion of pro-inflammatory cytokines, the secretions induced by BP/TPP/RGD/R848-based PA therapy were noticeably higher; this was favorable for triggering an anti-tumor immune response. These results suggest that BP/TPP/RGD/R848-based PA therapy could induce immunological stimulation effects *in vivo*.

3.6 *In vivo* PA therapy combined with CTLA-4

Next, we assessed whether such robust immunological responses triggered by PA therapy with BP/TPP/RGD/R848 would be able to inhibit the growth of tumor cells left behind after PA therapy at distant sites. The blockade of CTLA4 has been approved by the US Food and Drug Administration (FDA) for cancer treatment and may suppress the immuno-suppressive regulatory capacity of T_{regs} [56–58]. Thus, in our animal experiments, CTLA-4 blocking therapy was introduced to enhance the anti-cancer immuno-therapy efficacy generated *in situ* after precise PA ablation of primary tumors based on

BP/TPP/RGD/R848. Mice that bore bilateral 4T1 breast tumor flank tumors underwent CTLA4 treatment. The tumors in the left flanks (primary tumors) were designed for BP/TPP/RGD/R848-based PA therapy, and the tumors in the right flanks were designed without direct treatment as an artificial model of abscopal tumor. On the 1st day, nanoparticles (1 mg·mL⁻¹ 0.2 mL) were intravenously injected, and the left tumor received PA therapy. On the 2nd, 4th, and 6th day, the mice were intravenously injected with anti-CTLA4 (1 mg·mL⁻¹ 15 μ L) (Fig. 7(a)).

The tumor sizes on both sides that primary tumors with direct PA therapy treatment and distant tumors without PA therapy treatment were then measured (Figs. 7(b) and 7(c)). The control, Laser, BP/TPP/RGD/R848, and anti-CTLA4 injection alone showed no appreciable inhibitory effect on both primary and distant tumor growth. The tumor growth was partly delayed in BP/TPP/RGD/R848+anti-CTLA4 due to the non-specific immune responses elicited by adjuvant nanoparticles and anti-CTLA4. PA therapy (BP/TPP/RGD/R848+Laser) could only ablate primary tumors, not effectively inhibit distant tumors. In contrast, combining PA therapy with anti-CTLA4 therapy not only significantly suppressed their growth within the first 12 days post-treatment, but also offered remarkably synergistic effects to eliminate abscopal tumors, whose sizes gradually shrunk after their primary tumors on the contralateral side were treated by PA therapy and then disappeared after about 10 days.

We also found that 80% of 4T1 tumor-bearing mice could survive for 58 days after BP/TPP/RGD/R848-based PA therapy and anti-CTLA4 therapy (Fig. 7(d)); this is in marked contrast to mice in the other six control groups which all died within 36–48 days. The body weight of the mice in the seven treatment groups had no significant changes throughout the entire treatment, suggesting negligible systemic toxicity (Fig. 7(e)). In addition, hematoxylin-eosin staining (H&E) analysis of tumor tissues revealed that the PA therapy group combined with CTLA4 blockade exhibited much more pyknotic cells with highly condensed nuclei (apoptotic/necrotic) (Fig. 7(f)). Then, tumor-bearing mice irradiated with pulsed laser, also displaying non-obvious temperature increasing. Thus, minimal heating effect was observed upon pulsed laser irradiation *in vivo*, suggesting the non-thermal property of photoacoustic modality (Fig. S11 in the ESM). The highest *in vivo* antitumor therapeutic efficacy likely resulted from the integrated advantages of PA therapy combined with CTLA4 blockade, including strong PA cavitation that could mechanically disrupt intracellular organelles.

Immune cells in secondary 4T1 tumors were studied on day 7 to understand how the mechanism of synergistic anti-tumor effects triggered by BP/TPP/RGD/R848-based PA therapy in combination with anti-CTLA4 therapy interact with the immunological system (Fig. 8(a)). CTLs (CD3⁺CD4⁺CD8⁺) play a positive role in attacking tumor cells and could directly kill targeted tumor cells by releasing the cytotoxins-IFN- γ , perforin, granzymes, and granulysin [59, 60]. In our experiments, treatment with free anti-CTLA-4 or BP/TPP/RGD/R848 alone failed to promote CD8⁺ CTL infiltration into secondary tumors (Fig. 8(b)). In contrast, the percentage of total T cells in secondary tumors showed a significant increase after treatment with BP/TPP/RGD/R848 in combination with anti-CTLA-4 (Fig. S12 in the ESM). Of note, the percentage of CD8⁺ CTL in secondary tumors in mice after BP/TPP/RGD/R848-based PA therapy combined with anti-CTLA-4 treatment dramatically increased to 25% (Figs. 8(b) and 8(e)) and was significantly enhanced with indirect immunohistochemistry on frozen sections of secondary tumors in mice (Fig. 8(c)).

Figure 7 *In vivo* PA therapy combined with CTLA-4. (a) Schematic illustration to show the experimental design of combining PA therapy with anti-CTLA4 therapy *in vivo*. (b) The tumor growth curves for primary tumors (left) after various treatments. (c) The tumor growth curves for abscopal tumors (right) without direct treatments. (d) The survival rate for various treatment groups. (e) The weight of mice after various treatments. (f) Images of H&E-stained tumor sections harvested from the mice after various treatments on day 21. Data are shown as mean $\pm$ SD ($n = 4$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

CD4⁺ helper T-cells (CD3⁺CD4⁺CD8⁻) play important roles in the regulation of adaptive immunity; they can be classified into effector T-cells (T_{eff}) and may stimulate and interact with other leukocytes by secreting cytokines as well as T_{regs} that suppress antitumor immunity [61–63]. On day 7, abscopal tumors were harvested for analysis by flow cytometry. A large amount of T_{regs} remained in the secondary tumors of mice after ablation of primary tumors based on BP/TPP/RGD/R848-based PA therapy. Thus, although a vaccine-like immune response is generated based on BP/TPP/RGD/R848-based PA therapy, the anti-tumor efficacy of this group is still less effective due to the presence of large amounts of T_{regs}.

CTLA-4 blockade treatment can significantly reduce Tregs cells (CD3⁺CD4⁺Foxp3⁺) in secondary tumors (Figs. 8(d) and 8(e)).

Thus, the ratio of CD8⁺ CTL/Tregs and CD4⁺ T_{eff}/Tregs cells was significantly enhanced in secondary tumors in post-treatment mice based on BP/TPP/RGD/R848-based PA therapy plus anti-CTLA-4 treatment (Fig. S13 in the ESM). In addition, versus the four other groups shown in Fig. 8, BP/TPP/RGD/R848-based PA therapy combined with anti-CTLA4 induced the highest percentage of CD8⁺ CTL (also CD8⁺ CTL/Tregs ratio) responsible for cancer immunotherapy. We also tested cytokines, including TNF- α and IFN- γ in serum samples, from all mice on day 7 by ELISA. The results showed that the circulating cytokines of IFN- γ and TNF- α in serum were increased after PA therapy combined with anti CTLA4 treatment (Figs. 8(f) and 8(g)). The immune responses collectively suggest that tumor-associated antigens produced after PA

Figure 8 Abscopal effect of PA therapy with BP/TPP/R848/RGD in combination with checkpoint blockade immunotherapy. (a) Schematic illustration to show the experimental design of combining PA therapy with anti-CTLA4 therapy. (b) Representative flow cytometry plots showing different groups of T cells in secondary tumors. Tumor cell suspensions were analyzed by flow cytometry for T-cell infiltration (gated on CD3⁺ T cells). (c) Immunohistochemistry of CD8⁺ in tumor sections from mice with different treatments ($\times 400$). (d) Representative flow cytometry plots showing percentages of CD4⁺FoxP3⁺ T cells in secondary tumors after various treatments indicated (gated on CD25⁺ T cells). (e) Quantification of FoxP3, CD4 and CD8 expression by flow cytometry after various treatments. (f) and (g) The TNF- α and IFN- γ levels in sera from mice detected at 7 days after various treatments. Data are shown as mean $\pm$ SD ($n = 4$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

therapy can amplify tumor immune responses with the assistance of nanoparticles containing the immune adjuvant R848. Meanwhile, PA therapy combined with anti-CTLA4 antibody can effectively inhibit the immune suppression activity of the T_{regs} cells and increase the ratio of CTLs to T_{regs} cells, which can effectively inhibit abscopal tumors and greatly prolong the survival of mice.

3.7 Long-term immune-memory effects

An important feature of immunological memory is the ability of the immune system to protect organisms from previously encountered pathogens, including neoplastic diseases. Thus, it is necessary to assess immune memory induced by BP/TPP/RGD/R848-based PA therapy combined with anti-CTLA4. In our experiments, secondary 4T1 tumors were inoculated 40 days after BP/TPP/RGD/R848-based PA therapy to remove their first 4T1 tumor. Two rounds of treatment were given intravenously with anti-CTLA-4 antibody on different days

(1 mg·mL⁻¹ 25 μ L). The first round was given immediately after the first tumor was eliminated (1st, 3rd and 5th day). The second round was injected on days 41, 44, and 47 after re-inoculation of its secondary tumor (Fig. 9(a)).

We next carried out a series of analyses to understand whether a robust tumor immune memory was generated and how the antitumor immune memory was induced after PA therapy, especially when combined with the CTLA4 blockade. Memory T-cells are classified into central memory T-cells (T_{CM}) and effector memory T-cells (T_{EM}) based on effector function, proliferative capacity, and migration potential [64, 65]. After being stimulated by antigen, T_{CM} cells can rapidly proliferate, differentiate, and migrate to infected tissues [66]. In contrast to T_{CM} cells, T_{EM} cells can induce a strong immune memory effect by producing vital cytokines such as TNF- α and IFN- γ upon a second encounter with the same antigen [58, 67–69].

On day 50, we collected spleens from different groups of mice and measured changes in central memory CD8⁺ T-cells (T_{CM})

Figure 9 Long-term immune-memory effects induced by BP/TPP/RGD/R848 PA therapy and CTLA4 blockade combination therapy to inhibit cancer relapse. (a) Schematic illustration of BP/TPP/RGD/R848 PA therapy and CTLA4 blockade combination therapy to inhibit cancer relapse. (b) Proportions of effector memory T cells (T_{EM}) in the spleen analyzed by flow cytometry (gated on $CD8^+$ and $CD3^+$ T cells). (c) Quantification of T cells (T_{EM}) expression by flow cytometry after various treatments. (d)–(f) TNF- α , INF- γ and IL-12 levels in sera from mice isolated seven days after mice were re-challenged with secondary tumors (on day 47) (four mice per group). Data are shown as mean $\pm$ SD ($n = 4$). P values: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, ANOVA.

and effector memory $CD8^+$ T-cells (T_{EM}). Flow cytometry analysis showed that the percentage of T_{EM} cells ($CD3^+CD8^+CD44^+CD62L^-$) after PA therapy plus anti-CTLA-4 antibody to remove the first tumors was much higher than that of the control group in which tumors were treated with PA therapy, intravenous injection of nanoparticles, or intravenous injection of nanoparticles combined with anti-CTLA-4 (Figs. 9(b) and 9(c)). In contrast, the percentage of T_{CM} cells ($CD3^+CD8^+CD44^+CD62L^+$) in the combination therapy group was much lower than that of the three control groups (Fig. S14 in the ESM). Furthermore, concentrations of TNF- α , INF- γ , and IL-12 in sera increased after PA therapy combined with CTLA-4 blockade 1 week after mice were challenged with secondary tumors (Figs. 9(d)–9(f)). Our data strongly suggest that PA therapy plus CTLA-4 blockade can boost immune memory to effectively prevent tumor recurrence.

4 Conclusion

In this study, we describe PA tumor ablation with immune-adjuvant nanoparticles together with checkpoint-blockade therapy. This approach precisely eradicates primary tumors with minimal side effects on normal tissue. It also inhibits metastases and prevents tumor relapses. PA can precisely ablate tumors with multifunctional nanoparticles encapsulating both biodegradable and biocompatible BP. Immune-adjuvant toll-like receptor agonists can induce vaccine-like immune responses that may be combined with CTLA4 checkpoint blockade to realize highly effective cancer immunotherapy. Great anti-tumor efficacy has been observed using this strategy to treat an abscopal tumor model that can promote infiltrating CD8 and CD4 T-cells. Furthermore, a strong immune-memory effect was observed 50 days after PA tumor ablation with the nanoparticles. These data, along with anti-CTLA4 therapy, protected mice from tumor re-challenge. We further demonstrate that

such a treatment strategy may also be realized with systemic injection of all agents. Therefore, our study presents a new cancer treatment strategy, that can eliminate primary tumors, attack and kill spreading distant tumors, and offer immune-memory protection to prevent tumor relapse. Considering that most of the components of our nanoparticles are FDA-approved and biodegradable, our technique may be optimal for future clinical translation.

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Conflict of interest

The authors declare no conflict of interest.

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