

Singlet oxygen-responsive nanoplatform for synergetic photodynamic-immunotherapy guided by NIR-II imaging

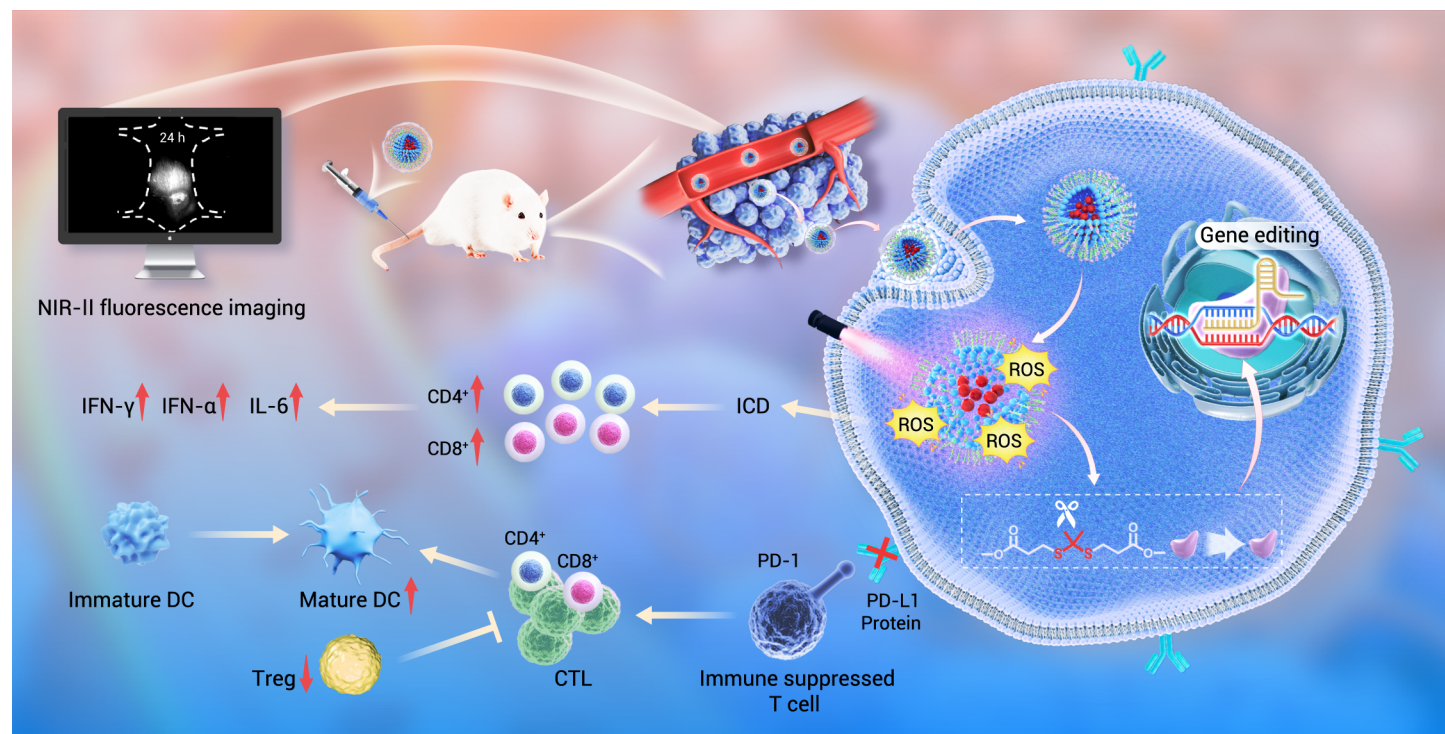
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- This study develops a light-activated system to improve therapy for triple-negative breast cancer.
- The nanoparticle uses CRISPR/Cas9 to edit the PD-L1 gene and a light-triggered therapy to kill tumor cells.
- The two methods work together to reshape the tumor's immune environment and enhance anti-tumor immunity.
- The system is monitored using advanced near-infrared imaging.

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Triple-negative breast cancer (TNBC) presents significant treatment challenges due to its lack of clear molecular targets and immunosuppressive tumor microenvironment (TME). This study reports the development of a light-triggered biomimetic nanodelivery system (OPTC-RNP@CM) to overcome these hurdles. The system integrates a semiconducting polymer for second near-infrared (NIR-II) imaging, a photosensitizer (Ce6), and CRISPR/Cas9 ribonucleoprotein targeting PD-L1 via a singlet oxygen-cleavable linker. Coated with homologous tumor cell membranes for enhanced targeting, the nanoparticles accumulate at the tumor site. Upon NIR irradiation, generated singlet oxygen simultaneously triggers the release of Cas9 RNP for PD-L1 gene editing and induces photodynamic therapy (PDT). This dual action promotes immunogenic cell death (ICD) and, synergistically with PD-1/PD-L1 pathway blockade, effectively remodels the immunosuppressive TME, amplifying the antitumor immune response. This versatile platform offers a promising strategy for precise and enhanced immunotherapy against TNBC.

INTRODUCTION

Triple-negative breast cancer (TNBC) lacks clear molecular targets and exhibits significant tumor heterogeneity.¹⁻³ The efficacy of standard treatments is severely limited due to significant side effects and their adverse impact on the host immune system.^{4,5} Particularly, the unique immunosuppressive tumor microenvironment (TME) of TNBC further limits the effectiveness of immunotherapy.⁶ The emergence of immune checkpoint blockade (ICB) strategies, such as utilized clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) ribonucleoprotein (RNP) targeting programmed cell death ligand 1 (PD-L1)⁷ to assisted immune-suppressive TME remodeling,⁸⁻¹¹ has brought new hope. This powerful and innovative tool holds unparalleled promise in permanently modifying target gene expression and stimulating the patient's immune system to fight against cancer cells.¹² Therefore, exploring and developing effective strategies to prolong the duration of immunotherapy and enhance its effectiveness is of great significance in cancer treatment.

Immunogenic cell death (ICD) releases damage-associated molecules, including adenosine-5'-triphosphate (ATP) and surface-exposed calreticulin (CALR),¹³ which induce an immune response and play a crucial key in activating the immune-suppressive TME.¹⁴⁻¹⁶ Specifically, when combined with ICB, ICD can effectively activate and significantly enhance tumor-specific immune responses.¹⁷ The commonly used photothermal therapy (PTT) can cause irreversible damage to normal tissues and tissue edema due to the high temperatures generated during the process.¹⁸ In contrast, photodynamic therapy (PDT) offers more controllable spatiotemporal properties and safety.^{19,20} PDT shows great potential in efficiently treating tumors by inducing ICD in tumors and combining with CRISPR technology to amplify the immune response.

The development of nanodelivery systems has provided an effective platform for the integration of multifunctional candidate materials.²¹ Currently, various nanoliposomes, polymers, and self-assembled nanomaterial delivery

systems have been developed.^{22,23} Semiconducting polymer nanomaterials, which are derived from polymers, have emerged as multifunctional near-infrared (NIR) absorbing and emitting biomaterials. These materials are extensively employed in various applications, including molecular imaging, phototherapy, and biological regulation.²⁴⁻²⁸ The low toxicity and high spatiotemporal resolution of light make it an ideal choice for the controlled release of drugs and RNPs, enabling on-demand release of the payload.²⁹⁻³² It is urgent to custom-develop polymer-based controlled drug delivery carriers to achieve specific light-triggered cleavage for remote activation of photosensitizer and CRISPR/Cas9 RNP. At the same time, the targeting issue of nanodelivery systems has also received significant attention. Nanoparticles coated with membranes, especially those disguised with biological cell membranes, can leverage their inherent biocompatibility and homotypic targeting properties to optimize their distribution and retention time in the body.³³⁻³⁵

In this paper, we report a biomimetic nanodelivery system (OPTC-RNP@CM) based on near-infrared photoregulation semiconductor polymers (OPT) that effectively inhibits TNBC tumor growth. This system is first assembled by semiconductor polymers OPT and photosensitizer (Ce6) to form OPTC, and then connected to Cas9/sgRNA_(PD-L1) (Cas9-RNP) via a thioketal (TK) bond (¹O₂-cleavable linker) to synthesize OPTC-RNP. Finally, the 4T1 cell membranes are wrapped around the OPTC-RNP to form a light-triggered biomimetic nanodelivery system. Meanwhile, the homologous targeting and camouflage properties of the cell membranes ensure that OPTC-RNP@CM is effectively delivered to the tumor site. It is noteworthy that, under the guidance of second near-infrared (NIR-II) fluorescence imaging, a large accumulation of biomimetic nanomaterials is observed at the tumor site. Polymer nanomaterial OPTC produce ¹O₂ under near-infrared light irradiation, and the side chains can safely release RNP through the cleavage of the linkers (sulfur-containing parts) by ¹O₂. Subsequently, the light-induced PDT effect activates ICD and combines with Cas9-RNP to synergistically enhance the immune response, making the anti-tumor effect more pronounced. Overall, this innovative biomimetic nanodelivery system demonstrates the potential of OPTC-RNP@CM as a nanoscale delivery system for TNBC. Not only does it improve the targeting and efficiency of therapeutic drugs, but it also specifically activates the immune-suppressive TME through the synergistic effects of multiple therapies, providing a novel and referable approach for the efficient clinical treatment of cancer.

MATERIALS AND METHODS

Materials

The following materials were commercially sourced: NH₂-PEG-NH₂ (J&K SCIENTIFIC, Beijing, China), thioglycolaldehyde (TK; Bidepharm, Shanghai, China), chlorin e6 (Ce6; Meilunbio, Dalian, China), calreticulin (CRT, Hanzhang Biotechnology, China), HMGB1 protein (Hanzhang Biotechnology, China), Cas9 proteins (Eastinno Biotechnology Co. Ltd., Ningbo, China), DAPI and DCFH-DA (Beyotime, Shanghai, China), and CM-Dil, CCK8 kit, Annexin V-FITC/PI Apoptosis Detection Kit, Calcein-AM/PI Double Stain Kit (Yeasten Biotechnology Co. Ltd.). sgRNA was synthesized in-house using the HiScribe T7 Quick High Yield RNA Synthesis Kit (New England Biolabs, Inc., USA). (Shanghai, China)

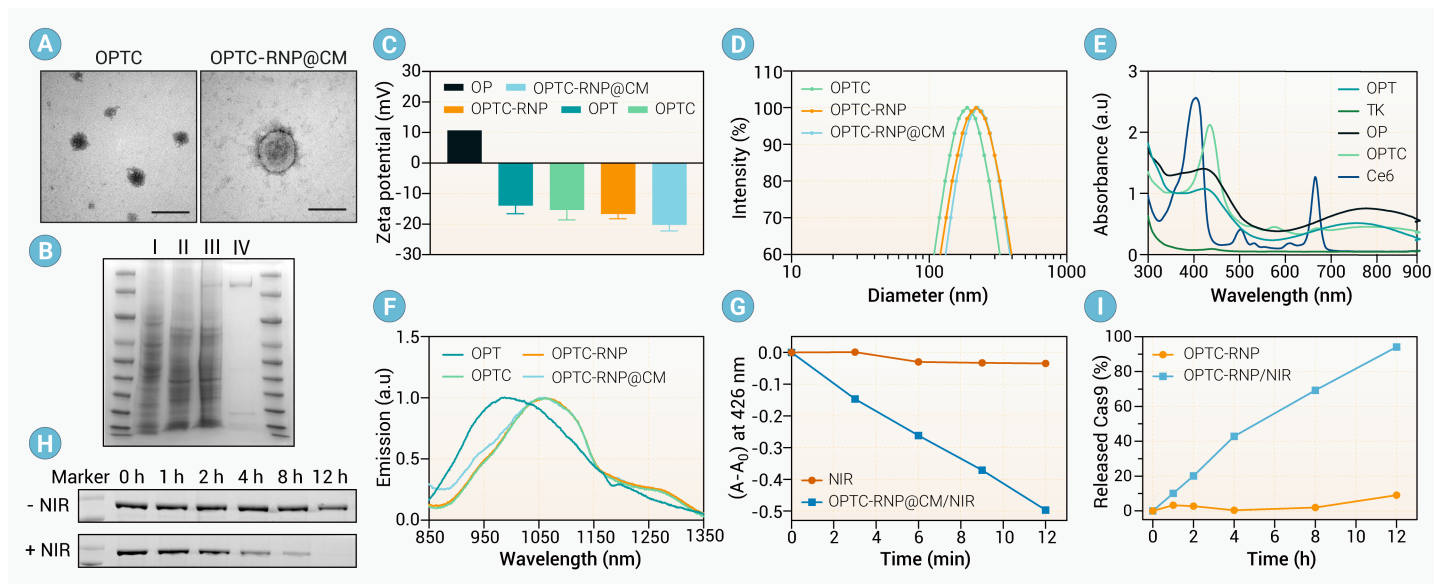


Figure 1. Basic characterization of the OPTC-RNP@CM synthesis process and in vitro protein release experiments (A) Representative TEM images of OPTC and OPTC-RNP@CM. (B) SDS-PAGE analysis of (I) marker, (II) 4T1 cell lysate, (III) 4T1 CM, (IV) OPTC-RNP@CM, (V) RNP and (VI) marker. (C) Zeta potential of OP, OPT, OPTC, OPTC-RNP, and OPTC-RNP@CM. (D) DLS of OPTC, OPTC-RNP, and OPTC-RNP@CM. (E) UV-Visible absorption spectra of OP, OPT, OPTC, Ce6, and TK. (F) Fluorescence emission spectrum of OPT, OPTC, OPTC-RNP, and OPTC-RNP@CM. (G) Relative absorbance of DPBF after adding OPTC-RNP@CM with or without NIR irradiation. (H) Residual amount of RNP in OPTC-RNP after NIR irradiation for different durations, determined by SDS-PAGE. (I) RNP release from OPTC-RNP corresponding to different durations of NIR irradiation. Scale bar: 200 nm.

BALB/c female mice (SPF grade, 4–6 weeks old) were purchased from Slac Animal (202401A023). These mice were raised in Laboratory Animal Center of Nanjing University of Chinese Medicine. All experiments conformed to animal ethics. A 660 nm laser (MDL-III-660D) from Changchun New Industries Optoelectronics Technology Co.Ltd.(Changchun, China). Avanti Polar Lipids were purchased from Merck & Co., Inc. (Alabama, USA).

Methods

Synthesis of monomer A. In a nitrogen-purged environment, a mixture was prepared comprising 2,7-Dibromofluorene (10.2 mmol), tert-butyl acrylate (41 mmol), and tetrabutylammonium bromide (0.78 mmol). Toluene (50 mL) and potassium hydroxide (2 M, 5 mL) were combined, and the resultant mixture was subjected to continuous stirring at ambient temperature for a duration of 12 hours. The reaction mixture was subsequently subjected to a sequential washing process, initially with deionized water and then with an aqueous solution of hydrochloric acid. Following this, the mixture was extracted using dichloromethane, and the resulting solution was dried over anhydrous sodium sulfate. The resultant mixture was subjected to filtration, and the filtrate was subsequently evaporated under vacuum conditions, yielding a yellow solid. This product was further refined through column chromatography, ultimately producing a purified yellow monomer A.

Synthesis of monomer B. In a round-bottom flask, mix Monomer A (3.7 mmol), bis(pinacolato)diboron (9.5 mmol), Pd(dppf)₂Cl₂, and KOAc (17 mmol). Dissolve in DMF and stir under nitrogen protection at 90°C for 12 hours under reflux. Purify by column chromatography to obtain the white product, Monomer B.

Synthesis of OSP-PEG. Mix monomer B (0.1 mmol) with 4,9-bis(5-bromothiophenyl)-6,7-bis(4-hexyloxyphenyl)-2-thia-1,3,5,8-tetrazacyclopentadiene naphthalene (0.1 mmol). Add tetrakis(triphenylphosphine)palladium and K₂CO₃ (120 mg) to the reaction flask, and finally add toluene and water. React at 90 °C for 20 minutes, and precipitate the polymer OS with methanol. Dissolve the polymer obtained from the previous step in DCM and react with trifluoroacetic acid for 24 hours to obtain the hydrolyzed product OSP. Subsequently, the hydrolysis product OSP (1 mmol) was mixed with NH₂-PEG-NH₂ (5 mmol) and reacted in the presence of NHS (20 mmol) and EDC (50 mmol). After reacting at room temperature for 48 hours, a dark green product, OSP-PEG, was obtained.

Synthesis of OSP-PEG-TK(OPT). The reaction of OSP-PEG (0.1 mmol) and TK (0.12 mmol), catalyzed by NHS/EDC at room temperature for 48 hours, was followed by 4 days of water dialysis and lyophilization, affording

the green compound OSP-PEG-TK.

Synthesis of OSP-PEG-TK-Ce6 (OPTC). OSP-PEG-TK and free Ce6 were mixed in a 1:1 molar ratio and dissolved in 1 mL of DMSO. The solution was then slowly injected into 9 mL of water under ultrasonication, followed by dialysis in water for 24 hours. After freeze-drying, a green product, OSP-PEG-TK-Ce6, was obtained.

Synthesis of OPTC-RNP. Incubate Cas9 protein with sgRNA in Tris-HCl buffer (pH=7.4) at a Cas9: sgRNA mass ratio of 1:3 for 10 minutes to form the Cas9/sgRNA complex. Add the activated OPTC dropwise to the excess Cas9/sgRNA complex, and incubate at 4°C with shaking overnight to generate OPTC-RNP.

Synthesis of OPTC-RNP@CM. First, extract a large amount of 4T1 cell membrane according to previously published methods. In brief, the cell membranes are centrifuged out after multiple cycles of cell disruption, then freeze-dried and quantified. To form uniform nanoparticles, the extracted cancer cell membrane (CM) is first extruded through a 400 nm porous polycarbonate membrane in Avanti Polar Lipids about 20 times. The collected cell membrane vesicles are then mixed with OPTC-RNP in a 1:1 mass ratio and suspended in 1 mL of H₂O. The mixture is then sonicated in an ice-water bath at 100 W for 1 minute to ensure complete mixing. Finally, the mixture is extruded through a 200 nm porous polycarbonate membrane in Avanti Polar Lipids about 20 times to obtain OPTC-RNP@CM.

Characterizations of OPTC-RNP@CM. For transmission electron microscopy (TEM; JEM-200CX, JEOL Ltd.) analysis, an aqueous solution of OPTC and OPTC-RNP@CM was deposited on a carbon-coated copper grid and dried prior to imaging. Measurements of particle size distribution and zeta potential were conducted via electrophoretic light scattering employing a Malvern Nano ZS zeta sizer (Malvern Instruments). UV-vis absorption spectra were acquired using a Shimadzu UV-3600 UV-vis-NIR spectrophotometer. To evaluate the retention of membrane proteins on OPTC-RNP, SDS-PAGE was performed. The samples with buffer were heated at 100°C in a metal bath for 10 minutes and then run on a polyacrylamide gel. After staining with Coomassie blue for 2 hours, the staining solution was washed overnight. Subsequently, the gel was imaged using an imaging system.

Evaluation of ¹O₂ generation. DPBF is used as a ROS molecule probe to detect the generation of ¹O₂. DPBF reacts irreversibly with ¹O₂, causing structural changes that lead to a decrease in its absorbance at 420 nm. DPBF dissolved in anhydrous ethanol is added to the OPTC, and then stirred in the dark for 12 minutes under either no irradiation or 660 nm laser (1 W·cm⁻²) irradiation. Every 3 minutes, 100 μL of the solution is removed for measurement. The

generation of singlet oxygen is detected by measuring the absorbance intensity at 420 nm in the UV-Vis absorption spectrum.

Evaluation of light-induced Cas9 RNP release. Dissolve 100 µg of OPTC-RNP nanoparticles in 1 mL of PBS. Collect the precipitate at different time points (0, 1, 2, 4, 8, and 12 hours) under either no light or with 660 nm laser (1 W cm^{-2}) irradiation. Determine the residual amount of RNP in OPTC-RNP using SDS-PAGE, and calculate the residual amount of the original OPTC-RNP by subtracting the residual amount using Image J.

OPTC-RNP@CM cellular internalization. We acquired the 4T1 cell line from the American Type Culture Collection. These cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, at 37°C in a humidified incubator with 5% CO₂. Cellular internalization of OPTC-RNP@CM was studied using CLSM. Transfection of EGFP-tagged ribonucleoproteins (RNPs) into 4T1 cell lines was performed, followed by a 6-hour incubation period. Subsequently, the cellular samples were rinsed thoroughly with phosphate-buffered saline (PBS). The cellular membranes were stained utilizing the CM-Dil dye (manifesting as red fluorescence), adhering strictly to the protocols provided by the manufacturer. Subsequently, the cells were rinsed with PBS and subjected to fixation using a freshly prepared 4% paraformaldehyde solution at ambient temperature for a duration of 15 minutes. Subsequently, the cell nuclei were stained utilizing the fluorescent dye DAPI (4',6-diamidino-2-phenylindole), which imparted a blue fluorescence, in strict adherence to the protocol provided by the manufacturer. After washing, cells were mounted with anti-fade reagent and observed using CLSM.

PD-L1 mutation frequency analysis. Following the application of various treatment protocols to 4T1 cells, genomic DNA was extracted from the treated 4T1 cells utilizing a standardized genomic DNA extraction kit. The DNA fragments targeted by the sgRNA were amplified utilizing a high-fidelity DNA polymerase, employing the specified primer pairs. Following gel purification via a DNA purification kit, the incidence of insertions and deletions was quantified utilizing a T7 endonuclease I assay kit. Purified DNA was analyzed by 2% agarose gel electrophoresis, and the frequency of insertions and deletions was calculated using ImageJ.

TA cloning and sequencing Analysis. PCR products with genome modifications were used for sequencing analysis. In summary, the ClonExpress II One-Step Cloning Kit was used to clone the PCR products into the pcDNA 3.1 vector. Sequencing of the cloned products was performed using BGH primers.

Protein blot analysis. 4T1 cells seeded in 24-well plates were treated using different methods. Cell lysis was performed with 1× cell lysis buffer containing a 1× protease inhibitor cocktail and 1 mM phenylmethylsulfonyl fluoride. Subsequently, a standard curve was generated utilizing the BCA Protein Assay Kit, facilitating the quantification of total protein content within the lysates. The lysate was combined with LDS sample buffer and a reducing agent, subsequently subjected to boiling for denaturation. Following loading with equivalent amounts of total protein (40 µg per sample) onto a 10% SDS-PAGE gel, samples underwent electrophoretic separation. The separated proteins were then electrotransferred onto a polyvinylidene difluoride (PVDF) membrane. The membrane was then incubated at ambient temperature with a blocking solution comprising 5% skim milk for a duration of 2 hours to prevent non-specific binding. Post-blocking, the membrane undergoes three 10-min TBST washes prior to incubation with the relevant primary antibody. The incubation is done overnight at 4°C. Post-incubation, the membrane is subjected to a triple washing regimen with TBST, each cycle lasting 10 minutes. Subsequently, the membrane is incubated with the secondary antibody under ambient temperature conditions for a duration of 1 hour. Finally, the target protein bands are visualized using ECL, and images are obtained and analyzed with an imaging system.

In vitro cytotoxicity and apoptosis induction. After plating 4T1 cells in 96-well plates for 24 hours, they were categorized into distinct experimental groups. CCK-8 reagent was added to all wells, followed by a 2-hour incubation. Optical density (OD) at 450 nm was measured with a microplate reader. Cell viability is expressed as a percentage, calculated by: $[(\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}) / (\text{OD}_{\text{control}} - \text{OD}_{\text{blank}})] \times 100\%$. In order to further analyze the apoptosis of cells, the cells were pre-seeded in a 24-well plate and incubated for 24 hours. Different nanoparticles were then used to treat the cells,

followed by irradiation with a 660 nm laser. Following PBS rinsing, a 100 µL aliquot of the Calcein AM/Propidium Iodide (PI) detection working solution was introduced into each well. The plate was subsequently incubated at 37°C under dark conditions for a duration of 30 minutes, and the staining results were observed under a Thunder microscope. Calcein AM exhibited green fluorescence, while PI showed red fluorescence. Apoptosis induction by the drug was evaluated using an Annexin V-FITC/PI dual staining assay. Cells were first seeded in a 6-well plate and incubated for 24 hours. Following treatment with various nano drugs and irradiation using a 660 nm laser. The cellular samples were harvested and subsequently resuspended in a volume of 100 µL of 1× Binding Buffer. To each experimental tube, 5 µL of Annexin V-FITC and 10 µL of propidium iodide (PI) were introduced. Concurrently, corresponding single-stain control tubes were prepared to ensure accurate fluorescence calibration. Following a 15-minute incubation period at ambient temperature under dark conditions, 400 microliters of 1× Binding Buffer was introduced to each respective reaction tube. Subsequently, the tubes were thoroughly mixed and incubated on ice. Apoptotic events were subsequently quantified through the application of flow cytometric analysis.

Production of ROS in cells. 4T1 cells were initially pre-seeded into a 24-well plate and maintained under standard culture conditions for a duration of 24 hours. Subsequently, these cells were subjected to co-incubation with various treatment groups within a 37°C incubator for an interval of 6 hours. After the washing step, serum-free DMEM supplanted the culture medium. The wells were subsequently loaded with the DCFH-DA fluorescent probe and incubated in the dark for 30 minutes. Subsequently, the 4T1 cell line was subjected to irradiation using a 660 nm wavelength laser. After irradiation, DCFH-DA can be oxidized by reactive oxygen species to produce highly fluorescent DCF, and the green fluorescence within the cells was observed and recorded using the Thunder imaging system.

Validation of DAMPs release. Intracellular CRT and HMGB1 Detection by Western Blot Cells were lysed on ice for 30 minutes using whole-cell lysis buffer containing protease inhibitors. After low-temperature high-speed centrifugation, the supernatant was collected, and the protein concentration was quantified using the BCA protein assay. The protein samples were mixed with Loading Buffer and denatured at 100°C using a metal bath. Equal amounts of protein from each group (20 µg per lane) were separated by 10% SDS-PAGE gel electrophoresis. The proteins were then transferred to a nitrocellulose membrane. Following transfer, the membrane was washed with TBST for 10 minutes and blocked with 5% skim milk for 2 hours to prevent non-specific binding. After blocking, the membrane was washed three times with TBST (10 minutes each). Primary antibody incubation was performed overnight at 4°C using anti-calreticulin (CRT, 1:1000 dilution) and anti-HMGB1 (1:2000 dilution). Subsequently, the membrane was incubated with HRP-labeled secondary antibody (1:5000 dilution) at room temperature for 1 hour. Protein bands were visualized using ECL, and grayscale values were analyzed.

NIR-II fluorescence spectroscopy. Subjects in this study were female SPF BALB/c mice, aged 4 to 6 weeks, procured from Shanghai Slack Laboratory Animal Co., Ltd. All animal experiments rigorously adhered to the institutional guidelines for animal care and use set forth by Nanjing University of Chinese Medicine (Ethics Approval: 202401A023). The 4T1 cells, cultivated in petri dishes, were subjected to trypsin digestion followed by centrifugation at 700 rpm for 3 minutes to facilitate cell collection. Sterile 1×PBS was used to resuspend cells prior to their subcutaneous implantation into female BALB/c mice (5×10^6 cells per animal). The OPTC-RNP@CM nanomedicine (1 mg/mL 150 µL) was administered via intravenous injection to the tumor-bearing mice, and fluorescence signals in the mice were recorded using an Edinburgh FLSP920 fluorescence spectrophotometer in the 900-1500 nm range when excited by an 808 nm laser. Terminal procedures involved euthanasia followed by collection of major organs and tumor tissues for imaging analysis.

In vivo anti-tumor effect assessment. BALB/c mice implanted with 4T1 tumor cells were randomly divided into 5 groups (5 mice per group) and treated with PBS/NIR, OPTC, OPTC/NIR, OPTC-RNP@CM, and OPTC-RNP@CM/NIR, respectively. OPTC-RNP@CM (1 mg/mL, 150 µL) was administered via tail vein injection every two days. Forty-eight hours after the administration of the nanodrug, the tumor site was irradiated with a 660 nm

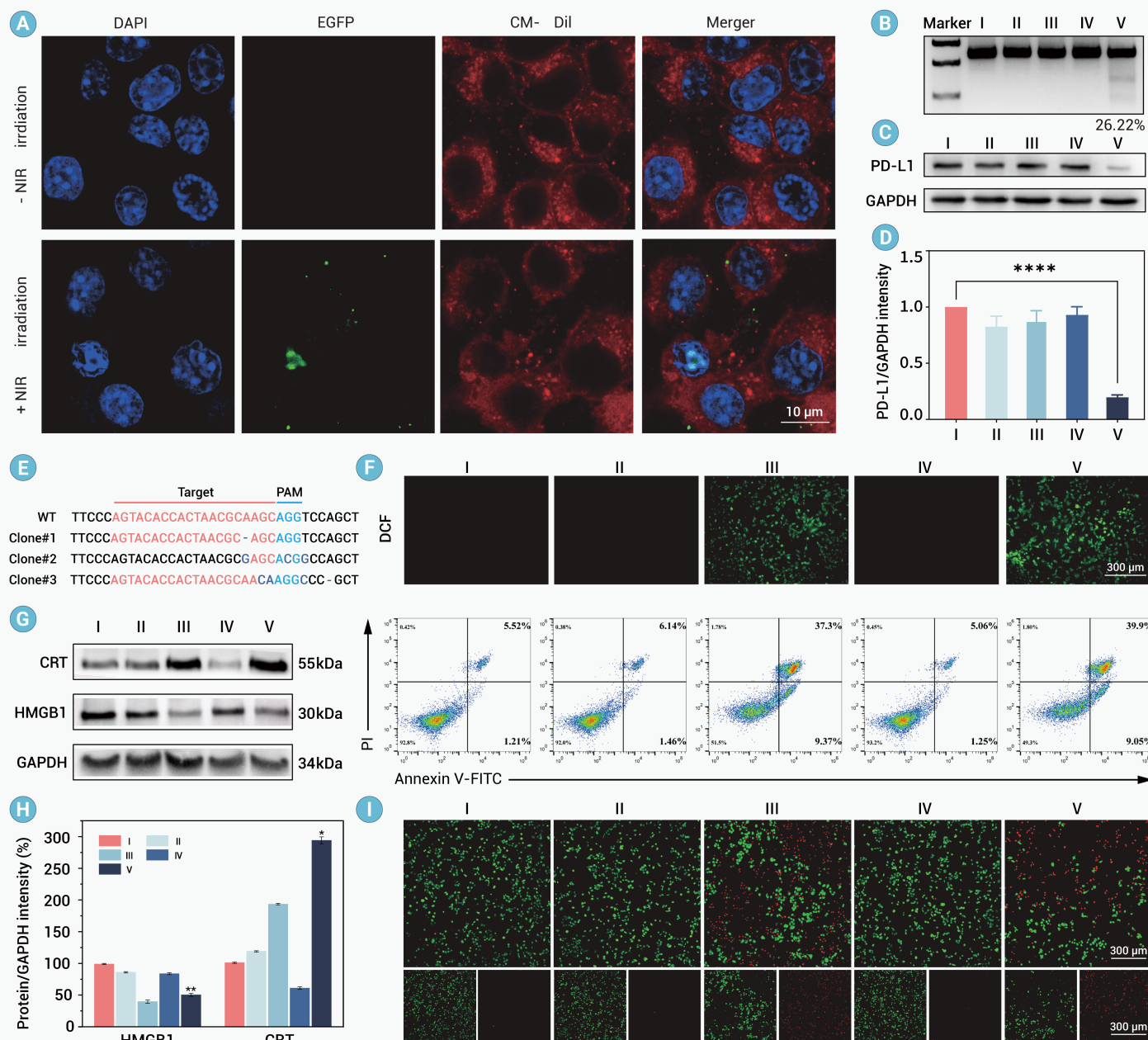


Figure 2. In vitro cell-based validation experiments (A) CLSM analysis of intracellular delivery of OPTC-RNP@CM carrying FITC-labeled Cas9 (green) to 4T1 cells under different conditions. Blue: DAPI; Red: CM-Dil. Scale bars: 10 μ m. (B) The T7 Endonuclease I (T7EI) assay was employed to evaluate the frequency of insertions and deletions (indels) at the PD-L1 locus in 4T1 cells following transfection with distinct treatment modalities. (C) Analysis of PD-L1 expression levels in 4T1 cells subjected to various treatment regimens via Western blotting. (D) Quantitative assessment the Western blot results of PD-L1/GAPDH across various treatments. (E) Identification of the representative DNA sequence of PD-L1 in mutant colonies following treatment with OPTC-RNP@CM/NIR. (F) Representative fluorescent micrographs depicting intracellular ROS levels in 4T1 cells, as visualized by DCFH-DA staining, following various treatment regimens (Scale bar: 300 μ m). (G) Protein expression of CRT and HMGB1 in different treatment groups. (H) Analyze the proportion of ICD-related proteins in the cells. (I) The depicted fluorescent micrographs illustrate the 4T1 cell response to various treatment regimens. Green fluorescence denotes calcein-AM staining, indicative of viable cells, whereas red fluorescence signifies PI staining, marking non-viable cells. The scale bar represents 300 μ m. (J) The induction of apoptosis in 4T1 cells subjected to various treatment regimens was quantitatively assessed through flow cytometric analysis. I: PBS/NIR, II: OPTC, III: OPTC/NIR, IV: OPTC-RNP@CM, V: OPTC-RNP@CM/NIR. (* P < 0.05, ** P < 0.01, *** P < 0.001).

laser (1 W cm^{-2}) for 5 minutes. Record the changes in body weight and tumor volume of the mice. The formula for calculating tumor volume is: Volume = (length $\times$ width²) / 2. On the 12th day after treatment, euthanize the mice and collect the tumor tissues, major organs, and peripheral blood.

Analysis of PD-L1 expression in tumor tissues after treatment. A portion of the tumor tissues collected from each group is taken and cut into small fragments. The tissues are then treated to extract proteins according to the instructions provided in the Western and IP cell lysis buffer manual by Shanghai Beyotime Biotechnology Co., Ltd. The extracted proteins are subjected to a WB experiment to analyze the expression of PD-L1 in the

tumor tissues. Another portion of the tumor tissues collected from each group is embedded and sectioned, followed by immunohistochemical analysis of the sections according to the manufacturer's instructions.

Flow cytometry analysis of immune cell infiltration in tumor sites. A portion of the tumor tissues collected from each group is cut into small fragments and treated with collagenase I and collagenase IV. The tissue is subsequently subjected to homogenization and filtered through a 70 μ m mesh. The resultant single-cell suspension is rinsed with pre-chilled 1 $\times$ PBS, followed by resuspension of the cells in a 2% BSA solution. The corresponding antibodies are added for staining, and finally, the samples are analyzed

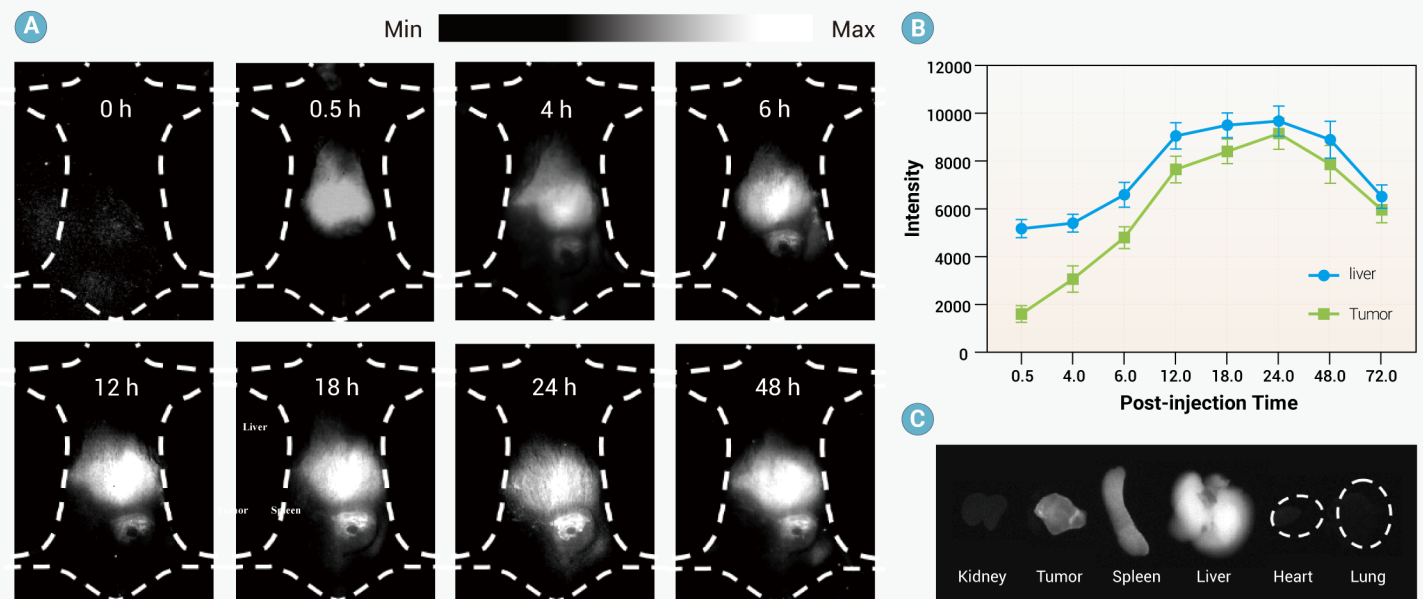


Figure 3. The distribution of OPTC-RNP@CM *in vivo* after intravenous injection in breast cancer-bearing mice (A) Representative *in vivo* NIR-II fluorescence imaging of nude mice bearing 4T1 breast cancer in BALB/C mice following intravenous injection of OPTC-RNP@CM. (B) NIR-II fluorescence signals of tumor, liver was tracked at different time points. (C) After 48 hours of drug administration, the mice were euthanized, and the main organs and tumor tissues were collected for two-region imaging analysis.

using a multicolor analytical flow cytometer.

Analysis of cytokine expression in mouse serum. Blood was drawn from mice in various treatment groups into non-anticoagulant tubes. Samples underwent natural clotting at room temperature (30–60 min) prior to centrifugation (3000 rpm, 10 min). The resulting supernatant was harvested for ELISA. Following the protocol, concentrations of the pro-inflammatory cytokines IFN- γ , TNF- α , and IL-6 were determined based on absorbance readings at 450 nm.

Nanomaterial biocompatibility analysis. A subset of the harvested tumor tissues and primary organs from each experimental cohort was preserved via fixation in a 4% paraformaldehyde solution. The excised tumors and organs were processed for microscopic evaluation by embedding in paraffin, fine sectioning, and hematoxylin and eosin (H & E) staining. Additionally, we will conduct a hemolysis assay. Whole blood samples were procured from healthy BALB/c mice via the orbital blood sampling technique and subsequently collected into anticoagulant-coated tubes. Following thorough agitation, blood samples were centrifuged at 3000 rpm for 15 minutes to separate the cellular components. The isolated blood cells were then incubated for 4 hours at 37°C with varying material concentrations and subsequently centrifuged again under identical conditions (3000 rpm, 15 min). Ultimately, the absorbance of the supernatant was quantified at a wavelength of 542 nm employing a microplate reader. Hemolysis rate (%) = $[\text{OD}(\text{sample group}) - \text{OD}(\text{PBS group})] / [\text{OD}(\text{ddH}_2\text{O group}) - \text{OD}(\text{PBS group})] \times 100\%$.

Statistical analysis: Statistical analysis of group differences employed either the Student's two-tailed t-test or Analysis of Variance (ANOVA). Significance thresholds for one-way ANOVA were set at * $p < 0.05$ (significant), ** $p < 0.01$ (significant), and *** $p < 0.001$ (extremely significant).

RESULTS AND DISCUSSION

Synthesis and characterization of OPTC-RNP@CM

We constructed a biomimetic tumor membrane-enclosed light-responsive nanodelivery platform (OPTC-RNP@CM). The synthesis route of OPT is shown in Figure S1. First, monomer A and monomer B were synthesized according to previous reports³⁶ (Figures S2–S3). Monomer B was employed to synthesize OSP (Figures S4–S5), which was then reacted with PEG-NH₂ to yield water-soluble OSP-PEG. The resulting OSP-PEG exhibited characteristic peaks corresponding to the -CH₂CH₂O- moiety (3.6 ppm) from NH₂-PEG-NH₂ in the ¹H NMR spectrum (Figure S6). Next, a ROS-sensitive thioketal (TK) linker was introduced to obtain OSP-PEG-TK (OPT). The successful attachment of the TK linker was confirmed by the appearance of characteristic peaks (-SCCH₃CH₃S-, 1.6 ppm) in the ¹H NMR spectrum (Figure S7). OPT and

Ce6 were co-assembled to form OSP-PEG-TK-Ce6 (OPTC), and the successful encapsulation of Ce6 was confirmed through UV-visible absorption spectrum. Transmission electron microscopy (TEM) analysis demonstrated that OPTC spontaneously organized into spheroidal nanoparticles, exhibiting an average diameter of 112 nm, as depicted in Figure 1A, a finding further supported by dynamic light scattering (DLS) analysis (Figure 1D). Next, the carboxyl group of TK was conjugated with Cas9 RNP targeting PD-L1, forming OPTC-RNP. To optimize the loading efficiency of Cas9 RNP, OPTC-RNP nanoparticles with varying mass ratios of OPTC to Cas9 RNP were synthesized. The Cas9 RNP content in the OPTC-RNP nanoparticles was assessed via agarose gel electrophoresis, which identified the optimal OPTC to Cas9-RNP ratio as 50 (Figure S8). Finally, based on previous reports,^{37,38} cancer cell-mimicking vesicles (CMs) were prepared using 4T1 breast cancer cells and used to coat OPTC-RNP, forming OPTC-RNP@CM. This modification enabled selective targeting of homologous tumor cells.

To assess the stability of the OPTC-RNP@CM complex, we replicated intricate vascular conditions and performed dynamic light scattering (DLS) analyses within a phosphate-buffered saline (PBS) solution at a pH of 7.4. At various designated time intervals, Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and deionized water were utilized. In Figure S9 indicating that these membrane-coated, self-assembled nanoparticles exhibit good stability. TEM images clearly reveal that the OPTC-RNP is encapsulated within a low-contrast bio-membrane structure, likely derived from the 4T1 cell membrane. To verify the retention of membrane proteins on OPTC-RNP@CM, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed. Remarkably, identical protein profiles were detected in both the 4T1 cell membrane fraction and the OPTC-RNP@CM complex, thereby substantiating the presence of adhesion molecules integral to the mediation of homotypic cell-cell interactions. (Figure 1B). This encapsulation was further confirmed by DLS in Figure 1D. Additionally, zeta potential measurements indicated a significant change in surface charge from -14.80 mV to -22.43 mV (Figure 1C). Additionally, UV-Vis absorption spectra and normalized fluorescence emission data revealed that OPTC possesses strong absorption in the near-infrared (NIR) region and emits in the second near-infrared window (NIR-II). After loading with RNP and coating with CM, the nanoparticles demonstrated enhanced targeting capability, enabling *in vivo* light modulation and tracking. (Figures 1E–F).

In vitro light-responsive drug release assessment

To ascertain the efficacy of OPTC-RNP@CM nanoparticles in producing photodynamic therapeutic effects, we conducted an *in vitro* assessment of

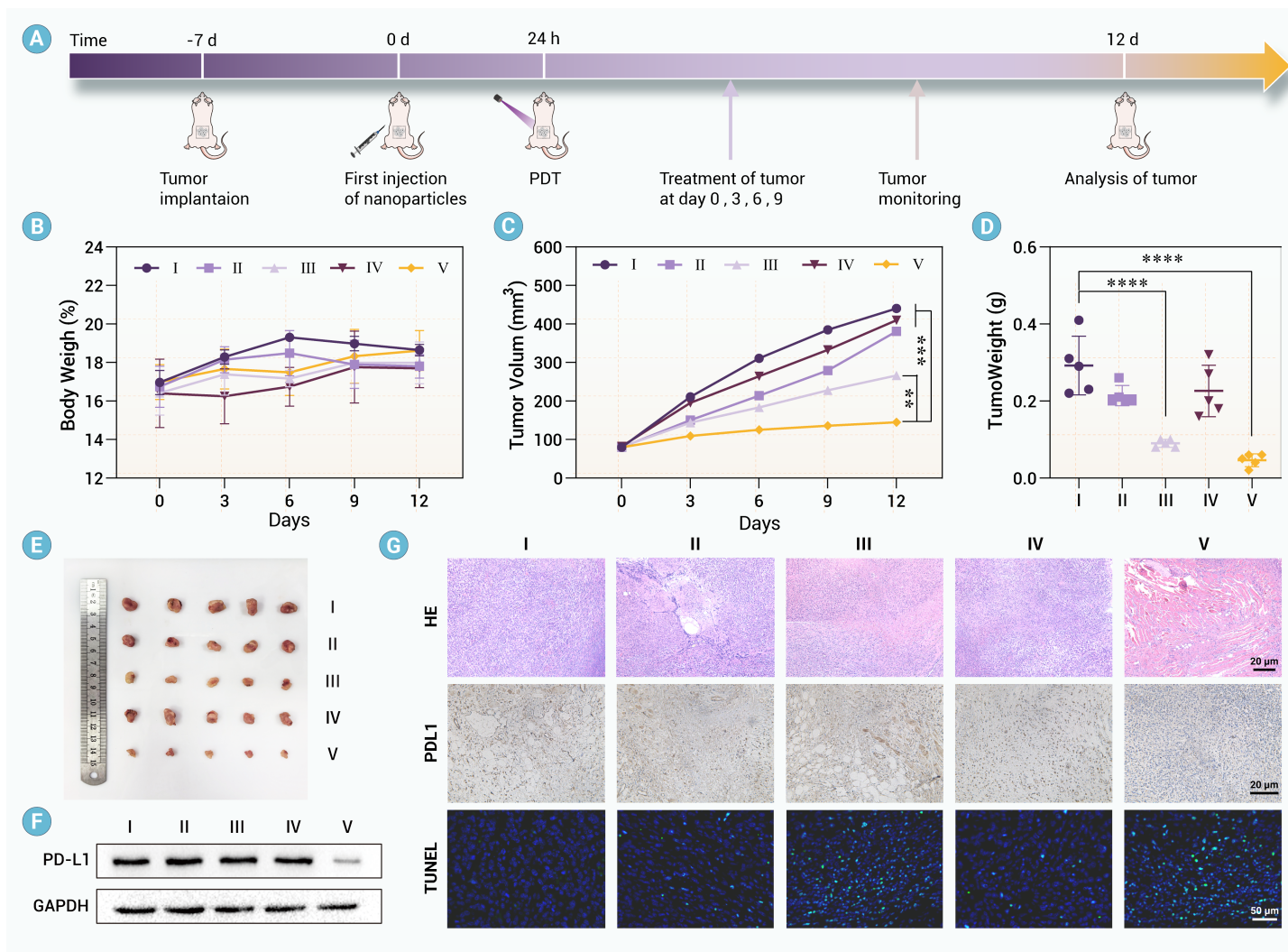


Figure 4. Synergistic triple-negative breast cancer immunotherapy via gene editing and photodynamic therapy utilizing OPTC-RNP@CM in vivo (A) Diagrammatic Representation of the Treatment Protocol Across Various Time Points in In Vivo Experiments Involving Female BALB/C Mice. (B-D) Variations in tumor mass, tumor volume, and murine body weight were observed following distinct therapeutic interventions. (E) Visual representations of neoplastic lesions subjected to various treatment modalities. (F) Evaluation of PD-L1 expression in tumor tissues subjected to various therapeutic regimens via Western blot analysis. (G) H&E, PD-L1, TUNEL staining of tumor sections from different treatments. Scale bars: 20 μm (H&E, PD-L1) and 50 μm (TUNEL). I: PBS/NIR, II: OPTC, III: OPTC/NIR, IV: OPTC-RNP@CM, V: OPTC-RNP@CM/NIR. (*P < 0.05, **P < 0.01, ***P < 0.001).

their capacity to generate singlet oxygen (1O_2). The absorbance of the distinctive peak of DPBF at 420 nm exhibited a marked reduction, thereby demonstrating the efficient generation of singlet oxygen (1O_2) by OPTC-RNP@CM (Figures 1G & S10). This is a prerequisite for achieving controlled release of CRISPR RNP through ROS-triggered cleavage of the thioether bond in OPTC-RNP@CM. Considering the high efficiency of 1O_2 generation by OPTC-RNP@CM, we evaluated the in vitro release behavior of Cas9-RNP from OPTC-RNP@CM triggered. The OPTC-RNP were irradiated with NIR, and the residual amount of Cas9-RNP in OPTC-RNP was detected using SDS-PAGE at different time points. Compared to the group without near-infrared irradiation, the residual amount of Cas9-RNP in OPTC-RNP rapidly decreased with prolonged incubation time. Approximately 70% of Cas9-RNP was released 8 hours after irradiation (Figures 1H-I). This reflects that the Cas9-RNP can be effectively released from OPTC-RNP nanoparticles through 1O_2 -mediated activation upon NIR irradiation.

In vitro cellular uptake and gene editing effects

We investigated the intracellular delivery capability of this nanoplatfrom and assessed its gene-editing efficiency. First, we co-incubated OPTC-RNP@CM with 4T1 cells, where the RNP in OPTC-RNP@CM was labeled with EGFP. We observed a clear green ERFR fluorescent signal of Cas9-RNP in the near-infrared light irradiation group, indicating that the OPTC-RNP@CM nanoparticles were successfully taken up by the cells and delivered into the

nucleus (Figure 2A). Subsequent targeted gene editing can only proceed after the release of Cas9 RNP from the nanoparticles. The 1O_2 generated by OPTC-RNP@CM under light irradiation can cleave the thioether bonds, thereby promoting the release of Cas9-RNPs from the nanoparticles and efficiently delivering them to the cell nucleus. To assess the in vitro gene-editing efficacy of the therapeutic platform, the four T1 cell lines were allocated into distinct treatment cohorts, followed by verification of the gene-editing outcomes within the in vitro setting. The mutation frequency was initially quantified through the application of the T7 endonuclease 1 (T7E1) assay. In the OPTC-RNP@CM/NIR treatment group, the detected mutation frequency of PD-L1 was recorded at 26.22% (Figure 2B). To further verify, we used Western blot (WB) analysis to detect the expression of PD-L1 protein in cells. The expression level of PD-L1 protein in group of OPTC-RNP@CM/NIR was lower than that in the control group (Figure 2C). We performed quantitative analysis of the Western blot data, which clearly visualized the expression levels of PD-L1 across different treatment groups, as shown in Figure 2D. The experimental outcomes exhibited congruence with the T7E1 assay findings, thereby corroborating the accurate excision of the PD-L1 gene within the 4T1 cellular population. Finally, to further demonstrate that OPTC-RNP@CM can accomplish gene editing of PD-L1, we performed DNA sequencing of the PD-L1 gene in the OPTC-RNP@CM/NIR group. The target gene showed three representative deletions and insertions near the PAM sequence, further confirming the CRISPR/Cas9-mediated knockout of PD-L1

(Figure 2E).

In vitro ROS generation evaluation and cytotoxicity

To confirm that OPTC-RNP@CM can generate ROS under NIR light after entering cells. Intracellular green fluorescence was not detected in cells following treatment with either OPTC or OPTC-RNP@CM without NIR light exposure. In contrast, intracellular green fluorescence was observed in the OPTC/NIR and OPTC-RNP@CM/NIR groups (Figure 2F). The fluorescence signal intensity in the OPTC-RNP@CM/NIR group is higher than that in the OPTC-RNP@CM group. The OPTC-RNP@CM nanoparticles produce a large amount of $^1\text{O}_2$ under near-infrared light irradiation, causing DNA damage to tumor cells, leading to apoptosis and activating immunogenic cell death. Among them, ROS can effectively oxidize the intracellular organic molecules within the cells, triggering ICD, releasing the HMGB1 protein and exposing the calreticulin (CRT) protein. We examined the CRT levels and HMGB1 protein expression in different treatment groups. The WB results showed that the CRT protein in the cells treated with OPTC-RNP@CM/NIR was significantly increased, while HMGB1 was significantly decreased. This indicates that a strong ICD effect occurred in the cells after OPTC-RNP@CM/NIR treatment (Figures 2G–H). Utilization of near-infrared-mediated genome editing to ablate the PD-L1 gene significantly potentiates the efficacy of immunotherapy, thereby augmenting the immune system's capacity to identify and eliminate tumor cells. To validate the anti-tumor potential of OPTC-RNP@CM nanoparticles under near-infrared light irradiation, we first performed a standard CCK-8 assay to assess the viability of 4T1 cells after co-incubation with different concentrations (0, 10, 20, 40, 80 $\mu\text{g}/\text{mL}$) of OPTC and OPTC-RNP@CM without near-infrared light exposure. The cell survival rates were all above 90%, indicating no significant cytotoxicity.

When 4T1 cells were exposed to different power levels, there was no significant decrease in cell viability (Figure S11). These results indicate that OPTC-RNP@CM, as a CRISPR-Cas9 delivery system, has good biocompatibility. To further validate the enhanced PDT of the genomic editing nanoplat-form, we evaluated the in vitro therapeutic effects of OPTC-RNP@CM at different concentrations. OPTC-RNP@CM demonstrated higher therapeutic efficacy compared to OPTC at the same incubation concentration. This indicates that OPTC-RNP@CM enhances PDT through genomic editing, showing promising therapeutic effects. Next, the killing effect was evaluated by detecting live cells and dead cells. All observed cells demonstrated robust green fluorescence signals, suggesting that the incurred damage to the tumor cells was minimal. However, in the OPTC-RNP@CM/NIR group, bright red PI fluorescence was distinctly observed, indicating the strongest 4T1 cell killing capability (Figure 2I). Finally, we performed flow cytometry to detect cell apoptosis, which also supports this trend (Figure 2J). We demonstrated that OPTC-RNP@CM/NIR exhibits good in vitro antitumor effects. This preliminary evidence confirms the feasibility of our CRISPR gene editing-enhanced near-infrared light therapy strategy and provides a basis for further exploration of its in vivo antitumor effects.

Biodistribution of OPTC-RNP@CM in vivo

NIR-II imaging can be used for intelligent real-time monitoring and evaluation of breast cancer progression after treatment, as well as metabolic detection, making it highly valuable for prognosis.^{39,40} To evaluate the in vivo combined therapeutic effect of OPTC-RNP@CM nanodrugs, establishing an in situ mammary cancer mouse model. First, to assess the targeting potential of OPTC-RNP@CM nanodrugs to tumors in vivo. Utilization of a NIR-II in vivo imaging system to monitor the distribution of OPTC-RNP@CM within tumor-bearing murine models at various temporal intervals subsequent to administration via tail vein injection. (Figure 3A). Over the course of the observation period, the signal intensity at the tumor locus exhibited a progressive enhancement, reaching its zenith at the 24-hour mark and maintaining detectability up to and beyond the 72-hour interval, as illustrated in Figure 3B. The data suggest that the OPTC-RNP@CM nanomedicine possesses the capability to precisely target the tumor locus and maintain localization for an extended duration, thereby facilitating subsequent therapeutic interventions. Furthermore, we euthanized the mice 48 hours after tail vein injection of OPTC-RNP@CM nanomedicine and performed ex vivo organ imaging to observe the fluorescence in the tumor and major organs. The fluorescence

was found to be primarily distributed in the liver, tumor, and spleen (Figure 3C).

Antitumor effects of OPTC-RNP@CM in vivo

Based on the excellent tumor-targeting capability of the OPTC-RNP@CM, we proceeded with in vivo antitumor experiments. The mice harboring tumors were randomly allocated into five distinct experimental groups, each comprising five individuals ($n=5$): (I: PBS/NIR, II: OPTC, III: OPTC/NIR, IV: OPTC-RNP@CM, V: OPTC-RNP@CM/NIR). Laser irradiation (660 nm , $1\text{ W}/\text{cm}^2$, 5 min) was administered to the mice 24 h after IV injection. After 72 hours, the nanodrug was injected into the mice again. This treatment cycle was repeated four times, with the mice being euthanized on the 12th day (Figure 4A). We systematically documented the body weight, as well as the tumor volume and mass, of mice across all experimental cohorts. Significantly, the OPTC-RNP@CM/NIR cohort demonstrated the most robust tumor suppression, suggesting that the integration of photodynamic therapy (PDT) with PD-L1-targeted gene editing immunotherapy yields a potent therapeutic outcome (Figures 4B–E). To determine the in vivo efficacy of PD-L1 knockout, tumor tissues were excised from mice within diverse treatment groups. Subsequently, WB assays were performed to evaluate the expression levels of PD-L1 protein within these tumor specimens. In the OPTC-RNP@CM/NIR group, the PD-L1 protein level was significantly reduced, indicating that the PD-L1 gene was successfully edited in vivo (Figure 4F). To validate the efficacy of PD-L1 gene editing, we conducted immunohistochemical staining on tumor tissue samples. The findings corroborated a reduction in PD-L1 protein expression within the OPTC-RNP@CM/NIR treatment group. In accordance with the established experimental protocols, tumor tissue samples were processed for histological analysis through staining with Hematoxylin and Eosin (H&E) and further evaluated for apoptosis using the Terminal Deoxynucleotidyl Transferase dUTP Nick End Labeling (TUNEL) assay. The outcomes derived from these staining methodologies corroborated that the OPTC-RNP@CM/NIR treatment group elicited significant tumor cell damage and apoptosis, thereby demonstrating a pronounced inhibitory impact on tumor proliferation, as illustrated in Figure 4G. Additionally, we evaluated the potential toxic side effects in vivo under near-infrared light-regulated OPTC-RNP@CM combined immunotherapy. H&E staining of principal organs revealed an absence of substantial organopathy (Figure S12). To evaluate the biocompatibility of the OPTC-RNP@CM nanocomposite material, a hemolysis assay was performed. The observed reduction in hemolysis rate for the nanocomposite material across various concentrations compared to the ddH_2O positive control confirms its favorable blood compatibility. (Figure S13). Therefore, the OPTC-RNP@CM nanocomposite material not only demonstrates strong antitumor immunotherapeutic effects in vivo but also possesses high biosafety.

In vivo immune response

To elucidate the synergistic activation of tumor immunity mediated by OPTC-RNP@CM nanoparticles, we conducted a comprehensive evaluation of the primary immune cell populations present within the tumor microenvironment. The findings indicated a statistically significant elevation in the proportion of $\text{CD3}^+\text{CD8}^+$ T cells within tumor tissues in the treatment groups relative to the control group (PBS/NIR). Significantly, the OPTC-RNP@CM/NIR cohort exhibited the maximal infiltration of $\text{CD3}^+\text{CD8}^+$ T cells within tumor tissues, suggesting that the synergistic application of PDT-induced immunogenic cell death and PD-L1 gene editing markedly augmented anti-tumor immune responses in murine models (Figure 5A). In contrast, treatment groups exhibited lower proportions of immunosuppressive regulatory T cells (Tregs) relative to the PBS/NIR control group. Furthermore, the OPTC-RNP@CM/NIR group had the lowest Tregs content, suggesting a substantial improvement in the immune system of these mice (Figure 5B). The capacity of OPTC-RNP@CM/NIR to enhance dendritic cell (DC) maturation within tumor tissues was subsequently evaluated against the control cohort. The findings indicated that the OPTC-RNP@CM/NIR group exhibited a substantially elevated concentration of mature dendritic cells within the tumor tissues relative to both the control group and the other experimental cohorts. The data suggest that the OPTC-RNP@CM/NIR therapeutic regimen efficiently mobilized a substantial population of dendritic cells (DCs) to the tumor microenvironment and induced their maturation, thereby markedly augmenting their antigen-presenting functionality (Figure 5C). To further elucidate the

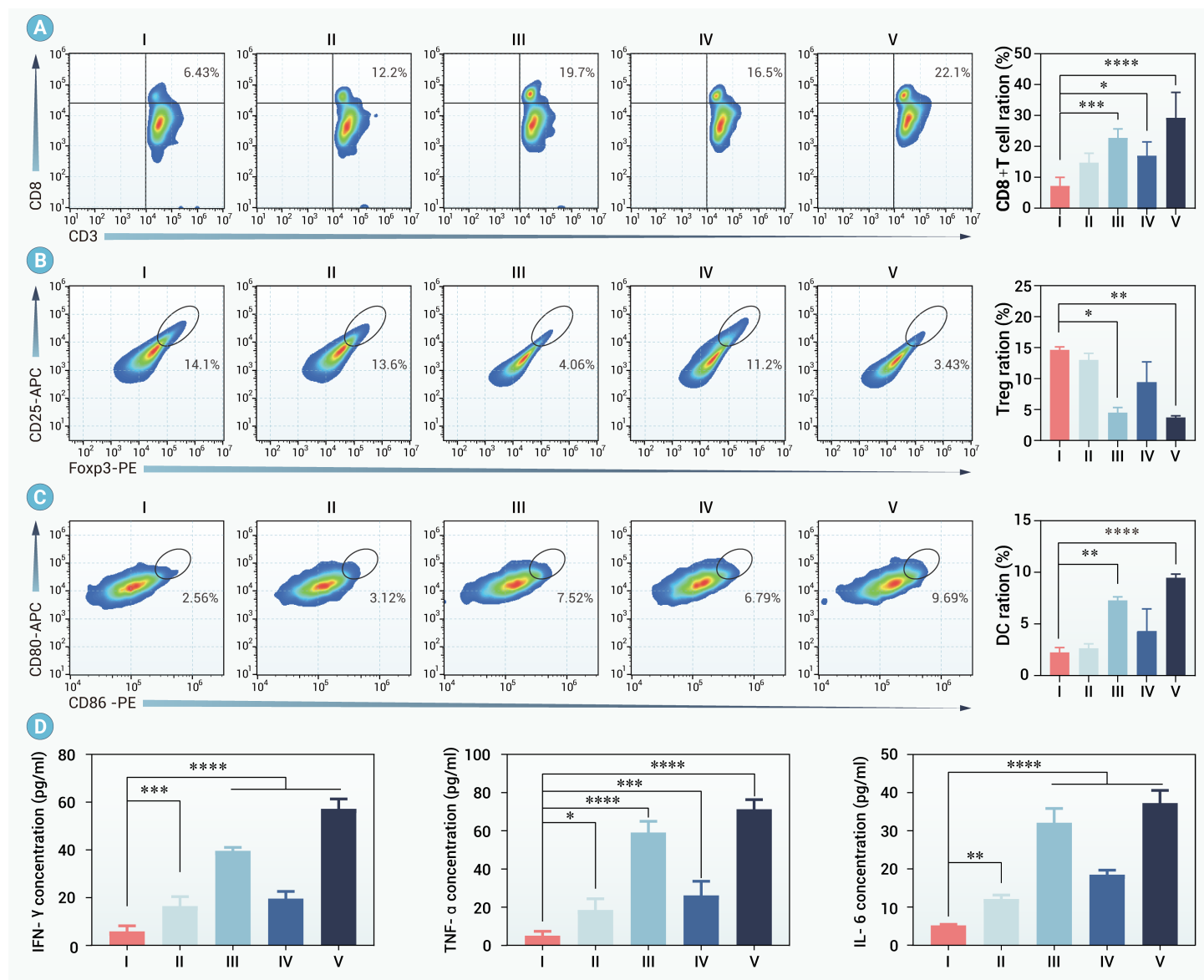


Figure 5. Synergistic activating T cell-mediated anti-tumor immunity by targeting PD-L1 and PDT (A) The figure presents representative flow cytometry histograms alongside the quantitative assessment of CD8⁺ T cell populations (subgated within CD3⁺ T cells) within the tumor tissues of murine subjects across various treatment cohorts. (B) Presentation of representative flow cytometry histograms accompanied by quantitative assessments of regulatory T cells (Tregs) within tumor tissues harvested from murine subjects across various treatment cohorts. (C) Depicted are representative flow cytometry histograms and the corresponding quantitative assessment of mature dendritic cells (de-fined as CD80⁺ CD86⁺ populations within CD11c⁺ gated cells) within the primary tumor following various treatment regimens. (D) The serum concentrations of IFN-γ, TNF-α, and IL-6 were quantified utilizing ELISA methodology. Group I: PBS/NIR, II: OPTC, III: OPTC/NIR, IV: OPTC-RNP@CM, V: OPTC-RNP@CM/NIR. (*P < 0.05, **P < 0.01, ***P < 0.001).

immunotherapeutic efficacy of photodynamic therapy (PDT) in conjunction with targeted programmed death-ligand 1 (PD-L1) inhibition, we quantified the expression levels of pertinent inflammatory cytokines in murine serum via enzyme-linked immunosorbent assay (ELISA). The experimental outcomes were in alignment with the trends identified in the previously discussed flow cytometry data (Figure 5D). In summary, the administration of OPTC-RNP@CM nanoparticles via tail vein injection, which concurrently induces immunogenic cell death (ICD) and targets PD-L1 gene editing, significantly augmented the tumor immune response in murine models.

CONCLUSION

In summary, we have rationally designed and established a biomimetic targeted and synergistic treatment strategy by ingeniously combining the multifunctional polymer OPT, photosensitizer Ce6, CRISPR/Cas9_{PD-L1}, and 4T1 tumor cells to form light-remote-controlled OPTC-RNP@CM nanoparticles. Under NIR light irradiation, OPTC generated ¹O₂, which cleaved the ¹O₂-cleavable linker, enabling the safe and efficient release of the gene carrier

RNP. This nanoparticle, under NIR-II imaging-guided NIR light remote control, induced PDT-induced ICD and combined with PD-L1 to not only prevent immune escape but also synergistically amplify the immune response, ultimately activating key cytokines IFN-γ, TNF-α, and IL-6, resulting in significant antitumor immunotherapy. This multimodal approach simultaneously targets tumors and enhances the overall antitumor immune response, providing a promising multifunctional platform for targeted tumor therapy in clinical settings.

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AUTHOR CONTRIBUTIONS

All authors contributed to the manuscript and approved the final version.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

The authors declare that the main data supporting the findings of this study are available within the paper and its Supplemental Information.

SUPPLEMENTAL INFORMATION

It can be found online <https://doi.org/10.59717/j.xinn-mater.2026.100190>.