Research article

Zhe Sun, Taojian Fan, Quan Liu, Luodan Huang, Weibin Hu, Lulin Shi, Zongze Wu, Qinhe Yang*, Liping Liu* and Han Zhang*

Autologous tumor antigens and boron nanosheet-based nanovaccines for enhanced photo-immunotherapy against immune desert tumors

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Zhe Sun, Taojian Fan, and Quan Liu are contributed equally to this work.

***Corresponding authors: Han Zhang**, Institute of Microscale Optoelectronics, Collaborative Innovation Centre for Optoelectronic Science & Technology, Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Physics and Optoelectronic Engineering, Shenzhen Key Laboratory of Micro-Nano Photonic Information Technology, Guangdong Laboratory of Artificial Intelligence and Digital Economy (SZ), Shenzhen University, Shenzhen, 518060, P. R. China; and **Liping Liu**, Department of Hepatobiliary and Pancreatic Surgery, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Shenzhen, 518020, P. R. China; and **Qinhe Yang**, School of Traditional Chinese Medicine, Jinan University, Guangzhou, Guangdong, 510632, P. R. China, E-mail: hzhang@szu.edu.cn (H. Zhang), liu.liping@szhospital.com (L. Liu), tyangqh@jnu.edu.cn (Q. Yang). <https://orcid.org/0000-0002-9131-9767> (H. Zhang). <https://orcid.org/0000-0003-2616-5973> (L. Liu)

Zhe Sun, Department of Hepatobiliary and Pancreatic Surgery, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Shenzhen, 518020, P. R. China; School of Traditional Chinese Medicine, Jinan University, Guangzhou, Guangdong, 510632, P. R. China; and Integrated Chinese and Western Medicine Postdoctoral Research Station, Jinan University, Guangzhou, Guangdong, 510632, P. R. China. <https://orcid.org/0000-0002-7967-8185>

Taojian Fan, Institute of Microscale Optoelectronics, Collaborative Innovation Centre for Optoelectronic Science & Technology, Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Physics and Optoelectronic Engineering, Shenzhen Key Laboratory of Micro-Nano Photonic Information Technology, Guangdong Laboratory of Artificial Intelligence and Digital Economy (SZ), Shenzhen University, Shenzhen, 518060, P. R. China. <https://orcid.org/0000-0001-7317-2610>

Quan Liu, Luodan Huang, Weibin Hu, Lulin Shi and Zongze Wu, Department of Hepatobiliary and Pancreatic Surgery, The Second Clinical Medical College (Shenzhen People's Hospital), Jinan University, Shenzhen, 518020, P. R. China. <https://orcid.org/0000-0001-9994-3743> (Q. Liu)

Abstract: Personalized therapeutic vaccines against immune desert tumors are an increasingly important field in current cancer immunotherapy. However, limitations in neoantigen recognition, impotent immune cells, and a lack of intratumoral infiltrated lymphocytes pose challenges for the cancer vaccines. Resected tumors contain various of patient-specific tumor autoantigens (TA), and its derived photonanovaccines have unique competency to overcome abovementioned barriers. We constructed a novel personalized photonanovaccine (B@TA-R848) with surgically sourced TA modified on two-dimensional boron nanosheets (BNSs) via polydopamine coating and loaded with immune adjuvant R848. B@TA-R848 has good properties of drug delivery and release, photoacoustic imaging, photothermal effect, and biocompatibility. In a mouse triple-negative breast cancer model, B@TA-R848-based photonanovaccine induced effective systemic antitumor immune responses, altered the local tumor microenvironment, and increased the intratumoral infiltration of immune cells. The combined photo immunotherapy could significantly inhibit tumor growth, recurrence, and metastasis. This work develops a novel photonanovaccine for low immunogenicity and high metastatic potential tumors, which is of great significance for exploring the clinical development of personalized tumor vaccines against immune desert tumors.

Keywords: 2D boron nanosheet; immunotherapy; nanovaccine; photothermal therapy; tumor autoantigen.

1 Introduction

Cancer is a serious threat to global public health [1]. Tumor immunotherapy aims to stimulate or regulate the body's immune system to eliminate tumor cells, especially with the help of enhanced antitumor immunity in the local tumor microenvironment (TME) [2, 3]. Personalized therapeutic tumor vaccine has become a research focus in the field of cancer immunotherapy. As specific and immunogenic tumor-associated antigens (such as polypeptides, DNA and

RNA, etc.), tumor vaccines activate or strengthen the host's specific antitumor immune responses with the assistance of cytokines, chemokines, or other adjuvants [4, 5]. However, the difficulties of identifying neoantigens with next generation sequencing (NGS) and computational algorithm, as well as the variability of neoantigens across patients by tumor heterogeneity and immunoediting, have greatly increased the cost of designing commercial vaccines [6]. In addition, for some immune desert tumors, current tumor vaccines are not effective because no appropriate tumor neoantigens have been identified, such as triple-negative breast cancer (TNBC), which is characterized by negative estrogen receptor (ER), progesterone receptor (PR), and proto oncogene (Her-2) [7, 8]. The immuno-oncology landscape in solid tumors is shockingly complicated and uncharted for tumor penetrating lymphocytes, gene mutations, and unidentified patient-specific neoantigens [9–11]. Many researches have showed that the development of tumor vaccines using autologous tumor antigens derived from tumor tissues or whole tumor cells is feasible and of great importance, due to that tumor tissues and tumor cells contain a lot of important information about the patient inside, including tumor penetrating lymphocytes, gene mutations that act as neoantigens, and have many patient-specific neoantigens [12–14]. In addition, neoantigen-based vaccines comprising more than 10 kinds of synthetic long peptides, such as NeoVax, GEN-009, and PGV001, have acquired well-pleasing and promising progresses in clinical research [15]. Thus, the surgically removed tumor-derived polyantigen vaccines provide a promising personalized treatment against tumors whose autoantigens are identified insufficiently, such as TNBC.

Effectively eliminating solid tumors and inhibiting tumor recurrence and metastasis are major challenges in tumor therapeutic vaccines. However, antitumor effects of vaccines are often diminished by physical and immunosuppressive TME in immune desert tumors [16–20]. Studies showed that the patient overall survival (OS) is correlated with factors of both the antigen load and CD8⁺ T cell infiltration, and treatments with a single factor are not always enough [15]. Therefore, many combination therapies have been applied to overcome the obstacles, including surgery- [13], radiation/chemo- [21], optical- [22–25], and check-point blockade inhibitors-combination therapeutic tumor vaccines [26–28]. Recently, functional nanoparticles have provided an excellent platform for tumor nanovaccine in cancer immunotherapy with unique delivery, imaging, therapeutic and theragnostic properties [29, 30]. More importantly, photosensitive nanoparticles smartly deliver tumor antigens/adjuvants into targeted cells and locations with a light controlling manner, induced photothermal therapy (PTT) can also kill most of tumor cells, activate host immune system and remodel TME,

especially for the higher tumor infiltration of CD8⁺ lymphocytes [31–33]. Therefore, a combination strategy with a versatile biophotonic nanovaccine platform attracts extensive interests in cancer precision treatments.

Two-dimensional nanomaterials have been exploited as novel nanomedicine, such as black phosphorus, MoSe₂, and tellurene, etc. [34]. These nanomaterials have a large surface to mass ratio and high drug loading rate, which facilitate adequate superiority for carrying and exposure of tumor tissue antigens and adjuvants in nanovaccines [34–36]. Among them, two-dimensional boron nanosheets (BNSs), as the lightest single-element two-dimensional material in terms of relative molecular mass, have a higher specific area and more drugs per unit mass. The good modifiability and ultrahigh drug loading rate of BNSs offer further potential for the biomedical application [34]. BNSs have favorable photothermal conversion efficiency and photoacoustic imaging capability, which can be used as a tumor diagnostic agent and photothermal agent [37, 38]. BNSs-induced local PTT triggers the immunogenic cell death and stimulates the maturation of dendritic cells (DCs) [39–41]. Moreover, it also increases blood flow and vascular permeability in the tumor tissues and alters interstitial pressure, thus promoting solid tumor infiltration of T cells [42, 43]. In addition, compared with other single-element two-dimensional materials, BNSs have excellent physiological environmental stability and biosafety, and are therefore highly exploitable and clinical applications for *in vivo* tumor diagnosis and treatments.

Herein, we developed a two-dimensional boron nanosheet-based photo nanovaccine that destroyed solid tumors and activated immunity, enabling higher tumor infiltration of lymphocytes for enhanced cancer immunotherapy, using a mouse triple-negative breast cancer model (TNBC, a kind of immune desert tumor model). BNSs were firstly coated with a layer of polydopamines, then modified with autologous tumor antigens (TA) from surgically sourced TNBC, and the modified nanosheets were loaded with immune adjuvant R848 at end to construct the nanovaccines (B@TA-R848, Figure 1(A)). B@TA-R848 enhanced DCs internalization through nanoparticle-mediated endocytosis to mature DCs and thus promoted antigen presentation. B@TA-R848 had a higher enrichment at the tumor site and performed the desired photoacoustic imaging and PTT effect after the vein injected. Results showed that enhanced tumor local and host systemic immunity were detected with an increased splenic tumor-specific CD8⁺ T lymphocytes and tumor infiltrated lymphocytes in mice after B@TA-R848-based photo immunotherapy. The antitumor effects were further studied in the TNBC model, and the results showed that tumor growth, metastasis and recurrence were

significantly inhibited by B@TA-R848 after tumor hyperthermia (Figure 1(B)). Therefore, this project developed a novel photo nanovaccine against solid tumors, especially against immune desert tumors with low immunogenicity.

2 Materials and methods

2.1 Materials

Boron powder (99.9%) and isopropanol (99.5%) were purchased from MACKLIN (China). Cy7-PEG-NH₂ was purchased from Yare (China). The molecular weight (MW) of the PEG chain in Cy7-PEG-NH₂ is 2 k Da. R848 was purchased from Meilunbio Co.Ltd (China). Calcein-AM and PI were bought from Sigma-Aldrich (USA). ELISA kits were bought from Biolegend Inc. (USA). Fluorochrome-conjugated antimouse antibodies were bought from Thermo Fisher Scientific (USA). Fetal bovine serum (FBS), PBS (pH 7.4), DMEM and RPMI mediums, and trypsin-EDTA solutions were purchased from Gibco Life Technologies (Switzerland).

2.2 Preparation of boron nanosheets (BNSs)

BNSs were prepared through a top-down approach by liquid exfoliation technologies. Firstly, the commercial boron powder with the

concentration of 5 mg mL⁻¹ was dispersed in isopropanol (IPA). The suspension was then probe sonicated for 10 h in ice-bath at 500 W. Then, the solution was then treated by bath sonication with a power of 1050 W for 3 h in ice-bath. After that, the solution was centrifuged (3000 rpm, 20 min) to discard the large chunks of bulk boron. The resulting suspension was then centrifuged (12,000 rpm, 30 min), and washed 3 times using IPA. At last, the precipitates were collected for future use.

2.3 Autologous tumor antigen extraction

The tumor-bearing mouse model was established by inoculating mouse TNBC cells (4T1) into Balb/c mice. The tumor tissues were dissected from the mice when the tumor sizes grew up to about 10 mm in diameter, and the tumor specimens were put into HBSS containing 50 µg mL⁻¹ gentamycin, which was then sent to the cell laboratory quickly under sterile condition. To get the single-cell suspension, the tumors were mashed and passed through a cell filter (70 µm). Afterward, tumor cells were centrifugation (3000 rpm, 2 min) and collected, then dispersed in 20 mL of PBS, frozen in liquid nitrogen for 1 h, and lysed five times at room temperature for 30 min. Finally, after centrifugation (8000 rpm, 5 min) to remove the massive tumor fragment, and the supernatant was collected and tumor antigen was obtained. The tumor antigen content was tested by bicinchoninic acid (BCA) assay kit with the manufacturer's protocol (Thermo Fisher Scientific).

Figure 1: Schematic depiction of B@TA-R848 for enhanced personalized photothermal-immunotherapy.

(A) The preparation of B@TA-R848. (B) The systemic delivery of B@TA-R848 as a photo nanovaccine for multimodal imaging-guided cancer therapy (BNSs: Two-dimensional boron nanosheets; TA: Tumor autoantigens; DCs: Dendritic cells; and CTL: Cytotoxic T lymphocytes).

2.4 Preparation of tumor autoantigen modified boron nanosheet (B@TA)

B@TA was prepared through a facile and simple method. In brief, 20 μL , 15 mg mL^{-1} of NaOH was added into 0.2 mg of dopamine and 1 mg of BNSs suspension in 1 mL of ethyl and stirred for 15 min. After that, 10 μL , 100 mg mL^{-1} autologous tumor antigen solution was added into the mixture, and it was magnetically stirred for 12 h at 4 °C away from light. The extra reactants were separated by dialysis (the molecular weight cutoff: 8–14 kDa), and the purified B@TA were acquired by lyophilization. The coating amount of tumor antigen was calculated based on the BCA protein determination method. The prepared B@TA suspension was centrifuged (12,000 rpm, 5min) and the supernatant was taken. The supernatant was dialyzed (molecular weight cutoff: 5 kDa) overnight at 4 °C. After proportional dilution, the absorbance of the supernatant samples was determined by UV-vis spectrophotometer, and the concentration was calculated by BCA standard curve. The coating amount (CA) of tumor antigen was calculated as

$$\text{CA (wt\%)} = (W_{\text{TA, supernatant}} / W_{\text{TA}}) \times 100\%$$

Where, $W_{\text{TA, supernatant}}$, and W_{TA} are the weight of TA in the supernatant and the weight of added TA, respectively.

2.5 Drug loading (B@TA-R848) and release

For R848 loading, 100 μL 10 mg mL^{-1} R848 methanol solution was added into 900 μL of B@TA suspension with different B@TA quality (200, 180, 160, 140, and 120 μg) and stirred for 12 h. After centrifugation, the supernatant was reserved for detection. The loading rate of R848 was measured by testing the supernatant using high-performance liquid chromatography (HPLC, mobile phase acetonitrile, and 0.1% phosphoric acid aqueous solution, determine wavelength 254 nm). The drug loading rate (DL) was calculated as

$$\text{DL (wt\%)} = (W_{\text{R848, L}} / W_{\text{B@TA}}) \times 100\%$$

Where, $W_{\text{R848, L}}$, and $W_{\text{B@TA}}$ are the weight of R848 in the nanovaccines and the weight of added B@TA-R848, respectively.

To investigate R848 release kinetics, 1 mg B@TA-R848 samples were redispersed in 1 mL PBS buffer solution (pH = 7.4 or 6.5) and incubated at different times. After centrifugation at 12,000 rpm, the supernatants were collected and an equal volume of methanol was mixed to dissolve the drug. The mixtures were then detected by HPLC and the amount of released R848 was calculated. To investigate NIR-triggered release of R848, the process was performed at pH 6.5 under identical conditions with different incubation times, and exposed to an 808 nm NIR laser (2 W cm^{-2} , 10 min).

2.6 Characterization

The morphology of BNSs and B@TA were characterized using transmission electron microscopy (TEM, JEM-3200Fs, JEOL, Japan). The morphology and thickness of BNSs and B@TA were studied using atomic force microscopy (AFM, FASTSCANBIO, Germany). The size and surface charge (zeta potential) of BNSs and B@TA were detected by Malvern Mastersizer 2000 (Zetasizer Nano ZS90, Malvern Instruments Ltd., UK). The chemical compositions of the NSs were analyzed with Fourier transform infrared spectrophotometry (FTIR,

Nexus 470, Nicolet, Madison, WI, USA) and X-ray photoelectron spectroscopy (XPS, ESCALAB 250Xi, Japan). Elemental mapping was applied to investigate the elementary composition of BNSs and B@TA using an energy-dispersive X-ray spectroscope (EDS) (Inca X-MAX, Oxford, UK) and a transmission electron microscope (JEM-3200Fs, JEOL, Japan). UV-Vis-NIR spectra of NSs were detected on an Infinite M200 PRO spectrophotometer.

2.7 Cell culture

Mouse breast neoplasm cell line (4T1 cells) and human umbilical vein endothelial cell line (HUVEC cells) were obtained from the Chinese Academy of Science Cell Bank for Type Culture Collection. 4T1 cells and HUVEC cells were cultured using RPMI 1640 medium and DMEM medium combined with 10% FBS (GIBICO) respectively at 37 °C in a humidified incubator with 5% CO_2 .

2.8 In vitro toxicity studies of BNSs, B@TA, and B@TA-R848

The cytotoxicity of BNSs, B@TA, and B@TA-R848 was evaluated by measuring the viability of different cancer cells, including 4T1 and HUVEC. Briefly, these two different cell lines were cultured in 96-well plates (5000 cells/well) and incubated for 24 h. Subsequently, the cell culture medium was replaced by new ones with different pre-dissolving concentrations of BNSs, B@TA, and B@TA-R848 (10, 25, 50, 100, and 200 $\mu\text{g mL}^{-1}$), and cells were cultured for another 24 h. Finally, the surviving cells were detected by a CCK-8 kit (Dojindo Molecular Technologies, Japan), and cell viabilities were calculated based on absorbance.

2.9 In vitro photothermal cytotoxicity

4T1 cells were seeded in a 27 mm glass-bottom dish and cultured for 24 h. The B@TA-R848 was then added into the wells and the concentration of B@TA-R848 was 100 $\mu\text{g mL}^{-1}$. After 12 h, the cells were irradiated with 808 nm laser (2 W cm^{-2} , 5 min). Four hours later, the cell viability was detected with live/dead cell double staining kit (calcein-AM/PI, Dojindo Molecular Technologies, Japan), and laser scanning confocal microscopy (CLSM, Zeiss 710 NLO) was used to take fluorescent photographs of the stained cells.

To quantify the photothermal cytotoxicity, different concentrations of B@TA-R848 were added (6.25, 12.5, 25, 50, and 100 $\mu\text{g mL}^{-1}$) into 96-well plates which plated with 4T1 cells. Cells were incubated for 12 h and then irradiated with NIR laser (808 nm, 2 W cm^{-2} , 5 min). The surviving cells were detected by CCK-8 kit (Dojindo), and cell viabilities were calculated based on absorbance.

2.10 Cell internalization of B@TA-R848-Cy7

To detect the cell internalization, B@TA-R848 was labeled with Cy7-PEG-NH₂ (2 kDa) and incubate with adherent growth 4T1 cells in a 27 mm glass-bottom dish. After that, Hoechst 33342 and lysosome tracker were added for staining cell nucleus and lysosome, respectively. The confocal microscopy (CLSM, Zeiss 710 NLO) was used to take fluorescent photographs of the stained cells.

2.11 *In vitro* dendritic cells activation

Mouse bone marrow-derived dendritic cells (BM-DCs) from BALB/C mice were first obtained. Immature BM-DCs (4×10^6) were cultured for seven days in complete medium with RPMI-1640, mouse granulocyte macrophage colony-stimulating factor (GM-CSF, 20 ng mL^{-1} , Pepro-Tech), and 2-mercaptoethanol (2-ME, 0.8 ng mL^{-1} , SigmaAldrich). 5×10^5 cells/well BM-DCs were seeded into 6-well plates and treated as follows: 1) Control group, cultured with bare complete RPMI-1640; 2) BNSs group, cultured with BNSs ($50 \text{ } \mu\text{g mL}^{-1}$) and complete RPMI-1640; 3) B@TA group, cultured with B@TA ($50 \text{ } \mu\text{g mL}^{-1}$); 4) B@TA-R848 group, cultured with B@TA-R848 ($50 \text{ } \mu\text{g mL}^{-1}$). Mature BM-DCs were stained with fluorescent labeled antibodies of CD11c-FITC, CD80-PerCP-Cy5.5, CD86-APC (Thermo Fisher Scientific, USA) after co incubation for another 24 h, and the results were analyzed by Flow cytometry (BD FACS Arial flow cytometer) and CytExpert software (Beckman Coulter, Inc.).

2.12 *In vivo* biocompatibility

All animal manipulations in this study were carried out in accordance with the requirements of the Laboratory Animal Ethics Committee of Jinan University (Approval No. LL-KY-2019259). Female BALB/c mice were randomly separated into three groups of three mice each, saline, BNSs and B@TA-R848 treated mice, respectively. For the saline-treated group, mice were intravenously injected with $100 \text{ } \mu\text{L}$ saline. For the BNSs and B@TA-R848 treated group, the suspension of BNSs or B@TA-R848 (1 mg mL^{-1} , 5 mg kg^{-1} body weight) was injected. After 7 and 14 days, these mice were sacrificed, and then their blood (about 0.8 mL) and major mouse organs were collected for biochemistry studies and pathological studies. The hematology parameters were acquired using a standard biochemistry test through serum samples. The major mouse organs were used for hematoxylin and eosin (H&E) histological examination.

2.13 Animal tumor model

Female BALB/c mice ($14 \pm 2 \text{ g}$, 5–6 weeks old) were purchased from the Model Animal Research Centre (Nanjing, China), and were selected to establish the xenograft 4T1 tumor model. Mice were given freely available fresh food and water daily and kept for at least seven days prior to the experiment. 4T1 cells (5×10^7) in PBS were injected into the right dorsal subcutis of mice. After approximately one week, tumors were fully established.

2.14 *Ex vivo* imaging studies

Free Cy7 and B@TA-R848-Cy7 (dose: $100 \text{ } \mu\text{L}$, 5 mg kg^{-1} body weight) were injected into the 4T1 xenograft tumor-bearing mice *via* tail intravenous injection, respectively. After 12 and 24 h, mice were determined using *ex vivo* NIR fluorescence imaging. Mice were sacrificed and major organs, such as lungs, heart, spleen, liver, kidneys, and tumors were obtained 24 h post injection, and the distribution of drugs were detected by the animal living imaging system. Mice were injected subcutaneously with B@TA-R848-Cy7 in the right calf, and the fluorescence signal was observed using *ex vivo* fluorescence imaging 6 h later. Mice were

dissected for bilateral inguinal lymph nodes and observed for fluorescence signals.

2.15 Photoacoustic imaging

For *in vitro* photoacoustic imaging, the excitation light along the wavelength 680–850 nm was first scanned for boron samples to collect photoacoustic signals, and the excitation light selected for the following photoacoustic imaging test was determined to be 780 nm. After that, photoacoustic signal detection was performed with different concentrations of BNSs and B@TA-R848 (0, 0.031, 0.062, 0.125, 0.25, and 0.5 mg mL^{-1}) dispersed in deionized water.

For *in vivo* photoacoustic imaging, the 4T1 tumor-bearing mice were intravenously injection of BNSs and B@TA-R848 (dose: $100 \text{ } \mu\text{L}$, 5 mg kg^{-1} body weight). After the injection, the signal was recorded on the photoacoustic instrument at 12 h. The photoacoustic signal from mice injected with saline served as a control. For imaging, the region of interest is 20 mm. All the photoacoustic imaging was obtained by the Vevo LAZR photoacoustic imaging system (Visual-Sonics Co.).

2.16 *In vivo* photothermal-immune combined cancer therapy

When the tumor size reached $\sim 100 \text{ mm}^3$, mice were randomly divided into five groups with different treatment ($n = 6/\text{group}$): 1) Saline ($100 \text{ } \mu\text{L}$, tail vein injection); 2) BNSs ($100 \text{ } \mu\text{L}$, tail vein injection, 5 mg kg^{-1}); 3) B@TA-R848 ($100 \text{ } \mu\text{L}$, tail vein injection, 5 mg kg^{-1}); 4) BNSs ($100 \text{ } \mu\text{L}$, tail vein injection, 5 mg kg^{-1}); and 5) B@TA-R848 ($100 \text{ } \mu\text{L}$, tail vein injection, $[\text{B@TA-R848}] = 5 \text{ mg kg}^{-1}$, $[\text{R848}] = 2.4 \text{ mg kg}^{-1}$). Each group was administered three times on day 1, 7, and 15. The first dose of each group was administered via tail vein injection, and the second and third doses were administered via subcutaneous paracancer injection at the same dose as the first. Group 4 and 5 were received photothermal treatment on day 1 (NIR irradiation by 1 W/cm^2 and 808 nm for 3 min at 12 h post-injection). The tumor sizes and the mouse weight in each group were measured and recorded. Relative tumor volumes were calculated as $V = ab^2/2$, where a and b represent the maximum and minimum diameters, respectively. The tumor growth inhibition rate (TGI, %) was measured with $\text{TGI} = (1 - V_{\text{treatment}}/V_{\text{control}}) \times 100\%$.

2.17 Histological examination and immunohistochemical analysis

The removed mouse organs and tumors were fixed with 4% para-formaldehyde and paraffin embedded to make $5 \text{ } \mu\text{m}$ thick tissue sections. After deparaffinization, tissue sections were stained using hematoxylin and eosin reagents (H&E) for histological examination; tumor sections were stained with Ki-67. The images were captured using the Leica DMi8 automatic inverted fluorescence microscope (Leica, Germany).

2.18 ELISA assay and flow cytometric analysis

The 4T1 tumor-bearing mice were allocated into four groups randomly, which were immunized with three injections of (1) Saline; (2) BNSs; (3) B@TA-R848 (5 mg kg^{-1}); and (4) B@TA-R848 (5 mg kg^{-1}); on day 7, 11,

and 15. The first dose of each group was administered via tail vein injection, and the second and third doses were administered via subcutaneous paracancer injection at the same dose as the first. The mice in group 2 and 4 were injected with BNSs and B@TA-R848 (5 mg kg^{-1}) in the tail vein and treated with photothermal therapy (laser irradiation 808 nm , 1 W cm^{-2} , 5 min) 12 h post injection on day 7. The mice were sacrificed on day 21 and blood was collected. Immediately, the mice were soaked in 75% alcohol solution for 3 min, and the lymph node and spleens were surgically removed and collected. For blood samples, blood was centrifuged (15 min , 1500 rpm) at room temperature to remove blood cells. IFN- γ , IL-2, IL-6, TNF- α , and IL-10 levels were analyzed using ELISA kits. The lymph node and spleen were harvested and minced into small pieces using scissors. The pieces were passed through $70 \mu\text{m}$ cell strainers supported by 50 mL of polypropylene pipe using 2 mL of RBCs lysis buffer. The additional 3 mL of RBC lysis buffer was added into centrifuge tubes and the obtained single-cell suspension was stood for 20 min to lyse RBCs at room temperature. The reaction was stopped by adding 5 mL of 1640 complete medium. Then the cells were washed twice with PBS. The cells were cultured into 100 mm of culture dish at a density of 1×10^7 cells in 15 mL of 1640 complete medium for 3 d. For dendritic cell maturation, cells from lymph node were obtained and then stained with anti-CD11c-FITC, anti CD80-Cy5.5, and anti CD86-APC antibodies (Thermo Fisher Scientific, USA) by the guidance of the manufacturer's protocols. CD11c + CD80 + CD86+ cells were defined as mature dendritic cells. For splenic lymphocytes, cells were obtained and then stained using isotype control (RatIgG2a), anti CD3-APC, anti CD4-FITC, and anti CD8a-PE antibodies (Thermo Fisher Scientific).

3 Results and discussion

3.1 Morphological and physical property characterization

Two-dimensional nanomaterial is defined as the nanomaterials in which the electron motions are confined to a two-dimensional plane, which means, the thinner the two-dimensional nanomaterial is, the higher the surface mass ratio it has [44]. Besides, the ultrathin thickness and high surface area-to-mass ratio of two-dimensional nanomaterials contributed to a controlled drug release profile of these materials, allowing them to promptly respond under environmental stimuli including light and pH, which is of significance for various optical diagnostic and therapeutic approaches [45]. In this study, we used the liquid phase sonication method combined with ultrahigh-speed cascade centrifugation to obtain boron nanosheets. The BNSs were prepared via a top-down method from bulk boron by liquid exfoliating processes according to our previous work [46]. The boron block is preferentially cut into slices by the resulting vertical ultrasonic wave under the anisotropically processing effect of probe sonication. Subsequently, boron nanosheets were obtained by further stripping of boron slices under the water bath sonication treatment. Finally, after the controlled velocity centrifugation, the boron nanosheets

were obtained with a uniform particle size of about 140 nm (Figure 2(A) and (B)) and a thickness of only about $0.8\text{--}1 \text{ nm}$ (Figure 2(C)). The crystalline nature of boron sheets was identified based on the diffraction pattern of typical individual boron sheets by high-resolution transmission electron microscopy (HRTEM) imaging, as demonstrated by distinct interference striated with a d-spacing of 0.51 nm , which corresponded to the (104) plane of a β -rhombohedral hexahedral boron structure. The photo images showed the Tyndall effect in BNSs due to the excellent dispersion in water (Figure 2(B)).

After obtaining BNSs, a dopamine-mediated polymerization was used to modify the tumor autoantigens on the BNSs. Dopamine molecules were polymerized under alkaline conditions and tend to form a layer of polydopamine film on the surface of inorganic materials. When proteins were present in the reaction system, polydopamine bound to the amino group in protein molecules, and modified the protein in its outermost layer. After modified the TA, average particle size of the prepared nanovaccines was about 200 nm (Figure 2(A)) and a faint outline around the periphery of the boron nanosheets could be observed on the transmission electron micrographs in Figure 2(B). The thickness was $6\text{--}8 \text{ nm}$ (Figure 2(C)). Digital photos showed that the B@TA suspension was darker than that of BNSs, which was due to the color brought by polydopamine. Similarly, the laser beam passing through the suspension showed a Tyndall effect, demonstrating that the B@TA could also be well dispersed in the aqueous phase.

Next, a series of characterization was performed to demonstrate the dopamine-mediated protein coating in B@TA. The element mapping (Figure 2(D)) and X-ray photoelectron spectroscopy (XPS, Figure 2(E)) were used to examine the elemental composition of BNSs and B@TA. According to the results in Figure 2(C), significant elemental boron and little elemental carbon could be observed in BNSs, while elemental nitrogen were hardly observed. It indicated that the BNSs were relatively pure boron nanosheets and contained only a small amount of impurities. In the B@TA results, not only boron element was present, but also obvious carbon and nitrogen elements could be observed on it. It indicated that an exotic carbon and nitrogen containing material had been bound to it. The XPS survey spectra of BNSs showed the peaks of boron, carbon, and oxygen, while the curve of B@TA demonstrated a new peak of nitrogen when compared to BNSs (Figure 2(E)), which was the same as the detection result of element mapping in Figure 2(C). The high-resolution XPS spectrum of BNSs proves that there is no obvious oxidation during the exfoliation process (Figure S1). The Fourier transform infrared (FTIR) spectra (Figure 2(F)) showed three

Figure 2: Preparation and characterization of BNSs and B@TA.

(A) Size, dispersity, and photo images of BNSs and B@TA; (B) TEM image of BNSs and B@TA (inset: HRTEM image of BNSs. The scale bars represent 500 and 200 nm, respectively. The scale bar of HRTEM represents 10 nm); (C) AFM image of BNSs and B@TA; (D) The element mapping images of BNSs and B@TA (The scale bars represent 100 nm). (E) XPS spectra of BNSs and B@T; (F) FTIR spectra of BNSs and B@TA; (G) Photothermal conversion stability of BNSs and B@TA. The laser was turned on for 10 min and then turned off for each cycle. The concentration of BNSs and B@TA are $200 \mu\text{g mL}^{-1}$ and the laser power density is 2 W cm^{-2} ; (H) Photothermal heating curves of BNSs and B@TA at different concentrations under 808 nm laser irradiation (2 W cm^{-2}) for 10 min; (I) Release profiles of R848 at different conditions with or without 808 nm NIR laser (2 W cm^{-2}).

absorption peaks at 1220 , 1540 , and 1650 cm^{-1} observed in B@TA, which may carry with tumor autoantigen proteins attributed to amide I/II/III bands or polydopamine [47, 48]. This suggested that BNSs are encapsulated by polydopamine or protein. To determine whether there was protein encapsulation in B@TA, we performed BCA and SDS-PAGE experiments. According to the results in Figure S2, the BCA produced a color response and based on the standard curves we could calculate the modifying rate of tumor fragment protein, which was $50.4 \pm 7.9\%$. The SDS-page in Figure S3 showed protein bands in the B@TA group at pH 6.5 water-acetic acid solution, which suggested that the modified proteins in B@TA could be released after degradation in the acid environment. These characterizations above demonstrated that tumor fragmentation proteins could be modified around boron

nanosheets via dopamine-mediated reactions and could be released under acidic conditions.

Subsequently, the NIR-induced hyperthermia potential of photo nanovaccines was explored. The photostability of BNSs and B@TA was examined by exposure of the BNSs and B@TA suspension to the near-infrared laser for six periods of irradiation. The data presented in Figure 2(G) showed that a power density of NIR with 2 W cm^{-2} , the change of the photothermal effect was considered insignificant during the six cycles of heating and cooling, thus indicating that the BNSs and B@TA had high photostability. The photothermal conversion efficiencies (η) of BNSs and B@TA were determined by previously reported processes [49], of which the details are described in the Supplemental Information. The UV-vis-NIR absorbance spectra and standard curve of photothermal conversion of both nanoparticles were shown in

Figure S4(A)–(D). The photothermal conversion efficiency of BNSs and B@TA were calculated as 23.2 and 33.8%, respectively (Equation (4) in supplementary Material, Figure S4(E) and (F)). The result in Figure 2(H) demonstrated that the temperature increased of both BNSs and B@TA at increased concentrations in aqueous solution under laser irradiation, which all exhibited a robust photothermal effect with concentration dependence under the same irradiation power. The maximum temperature gradient ($\Delta T_{\max}$) were 39.5 and 43.2 °C. Interestingly, the photothermal conversion efficiency and the $\Delta T_{\max}$ of B@TA were higher than those of BNSs, which may attribute to polydopamine [50]. This high photothermal conversion efficiency and excellent photothermal stability suggested that the BNSs and B@TA could be used as photothermal therapy reagents. The storage stabilities of BNSs and B@TA were also measured. As illustrated in Figure S5(A) and (B), no significant variations in the particle size of BNSs and B@TA were detected at each time point. In Figure S5(C), after storage at room temperature for 48 h, the absorption of BNSs slightly decreased, indicating the degradation of BNSs. In contrast, no significant changes were observed in the B@TA absorption curve, indicating increased stability after polydopamine encapsulation and antigen modification.

Vaccines often need to be used in conjunction with immune adjuvants to achieve the effect of activating the host's immunity. However, most immune adjuvants are hydrophobic small molecules and their *in vivo* delivery remains a major challenge. Thus, we next investigated the drug loading and controlled release capabilities of B@TA. R848 (Toll-like receptor 7/8 agonist) is a hydrophobic drug molecule and acts as an immune adjuvant (Figure S6). B@TA could efficiently load R848 with a maximum loading efficiency of about 73% (Figures S7 and S8), which may be related to the π - π stacking effect generated between BNSs, polydopamine and drug molecules. The smallest molecular mass of boron among the two-dimensional nanomaterials resulted in a high drug loading rate of boron-based nanomaterials. The encapsulated drugs could be released under acidic (pH 6.5) and NIR irradiation triggered due to the breakdown of the protein shell caused by polydopamine degradation [51]. In our study (Figure 2(I)), the R848 release rate of B@TA-R848 was below 20% of the total drug for 24 h at pH = 7.4 and without NIR irradiation, and was slightly higher than 20% at pH 7.4 plus NIR irradiation. At pH = 6.5 and no NIR irradiation, the drug was released nearly 60% at 24 h, while at pH = 6.5 plus NIR irradiation, the drug was released about 80% at 24 h. This indicated that acidic conditions can accelerate the degradation of polydopamine and lead to a large release of the drug, and the maximum release of the drug is achieved under acidic conditions with NIR

irradiation. The results of this experiment suggested that B@TA-R848 can release adjuvant well in simulated tumor microenvironment or lysosomes under NIR irradiation. Thus, B@TA-R848 achieved controlled release of adjuvants, overcomes the nonspecific distribution of drug and therapeutic components in the body, reduces toxic side effects and increases the therapeutic effect.

3.2 *In vitro* cytotoxicity

The biocompatibility and cytotoxicity of B@TA-R848 *in vitro* were investigated, which are prerequisites for the application of a nanotherapeutic system. The cytotoxicity of B@TA-R848 was evaluated by CCK-8 assay in both healthy HUVEC and 4T1 cancer cells. HUVEC were incubated with different nanoparticles (including BNSs, B@TA, and B@TA-R848) at increased concentrations for 24 h (Figure 3(A)). The Figure 3(A) showed that no obvious cytotoxicity was observed against normal HUVEC treated with BNSs, B@TA, and B@TA-R848 at the indicated concentrations, as demonstrated by relative cell viabilities of these nanoparticles were calculated around 1.0. The biocompatibility and cytotoxicity of BNSs and B@TA-R848 with mouse bone marrow-derived dendritic cells was also detected and no obvious cytotoxicity was observed (Figure S9). Figure 3(B) presented the cytotoxicity of different nanomaterials against 4T1 cells under NIR irradiation. In brief, 4T1 cells were incubated with BNSs, B@TA, and B@TA-R848 individually for 12 h and then exposed by an NIR laser (808 nm, 5 min). After post photothermal processing, cells were kept for another 4 h incubation, followed by quantitatively detection and calculation of the cell viability. The results in Figure 3(B) demonstrated that, with the assist of NIR, the cell survival rates of all material groups were lower than that of the control group, indicating that these groups of materials can photothermally kill tumor cells. Besides, both B@TA and B@TA-R848-treated groups exhibited obvious lower survival rates in 4T1 cancer cells at all used drug concentrations from 6.25 to 100 $\mu\text{g mL}^{-1}$, compared to the BNSs group, owing to the improved photothermal effect after polydopamine coating with tumor autoantigens. And the cytotoxicity of B@TA and B@TA-R848 combined with NIR radiation showed a concentration dependent increase, as demonstrated by reduced cell viabilities with increased concentrations, suggesting that the effect of photothermal killing of tumor cells was enhanced with increasing material concentration. Photothermal destruction of tumor cells were further illustrated by laser scanning confocal microscopy (LSCM) images after co-staining with calcein-AM and propidium iodide via a double-staining kit. The cells were expected to

fluoresce in green or red after costaining, representing living or dead cells, respectively. A large area of dead cells were observed at the site of laser irradiation, whereas no significant cell death was observed in the unirradiated area, clearly noticed by the distinct distribution of green and red-fluorescent cells in the image, which also exhibited excellent photothermal destruction ability of the B@TA-R848 in cancer cells (Figure 3(C)). Cell uptake and intracellular trafficking of B@TA-R848 in 4T1 cells were investigated by CLSM. Cy7-labeled B@TA (B@TA-R848-Cy7) was efficiently internalized and localized in lysosomal compartments after incubation for 6 h, as demonstrated by colocalization of Cy7 with lysotracker label fluorescence, shown as yellow fluorescence in the merge image (Figure 3(D)).

3.3 *In vivo* tumor-targeting efficacy

A 4T1 xenograft tumor model was established for the investigation of *in vivo* therapeutic effects of B@TA-R848, thus exploring the possibility of further pre-clinical application. The *in vivo* biodistribution and tumor accumulation of B@TA-R848-Cy7 after intravenous injection were determined via *in vivo* fluorescence imaging. In free Cy7 group, intense fluorescent signals from free Cy7 were detected in the liver and kidney but not in the tumor at 12 and 24 h postinjection, indicating that free Cy7 were nonspecific in

distribution *in vivo* (Figure 4(A)). In contrast, B@TA-R848-Cy7 exhibited significantly higher accumulation levels in tumors at both measured time-points, which could generally be attributed to the enhanced permeability and retention effect (EPR). In addition to fluorescent signals detected at tumor sites, they were also detected in the major mouse organs including the liver, kidney, and lung. The accumulation of B@TA-R848-Cy7 in the liver and kidney could be attributed to the metabolism, including a quick recognition and clearance of the mononuclear phagocyte system and renal excretion. Moreover, accumulation in the lungs was presumably caused by mechanical stagnation of bulky particles. Figure 4(B) showed that the semiquantitative fluorescence statistics and the results illustrated that B@TA-R848-Cy7 had more accumulation in tumors compared to free Cy7. At both 12 and 24 h after B@TA-R848-Cy7 injection, significant fluorescent signals were detected in the tumor tissues (Figure 4(A)), and there was little difference in fluorescence intensity in the semiquantitative fluorescence statistics (Figure 4(B)), indicating that the nanovaccine had accumulated in the tumor in a certain amount. Therefore, in the subsequent experiments, we chose the time point of 12 h after injection to perform photothermal treatment on mice. Six hours after subcutaneous injection of B@TA-R848-Cy7 via the right calf, a significant fluorescent signal could be detected at the right inguinal lymph node in mice (Figure S10). This

Figure 3: *In vitro* experiments.

(A) Relative viability of HUVEC cells after incubation with BNSs, B@TA, and B@TA-R848 (concentrations of 10, 25, 50, 100, and $200 \mu\text{g mL}^{-1}$) for 24 h. (B) Relative viability of 4T1 cells after incubation with BNSs, B@TA, and B@TA-R848 (concentrations of 6.25, 12.5, 25, 50, and $100 \mu\text{g mL}^{-1}$) for 4 h after irradiation with 808 nm laser (2 W cm^{-2}) for 5 min. (C) Corresponding laser scanning confocal microscopy of 4T1 cells stained with calcein AM (live cells, green fluorescence) and PI (dead cells, red fluorescence), scale bar: $100 \mu\text{m}$. (D) Confocal images of 4T1 cell internalization of B@TA-R848-Cy7 (blue: Cell nucleus stained with hoechst33342; green: Lysosome stained with lysosome tracker; red: B@TA-R848-Cy7), scale bar: $20 \mu\text{m}$.

may help immune-related cells to capture the subcutaneously administered nanovaccine and complete lymphocyte homing.

Photoacoustic imaging is a novel non-invasive and non-ionizing biomedical imaging approach that evolved in recent years to enable 50 mm deep *in vivo* tissue imaging, which has

Figure 4: *In vivo* imaging and biodistribution.

(A) *Ex vivo* fluorescence images of the tumor at 12 and 24 h post injection and major organs at 12 and 24 h post injection. (B) Semiquantitative biodistribution of free Cy7 or Cy7-labeled B@TA-R848 in BALB/C mice detected by the average fluorescence intensity of tumors and major organs per gram. (C) *In vitro* photoacoustic images of BNSs and B@TA-R848 as a function of concentration (0, 0.031, 0.062, 0.125, 0.25, and 0.5 mg mL⁻¹); (D) Photoacoustic values of BNSs and B@TA-R848 as a function of concentration (0, 0.031, 0.062, 0.125, 0.25, and 0.5 mg mL⁻¹). (E) Photoacoustic images of the tumor site in control, BNSs, and B@TA-R848 groups at 12 h post injection. (F) Quantitative analysis of photoacoustic values in (E). (G) Infrared thermographic maps and time-dependent temperature increase in the 4T1 tumor-bearing mice irradiated by the 808 nm laser (1 W cm⁻², 5 min) injection with 100 μL of saline, BNSs (1 mg mL⁻¹) and B@TA-R848 (1 mg mL⁻¹). (H) Time dependent temperature changes in the 4T1 tumor-bearing mice after different treatments in (G).

become one of the most promising biophotonic diagnostic methods that contribute to imaging-guided cancer therapy. In this work, we tested the *in vitro* and *in vivo* photoacoustic imaging capability of BNSs and B@TA-R848, respectively. A strong concentration-dependent photoacoustic signal was found in both BNSs and B@TA-R848 groups at 780 nm wavelengths (Figure 4(C)), which means B@TA-R848 is capable of being a photoacoustic agent to facilitate imaging-guided malignancy treatment. The results in Figure 4(D) showed a good linear relationship between the photoacoustic signals of BNSs and B@TA-R848 and the material concentration, indicating that the protein-modified nanovaccines also had a high photoacoustic imaging sensitivity. In photoacoustic imaging results, intense photoacoustic signals were detectable at the tumor sites after intravenous injection of BNSs and B@TA-R848 in tails when compare to the control group, suggesting that both BNSs and B@TA-R848 can be used for intratumorally photoacoustic imaging (Figure 4(E)). Also of note, most of signals in both groups were concentrated near the vessels along the outer edge of the tumor (Figure 4(E)). It indicated that nanovaccines could be accumulated in tumors, but still had difficulty to get inside solid tumors. Figure 4(F) showed the quantitative analysis of each photoacoustic signal of Figure 4(E). From the results, it was clear that the signal intensity of B@TA-R848 was stronger than that of BNSs, indicating that B@TA-R848 had better tumor enrichment effect after wrapping polydopamine and tumor antigen protein.

The photothermal therapeutic efficacy mediated by B@TA-R848 *in vivo* was further investigated. 4T1 tumor-bearing mice were grouped randomly and administered with BNSs, B@TA and B@TA-R848, respectively. Afterwards, the mice were performed with photothermal therapy. The photothermal effect *in vivo* was monitored by measuring the temperature variations of tumors (Figure 4(G)). Five minutes after irradiation, the temperature at the tumor site treated with saline or BNSs only raised to 38.1 or 47.5 °C. Comparatively, in mice injected with B@TA-R848, the tumor temperature rapidly increased rapidly during a continuous irradiation, reaching up to 53.5 °C, which is sufficient to cause tumor cell death. The temperature changes of different groups were recorded in Figure 4(H), and the results showed that the temperature of mice in the saline group increased by only 4 °C after NIR irradiation, while the temperatures of the BNSs and B@TA-R848 groups increased by about 10 and 15 °C.

3.4 *In vivo* combined PTT and immunotherapy efficacy

We next detected the *in vivo* biotoxicity of the nanovaccines. Saline, BNSs, or B@TA-R848 were injected into three groups

of healthy mice by tail vein administration, respectively. Blood was collected from mice in each group at day 1, 7, and 14 after administration and used for hematological and histological purposes. Various serum biochemical parameters were measured including creatinine (Cr), blood urea nitrogen (Urea), globulin (GLB), albumin (ALB), alanine aminotransferase (ALT), total protein (TP), uric acid (UA), and aspartate aminotransferase (AST) (Figure S11). Besides, the blood routine indexes were examined, including red blood cells (RBC), white blood cells (WBC), hematocrit (HCT), platelets (PLT), mean corpuscular volume (MCV), hemoglobin (HGB), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC), (Figure S11). Taken together, these results displayed that no significant differences in statistic were observed among the three groups. Results from H&E stained sections showed that no apparent tissue inflammation or local damage signals were detected in the liver, heart, spleen, kidney, and lung, all of which are major organs (Figure S12). Thus, the results demonstrated that BNSs and B@TA-R848 did not generate significant hemotoxicity, infection or inflammation in mice.

After confirming the tumor-targeted performance and *in vivo* biotoxicity of photo nanovaccines, an *in vivo* anti-tumor study was undertaking to assess the antitumor effects of each single therapy and the combined therapy. In brief, 4T1 tumor-bearing mice were randomly grouped and performed with different treatments: (1) Saline, (2) BNSs, (3) B@TA-R848 (immunotherapy), (4) BNSs + NIR (photothermal therapy), and (5): B@TA-R848 + NIR (photothermal and immunotherapy). Each group was administered via tail vein at the first dose and subcutaneously via paraneoplastic at the second and third doses. The dose of intravenous injection of nanomaterials was 5 mg kg⁻¹ body weight per group. Groups 4 and 5 underwent 5 min NIR laser irradiation at 12 h post-injection. After that, the mice were immunized by subcutaneous injection of each material every four days. The tumors were inoculated subcutaneously on the contralateral side as distal tumors on day 15, and the treatment cycle ended on day 21. The flow chart was shown in Figure 5(A). The tumor volumes were recorded and measured by a digital caliper. As expected, the tumor growth decreased in these groups (group 3–5), when compared with saline and BNSs groups (Figure 5(B)). Group 4 and 5 had a better therapeutic effect than group 3, suggesting that single photothermal therapy is more effective against solid tumors than using immunotherapy alone. After about 14 days, tumors in group 4 gradually resumed growth, thereby indicating that photothermal therapy alone did not prevent tumor recurrence. It is worth noting that the highest tumor growth inhibition was presented in the mice of group 5, compared to other groups, and the tumors of the five mice in this group disappeared

without recurrence, indicating the remarkably synergetic effect of combined PTT-immunotherapy. In none of the groups, obvious side effects were noted, including unusual weight loss and eating or activity disorders (Figure 5(C)). Tumor growth inhibition rate (TGI) showed that the B@TA-R848 group had the highest rate of tumor inhibition (Figure 5(D)). Images of H&E and Ki-67 staining showed that group 5 had the largest number of tumor cell death among all experimental groups

(Figure 5(E)). The growth results after distal tumor inoculation showed that the tumor volume was smaller in the BNSs + NIR and B@TA-R848 groups than in the control and BNSs groups, and no growth was observed in the B@TA-R848 + NIR group. This suggested that combined photothermal and immunotherapy can activate strong *in vivo* antitumor immunity and inhibit distal tumor growth. The H&E and digital images of lung tissue sections showed that tumor metastases were found

Figure 5: *In vivo* combined cancer therapy effect.

(A) Scheme of antitumor study. (B) Tumor growth curve of 4T1 tumor-bearing mice with various treatments. (C) Bodyweight of mice over time to the various treatments. (D) Tumor growth inhibition rate. (E) Representative H&E staining and immunohistochemical staining of Ki-67 in treated tumor tissues (scale bar, 100 and 50 μm). (F) Distal tumor growth statistics in different treatment groups. (G) The H&E staining and photo images of lungs in different treatment groups (scale bar 100 μm). (D, E, F) $n = 6$; ** $p < 0.01$, *** $p < 0.001$ and ns: Not significant ($p > 0.05$), analyzed by *t*-test, followed by Dunnett's multiple comparisons test. Data represent mean $\pm$ s.d.

in group 1 and 2 and no obvious tumor metastases were found in group 3, 4, and 5 (Figure 5(G)), thereby indicating that both photothermal and immunotherapy inhibited tumor metastasis to a certain extent.

3.5 Dendritic cells immuno-stimulation and T cell responses elicited by B@TA-R848

The immunoregulatory effect of B@TA-R848 as a photo nanovaccine was further studied. Dendritic cells are a class of antigens-presenting cells, linking innate and adaptive immune responses. CD8 α ⁺ dendritic cells direct the differentiation of Th1-type cells and CD8⁺T cells. And high expression of CD80/86 molecules is associated with the dendritic cells maturation, which plays key roles in activating antitumor immune responses in tumor vaccine applications [52]. Specifically, murine granulocyte-macrophage colony-stimulating factor (GM-CSF) and 2-mercaptoethanol (2-ME) were cultured with BM cells for seven days to obtain BM-DCs. The CLSM and TEM images of Figure 6(A) and (B) showed that more B@TA-R848-Cy7 than free Cy7 were taken up by BM-DCs. This suggested that autologous tumor antigen-modified nanovaccines were more likely to be taken up by DCs. We then examined the effects of nanovaccines on the *in vitro* polarization and maturation of BM-DCs. BM-DCs were divided into four groups with different treatments. Notably, the B@TA-R848 group induced the highest expansion of the CD80 + CD86⁺ dendritic cells subset compared to other experimental groups (Figure 6(C)), indicating that B@TA-R848 stimulated BM-DCs polarization and maturation *in vitro* with a high immunogenicity. The *in vivo* experiments were executed as described in Figure 6(D). 4T1 tumor bearing mice were split into four groups randomly, and immunized three times every 4 days. Each group was administered by the following methods: (1) Saline, (2) BNSs + NIR, (3) B@TA-R848, and (4) B@TA-R848 + NIR. Each group was administered via tail vein at the first dose and subcutaneously via paraneoplastic at the second and third doses. Group 2 and group 4 were received the photothermal treatment after 12 h of the first injection. Mice were sacrificed on day 21 and then the blood, lymph nodes in the groin, and spleens were collected. Serum was prepared and the concentration of serum cytokines were determined using related mouse enzyme-linked immunosorbent assay (ELISA) kits by the manufacturer's instructions.

Varying degrees of increased secretion levels of IFN- γ , TNF- α , IL-2, and IL-6 were presented in all nanoparticle-based treated groups, and the highest expression levels of these pro-inflammatory cytokines were exhibited in the combined therapy group (group 4) among these four groups

(Figure 6(E)). It indicated that the combined treatment can achieve a significant activation of pro-inflammatory cytokines synergistically influenced by photothermal and immunotherapy, which may explain why the best antitumor effects was present in this group. However, no statistical difference in the expression changes an anti inflammatory cytokine (IL-10) among all injected groups. Next, single-cell suspension of lymph nodes and spleens were obtained. The isolated immune cells were incubated with appropriate fluorophore-bonded antimouse antibodies, and then immunological responses of the four groups were analyzed by flow cytometry.

As presented in Figure 6(F), the percentage of CD80 + CD86⁺ cells was higher in the group 2 than in the group 1, indicating an increase in mature DCs, which suggested that the photothermal treatment could promote DCs maturation in the tumor-draining lymph node. Similarly, mice in group 3 which were treated with B@TA-R848 alone also showed an increase in mature dendritic cells, indicating that B@TA-assisted immune adjuvants stimulated DCs maturation. The fourth group had the highest percentage of CD80 + CD86⁺ cells, suggesting that combined photothermal and immunotherapy can most efficiently stimulate DCs maturation.

Finally, the effect of B@TA-R848 on regulating T lymphocytes was also evaluated. As is well-known, CD4⁺ T cells and CD8⁺T cells are considered "helper" and "killer" cells that recognize and kill tumor cells, respectively. Mature dendritic cells can present antigens to these T lymphocytes for tumor suppression. As shown in Figure 6(H) and (I), mice administrated with B@TA-R848 under 808 nm NIR irradiation exhibited the highest percentage of CD8⁺ T cells in the splenic CD3⁺ T cells. By contrast, the percentages of CD8⁺ T cells in the BNSs + NIR group and B@TA-R848 group were lower than those of mice in the B@TA-R848 + NIR group, thereby indicating that combining photothermal and immunotherapy has a better effect of activating the immune system compared to a single treatment. The results in Figure 6(J) and (K) demonstrated the intratumoral infiltration of CD8⁺ T lymphocytes. The data showed that the group that underwent laser irradiation (BNSs + NIR and B@TA-R848 + NIR group) had a better lymphocyte tumor infiltration when compared with the group that did not receive laser irradiation (saline and B@TA-R848 group). Thus, the findings indicated that infiltration of lymphocytes was promoted in the solid tumors after photothermal treatment. Taken together, B@TA-R848 promoted dendritic cells maturation and antigen presenting, and generated more CD4⁺, CD8⁺ T cells for antitumor immunity. After being targeted to a solid tumor, B@TA-R848 can destroy the solid tumor microenvironment through NIR laser irradiation

Figure 6: Antitumor immune response achieved by synergistic photo immunotherapy with B@TA-R848.

(A) Confocal images of dendritic cell internalization of B@TA-R848-Cy7 (blue: Cell nucleus stained with hoechst33342; green: Cytoplasm stained with FITC; red: B@TA-R848-Cy7), scale bar: 20 μ m. (B) TEM image of dendritic cell internalization of B@TA-R848, scale bar: 2 μ m. (C) Measurement of *in vitro* BM-DCs maturation (CD80+, CD86+) in total CD11c + dendritic cells with four different treatments (Control, BNSs, B@TA, and B@TA-R848). (D) Experimental procedure and schematic diagram for the cytokine, dendritic cell, and T cell analysis with different treatments (Saline, BNSs + NIR, B@TA-R848, and B@TA-R848 + NIR). (E) Quantitative analysis of Th-1 (IFN- γ , IL-2, TNF- α) and Th-2 (IL-10) associated cytokines in serum using ELISA kits. (F, G) Flow cytometry determination of dendritic cells maturation (CD80 + CD86+ gated on CD11c+) in tumor-draining lymph node. (H, I) CD3+ CD8+ T cells in spleen (CD4-CD8+ gated on CD3+). (J, K) Confocal imaging of tumor immunofluorescence showing CD8+ T cell infiltration (scale bar, 100 μ m). Red fluorescence represents CD8+ positive T cells (scale bar, 100 μ m). (b, c, d, e, f, g, h) $n = 3$; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, and ns: not significant ($p > 0.05$), analyzed by *t*-test, followed by Dunnett's multiple comparisons test. Data represent mean $\pm$ s.d.

treatment, and increase lymphocyte infiltration. Besides, during photothermal therapy of tumors, tumor cells can experience immunogenic cell death, i.e., tumor cells are converted from nonimmunogenic cells to cells with immunogenicity while undergoing apoptosis, and antitumor immune effects are stimulated in the organism. The synergetic effect of combined immunogenic cell death and tumor autologous antigen supported by B@TA-R848-mediated immune responses exhibited a better immunopotential action when compared to monotherapy, thereby showing an optimal anti-tumor effect. Moreover, local hyperthermia increased blood flow and vascular permeability, change interstitial pressure, and promoted T cells to enter the tumor to fight against cancer cells. As a result, the combination treatment group better promoted DCs maturation and T cells activation than the monotherapy, and achieved the best therapeutic effect.

4 Conclusions

In summary, we reported a novel strategy to prepare photo nanovaccines (B@TA-R848) based on single-element 2D boron nanosheets and tumor autoantigens (TA) as a combination treatment platform for cancer photo immunotherapy against immune desert tumor TNBC. The photo nanovaccines had nanoscale dimensions and exhibited higher photothermal conversion efficiency than boron nanosheets under 808 nm laser irradiation. Tumor autoantigens were obtained from surgically excised tumor fragments. Thus, the photo nanovaccines can provide an abundance of autologous tumor antigens compared to traditional single-antigen vaccines limited by individualized epitope recognition. The systematic studies also showed the potential of photo nanovaccines for multimodal imaging capability including fluorescence imaging, photoacoustic imaging, and photothermal imaging. Both *in vitro* and *in vivo* evaluation demonstrated that the photo nanovaccines had the potential to serve as personalized vaccines to promote dendritic cells maturation and triggered robust antitumor T-cell immune responses. In TNBC mouse model, the photo nanovaccines not only eliminated solid tumors, but also prevented tumor metastasis under combined photothermal immunotherapy. We believe that this photo nanovaccine system can be used for novel therapeutic functions by changing the functional proteins modified in the outer layer or by changing the drugs loaded, and follow-up studies are expanding.

Although in this study we are mesmerized by the results of B@TA-R848 as a photo nanovaccine in the TNBC treating experiment, there were still a fraction of tumor-bearing mice that had the signs of tumor recurrence even by combined therapeutic tumor vaccine with PTT. One of the prevailing

perceptions is the complexity of the tumor immune micro-environment. Another important reason is that tumor immunologic heterogeneity among different individuals affects the immune response to tumor vaccines. Therefore, it is still worth exploring to combine the new technologies of immunotherapy with the excellent properties of 2D nanomaterials. Moreover, it is highly conceivable that combination therapy consisting of B@TA-R848 and other therapeutics would improve the greater clinical anticancer effects, such as the combination of B@TA-R848 with chemotherapy or the drugs of immune checkpoint blockade (ICB), such as CTLA-4 and PD-1/PD-L1 blocking drugs, which would likely produce synergistic effects on account of the multifaceted antineoplastic mechanisms.

Together, this study suggested the great potential of B@TA-R848 in the elimination of solid tumors and induction of an anti-tumor immune response against immune desert tumor. It is expected that this study would spearhead the development of a novel photosensing material-based multifunctional nanotherapeutic platform for efficient personalized synergistic treatments against cancer.

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Detailed methods contain photothermal conversion efficiency, immunofluorescence, and statistical analysis. These materials are available free of charge on publications.