COMMUNICATION

Tumor-Targeted Drug and CpG Delivery System for Phototherapy and Docetaxel-Enhanced Immunotherapy with Polarization toward M1-Type Macrophages on Triple Negative Breast Cancers

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Cancer immunotherapy has achieved promising clinical responses in recent years owing to the potential of controlling metastatic disease. However, there is a limited research to prove the superior therapeutic efficacy of immunotherapy on breast cancer compared with melanoma and non-small-cell lung cancer because of its limited expression of PD-L1, low infiltration of cytotoxic T lymphocytes (CTLs), and high level of myeloid-derived suppressor cells (MDSCs). Herein, a multifunctional nanoplatform (FA-CuS/DTX@PEI-PPIX-CpG nanocomposites, denoted as FA-CD@PP-CpG) for synergistic phototherapy (photodynamic therapy (PDT), photothermal therapy (PTT) included) and docetaxel (DTX)-enhanced immunotherapy is successfully developed. The nanocomposites exhibit excellent PDT efficacy and photothermal conversion capability under 650 and 808 nm irradiation, respectively. More significantly, FA-CD@PP-CpG with no obvious side effects can remarkably inhibit the tumor growth in vivo based on a 4T1-tumor-bearing mice model. A low dosage of loaded DTX in FA-CD@PP-CpG can promote infiltration of CTLs to improve efficacy of anti-PD-L1 antibody (aPD-L1), suppress MDSCs, and effectively polarize MDSCs toward M1 phenotype to reduce tumor burden, further to enhance the antitumor efficacy. Taken together, FA-CD@PP-CpG nanocomposites offer an efficient synergistic therapeutic modality in docetaxel-enhanced immunotherapy for clinical application of breast cancer.

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mechanisms to stimulate tumor-specific immune responses.\cite{17} Immunogenic death of tumor cells or engage immune effector effect on immune system for patients. Recent research revealed immunotherapy is to promote the infiltration of CTLs, reduce of tumor cells.\cite{19} As for photothermal therapy, PTT usually or DNA damage, subsequently causing apoptosis and necrosis ablation of tumor cells after photoirradiation.\cite{20} Although PDT (NIR) absorbance to generate heat energy and cause thermal sensitizers can transform surrounding oxygen molecules into cytotoxic reactive oxygen species (ROS) under light irradiation, especially singlet oxygen ($^{1}\text{O}_2$), leading to irreversibly protein or DNA damage, subsequently causing apoptosis and necrosis of tumor cells.\cite{39} As for photothermal therapy, PTT usually involves light absorbing agents with a strong near-infrared (NIR) absorbance to generate heat energy and cause thermal ablation of tumor cells after photoirradiation.\cite{20} Although PDT and PTT are promising tumor-therapeutics modalities, there inherent shortcomings, such as nonspecific accumulation at health tissues for nontargeted photosensitizers and hardness to decompose in living systems with potential toxicity problems severely limit their extensive applications.\cite{21} So rational design of smart nanomaterial that could combine multimodal therapy and overcome their own inherent limitations is of great interest and significance toward successful cancer treatment.

In this work, we constructed a tumor-targeted drug and CpG delivery system for near-infrared induced phototherapy and docetaxel-enhanced immunotherapy. Mesoporous CuS nanoparticles (NPs) were chosen as the drug nanocarrier and PTT agents owing to the high photothermal conversion efficiency and excellent biocompatibility.\cite{22} Then, the surface modification with tumor target ligand folic acid (FA) was favorable for improving active delivery of nanoparticles, further enhancing the transport efficiency of DTX at tumor sites through targeting effect of FA to overexpressed FA receptors on the tumor cells.\cite{24} To improve the water solubility of nanoparticles and further realize CpG delivery, PEI-PpIX (polyethyleneimine–protoporphyrin IX) conjugates (Figure S1, Supporting Information) and CpG were anchored alternatively to fabricate FA-CuS/DTX@PEI-PpIX-CpG (denoted as FA-Cd@PP-CpG) nanocomposite (Scheme 1). On the basis of the scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images, the as-synthesized CuS NPs showed monodisperse spherical shape and uniform size about 100 nm (Figure 1a,d). Next, after conjugation of FA and DTX loading, there was negligible change of the morphology compared to CuS (Figure 1b,e). The average size of FA-Cd@PP-CpG nanocomposite increased to 117 nm; furthermore, there was a thin polymer shell wrapped around the FA-CuS/DTX core, which might result from the anchor of PEI-PpIX conjugate (Figure 1c,f). The average diameter of nanoparticles could also be determined by dynamic light scattering (DLS) analysis, which showed the size increased from 102.9 ± 2.7 nm of CuS to 115.6 ± 2.3 nm of FA-Cd@PP-CpG (Figure S2, Supporting Information). Energy dispersive spectrometer (EDS) and X-ray photoelectron spectroscopy (XPS) revealed that Cu, S, O, C, N were presented in the sample, indicating the successful preparation of FA-Cd@PP-CpG nanocomposite (Figures S3a,b and S5a, Supporting Information). And the content of polymer measured by thermogravimetric analysis (TGA) was confirmed to be 20.15% (Figure S3c, Supporting Information). Also, FA-Cd@PP-CpG nanocomposite dispersed well in water, phosphate buffer saline (PBS), Dulbecco’s modified Eagle medium (DMEM), and fetal bovine serum (FBS) (inset of Figure 1f), which was verified by the hydrodynamic size and zeta-potential of FA-Cd@PP-CpG in different solutions (Figure S4a, Supporting Information). Moreover, the stability of the particles was monitored for ten days through DLS measurement. No obvious changes about the size of FA-Cd@PP-CpG were observed during the tracking period, demonstrating the good stability of the nanocomposite in water and physiological solution (Figure S4b, Supporting Information).

X-ray powder diffraction (XRD) pattern (Figure S5b, Supporting Information) demonstrated a covellite CuS phase (JCPDS No. 06-0464). The XRD data of FA-Cd@PP-CpG nanocomposite showed that the peaks intensity decreased, which may be attributed to the filling of pores with the drug and the high degree of disorder.\cite{24} The final FA-Cd@PP-CpG nanocomposite showed a decrease in potential to ~16 mV compared to the positive potential of FA-Cd@PP (+ 20.2 mV), indicating the successful anchor of CpG due to the negative charge of DNA (Figure S5c, Supporting Information). From UV–vis–NIR absorption spectra, FA-Cd@PP exhibited new absorption peak of FA at near 280 nm, as well as the characteristic peak of PEI-PpIX (Figure S6a,b, Supporting Information). The results were also validated from the change of the color before and after functionalization (inset of Figure S6b, Supporting Information). Then we studied the loading efficiency of DTX with high-performance liquid chromatography (HPLC). According to the standard curve of DTX at 227 nm (Figure S6c, Supporting Information), the drug loading efficiency was determined to be about 23%. Such high loading capacity for the hydrophobic drug suggested that CuS NPs held great potential as a promising nanocarrier for drug delivery.

Subsequently, diphenylisobenzofuran (DPBF) was utilized to evaluate the singlet oxygen production ability of FA-Cd@PP-CpG under 650 nm irradiation (Figure 1g). In the presence of FA-Cd@PP-CpG, DPBF absorption decayed gradually along with the irradiation time, although the $^{1}\text{O}_2$ generation ability was weaker than that of free PpIX and PEI-PpIX (containing equal amount of PpIX) owing to the irradiation light absorbed partly by the carrier CuS.\cite{25} The photostability of free
PpIX, PEI-PpIX, and FA-CD@PP-CpG after laser irradiation was then investigated (Figure S7a, Supporting Information). Considering the optical adsorption of CuS in the NIR region, the photothermal response of FA-CD@PP-CpG was evaluated under 808 nm irradiation. In marked contrast to PBS and PEI-PpIX with negligible temperature variation, CuS and FA-CD@PP-CpG solutions showed considerable temperature rise of 35.8 and 34.4 °C, at a concentration of 100 µg mL\(^{-1}\), respectively (Figure 1h). The temperature rise of FA-CD@PP-CpG was found to be concentration- and irradiation-time-dependent (Figure 1i,j). Besides the excellent photothermal conversion ability, FA-CD@PP-CpG also exhibited high photostability and good reproducibility even after three laser on/off cycles (Figure 1k and Figure S7b, Supporting Information). Therefore, FA-CD@PP-CpG could serve as an efficient photothermal agent for potential application in cancer PTT.

Next, the cumulative release profiles of DTX in PBS at pH 7.4 (the physiological environment with neutral pH) and pH 5.0 (the acidic condition of endosome/lysosome in tumor cell) were investigated. FA-CD@PP-CpG nanocomposite at pH 7.4 and pH 5.0 both exhibited the apparent sustained release behaviors for DTX, especially in the early stage with a relatively fast release (Figure 1l). Additionally, FA-CD@PP-CpG presented faster release at pH 5.0 compared with that at pH 7.4 condition for DTX, which might be due to the faster breakdown of amide carbonyl between PEI and PpIX in the acidic condition, confirmed by the change of Fourier transform infrared (FTIR) spectroscopy in Figure S8 (Supporting Information). Specifically, the accumulative release amount of DTX from the drug carrier reached 90.0% after being immersed at pH 5.0 condition for 48 h. So the FA-CD@PP-CpG nanocomposite possessed pH-responsive release behavior for DTX, resulting in the enhanced immunization.

A high biocompatibility and phagocytosis of nanomaterials is required as a precondition for cancer applications. Compared with CuS nanoparticles, there was no obvious difference in the viability (>85% cell viability) on 4T1 cancer cells treated with FA-CuS@PP-CpG, whereas FA-CD@PP-CpG presented enhanced cytotoxicity than FA-CuS@PP-CpG at the high concentration ranges (Figure S9, Supporting Information, and Figure 2a), which is consistent that high dosage of chemotherapeutics can kill tumor cells but have little effects.
at low concentration. And FA-CD@PP-CpG exhibited negligible toxicity toward HBL-100 normal cells within the range of concentrations tested (Figure S10, Supporting Information). Then the targeting efficiency of FA-CD@PP-CpG on 4T1 cells was evaluated by confocal laser scanning microscopy (CLSM) images. FA-CD@PP-CpG exhibited red fluorescence in the cytoplasm of 4T1 cells after incubation for 4 h, while only weak red fluorescence could be observed in A549 lung cancer cells or in 4T1 cells treated with CD@PP-CpG without conjugation of FA (Figure 2b and Figure S11, Supporting Information). Next, the cellular uptake efficiency of FA-CD@PP-CpG was assessed. Both flow cytometry results and CLSM images
revealed that the red fluorescence intensity inside the cells increased remarkably with the prolonged time (Figure 2c,d and Figure S12, Supporting information). All these results demonstrated that FA-CD@PP-CpG with excellent biocompatibility could be internalized efficiently by 4T1 cells via FA-receptor mediated endocytosis.

We then investigated the generation of ROS in 4T1 cells treated with FA-CD@PP-CpG under 650 nm laser irradiation. 4T1 cells exhibited obvious green fluorescence, and the fluorescence signals were strengthened with an increase in the concentration of FA-CD@PP-CpG (Figure 2e). Next, we studied the combination therapy with different composites. Compared to PDT alone with CpG-free nanocomposites (FA-CD@PP), or PTT alone with FA-CD@PP, the combined treatment offered the most effective cancer cell killing. Moreover, the cell survival ratio of the group treated with FA-CD@PP-CpG for PDT, PTT, and immunotherapy exhibited negligible change compared to that of FA-CD@PP, which was due to the incapability of CpG to induce the apoptosis of 4T1 cells without invoking the antigen presenting cells (Figure 2f). The antitumor efficiency in vitro was also assessed by live/dead viability (Figure 2g and Figure S13, Supporting Information). For the group treated with free CpG, no apparent dead cells could be observed because of nontoxicity of CpG. And there was significant cell death in the group subjected to dual laser irradiation compared to DTX, PDT, PTT, indicating the significant inhibitory effect of FA-CD@PP-CpG nanocomposite on tumor cells in vitro.

Afterward, photothermal imaging and fluorescence imaging in vivo was recorded to assess the feasibility of FA-CD@PP-CpG inside the tumor. All animal procedures conformed to the Guide for the Care and Use of Laboratory Animals and the ethical approval number was TJLAC-019-137. The temperature in the tumor site increased to 44°C after irradiation for 10 min, which was high enough to kill tumor cells. Comparatively, the temperature of the tumor presented a feeble increase in the PBS group (Figure 3a,b). Weak fluorescence was observed in the tumor site after 6 h intravenous injection. And the fluorescence signal enhanced continuously with an increase in the housing time, reaching a maximum at 12 h. Subsequently, the fluorescence observed in the livers and tumors, more significantly, no fluorescence observed in other tissues or organs further demonstrated FA-CD@PP-CpG highly targeted on tumors (Figure 3c–e). Importantly, few uptake of FA-CD@PP-CpG by macrophages compared with 4T1 cells suggested that the accumulation in liver was not due to the uptake by the reticuloendothelial system, but the temporary retention because of the liver’s unique anatomical and physiological structure. (Figure S14, Supporting Information).

Next, we carried out the combination therapy in vivo using FA-CD@PP-CpG on 4T1-tumor-bearing mice. Compared with the rapid increase of tumor in PBS group, there was negligible antitumor activity in the group treated with CpG and aPD-L1 as well as group injected with free DTX. Importantly, the
group treated with FA-CD@PP-CpG + aPD-L1 + PDT + PTT (abbreviated as aPD-L1 + PDT + PTT) exhibited the remarkable tumor growth inhibition effect compared to various control groups (Figure 3f–h). Survival rate of different groups further demonstrated that aPD-L1 + PDT + PTT group presented the best antitumor efficacy (Figure 3i). Hematoxylin and eosin (H&E) staining revealed that tumor tissues in the aPD-L1 + PDT + PTT group were severely damaged in comparison with that of PBS group (Figure S15, Supporting Information). Simultaneously, all mice in the seven groups displayed no significant body weights difference, indicating negligible systemic side effect of FA-CD@PP-CpG (Figure 3j), which was also confirmed by images of H&E stained major organs (heart, liver, spleen, lung, and kidney) and the corresponding blinded scores of major tissues (Figure S15 and Table S1, Supporting Information).

Figure 3. a) The in vivo thermal images of the mice after intravenous injection of PBS and FA-CD@PP-CpG under 808 nm irradiation. b) Temperature change curve of tumor sites as a function of irradiation time. c) Fluorescence images of the mice after intravenous injection of FA-CD@PP-CpG and d) ex vivo imaging of tumor and major organs after 24 h postinjection. e) Quantitative fluorescence curve of tumor tissues. f) Tumor growth curves in different groups. g) The weight and h) representative photographs of tumor tissue in different groups obtained on day 14. i) The survival rate and j) the body weight changes in different groups. k–m) Physiological function assessment of liver and kidney toxicity from healthy, control, and aPD-L1 + PDT + PTT treated mice. The error bars are based on the SD of five mice. *p < 0.05, **p < 0.01, and ***p < 0.01.
In addition, all blood biochemical values, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), albumin (ALB), blood urea nitrogen (BUN), creatinine (CREA), and uric acid (UA), were not statistically different between mice treated with aPD-L1 + PDT + PTT and healthy mice, which suggested that no hepatic dysfunctions and abnormal renal functions were caused by FA-CD@PP-CpG (Figure 3k–m and Figure S16, Supporting Information).

We then investigated the specificity of FA-CD@PP-CpG on the CTLs generation. A ratio of 4.8:1 carboxyfluorescein succinimidyl ester (CFSE) losing on FA-CD@PP-CpG versus control group showed an average > fivefold multiplication (5 generations), higher than CpG + aPD-L1 (4 generations) and DTX (3.5 generations) (Figure 4a). To further explore the differentiation of MDSCs, we studied the presence of M1 (CCR7) or M2 (MR) markers on MDSCs after different treatments. Figure 4b showed that the level of CCR7 was upregulated and correspondingly, the level of MR was downregulated significantly by aPD-L1 + PDT + PTT compared with DTX group, which revealed the polarization of MDSCs population to an M1-like phenotype via DTX transported by nanocomposite. Subsequently, the result of cytokine secretion in MDSCs supernatants showed that MDSCs treated with aPD-L1 + PDT + PTT had significant upregulation of IL-12 but downregulation of IL-10 production compared with other three groups (Figure 4c,d). Next, we acquired various macrophage cell surface markers of different groups (CD40, CD80, CD86, MHC class II, and CD11c) (Figure 4e). For aPD-L1 + PDT + PTT group, MDSCs were found to express highest levels of all above mentioned cell surface markers compared with CpG + aPD-L1 treated, DTX-treated and the control group. The results showed that DTX transported by FA-CD@PP-CpG could polarize MDSCs toward an M1-like phenotype by expressing high levels of CCR7, increasing the generation of IL-12 and further kill cancer cells effectively.

Figure 4. a) The proliferation of CD3 + CD8 + T cells assessed by CFSE dilution, with unstimulated CD3 + CD8 + T cells as the control (gray shade) after 48 h incubation (left) and quantitative fluorescence curve of T cells (right). b) Histogram representation of CCR7 and MR levels in DTX and aPD-L1 + PDT + PTT treated tumors. c,d) Cytokine levels of IL-12 (c) and IL-10 (d) in MDSC supernatant from mice. e) Flow cytometric assay of CD40, CD80, CD86, MHC class II, and CD11c expression from CpG + aPD-L1, DTX, and aPD-L1 + PDT + PTT treated tumors. p values:*p < 0.05, **p < 0.01, and ***p < 0.01.
The significant inhibition of tumors in the group treated with aPD-L1 + PDT + PTT may contribute to the increased infiltration of CTLs in tumor sites. Flow cytometry result revealed that group treated with CpG + aPD-L1 or DTX had an improved level on T-cell infiltration compared with PBS group with limited T-cell infiltration. Comparatively, tumors treated with aPD-L1 + PDT + PTT were remarkably infiltrated by CTLs (Figure 5a,b). Additionally, for aPD-L1 + PDT + PTT group, tumor presented a highest level of TNF-α, IL-12 and IFN-γ because of the synergetic effect of CpG and DTX compared to other control groups (Figure 5c–e). The same outcomes of CTLs infiltration in tumor site were observed through immunofluorescence (Figure 5f and Figure S17, Supporting Information). Tumors from aPD-L1 + PDT + PTT treated mice were remarkably infiltrated by both CD4+ and CD8+ T cells, and the percentage of CD8+ T cells was threefold of that in the CpG + aPD-L1 group, twofold of that in the DTX group and 1.5-fold of that in the other groups (Figure 5g).

In summary, we report herein the tumor-targeted DTX and CpG delivery system as a smart platform for cancer PDT, PTT, and docetaxel-enhanced immunotherapy in 4T1 breast cancer cells bearing mice. FA-CD@PP-CpG nanocomposite presented negligible toxicity to normal tissues, but could cause remarkable damage to tumors in vivo when combined with aPD-L1 and dual laser irradiation (650 and 808 nm). Combination of FA-CD@PP-CpG with aPD-L1 enhanced the infiltration of CTLs, which was favorable of aPD-L1 to block the PD-1/PD-L1 checkpoint-blockade and further prevent CTLs from dysfunction and exhaustion, thus kill cancer cells efficiently. Furthermore, DTX transported by FA-CD@PP-CpG to tumor cells could directly induce an phenotype polarization of MDSCs contributes to more generation of proinflammatory cytokines, thus resulting in enhanced immunotherapy efficacy. Taken together, our study presented a novel strategy for highly superior antitumor efficiency by combining PDT, PTT, and docetaxel-enhanced immunotherapy, which might open new avenues in breast cancer immunotherapy for clinical translation.

Supporting Information
Supporting Information is available from the Wiley Online Library or from the author.
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Conflict of Interest

The authors declare no conflict of interest.

Keywords

CpG, docetaxel, immunotherapy, PD-L1/anti PD-L1, phototherapy

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