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Artificial “Nano-targeted Cells” for Bimodal Imaging-Guided Tumor Cocktail Therapy

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Abstract

Background: Single therapeutic modality always has its limitations in combating metastatic lesions with complicity. Although the emerging immunotherapy exhibits preliminary success, solid tumors are usually immunosuppressive, leading to ineffective antitumor immune responses and immunotherapeutic resistance. Rational combination of several therapeutic modalities may potentially become a new therapeutic strategy to effectively combat cancer.

Results: Poly lactic-co-glycolic acid (PLGA) nanospheres were constructed with photothermal transduction agents (PTAs)- Prussian blue (PB) encapsulated in the core and chemotherapeutic docetaxel (DTX)/ immune adjuvant- imiquimod (R837) loaded in the shell. Tumor cell membranes were further coated outside PLGA nanospheres (designated as “M@P-PDR”), which acted as “Nano-targeted cells” to actively accumulate in tumor sites, which was guided/monitored by photoacoustic (PA)/ magnetic resonance (MR) imaging. Upon laser irradiation, photothermal effects were triggered. Combined with DTX, PTT induced *in situ* tumor eradication. Assisted by immune adjuvant R837, the maturation of DCs were promoted. Besides, DTX polarized M2-phenotype tumor-associated macrophages (TAMs) to M1-phenotype, relieving immunosuppressive TME. Integrating the above processes, the infiltration of cytotoxic T lymphocytes (CTLs) increased. The primary tumors and metastasis were significantly inhibited when treated with “Nano-targeted cells” based cocktail therapy.

Conclusion: “Nano-targeted cells” based therapeutic cocktail therapy is a promising approach to promote tumor regression and counter metastasis/ recurrence.

Key Words: Cocktail therapy, Photothermal therapy, Immunosuppressive tumor microenvironment, Homologous targeting, Nanomedicine

Introduction

Cancer is one of the leading causes of death [1]. Conventional oncologic methodologies, such as surgical resection, radiotherapy, chemotherapy, *etc.*, may cause severe side-effects to normal tissues, and some patients may suffer from recurrence and metastases[2-4]. Therefore, it is crucial to develop effective therapeutic strategies to eradicate tumors. In addition to safety and high selectivity, recurrence and metastasis should also be prevented. In the past decades, we have witnessed preliminary efficacy of emerging hyperthermia therapy (HTT) against malignant tumors [5]. As one of the paradigms of HTT, photothermal therapy (PTT) takes advantage of localized photothermal transduction agents (PTAs) to convert light energy into heat and subsequently raises the temperature of the tumor site, thereby inducing cancer cell death. Powered by nanotechnologies, PTT offers unparalleled advantages, such as noninvasiveness and extremely low toxicity to normal tissues. In addition, PTAs can often warrant both diagnostic and therapeutic functions [6, 7]. Although PTT can inhibit the growth of primary tumors to some extent, it also exposed certain disadvantages, such as limited light penetration, which could result in inadequate tumor tissues ablation. Moreover, only one therapeutic modality always has its limitations in combating metastatic lesions with complicity [8, 9]. In fact, recurrences after hyperthermic ablation are common [10-12], which is urgent to be addressed.

Recently, cancer immunotherapy, which activates the inherent immunological systems to identify, attack, and eradicate cancer cells, has attracted increasing attention [13-16]. Many studies have demonstrated the striking anticancer performance of PTT combined with immunotherapy [17, 18]. Tumor-associated antigen (TAA) can be released from dying tumor cells induced by hyperthermia, recognized by antigen-presenting cells (APCs) and presented to naive T cells [19-22]. Dendritic cells (DCs) have been recognized as the most potent APC, and the efficacy of T cells activation mainly determine by the maturation stage of DCs. Therefore, immune adjuvants, which are non-specific immunopotentiators, are introduced to promote the maturation of DCs. Among various adjuvants, imiquimod (R837), a toll-like receptor-7 agonist, has been demonstrated to significantly stimulates the maturation of DC cells and enhances immune response [23-25]. On the other hand, due to the infiltration of immunosuppressive cells, including protumoral M2-phenotype tumor-associated macrophages (TAMs), solid tumors are usually immunosuppressive, which can lead to ineffective antitumor immune responses and immunotherapeutic resistance. Fortunately,

recent studies have demonstrated that certain chemotherapeutic drugs, such as docetaxel (DTX), can attack tumor cells and prompt the release of TAAs, as well as effectively reverse immunosuppressive TME by polarizing protumoral M2-phenotype TAMs to tumoricidal M1-phenotype TAMs [26, 27].

In the above-mentioned modalities, although when they are applied individually, inadequate hyperthermia ablation and immunosuppressive TME are their predominant therapeutic obstacles, respectively, PTT combined with chemotherapy and immunotherapy could seemingly compensate the drawbacks for each other, achieving a synergistic therapeutic outcome [28, 29]. However, it is worth mentioning that to rationally integrate these modalities into one nanoplatform, any off-target delivery must be avoided [6, 30]. Owing to the stealthy and surface properties, cell-membrane-coating is introduced for extended blood circulation and highly specific targeting [31-35]. In particular, cancer cell membranes, which can be readily extracted from various cancer cells, are known to promote ligand-based active homologous targeting towards cancer cells [36-39].

Inspired by these findings, we report a homologous targeted cocktail therapy that integrates several therapeutic modalities. Just like a cocktail is a rational combination of several different wines to get the best taste, this study integrates several therapeutic modalities to achieve the best therapeutic effect. In this study, Prussian blue (PB) was selected as the PTA because of its good biocompatibility and excellent photothermal conversion efficacy [40, 41]. In addition, PB can also act as contrast agents for the enhanced photoacoustic (PA) imaging and T1-weighted magnetic resonance (MR) imaging, providing guidance/monitoring during the treatment. Cell-membrane-coated poly (lactic-co-glycolic acid) (PLGA) nanospheres are constructed with PB encapsulated in the core and DTX/ R837 loaded in the shell (designated as “M@P-PDR”). M@P-PDR nanospheres act as “Nano-targeted cells” to actively accumulate in tumor sites due to the homologous targeting capability. Upon laser irradiation, combined with DTX, PTT induces in-situ tumor eradication, releasing TAAs, and further enhancing tumor cell immunogenicity. Besides, DTX can relieve immunosuppressive TME by polarizing protumoral M2-phenotype oncogenic TAMs to tumoricidal M1-phenotype oncogenic TAMs. Furthermore, immune adjuvant R837 promotes the maturation of DCs, which can more effectively present TAAs and further stimulate the host immune response. Cocktail therapy, namely PTT combined with chemotherapy and immunotherapy, creates a

“doomsday storm” for tumors. This study provides a feasible approach to promote tumor regression and counter metastasis/ recurrence. Considering that all ingredients in these “Nano-targeted cells” are Food and Drug Administration (FDA)-approved and that the biosafety/biocompatibility has been systematically studied and evaluated, these as-synthesized nanospheres hold great potential for further clinical translation.

2 Materials and Methods

2.1 Materials

All reagents used in this work were of analytical grade. Citric acid, FeCl_3 , and $\text{K}_4[\text{Fe}(\text{CN})_6]$ were purchased from Shanghai Jingchun Biological Technology Co., Ltd. (Shanghai, China). PLGA (lactide: glycolide = 50:50, PLGA 12000 Da Mw) was obtained from Shandong Daigang Biology Engineer Corp. (China). Imiquimod (R837), docetaxel (DTX), Poly (vinyl alcohol) (PVA 25,000 Mw), calcein-AM (CAM), propidium iodide (PI), 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) and fluorescence dyes 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) were purchased from Sigma-Aldrich (Shanghai, China). Membrane protein extraction kits, phenylmethanesulfonyl fluoride (PMSF), penicillin-streptomycin solution, and trypsin were purchased from Beyotime (Shanghai, China). Trichloromethane (CHCl_3) was purchased from Chongqing Chuandong Chemical Corp. (Chongqing, China). Cell Counting Kit-8 (CCK-8) was obtained from MedChemExpress (Monmouth Junction, NJ, USA). Enzyme-linked immunosorbent assay (ELISA) kits including mouse IL-6, L-12, $\text{TNF-}\alpha$ and IL-10 were purchased from Meimian Industrial Co., Ltd. (Jiangsu, China).

2.2 Synthesis of P-PDR

The synthesis of PB nanoparticles (NPs) is described in detail in the supporting information. PLGA nanospheres loaded with PB NPs, DTX and R837 (designated as P-PDR) were synthesized using a typical double-emulsion method (water/oil/water). Briefly, DTX (6 mg), R837 (2 mg) and PLGA (50 mg) were dissolved in 2 mL of trichloromethane (CHCl_3), followed by adding 400 μL of PB NPs (20 mg mL^{-1}). Then the mixture was emulsified by using an ultrasonic probe (Sonics & Materials, Inc., USA) at 78 W for 2 min to form the first emulsion. Subsequently, 6 mL of PVA solution (4% w/v) was added to the above emulsion for the second emulsion with sonication power

at 65 W. Afterwards, isopropanol solution (6 mL, 2% w/v) dissolved in the second emulsion for magnetic stirring. Finally, after centrifugation at 10,000g for 6 min, the P-PDRs were collected from the slurry and stored at 4°C for further use. PLGA nanospheres loaded with different ingredients, such as P-P (PLGA loaded with PBs), P-DR (PLGA loaded with DTX and R837), P-PD (PLGA loaded with PBs and DTX), and P-PR (PLGA loaded with PBs and R837) were prepared by the same process as described above by replacing the cargo. DiI or DiR-labeled PLGA nanospheres were fabricated in the same manner, except that DiI or DiR was added to the CHCl₃ mixture.

2.3 Extraction of Cancer Cell Membrane and Synthesis of M@P-PDR

Murine breast cancer cells (4T1) were purchased from Zhongqiaoxinzhou Biotechnology Co., Ltd. (Shanghai), and cultured in Roswell Park Memorial Institute (RPMI) 1640 complete medium (Zhongqiaoxinzhou Biotechnology Co., Ltd., ZQ-Z201) containing 10% fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator at 37°C with 5% CO₂. The cell membrane was extracted using a membrane protein extraction kit from Beyotime Biotechnology. Cancer cell membranes-coated PLGA nanospheres were constructed according to the previous method with slight modifications [42]. Briefly, 4T1 cell membranes suspension (1 mg mL⁻¹) was mixed with P-PDR nanospheres under probe sonication (2 min, 40 W). Afterward, the mixture was centrifuged (10,000 g, 4°C, 10 min) and the slurry was washed twice to obtain M@P-PDR nanospheres.

2.4 Characterization of M@P-PDR

The morphology of M@P-PDR was observed with a transmission electron microscope (TEM, Hitachi H-7600, Japan) and a scanning electron microscope (SEM, Zeiss SUPRA™ 55, Germany). The protein content of M@P-PDR was analyzed by the SDS-PAGE method. The particle size distribution and zeta potential of M@P-PDR were measured with a dynamic laser scattering (DLS) particle sizer (ZEN3600, Malvern Instruments, UK). The contents of R837 and DTX in M@P-PDR were measured by liquid chromatography-mass spectrometry (LC-MS, Chromatograph: UltiMate 3000 RS, Mass spectrometer: TSQ Quantum GC, China). The UV absorption spectrum of PB was obtained by a spectrophotometer (UV-3600, Shimadzu, Japan). The loading efficacy of PB, R837 and DTX was calculated by the following formula:

$$\text{Loading efficacy (\%w/w)} = (\text{mass of PB/R837/DTX in M@P-PDR}) / \text{total mass of PB, R837 or DTX input} \times 100\%$$

2.5 *In Vitro* Photothermal Performance and PA/MR Bimodal Imaging of M@P-PDR

To study the photothermal performance of M@P-PDR, the temperature changes of M@P-PDR (with concentration at 1, 2, 3, 4 and 5 mg mL⁻¹) after 808 nm laser irradiation were monitored by an infrared thermal camera (Fotri226, Shanghai, China). The laser power intensity was set at 1.5 W cm⁻², 5 min. Besides, 5 mg mL⁻¹ of M@P-PDR nanospheres were irradiated with different power intensities (0.75, 1.00, 1.25 and 1.50 W cm⁻²) for 5 min. In addition, the photothermal stability of M@P-PDR was analyzed by exposing M@P-PDR to five cycles of laser irradiation on/off. For the *in vitro* PA imaging, M@P-PDR nanospheres at a concentration of 2 mg mL⁻¹ was scanned by PA laser with the excitation wavelength ranging from 680 to 970 nm (interval = 5 nm) to detect the optimum excitation wavelength using a PA imaging system (Vevo LAZR, CA). Then, M@P-PDR nanospheres at different concentrations (2, 4, 6, 8 and 10 mg mL⁻¹) were imaged with the optimal settings (λ = 740 nm). The acquired PA images were then analyzed by Vevo LAZR software to quantify the PA signal intensities within the region of interest (ROI). For T1-weighted MR imaging, different concentrations (1.25, 2.5, 5, 10 and 20 mg mL⁻¹) of M@P-PDR nanosphere were placed in 2 mL Eppendorf tubes for MR imaging using an MR imaging scanning device (Philips Achieva 3.0 T, Netherland). T1-weighted images of all samples were obtained using the following parameters: fast field echo (FFE), repetition time (TR) = 494 ms, echo time (TE) = 10 ms, flip angle = 90°, field of view (FOV) = 190 mm, and slice thickness = 1.0 mm. the T1 signal intensities within the ROI were also measured.

2.6 Targeting Capability and Cytotoxicity Evaluation of M@P-PDR

To investigate the targeting capability of these M@P-PDR nanospheres, 4T1 cells were seeded in confocal-specific dishes for 24 h to allow adhere, and then DiI-labeled M@P-PDR or P-PDR nanospheres suspensions were added at an equivalent PLGA concentration of 50 μ g mL⁻¹. After various incubation times (0.5, 1, 2, 3, and 4 h), the nuclei were stained with DAPI for confocal laser scanning microscope (CLSM, LSM710. Carl Zeiss, Germany) observation. Flow cytometry (BD FACSVantage SE, USA) was also carried out for quantitative analysis.

To observe the cytotoxicity of M@P-PDR against 4T1 breast cancer, 2×10^4 cells per well were cultured in a 96-well for 24 h. Then, different concentrations of P-PDR and M@P-PDR (equivalent PLGA concentrations at 20, 40, 60, 80, 100, 200 and 400 μ g mL⁻¹) dispersed in RPMI

1640 medium were added and cocultured for 24 h. And then the cell viabilities were tested via a typical CCK-8 assay. Additionally, 4T1 cells seeded in 96-well plates (2×10^4 cells per well) were randomly divided into seven groups including (i) control group, (ii) laser-only group, (iii) M@P-PDR group, (iv) M@P-DR + laser group, (v) M@P-PR + laser group, (vi) P-PDR + laser group, and (vii) M@P-PDR + laser group. The cells in various groups were treated with the corresponding nanospheres (the equivalent PLGA concentration is $400 \mu\text{g mL}^{-1}$) for another 4 h, followed by 808 nm laser irradiation (1.5 W cm^{-2} , 5 min). Then the cell viabilities were tested by a typical CCK-8 assay. For flow cytometry assessments, 4T1 cells were cultured in 6-well plates (8×10^4 cells per well) overnight. After various treatments, the proportions of apoptosis in each group were analyzed. Furthermore, cell viabilities were also visualized by CAM /PI staining.

2.7 *In Vitro* DC Maturation Analysis

BALB/c mice bone marrow-derived DCs were purchased from Otwo. Biotech. Inc. (Shenzhen, China). To evaluate the *in vitro* DCs activation, a transwell system was used. Initially, 4T1 cells were seeded in the upper chambers, and the immature DCs were seeded in the lower plates. They were randomly divided into six groups including (i) control group, (ii) M@P-PDR group, (iii) M@P-PD + laser group, (iv) M@P-PR + laser group, (v) P-PDR + laser group, and (vi) M@P-PDR + laser group, and received the corresponding treatment, respectively. Then the 4T1 cells in the upper chambers were harvested and incubated with the lower plates for another 24 h. Finally, DCs were collected and stained with anti-CD11c-FITC, anti-CD86-PE and anti-CD80-APC (eBioscience, Thermo Science, USA) for flow cytometry analysis. Otherwise, the supernatant was assayed by the ELISA kit for the detection of IL-6, IL-12 and TNF- α .

2.8 Animal Models

Female BALB/c mice (6 weeks old) were purchased from Enswell Biotechnology Ltd (Chongqing, China). All experimental protocols in this study were approved by the Animal Ethics Committee of the Second Affiliated Hospital of Chongqing Medical University. To inoculate the 4T1 breast cancer model, 4T1 cells (1.2×10^6 cells per mouse) suspended in RPMI-1640 medium were subcutaneously injected into the fifth mammary fat pad on the left side.

2.9 *In Vivo* Biosafety and Biodistribution of M@P-PDR

To evaluate the *in vivo* biosafety of these M@P-PDR “Nano-targeted cells”, healthy BALB/c

mice were intravenously administrated with M@P-PDR nanospheres suspension (3 mg mL⁻¹, 200 μL per mouse). Mice were sacrificed at 1 d, 3 d, 7 d, 15 d and 30 d (n = 5) post the injection, and then blood samples were collected for hematology analysis and serum biochemical tests, respectively. Major organs (heart, liver, spleen, lung, and kidney) were subjected to H&E staining. Mice injected with saline were set as control.

To explore the biodistribution and *in vivo* targeting behavior of these “Nano-targeted cells”, tumor-bearing mice were randomly divided into two groups (n = 3), they were then injected with DiR-labeled M@P-PDR or P-PDR nanospheres (equivalent PLGA concentration at 3 mg mL⁻¹, 200 μL), respectively. Then, these mice were subjected to an *in vivo* fluorescence imaging system at various time intervals post above administration to record the DIR fluorescence imaging. In the meantime, the corresponding fluorescence intensities were analyzed. Finally, animals were sacrificed to harvest the major organs (heart, liver, spleen, lung, and kidney) and tumors for *ex vivo* fluorescence evaluation.

2.10 *In Vivo* MR/PA Bimodal Imaging

4T1 tumor-bearing mice were randomly divided into two groups (n = 3), each mouse was injected with M@P-PDR or P-PDR nanospheres (equivalent PLGA concentration at 3 mg mL⁻¹, 200 μL), respectively. The PA images in the tumor region were acquired with a prolonged post-injection period (0, 1, 2, 4, 6, 8, and 24 h). Likewise, the corresponding PA intensities were quantitatively analyzed. T1-weighted MR imaging was also carried out after intravenous injection of these developed M@P-PDR. The greyscale images were converted to pseudo-color using MATLAB (2016). The signal intensities (SI) of the tumor tissues were measured, and the percentage of signal intensity enhancement (PSIE) was simultaneously calculated. PSIE was calculated as follows: $(SI_{\text{post}} - SI_{\text{pre}}) / SI_{\text{pre}} \times 100\%$.

2.11 *In Vivo* Photothermal Performance and Tumor Growth Inhibition Evaluation

To mimic distant tumors, after 6 days of primary tumor incubation (day-7), the equivalent 4T1 cells were subcutaneously injected into the right mammary fat pads at day -1. Then, all tumor bearing-mice were randomly divided into eight groups (n = 5) including: (i) saline group (control), (ii) M@P-PDR group, (iii) M@P-DR + laser group, (iv) M@P-P + laser group, (v) M@P-PR + laser group, (vi) M@P-PD + laser group, (vii) P-PDR + laser group, and (viii) M@P-PDR + laser group.

The mice were intravenously injected with the corresponding nanospheres (equivalent PLGA concentration at 3 mg mL⁻¹, 200 µL), respectively. After 8 h of the injection, the tumors were irradiated by an 808 nm laser (1.5 W cm⁻², 10 min). The temperature changes in tumor areas were recorded by a thermal camera. On the 3rd-day post-treatments, one mouse in each group was sacrificed for primary tumor and major organs (heart, liver, spleen, lung, and kidney) dissection. Then, H&E staining and examination were performed. Besides, the tumor tissues were further stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and heat shock 70 kDa protein (HSP70). On day 9, the distant tumors were collected for proliferating cell nuclear antigen (PCNA) staining. To monitor tumor progression, the mice were photographed, and the tumor volume changes were measured. During the treatment periods, the bodyweight of the mice was also recorded.

2.12 Analysis of Infiltrating Immune Cells

To detect the *in vivo* immune response, 4T1 xenotransplant tumors were divided into eight groups and received treatments identical to *in vivo* therapeutic evaluation. On the 9th day, both primary tumors and distant metastases were harvested for single-cell suspensions fabrication. The prepared cells were further stained with CD11c-FITC (eBioscience, Catalog: N418), CD86-PE (eBioscience, Catalog: GL1), CD80-APC (eBioscience, Catalog: 16-10A1), F4/80-APC (Biolegend, Catalog: BM8), CD11b-PE (Biolegend, Catalog: M1/70), CD80-FITC (Biolegend, Catalog: 16-10A1), CD206-FITC (Biolegend, Catalog: C068C2), CD3-FITC (Biolegend, Catalog: 17A2), CD8a-APC (Biolegend, Catalog: QA17A07) and CD4-PC5.5 (Biolegend, Catalog: RM4-5) antibody and then analyzed by flow cytometry. Serum was collected from different groups of mice, and cytokines including IL-6, IL-12, TNF-α and IL-10 were analyzed using ELISA kits according to the manufacturer's protocols. In addition, immunofluorescence staining was further conducted to investigate the infiltrating immune cells in tumor tissues.

2.13 Statistical Analysis

Data are expressed as mean ± standard deviation, and the significance of the differences between the two groups was analyzed using the Student's two-tailed t-test (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001).

3 Results and Discussion

3.1 Design, Synthesis and Characterization of M@P-PDR

M@P-PDR nanospheres, unique “Nano-targeted cells” with PB NPs encapsulated in the core, DTX/R837 loaded in the shell, and cancer cell membranes coated on the surface, were constructed for a homologous targeted cocktail therapy that concurrently integrates PTT, chemotherapy and immunotherapy to achieve the optimum anti-tumor effect. These “Nano-targeted cells” actively accumulated in tumor sites due to the homologous targeting capability, which was guided/monitored by PA/MR bimodal imaging. Upon laser irradiation, photothermal effects were triggered. Combined with DTX, PTT induced in situ tumor eradication, releasing TAAs. Assisted by immune adjuvant R837, TAAs could promote the maturation of DCs, accomplished with an increased secretion of TNF- α , IL-6, and IL-12. Besides, DTX polarized M2-phenotype TAMs to M1-phenotype and decreased the secretion of IL-10, relieving immunosuppressive TME. Integrating the above processes, infiltration of cytotoxic T lymphocytes (CTLs) increased to treat metastasis (**Scheme 1**).

P-PDR nanospheres were prepared by a simple double emulsification method [34, 40]. The obtained P-PDR nanospheres were further coated with cancer cell membranes to construct M@P-PDR “Nano-targeted cells” with homologous targeting capability (**Fig. 1A**). The SEM image indicated that M@P-PDR displayed a uniform and spherical morphology (**Fig. 1B**). M@P-PDR “Nano-targeted cells” showed a more obvious coating compared to P-PDR (**Fig. 1C**), which could be ascribed to the coverage by cancer cell membranes. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to further analyze the protein composition of these M@P-PDR “Nano-targeted cells”, and the results showed that M@P-PDR nanospheres had almost the same protein composition as the original 4T1 cell membrane (**Fig. 1D**), which further demonstrated the success of cell membrane coating. The zeta potential of the P-PDR and M@P-PDR nanospheres were -24.2 ± 0.85 mV and -17.0 ± 1.40 mV, respectively (**Fig. 1E**), which could potentially prolong the blood circulation as well as benefit other applications in the biological milieu [43]. Dynamic light scattering (DLS) showed that the average hydrodynamic diameter of the nanospheres slightly increased from 297 nm to 326.4 nm after the cell membrane coating (**Fig. 1F**). Compared to the UV-Vis spectrum of M@P-PDR, the spectrum of M@P-PDR suspension presented a characteristic absorption band of PB at 700 nm (**Fig. 1G**), indicating the successful loading of PB NPs in M@P-

PDR. The loading efficacy of PB NPs, R837 and DTX were calculated to be 37.28%, 78.57% and 69.67%, respectively, according to the standard curves (**Fig. S1A, S1B**) and liquid-mass spectrometry analysis (**Fig. S1C, S1D**). Such high loading capacities for these drugs demonstrated that PLGA nanospheres held great potential as promising nanocarriers for drug delivery, which also has been reported by many previous studies [44].

3.2 In Vitro Photothermal Performance and PA/MR Bimodal Imaging of M@P-PDR

The distinctive absorbance of PB NPs in the NIR region indicated the potential for M@P-PDR to boost photothermal therapeutics [45]. Therefore, *in vitro* photothermal performance of M@P-PDR was systemically studied. The photothermal conversion of M@P-PDRs was evaluated at different laser power densities (0.75, 1.00, 1.25 and 1.50 W cm⁻²) and different M@P-PDR concentrations (1, 2, 3, 4 and 5 mg mL⁻¹), respectively. Significant laser-power-dependent (**Fig. 2A, 2B**) and concentration-dependent (**Fig. 2C, 2D**) photothermal effects were observed. Moreover, excellent photothermal heating/cooling-cycling stability was also demonstrated (**Fig. 2E**). On this ground, M@P-PDRs can be used as PTCAs for subsequent PTT.

The high sensitivity and high spatial resolution of PA imaging facilitate the visualization of nanocarriers *in vivo* [46]. The multi-wavelength PA signal spectrum of M@P-PDR nanospheres showed that 740 nm was the optimal wavelength for PA imaging (**Fig. S2**). As shown in **Fig. 2F**, the PA signal intensities of M@P-PDR suspensions increased in a significant concentration-dependent manner. MR imaging performance was also investigated. As shown in **Fig. 2G (inset)**, the brightness of the T1-weighted MR images increased with the concentration of M@P-PDR nanospheres, and the pseudo-colored T1-mapping images also showed the same tendency. The relaxation rate (R1 value) was calculated to be 0.113 mM⁻¹ s⁻¹ by measuring the relaxation time (**Fig. 2G**). With the enhanced PA/MR dual-modal imaging capacity, the metabolic profiles of these M@P-PDR nanospheres at tumor sites can be visualized, providing guidance/monitoring for the subsequent cocktail therapy.

3.3 Biocompatibility assay of M@P-PDR

As a prerequisite for any clinical development, the biocompatibility of M@P-PDRs was investigated both *in vitro* and *in vivo*. First, the cytotoxicity of M@P-PDR and P-PDR nanospheres toward 4T1 cells was evaluated using a standard CCK-8 assay. After 24 h of coincubation, both

M@P-PDR and P-PDR nanospheres showed negligible toxicity to 4T1 cells when the PLGA concentration was lower than 400 $\mu\text{g mL}^{-1}$ (**Fig. S3**). To further investigate the biocompatibility of M@P-PDR, the *in vivo* acute and relatively long-term toxicity of M@P-PDR were evaluated in healthy BALB/c mice. Routine blood tests and serum biochemical assay were performed on days 1, 3, 7, 15 and 30 after intravenous administration of M@P-PDR (**Fig. S4A, S4B**). Compared with the reference range of hematology data, all indicators of the treated mice and the control group remained at normal levels. In addition, the major organs (heart, liver, spleen, lung and kidney) were collected for H&E staining (**Fig. S4C**), and negligible histomorphological or pathological changes were observed. All these results strongly demonstrated that the ideal high biocompatibility of M@P-PDR nanospheres as a multitasking therapeutic agent, providing great potential for their further clinical translation.

3.4 *In Vitro* Homologous Targeting Capacity of M@P-PDR

The effective intracellular uptake of M@P-PDR nanospheres is the key to improve their therapeutic efficacy. Functionalized by adhesion proteins of cancer cells on the surface, cancer cell biomimetic nanoplateforms are expected to exhibit specific homologous targeting capacity[31, 34, 37]. Therefore, the targeting capacity of these M@P-PDR “Nano-targeted cells” was evaluated using CLSM. As shown in (**Fig. 3A, S5A, and S5B**), 4T1 cells treated with M@P-PDR “Nano-targeted cells” exhibited stronger red fluorescence than that of P-PDR nanospheres, indicating that the cancer cell membranes-coating promoted the intracellular uptake of nanocarriers. Moreover, the red fluorescence enhanced with the extension of coincubation time. This phenomenon was further confirmed by flow cytometry quantitative analyses (**Fig. 3B**). For instance, after 2 h of incubation, the intracellular uptake rate of the “Nano-targeted cells”-treated group reached to 61.67%, while the P-PDR nanospheres-treated group was only 12.54%. The above results indicated that the presence of cancer cell membranes facilitated the intracellular uptake of nanocarriers thus exerting more effective therapeutic effects.

3.5 *In Vitro* Therapeutic Effects

M@P-PDR nanospheres have been demonstrated to act as PTCAs to convert light energy into thermal energy. The photothermal effects combined with chemotherapeutic drug (DTX) of M@P-PDR nanospheres against 4T1 cell was evaluated next. According to the results of the CCK-8 assay

(**Fig. 4A**), the cell viability in M@P-PR + L group was $46.14 \pm 5.62\%$, showing the high efficacy of PTT against tumor cells. The M@P-PDR + L group showed a lower cell viability ($18.75 \pm 6.21\%$), probably because the released DTX had a certain killing effect on cancer cells. The cell viability of the P-PDR + L group was $30.93 \pm 2.11\%$, which was lower than that of the M@P-PDR + L group, as the cancer cell membrane modification could have promoted more therapeutic agents to accumulate into the tumor cells to mediate the therapeutic processes. The cell viabilities of the laser only group, M@P-PDR only group and the M@P-DR + L group were $91.67 \pm 6.08\%$, $91.26 \pm 6.70\%$ and $90.43 \pm 5.87\%$, respectively, which were not statistically different compared with that of the control group ($95.30 \pm 7.30\%$). The cell damages were also analyzed by flow cytometry (**Fig. 4C**), and the results were consistent with CCK-8 results. Furthermore, cells after various treatments were also stained with CAM/PI to distinguish the live (green fluorescence) and dead (red fluorescence) cells. As shown in **Fig. 4B**, in the M@P-PDR + L group, almost all cancer cells died, showing bright red fluorescence, while in the P-PDR + L group, part of the cancer cells appeared green due to the lack of efficient intracellular uptake. According to the above results, PTT combined with chemotherapy can inhibit the activity of tumor cells, and the presence of cancer cell membranes optimizes the therapeutic effect of tumors.

3.6 Activation of DCs *In Vitro*

It is well known that the dead or dying cells would release TAAs and damage-associated molecular patterns (DAMPs) such as heat shock protein 70 (Hsp70) and calreticulin, which can activate anti-tumor immune responses [47-49]. These processes are also likely to happen as a result of the above-mentioned therapeutic effects. Immune adjuvants such as R837 can further enhance this response. Recognition of the R837 by immune cells, such as DCs, that express TLR 7 can significantly promote DC maturation and produce a range of pro-inflammatory cytokines, including TNF- α (a key marker of cellular immune activation), IL-6 and IL-12 (key markers of innate immunity), thereby stimulating T cell responses [25]. DCs, as the most powerful antigen-presenting cells, plays an essential role in activating anti-tumor immune responses [50]. In general, DCs capture TAAs released from dead or dying cancer cells and process them for presentation on major histocompatibility complex class I (MHC-I) molecules. Antigen-loaded DCs then migrate from peripheral tissues to the T-cell zone of the draining lymph nodes, where antigen presentation

promotes the differentiation of naive T cells into CTLs. Eventually, antigen-stimulated T cells leave the lymph nodes and migrate to metastatic tumors, achieving immunotherapy [51, 52]. Notably, only mature DCs elicit CTLs anti-tumor responses [53]. The upregulation of typical markers, including co-stimulatory molecules (CD11c+, CD80+, CD86+), indicates the degree of DC maturation. Considering the strong cytotoxic effects induced by M@P-PDR, we investigated whether M@P-PDR-mediated therapy activates immune responses *in vitro* by using a transwell system in which differently treated 4T1 cancer cells and untreated bone marrow-derived DCs (naive) were seeded in the upper and lower chambers, respectively (**Fig. 5A**). The maturation efficacy of DCs was measured by flow cytometry (**Fig. 5B, 5C**). A slight increase in DC maturation was observed in the M@P-PDR-treated group, which was probably due to the inevitable release of a small amount of R837 from these nanospheres. Compared to the M@P-PD + L group (without R837), the level of DC maturation in the M@P-PDR + L group was greatly increased, which further indicated the role of R837 in promoting the maturation of DCs. Relevant cytokines (TNF- α , IL-6 and IL-12) that would be released by mature DCs were measured by ELISA assays next. It was found that the M@P-PDR group and the M@P-PDR + L group showed higher secretion levels than the M@P-PD + L group, which could be attributed to the pivotal role of R837. Compared to the untargeted P-PDR + L group, DCs in M@P-PDR + L group secreted much more cytokines probably due to homologous targeting capacity mediated by cancer cell membranes (**Fig. 5D-F**).

3.7 Biodistribution and *In Vivo* MR/PA Bimodal Imaging

To monitor the biodistribution and *in vivo* targeting behavior of these M@P-PDR “Nano-targeted cells”, fluorescence imaging of tumor-bearing mice was performed. DiR-labeled M@P-PDR and P-PDR nanospheres were intravenously injected, respectively. In the M@P-PDR-treated group, obvious fluorescence signals at the tumor sites were observed. The signals increased with injection time and reached a peak at 8 h (**Fig. 6A, 6B**). The mean fluorescence intensity of the tumors was $13.70 \pm 1.35 \times 10^3$, which was 2.49-fold higher than that of the P-PDR-treated group ($5.50 \pm 0.54 \times 10^3$), which could be resulting from the homologous targeting capacity of cancer cell membranes. More importantly, significant fluorescence signals were still clearly visible at 24 h post-injection, indicating long-term retention of these M@P-PDR “Nano-targeted cells”. Afterward, tumors and major organs (heart, liver, spleen, lung, kidney) were dissected for *ex vivo* fluorescence

imaging. The fluorescent signals of tumors in the M@P-PDR group were evidently stronger than that of the P-PDR group ($p < 0.05$) (**Fig. S6A, S6B**). These results clearly indicated that the cancer cell membrane-coated nanospheres were endowed with superior active targeting ability, showing promising possibilities for *in vivo* precise imaging and effective treatment.

The aforementioned *in vitro* experiments have confirmed that M@P-PDR could act as contrast agents to enhance both PA imaging and T1-weighted MR imaging. Therefore, bimodal PA and MR imaging performance were further investigated *in vivo*. As expected, in the M@P-PDR group, the PA signals within tumor regions gradually enhanced with prolonged time, and reached a peak at 8 h post-injection (0.479 ± 0.022) in comparison with those at pre-injection (0.089 ± 0.003) (**Fig. 6C, 6E, S6C**). At 24 h post-injection, the PA signal intensities (0.433 ± 0.022) slightly decreased due to the gradual clearance of these nanospheres from tumor tissues. In contrast, in the P-PDR group without homologous targeting, the PA signals were significantly weaker throughout the time course of the observation. The T1-weighted MR imaging results showed that the tumors in the M@P-PDR group were clearly demarcated from the surrounding normal tissues with clear anatomical structures.

In addition, obvious bright enhancements were observed at the tumor areas over time, reached to a peak at 8 h post-injection, and were sustained for 24 h (**Fig. 6D, 6F**). PSII was used for quantitative analysis of the T1-weighted MR imaging enhancement. Specifically, the average T1-weighted signal intensities in the M@P-PDR group increased by $159.632 \pm 8.549\%$ at 8 h post-injection, whereas only $76.784 \pm 3.346\%$ enhancement rate was observed in the P-PDR group. The pseudo-color images also clearly showed the enhancements (**Figure S6D**). The trend of MR imaging is consistent with that of PA imaging and the enrichment had been reflected by *in vivo* fluorescence imaging. These results indicated that surface modification of cancer cell membranes on P-PDR structures contributed to the efficient accumulation of nanocarriers in tumor sites. Additionally, the excellent PA/MR bimodal imaging performance of these M@P-PDR “Nano-targeted cells” can provide the therapeutic time window and guide the NIR laser irradiation, achieving more precise therapy delivery.

3.8 *In Vivo* Cocktail Therapy Evaluation

3.8.1 *In Vivo* Photothermal Performance

After the enrichment of the PTCAs in tumor areas, the local temperature would rise under laser

irradiation. The tumors were exposed to laser irradiation 8 h after intravenous injection of nanospheres, and the temperature changes were monitored by an infrared thermal imaging system. As shown in **Fig. 7B-D**, temperatures of tumors presented a feeble increase in the M@P-PDR and M@P-DR + L groups compared to the control group in the absence of laser or PB NPs. Significant temperature increase was observed in the groups with concurrent presence of laser irradiation and the PB components, demonstrating excellent *in vivo* photothermal performance. The temperature in the M@P-PDR + L group increased to 62.7 °C, which was much higher than that of the P-PDR + L group (51.7 °C) without homologous targeting capacity. Assisted by cancer cell membrane coating, PTCAs could accumulate in tumors more efficiently to achieve more efficient and uniform localized hyperthermia.

3.8.2 Immune Responses Evaluation

Encouraged by the activation of DCs *in vitro*, the *in vivo* immune responses were evaluated next. The experimental design is shown in **Fig. 7A**. Tumors were inoculated at both the left and right mammary fat pads of mice in chronological order, and set as primary tumor (1st) and artificial mimicked metastasis (2nd), respectively. The mice were randomly divided into eight groups and administered with different treatments. The day when the treatments designated was set as day 0. To analyze the DCs maturation level *in vivo*, primary tumors (1st) (**Fig. 8A, 8B**), metastatic tumor (2nd) (**Fig. S7A, S7B**) and lymph node (**Fig. S7A, S7C**) were collected to make single-cell suspensions for flow cytometry assay on day 9. Similar to the *in vitro* results, the integration of the R837 immune adjuvant endowed M@P-PDR “Nano-targeted cells” with a much stronger ability to promote DC maturation, accompanied by increased cytokine secretion *in vivo*. In detail, the M@P-PDR + L group induced the highest level of DC maturation ($66.56 \pm 2.78\%$), which was significantly higher than the M@P-PD + L group without R837 ($22.81 \pm 4.26\%$), M@P-PDR “Nano-targeted cells” alone ($32.65 \pm 2.84\%$), and P-PDR + L group without homologous targeting capability ($54.55 \pm 1.96\%$). After PTT combined chemotherapy, tumor tissues were damaged and tumor cell fragments released TAAs, showing an “autologous cancer vaccine-like” function [54]. Especially in the presence of immune adjuvant, it can promote the maturation of DCs more efficiently [55].

Although the activation could be established through multiple pathways, the immunosuppressed TME often results in suboptimal immune response. As an important component

of TME, TAMs play an important role in tumor immune regulation. As a major member of the TME, M2-phenotype macrophages promote tumor cell invasion, metastasis and suppress immune responses by secreting relevant cytokines (*e.g.*, IL-10), M1-phenotype macrophages counteract tumor growth, and promote inflammatory and immune responses by secreting relevant cytokines (*e.g.*, IL-6, IL-12 and TNF- α ,) [56-59]. In a pro-immune response pathway, M2-phenotype cancer-promoting TAMs can be repolarized to M1-phenotype cancer-suppressing TAMs under certain conditions [60]. In this study, DTX was introduced to promote the polarization of M1-phenotype to M2-phenotype TAMs. To analyze the polarization of M2-phenotype macrophages, we studied the presence of M1 (F4/80⁺ CD11b⁺ CD80⁺) and M2 (F480⁺ CD11b⁺ CD206⁺) makers on day 9 after various treatments. As shown in **Fig. 8C-F, S8A**, the expression of F4/80⁺ CD11b⁺ CD80⁺ was significantly upregulated in the groups integrated with DTX, accomplished with the downregulation of F480⁺ CD11b⁺ CD206⁺ expression, which demonstrated the potency of DTX to promote the polarization of M1-phenotype to M2-phenotype TAMs.

When the host immune status changes, the levels of cytokines *in vivo* will change correspondingly. Here, the levels of IL-6, IL-12, TNF- α and IL-10 in the eight groups were investigated by ELISA assay on day 9. As showed in **Fig. 9A-C**, the levels of these cytokines were consistent with the change of the host immune status (DC maturation and polarization of TAMs) discussed before. The groups integrated with DTX downregulated the production of IL-10 (**Fig. 9D**), further demonstrating that DTX has an excellent ability to promote the polarization of M1-phenotype to M2-phenotype TAMs.

CD8⁺ T cells, namely CTLs, are essential for the anti-cancer immune response. To evaluate the T cell response *in vivo*, the spleens of mice were collected on day 9 and T cells in the spleens were analyzed using flow cytometry. The results (**Fig. 9E, 9F**) showed that the infiltration of CD8⁺ T cells in the M@P-PDR+L group was $35.50 \pm 0.96\%$, which was significantly higher than the control group ($17.33 \pm 1.13\%$), the M@P-PDR group ($23.54 \pm 1.83\%$), the M@P-DR+L group ($25.70 \pm 1.57\%$), the M@P-P+L group ($18.01 \pm 1.77\%$), the M@P-PR+L group ($27.64 \pm 1.86\%$), the M@P-PD+L group ($22.99 \pm 2.18\%$), and the P-PDR+L group ($31.09 \pm 1.71\%$), indicating that the processes (PTT, chemotherapy, DC maturation and polarization of TAMs) mediated by M@P-PDR “Nano-targeted cells” triggered excellent anti-tumor immune responses. Consistently,

immunofluorescence images of the primary and metastatic tumors also revealed a substantial infiltration of CD8⁺ T cells (**Fig. S8B**).

3.8.3 *In Vivo* Antitumor Therapy for Primary and Metastatic Tumors

Encouraged by the satisfactory immune response, the M@P-PDR-based cocktail therapy could be a promising candidate to combat distant tumors. In the following study, we investigated whether such a strong immune response initiated by M@P-PDR was available for long-term inhibition of metastatic tumors. The therapeutic efficacy was evaluated by monitoring the growth of primary tumors and distant tumors. Compared with the primary tumors (1st) in the control group, all other treated groups exhibited certain inhibition effects on tumor growth (**Fig. 10A, 10B, S9, S10A**). In detail, no significant difference has been found between M@P-PDR group and M@P-DR + L group, whereas the limited tumor growth regression occurred as a result of the release of DTX and R837 in tumor sites. However, the photothermal effect of M@P-P greatly inhibited tumor progression, with 3.05 folds growing in comparison to primary tumor volume. Particularly, the primary tumors in the M@P-PDR + L group were remarkably inhibited, suggesting the excellent anti-tumor efficiency of such cocktail therapy that concurrently integrates PTT, chemotherapy and immunotherapy. As for the distant tumor growth in **Fig. 10A,10C, S9, S10B**, it was found that the tumors in M@P-PDR + L group were also effectively inhibited, which could be ascribed to the strong immune response resulted from R837-induced DCs maturation (immune activation) and DTX mediated polarization of TAMs (relief of immunosuppression). To compare the therapeutic effects more directly, the tumors in each group were collected at 3 d post-injection for TUNEL and HSP70 staining (**Fig. 10D**). The results showed that in the presence of both PTCAs and laser irradiation, the expression of HSP70 was higher than other groups, presenting obvious red fluorescence. The M@P-PDR + L group showed a higher expression of HSP70 than P-PDR + L, indicating the specific targeting effect of cancer cell membranes on the accumulation of nanotherapeutic agents in tumor sites. The H&E and TUNEL staining presented a similar tendency that the photothermal effects arose the massive tumor necrosis. The PCNA results of distant tumors revealed that the M@P-PDR + L group exerted extensive anti-tumor effects, with negligible tumor proliferation. In addition to the pathological examination, the survival rates of mice in each group was monitored until day 57 (**Fig. S10D**). The mice in M@P-PDR + L group survived without

obvious tumor recurrence. These results evidently confirmed that the powerful systemic immune response of M@P-PDR-based cocktail therapy effectively inhibited the growth of distant tumors, provided a new strategy for PTT/chemotherapy/immune therapy. The H&E staining of major organs (**Fig. S11**) and the negligible body weight changes (**Fig. S12**) further demonstrated the satisfactory biosafety of this synergistic therapeutic modality.

4 Conclusion

In summary, we rationally proposed a “Nano-targeted cells”-based cocktail therapy, where PTT combined with chemotherapy and immunotherapy, creating a “doomsday storm” for tumors. These as-synthesized “Nano-targeted cells” actively accumulate to the tumor sites due to the homologous targeting capability, which can be guided by PA/MR bimodal imaging. Upon laser irradiation, PTT will be triggered and TAAs will be subsequently released. The released TAAs, together with the immune adjuvant R837, drive the maturation of DCs, secreting cytokines including TNF- α , IL-6, and IL-12. Furthermore, chemotherapeutic drug DTX polarizes protumoral M2-phenotype oncogenic TAMs to tumoricidal M1-phenotype oncogenic TAMs, relieving immunosuppressive TME, accompanied by the decrease of IL-10. The above processes promote the infiltration of CTLs for treating distant metastasis. The primary tumors and metastasis are significantly inhibited. “Nano-targeted cells”-based therapeutic cocktail therapy is a promising approach to promote tumor regression and counter metastasis/ recurrence.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/>

Additional file: Fig. S1 Standard curve of PB, R837 and DTX. **Fig. S2** PA imaging intensities under full-spectrum scanning. **Fig. S3** Cell viabilities of 4T1 cells after co-incubation with of M@P-PDR and P-PDR for 12 h. **Fig. S4** *In vivo* biosafety of M@P-PDR. **Fig. S5.** Confocal microscopy images of 4T1 cells treated with P-PDR and M@P-PDR. **Fig. S6** (A) Fluorescence images of major organs and tumors in M@P-PDR and P-PDR treated groups 24 h after intravenous administration and (B) the corresponding fluorescence intensities. (C) *In vivo* PA images of tumors. (D) T1-weighted MR

images of 4T1 tumor-bearing mice. **Fig. S7** (A) Flow cytometric analysis of DCs maturation in distant tumors (2nd) and lymph nodes. (B) The corresponding quantification of DCs maturation in distant tumors (2nd) and (C) the corresponding quantification analysis of DCs maturation in lymph nodes (LNs). **Fig. S8** (A) Immunofluorescence images of CD80 and CD206 in distant tumors (2nd) on day 9. (B) Immunofluorescence images of CD8⁺ T cells in the distant tumors (2nd) on day 9 after different treatments. **Fig. S9** Images of mice during the 27-day treatment period. **Fig. S10** (A) Statistical analysis of primary tumors (1st) and (B) distant tumors (2nd) on day 27. (C) Time-dependent body weight curves of mice. (D) Survival curves of mice after different treatments. **Fig. S11.** H&E staining of the major organs of all groups collected on day 3 after different treatments. **Fig. S12.** Time-dependent body weight curves of mice.

Declarations

Consent for publication

Not applicable.

Availability of data and materials

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

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Authors' contributions

QQC and LZ conceived and carried out the experiments. TTS and HTR designed the research. QQC, MXT, WWL, SLL and LL analyzed the data. QQC, LZ, ZYX, WZ, ZGW and YC drafted and revised the manuscript. TTS, HTR and LZ supervised the research. All authors read and approved the final manuscript.

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Competing interests

The authors declare no conflicts of interest.

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Figures

Figure 1

Characterization of the M@P-PDR nanospheres. (A) Schematic illustration of the synthetic process for M@P-PDR nanospheres. (B) SEM image of M@P-PDR. (C1) TEM image of P-PDR; (C2) TEM image of M@P-PDR. (D) SDS-PAGE protein analysis results of cancer cell membrane vesicles, M@P-PDR and P-PDR. (E) DLS results of P-PDR and M@P-PDR nanospheres. (F) Zeta-potential of P-PDR and M@P-PDR nanospheres. (G) UV-Vis-NIR spectra of PB NPs, M@P-PDR and M@P-PDR suspensions.

Figure 2

(A) Infrared thermal images of M@P-PDR at a concentration of 5 mg mL⁻¹ under 808 nm laser irradiation at different power densities (0.75, 1.00, 1.25 and 1.50 W cm⁻²), and (B) the corresponding temperature-time curves of M@P-PDR at different power densities. (C) Infrared thermal images of M@P-PDR at different concentrations (0, 1, 2, 3, 4 and 5 mg mL⁻¹) under 808 nm laser (1.5 W cm⁻², 5 min) irradiation, and (D) the corresponding photothermal temperature-time curves of M@P-PDRs at different concentrations. (E) Temperature change curves of M@P-PDR over five laser irradiation on/off cycles. (F)

Linear relationship between PA intensities and M@P-PDR concentrations, and the corresponding in vitro PA images (inset). (G) T1 relaxation rate of M@P-PDR and the corresponding in vitro MR images (inset).

Figure 3

(A) CLSM images of 4T1 cells treated with M@P-PDR and P-PDR nanospheres for different times (0.5, 1, 2, 3 and 4 h), respectively (the blue indicates nucleus stained with DAPI, the red indicates M@P-PDR or P-PDR nanospheres stained with Dil), and (B) the corresponding flow cytometry quantitative analyses.

Figure 4

In vitro therapeutic effects of M@P-PDR. (A) CCK8 results after various treatments. (B) CLSM images of 4T1 cells co-stained with CAM and PI after various treatments to distinguish the live (green fluorescence) and dead (red fluorescence) cells. (C) Flow cytometry results after various treatments.

Figure 5

(A) The design scheme of the transwell system experiment. (B–C) The expression levels of CD11c⁺, CD86⁺ and CD80⁺ on the surface of DCs analyzed by flow cytometry after different treatments. (D–F) The secretion of IL-6, IL-12 and TNF-α in DC suspensions after different treatments.

Figure 6

Biodistribution and in vivo MR/PA bimodal imaging of M@P-PDR. (A) Fluorescence images of 4T1 tumor-bearing mice at different time points (pre-injection, 1, 2, 4, 6, 8 and 24 h), and (B) the corresponding fluorescence intensities of tumors. (C) In vivo PA images of tumors and (E) the corresponding signal intensities (D) T1-weighted MR images and (F) the corresponding PSIE of tumors.

Figure 7

In vivo photothermal performance of the M@P-PDR “Nano-targeted cells”. (A) Schematic illustration of the in vivo experimental design. (B) Infrared thermal images of 4T1 tumor-bearing Balb/c mice under different treatment groups. (C) Photothermal temperature-time curves of the eight groups under laser irradiation. and (D) of the corresponding temperature changes at tumor sites during irradiation.

Figure 8

Degree of in vivo DCs maturation and polarization of TAMs based on M@P-PDR “Nano-targeted cells”. (A) Flow cytometric analysis of DCs maturation in primary tumors (1st) of mice in different treatment groups. and (B) the corresponding quantification of DCs maturation. (C) Flow cytometric analysis of M2-TAMs (CD206⁺ F4/80⁺ CD11b⁺) in primary tumors (1st) and (D) the corresponding quantification of M2-TAMs. (E) Flow cytometric analysis of M1-TAMs (CD80⁺ F4/80⁺ CD11b⁺) in primary tumors (1st) and (F) the corresponding quantification of M1-TAMs.

Figure 9

In vivo immunostimulatory effects based on M@P-PDR “Nano-targeted cells”. (A-D) The secretion levels of IL-6, IL-12, TNF- α and IL-10 measured by ELISA assay. (E) Flow cytometric analysis of CD8+ T cell in the spleens of mice in different groups. (F) Quantification of CD8+ T cells.

Figure 10

Anti-tumor effects of cocktail therapy based on M@P-PDR “Nano-targeted cells”. (A) Digital photos of 4T1 tumors on both sides in vivo and ex vivo on day 27 after different treatments. (B) Growth curves of the primary tumors (1st) and (C) the distant tumors (2nd) in different groups. (D) H&E staining, TUNEL staining and HSP70 staining images of the primary tumor (1st) excised at day 3 after different treatments, and PCNA staining images of the distant tumor (2nd) excised at day 9 after different treatments.

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