A \text{Cu}_9\text{S}_5\text{ nanoparticle-based CpG delivery system for synergistic photothermal-, photodynamic- and immunotherapy}

Lulu Zhou$^{1,2}$, Lv Chen$^{1,2}$, Xiaochun Hu$^1$, Yonglin Lu$^1$, Wenjie Liu$^1$, Yanting Sun$^1$, Tianming Yao$^1$, Chunyan Dong$^{1,2}$ & Shuo Shi$^1$

Despite its great potential in cancer therapy, phototherapy, including photothermal therapy (PTT) and photodynamic therapy (PDT), often cause metastasis of tumors. Immunotherapy has revolutionized the cancer treatment owing to the capability of activating immune system to eliminate tumors. However, the integration of phototherapy and immunotherapy in a single nanoagent for cancer therapy is still a challenging task. Here, we fabricated (\text{Cu}_9\text{S}_5@\text{mSiO}_2-\text{PpIX@MnO}_2@\text{CpG} (\text{CSPM@CpG})) as a synergistic therapeutic model for phototherapy enhanced immunotherapy. The intracellular uptake of cytosine-phosphate-guanine (CpG) promoted the infiltration of cytotoxic T lymphocytes (CTLs) in tumor tissue, further stimulating the production of interferon gamma (IFN-$\gamma$) and remarkably elevating the immune response level. Excellent anti-tumor effects have been achieved by synergistic PTT/PDT/immunotherapy. The metastasis of tumors was effectively inhibited by the immune response of CpG. Thus, our proposed work provides a strategy to combine phototherapy with immunotherapy to enhance the therapeutic efficiency and further inhibit metastasis of tumors.

$^{1}$Shanghai Key Laboratory of Chemical Assessment and Sustainability, School of Chemical Science and Engineering, Breast Cancer Center, Shanghai East Hospital, Tongji University, 200092 Shanghai, P. R. China. $^{2}$Theses authors contributed equally: Lulu Zhou, Lv Chen $^{✉}$email: cy_dong@tongji.edu.cn; shishuo@tongji.edu.cn
The traditional and main strategies for cancer treatment, such as surgical resection, chemotherapy, and radiotherapy, always accompany with systemic side effects including high metastasis rate or toxicity to the immune system and normal tissues, so it is considerably urgent to develop accurate and efficient tumor-therapeutic modalities. As a novel and minimally invasive alternative to traditional therapeutic methodology, phototherapy, including photothermal therapy (PTT) and photodynamic therapy (PDT), has attracted widespread interest owing to its efficient cancer destruction ability, fewer side effects and minimal invasiveness.

The process of PTT involves that photothermal agents exposed to near infrared (NIR) light can efficiently convert the optical energy into thermal energy and ablate target tumors by hyperthermia. Recently, copper sulfide compounds have been receiving a great deal of interest due to their strong NIR absorption derived from p-type carriers in vacancy-doped nanocrystals. Low cost, easy synthesis, and high thermal conversion efficiency. In parallel, the photosensitizer during PDT can convert surrounding oxygen molecules into cytotoxic reactive oxygen species (ROS), such as singlet oxygen ($^{1}O_{2}$), water (H$_2$O), and superoxide (O$_2^{-}$). The rational design and preparation of CSPM@CpG are illustrated in Fig. 1a. First of all, the core of Cu$_9$S$_5$ nanocrystals were synthesized by a thermal decomposition. Then these nanocrystals were transferred into an aqueous phase using cetyltrimethylammonium bromide (CTAB). Subsequently, the synthesized Cu$_9$S$_5$-CTAB was coated with a mesoporous silica (msSiO$_2$) shell through the reduction of KMnO$_4$ on the silica layer. This hybrid silica layer was used for tumor microenvironment-responsive generation of O$_2$ to relieve tumor hypoxia, thereby achieving an enhanced photodynamic therapy. Lastly, Cu$_9$S$_5$ was successfully decorated on the surface of Cu$_9$S$_5$@msSiO$_2$@PPIX@MnO$_2$ (CSPM) to obtain CSPM@CpG through electrostatic interaction for later immunotherapy.

Transmission electron microscopy (TEM) image shows that Cu$_9$S$_5$ nanocrystals possess a uniform morphology with a mean size of ~20 nm (Fig. 1b). And the high crystallinity of Cu$_9$S$_5$ is evidenced by high-resolution TEM (HRTEM) image (Fig. 1c). It can be clearly observed that a silica layer on CSP owing to the different electron penetrability between Cu$_9$S$_5$ and SiO$_2$ shell (Fig. 1d). TEM image also indicates CSP nanoparticles were well-dispersed with average diameter of 47.6 nm. Further, the reduction of KMnO$_4$ and the adsorption of CpG on the surface of CSP yields CSPM@CpG nanoparticles with an unaltered core morphology.

Results

Fabrication and characteristics of CSPM@CpG nanocomposite. The rational design and preparation of CSPM@CpG are illustrated in Fig. 1a. First of all, the core of Cu$_9$S$_5$ nanocrystals were synthesized by a thermal decomposition. Then these nanocrystals were transferred into an aqueous phase using cetyltrimethylammonium bromide (CTAB). Subsequently, the synthesized Cu$_9$S$_5$-CTAB was coated with a mesoporous silica (msSiO$_2$) shell through the reduction of KMnO$_4$ on the silica layer. This hybrid silica layer was used for tumor microenvironment-responsive generation of O$_2$ to relieve tumor hypoxia, thereby achieving an enhanced photodynamic therapy. Lastly, Cu$_9$S$_5$ was successfully decorated on the surface of Cu$_9$S$_5$@msSiO$_2$@PPIX@MnO$_2$ (CSPM) to obtain CSPM@CpG through electrostatic interaction for later immunotherapy.

Transmission electron microscopy (TEM) image shows that Cu$_9$S$_5$ nanocrystals possess a uniform morphology with a mean size of ~20 nm (Fig. 1b). And the high crystallinity of Cu$_9$S$_5$ is evidenced by high-resolution TEM (HRTEM) image (Fig. 1c). It can be clearly observed that a silica layer on CSP owing to the different electron penetrability between Cu$_9$S$_5$ and SiO$_2$ shell (Fig. 1d). TEM image also indicates CSP nanoparticles were well-dispersed with average diameter of 47.6 nm. Further, the reduction of KMnO$_4$ and the adsorption of CpG on the surface of CSP yields CSPM@CpG nanoparticles with an unaltered core morphology.

Electron diffraction spectroscopy (EDS) spectrum indicates that CSPM@CpG consisted of the elements of Cu, S, Si, and Mn (Supplementary Fig. 1b). In addition, elemental mapping images showed that the elements mentioned above are simultaneously distributed in CSPM@CpG NPs, further confirming the successful formation of the as-aspected product (Fig. 1e). Moreover, X-ray photoelectron spectroscopy (XPS) was then measured to verify above results and determine the valence of copper and manganese. The survey spectrum demonstrated that the sample is composed of Cu, S, Si, and Mn atoms (Fig. 1f), which is consistent with Supplementary Fig. 1b. In particular, Cu L$_2$ peaks include Cu L$_2$ at 932.2 eV with the satellite peak at 941.4 eV and the Cu L$_2$ at 952.7 eV with the satellite peak at 960.5 eV, which are in accordance with the Cu L$_2$ orbital of Cu II. The high-resolution XPS showed that Mn 2p peaks are centered at 651.2 and 640.2 eV, assigned to Mn 2p$_{3/2}$ and Mn 2p$_{1/2}$.
peak, respectively (Fig. 1g), and this reveals that the main oxidation state of Mn is $+4$. The size distributions of Cu$_9$S$_5$, CSP, and CSPM@CpG were obtained by dynamic light scattering (DLS) (Supplementary Fig. 2). To test the stability of CSPM@CpG, the nanocomposite was dispersed in PBS buffer solutions at different pH values at 37 °C or 43 °C within 12 h. And there were negligible changes about the sizes and zeta-potential of CSPM@CpG in different solutions (Supplementary Fig. 3). Additionally, no obvious changes about the CpG concentration and zeta-potential of CSPM@CpG before and after laser irradiation were observed (Supplementary Table 1). The high stability of CSPM@CpG suggests their potential for in vitro and in vivo therapeutic applications.

X-ray powder diffraction (XRD) pattern of Cu$_9$S$_5$ core nanoparticles is consistent with that of standard Cu$_9$S$_5$ phase (JCPDS card No: 47-1748), with several well-defined characteristic peaks such as (0, 0, 15), (1, 0, 10), and (0, 1, 20) (Supplementary Fig. 4). And all the peak positions of as-prepared CSP and CSPM@CpG can be directly fitted with the standard of Cu$_9$S$_5$ (Fig. 1h). In comparison with CSP, no diffraction peaks of MnO$_2$ and meanwhile the weaken peak intensity were observed in the XRD pattern of MnO$_2$ shell-coated CSPM, implying the amorphous nature of MnO$_2$ layer. The N$_2$ adsorption/desorption isotherms (inset is the corresponding pre-size distribution) of CSPM@CpG (Fig. 1i). CSPM has a similar FT-IR spectrum to CSP because of the similar functional groups...
Temperature change of CSPM@CpG under different concentrations. CSPM@CpG.

The detection of CSPM@CpG nanocomposite (100 μg mL⁻¹) was faster under acidic conditions (pH 6.5) compared with that at pH 7.4 (Fig. 2c). Notably, the CpG release was slightly under the physiological condition (pH 7.4) at 37 °C or 43 °C (Fig. 2a and Supplementary Fig. 5a). The obtained CSPM@CpG nanocomposite, a solution containing 100 μg mL⁻¹ of DCFH under 650 nm laser irradiation at 1 W cm⁻² for 5 min (monitored once per minute).

Photothermal, photodynamic, and catalase-like performance with CSPM@CpG. The strong adsorption in the NIR region of CSP (Supplementary Fig. 6a) endows CSPM@CpG nanocomposite with a photothermal property. It was noteworthy that CSPM@CpG exhibited more excellent photothermal effect in comparison with CSP under the same conditions and the temperature of CSPM@CpG was 15 °C higher than that of CSP (100 μg mL⁻¹) after 5 min of 808 nm NIR irradiation (Fig. 2b). From the infrared thermal images (inset of Fig. 2b), it could be also clearly observed that CSPM@CpG showed higher temperature change than CSP. We speculated that surface plasmon coupling effect generated by CSPM@CpG nanocomposite highly improved local electromagnetic field, thus extending beyond the resonant plasmon excitation energy and resulting in an elevated heat-generating ability. Under the irradiation of 808 nm laser (2 W cm⁻²), the temperature change showed a concentration and irradiation time-dependent pattern, which meant the temperature increase accordingly with the increase of concentration and irradiation time (Fig. 2c and Supplementary Fig. 6b). After 5 min of irradiation, the temperature of all CSPM@CpG solutions increased obviously as high as ~80 °C, indicating the great potential of CSPM@CpG to be a photothermal agent (Fig. 2d).

Particularly, at a concentration of 100 μg mL⁻¹ of 808 nm irradiation (laser on) and a natural cooling process without NIR laser irradiation for 5 min (laser off) were applied. As expected, the repeated heating/cooling cycles had no significant impact on the temperature change (Fig. 2e), which demonstrated the great photostability and reproducibility of CSPM@CpG nanocomposite. These results successfully indicated that as obtained CSPM@CpG nanocomposite could serve as a good photothermal agent.

To further investigate the photodynamic effect of CSPM@CpG nanocomposite, a solution containing 100 μg mL⁻¹
nanocomposite and 2 mM 2′,7′-Dichlorofluorescin (DCFH) was exposed to NIR irradiation for 5 min (650 nm, 1 W cm\(^{-2}\)). DCFH was applied as a fluorescent probe to evaluate the production of ROS\(^{-1}\). In the presence of CSPM@CpG, the fluorescence of DCF increased obviously along with the increase of time, indicating the superior 1O\(_2\) generation ability of nanocomposite (Fig. 2f). Additionally, to identify whether the heat during PTT would affect the generation of 1O\(_2\), the 1O\(_2\) production of CSPM@CpG under 650 nm, 808 nm, and 650 nm + 808 nm laser irradiation was studied (Supplementary Fig. 6c). The fluorescence intensity after dual laser irradiation was enhanced dramatically compared with that after single laser irradiation, which was due to the combined effect originating from O\(_2\)− or HO\(_2\) generated by Cu,S\(^{-}\) and 1O\(_2\) produced by PpIX. So CSPM@CpG nanocomposite can serve as an efficient photosensitizer for PDT application in cancer treatment.

It is well known that there are excessive amounts of H\(_2\)O\(_2\) produced by malignant cancer cells. As hydrogen peroxide can be decomposed by MnO\(_2\) into oxygen, and then the catalase capacity of CSPM@CpG NPs was estimated by evaluating the consumption of H\(_2\)O\(_2\) (10 mM) through the decomposition. The obvious decrease of H\(_2\)O\(_2\) concentration in the sample containing CSPM@CpG NPs (100 μg mL\(^{-1}\)) under physiological pH condition (pH 7.4) was recorded by UV-Vis spectra (Fig. 2g, b). By contrast, there were negligible changes of the control group (only containing H\(_2\)O\(_2\)). In addition, as the catalytic activity of catalysts was affected by pH, the catalase-like capacity of CSPM@CpG was further measured at pH 6.5, which was the characteristic pH of the tumor microenvironment owing to the exuberant metabolism of cancer cells. As expected, CSPM@CpG also showed high catalytic activity at pH 6.5 (Supplementary Fig. 6d, e). All of these results indicated that CSPM@CpG possessed a catalase-like property at both pH 7.4 and pH 6.5 for inducing the decomposition of H\(_2\)O\(_2\) to produce O\(_2\).

**Cellular experiments.** Cellular experiments were further conducted to determine the effectiveness of our nanocomposite to serve as agents for PTT/PDT/immunotherapy. First, to make sure CSPM@CpG possess high biocompatibility and high phagocytosis for the further treatment, cell viability and cellular uptake efficiency of CSPM@CpG were evaluated on 4T1 cells. It was found that CSPM@CpG showed no obviously adverse effect on cells even at high concentrations up to 1000 μg mL\(^{-1}\), which reflected CSPM@CpG possessed an excellent biocompatibility for biological application (Fig. 3a). Such high biosafety was owing to the low toxicity of the precursor of the composites\(^{42}\). Then the cellular uptake efficiency of CSPM@CpG was assessed by confocal laser scanning microscope (CLSM) and Cu\(^{2+}\) content per well after incubation with cells for 4 h through ICP measurement. The results revealed that with prolonging of incubation time, an enhanced red fluorescence accumulated in the cells was observed (Fig. 3b and Supplementary Fig. 7). Meanwhile, with the increase of concentration, the more Cu\(^{2+}\) content taken by 4T1 cells was observed (Supplementary Fig. 8). It was indicated that CSPM@CpG was effectively internalized in 4T1 cells.

To evaluate the PDT efficacy of MnO\(_2\)-coated CSP nanoparticles, 4T1 cells were incubated with CSP and CSPM@CpG with or without exogenous 100 μM H\(_2\)O\(_2\), then irradiated by 650 nm laser for 30 min (1 W cm\(^{-2}\)) in either O\(_2\) or N\(_2\) environment. After incubation for another 24 h with fresh cell medium, the cell viability was used to determine the PDT efficacy (Fig. 3c). Although the PDT-induced cell killing under the nitrogen atmosphere was found to be much less effective, the PDT efficacy of CSPM@CpG was dramatically intensified in the case of exogenous H\(_2\)O\(_2\) adding but not for CSP NPs without MnO\(_2\) coating. Subsequently, the level of intracellular ROS induced by CSPM@CpG under irradiation was also investigated by CLSM using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as fluorescent probe. In comparison to CSP, an enhanced fluorescence of CSPM@CpG under hypoxic environment with the exogenous H\(_2\)O\(_2\) was observed (Fig. 3d), likely due to the additional supply of oxygen by MnO\(_2\)-triggered decomposition of H\(_2\)O\(_2\). Additionally, much stronger fluorescence was observed with the increasing concentration of CSPM@CpG in O\(_2\) atmosphere (Fig. 4a), which could be ascribed to the reaction of DCFH-DA with the larger amounts of ROS generated. All of the above results demonstrated that CSPM@CpG would offer a chance for enhanced-PDT to attain a more effective anti-tumor therapy.

The in vitro anti-tumor efficiency was determined by the viability of 4T1 cells incubated with different composites under light irradiation (Fig. 4b). Under 650 nm laser (1 W cm\(^{-2}\)) and 808 nm laser (2 W cm\(^{-2}\)) simultaneous irradiation for 5 min, the cell viability treated with CSPM NPs was significantly decreased, showing a dose-dependent manner. When the concentration of CSPM NPs reached 200 μg mL\(^{-1}\), the cell viability declined to 15.3%. It was also found that the combined treatment induced a dramatically higher cytotoxicity compared to those after treatment by either PDT or PTT alone. Take the concentration of 100 μg mL\(^{-1}\) as an example, the viability of 4T1 cells treated with CSPM nanocomposite for PDT, PTT, and combined PDT and PTT was 77.6, 59.2, and 41.6%, respectively. And the groups treated with PDT, PTT, and immunotherapy showed negligible change in cell viability (41.60% at 100 μg mL\(^{-1}\)) compared to those treated with combined PDT and PTT therapy (41.62% at 100 μg mL\(^{-1}\)). The results could be ascribed to the incapability of CGP to induce the apoptosis of 4T1 cells without invoking the antigen presenting cells in vitro\(^{41}\). The combination index (CI) of PTT-PDT-immunotherapy was calculated to be 0.9, according to the Chou-Talalay method\(^{49}\). The anti-tumor efficiency of CSPM@CpG in vitro was also evaluated by cell apoptosis assay with Annexin V-FITC Apoptosis Detection Kit (Supplementary Fig. 9). The involvement of dual staining, green emission from Annexin V-FITC bound to phosphatidyl serine and red emission from DNA binding PI dye to identify the cellular apoptotic (early or late) or necrotic pathways. During the early stage of apoptosis, cells bind to Annexin V-FITC and change their phospholipid asymmetry to show green fluorescence while necrotic cells bind only with PI. In late apoptosis, the cells lose their membrane integrity and display dual positive emission. Under combined 650 nm and 808 nm irradiation for 5 min, the ratio of dead cells and live cells for CSPM@CpG is 28.8%, much higher than 0% for single 650 nm irradiation (22.3% apoptotic cells) and 22.6% for 808 nm irradiation alone. These results were consistent with the cytotoxicity results. In addition, as observed in Supplementary Fig. 9, the apoptotic cells at an early stage with PDT treatment could be transformed to dead cells with the treatment of PTT, which demonstrate that PTT has an excellent anti-cancer effect.

Next, the in vitro immune-stimulatory activity of CSPM@CpG was evaluated. CpG, CSP, CSPM, and CSPM@CpG were incubated with DC 2.4 cells, respectively. CpG could be taken by DC cells (infiltration of phagocytosis by this population was shown in Supplementary Fig. 10). The secretion of cytokines, IL-12, as an indicator of immune response, was the most important stimulator of CTL and was measured by ELISA. For CSP and CSPM group, the production of IL-12 could be enhanced to some extent, but compared with free CpG alone, the production capacity was lower, which shows the negligible effect to induce the IL-12 release (Fig. 4c). However, the secreted IL-12 levels stimulated by CSPM@CpG were significantly higher than that of CSP, CSPM, or CpG alone. The enhanced intracellular uptake and low nuclease degradation of CpG in this delivery system
Fig. 3 In vitro experiments with CSPM@CpG. a The viabilities of 4T1 cells incubated with different concentrations CSPM@CpG (from 10 to 1000 μg mL⁻¹) for 24 h. b CLSM images of 4T1 cells incubated with CSPM@CpG for different time periods. c In vitro PDT treatment of 4T1 cells by CSP or CSPM@CpG with or without addition of H₂O₂ under 650 nm light irradiation in O₂ or N₂ atmospheres. d Detection of intracellular ROS in 4T1 cells incubated with CSP and CSPM@CpG upon the addition of H₂O₂ under 650 nm irradiation in N₂ atmospheres. Scale bar: 100 μm. Data are presented as means ± standard deviation (s.d.; n = 3). p values **<0.01, ***<0.001.

Fig. 4 In vitro experiments with CSPM@CpG. a Detection of intracellular ROS in 4T1 cells of PBS group and different concentrations of CSPM@CpG groups under 650 nm laser irradiation at 1 W cm⁻² for 5 min. Scale bar: 100 μm. b Cell viability of 4T1 cells incubated with different concentrations of CSPM@CpG NPs under PDT treatment (650 nm, 1 W cm⁻², 5 min), PTT treatment (808 nm, 2 W cm⁻², 5 min), and combined PDT treatment and PTT treatment. c The secretion of IL-12 from DC 2.4 cells stimulated with different treatments. The concentration of CpG was consistent with that loaded in the CSPM@CpG. Data are presented as means ± standard deviation (s.d.; n = 3). p values *<0.05, **<0.01, ***<0.001, ****<0.0001.
In vivo biodistribution, photothermal imaging, and combined PTT-PDT-immune treatment with CSPM@CpG. Prior to studying anti-tumor efficiency, quantitative biodistribution of 4T1-bearing mice at four different time points (2 h, 6 h, 12 h, and 24 h) after intravenously injected CSPM@CpG was analyzed by determining the Cu content in the major organs (heart, liver, spleen, lung, and kidneys) and tumors (Supplementary Fig. 12). The content of Cu was found to be 7.07% of the injected dose per gram tissue (% ID/g) in the tumor at 2 h, and reached the maximum level at 12 h post-injection (24.29% ID/g), then began to decline, reaching (17.45% ID/g) at 24 h post-injection. Importantly, few uptake of CSPM@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 caused by the liver’s unique anatomical and physiological structure (Supplementary Fig. 13). Next, the photothermal effect of CSPM@CpG in vivo was investigated. Briefly, tumor-bearing mice were exposed to 808 nm laser irradiation for 10 min at 12 h post-intravenous injection of CSPM@CpG and the temperature was recorded at selected time points by infrared thermal imaging camera (Fig. 5a). The local temperature of tumor sites in CSPM@CpG group at 200 μg mL−1 increased rapidly to higher than 58.6 °C, which was high enough to ablate tumor cells in PTT (Fig. 5b). By contrast, the tumor temperature of the PBS group showed a feeble increase under the same experimental condition. All these data demonstrated the excellent photothermal conversion of CSPM@CpG nanocomposites in vivo.

We further assessed the tumor-inhibiting efficiency of CSPM@CpG in the tumor-bearing mice was further tested. We could observe that the tumor treated with PBS showed a rapid growth, while tumor growth was remarkably inhibited in the CSPM@CpG + PDT + PTT (abbreviated as PDT + PTT) group (Fig. 5c). For the mice treated with PDT or PTT, tumor growth exhibited some inhibition. The group treated with CpG and aPD-L1 had some inhibition for tumor growth after 14 days treatment, indicating that immunotherapy alone was weak for large solid tumors. By contrast, the tumor of CSPM@CpG + aPD-L1 + PDT + PTT (abbreviated as aPD-L1 + PDT + PTT) group was smaller than that of PDT + PTT group and CpG + aPD-L1 group, suggesting the synergistic therapeutic efficiency of CSPM@CpG nanocomposite. The tumor weight and the real images of tumor after different treatments on day 14, also demonstrated a remarkable inhibition of tumors treated with aPD-L1 + PDT + PTT group compared with PBS group (Fig. 5d, e). Simultaneously, mice body weights during the treatment period were also recorded to evaluate the potential long-term toxicity in vivo. The mice weights of all groups remained normal without any obvious decrease (Fig. 5f). In addition, the toxicity was further assessed by histological tissue imaging in major tissues (heart, liver, spleen, lung, and kidney) after various treatment through hematoxylin and eosin (H&E) staining. There were no appreciable histopathological abnormalities on mice from the combination therapy group, indicating that CSPM@CpG nanocomposite induced no apparent toxicity to mice (Supplementary Fig. 14). We further photographed the lung of the mice (Fig. 5g). The lungs became atrophic obviously and there were pulmonary metastasis of cancer cells occurred heavily in the PBS-treated mice. Additionally, no pulmonary metastasis was observed from the CpG + aPD-L1 group and aPD-L1 + PDT + PTT compared with other groups because of the activation of immune system by immunotherapy.

Immunological responses after the combined PTT-PDT-immune treatment. The effective inhibition of tumors in aPD-L1 + PDT + PTT group was not only the effect of PDT and PTT, but also the immunotherapy (Fig. 6a). To explore the infiltration of CTLs in tumor site, tumors harvested on day 14 with various treatments were analyzed by the flow cytometry and immunofluorescence. The results revealed that for PBS group, tumors had limited T cells infiltration. Comparatively, tumors treated with aPD-L1 + PDT + PTT were remarkably infiltrated by CTLs (CD8 + Granzyme B + cells were defined as CTLs) (Fig. 6b). The percentage of CTLs was ~40-fold of that in the PBS group and 2-fold or 1.2-fold compared with that in the group treated with CpG + aPD-L1 and PDT + PTT (Fig. 6c), respectively, owing to the capacity of aPD-L1 to block the PD-1/PD-L1 pathway and further prevent CTls from dysfunction and exhaustion. To further investigate the activation of CTls, secretion of cytokines in serum were detected through ELISA (Fig. 6d–f). For aPD-L1 + PDT + PTT group, tumor presented the highest level of IL-12, TNF-α, and IFN-γ, owing to the capability of IL-12 to induce the secretion of IFN-γ. The same outcomes of infiltration of CTls in tumor site were observed through immunofluorescence (Fig. 6g and Supplementary Fig. 15). Tumors from aPD-L1 + PDT + PTT treated mice were considerably infiltrated by both CD8+ and Granz B + T cells in comparison with limited infiltration of PBS group.

Discussion

As depicted in Fig. 7, PTT, PDT, and immunotherapy were well combined in our developed CSPM@CpG nanocomposite for their complementary advantages to achieve a synergistic treatment effect. The nanocomposite with efficient anti-tumor efficiency could effectively release lots of heat and ROS under laser irradiation, causing apoptosis and necrosis of tumor cells. Meanwhile, CSPM@CpG nanocomposite could ameliorate the hypoxic condition in the tumor site via inducing the decomposition of H2O2 to further promote photodynamic efficiency. More significantly, treatment with CSPM@CpG combined with aPD-L1 led to the activation of dendritic cells followed by secreted of cellular cytokines and infiltration of CTls. Besides eliminating primary tumor cells with the synergic effect of PTT, PDT, and immunotherapy, the enhanced immunotherapy with CSPM@CpG could inhibit the metastasis of tumor through the long-term immunological response of CpG.

In summary, we have successfully developed a multifunctional nanocomposite to load CpG based on Cu9S5 nanoparticles (CSPM@CpG) for curing solid tumors and inhibiting metastasis. The obtained nanocomposite exhibited excellent NIR activated therapeutic performance both in vitro and in vivo. Effective tumor growth suppression has been achieved from combination of CSPM@CpG-based PTT-PDT with aPD-L1 group under the light irradiation. Subsequent CTls infiltration experiments demonstrate that the nanocomposite could also increase CpG uptake and enhance more production of immune cytokines, which effectively inhibited the metastasis of tumors. With excellent biocompatibility, our Cu9S5-based theranostic nanocomposite could be a promising candidate for combination phototherapy with immunotherapy in clinical applications.
Methods

Materials. Sodium diethyldithiocarbamate (SDEDTC), cetyltrimethylammonium bromide (CTAB), tetraethylorthosilicate (TEOS), and (3-aminopropyl) triethoxysilane (APTES) are analytically pure and were purchased from Aladdin Chemistry Co. Ltd., Shanghai, China. Anhydrous cupric chloride (CuCl₂), protoporphyrin IX (PpIX), and poly(allylamine hydrochloride) (PAH) were obtained from Meryer Chemical Technology Co. Ltd, Shanghai, China. All of the other reagents were obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). CpG (1826) oligodeoxynucleotides (5′-TCCATGACGTTCCTGACGTT-3′) were synthesized by Sangon Biotechnology Co., Ltd., Shanghai, China. All reagents were used without further purification. Ultrapure water was used throughout all experiments and to prepare all solutions. Cell counting kit-8 (CCK-8) was purchased from Zoman Biotechnology Co, Ltd (Beijing, China). High glucose Dulbecco’s modified Eagle’s medium (DMEM), 1% penicillin–streptomycin (PS), 0.25% trypsin–EDTA, phosphate and phosphate-buffered saline (PBS) were obtained from Hyclone. Fetal bovine serum (FBS) was purchased from Gibco Invitrogen.

Synthesis of copper diethyldithiocarbamate precursor. First, copper precursor solution (Cu(DEDTC)₂) was prepared by reacting CuCl₂ with SDEDTC as follows: a solution of SDEDTC in distilled water was added dropwise into another solution of CuCl₂ and distilled water under magnetic stirring at room temperature, forming a dark brown turbid solution, and then kept for 4 h of continuously stirring. Lastly, the dark brown product was obtained by filtration and dried under vacuum before use.

Synthesis of oleylamine-capped Cu₉S₅ nanocrystals. Cu₉S₅ nanocrystals were synthesized by a thermal decomposition method according to a previously reported procedure. In total, 15 mL of oleylamine in a three-necked flask (100 mL) was degassed at 80 °C in order to remove any water and oxygen and subsequently was slowly heated to 300 °C and kept at a constant temperature for 20 min under argon atmosphere. Then, another 5 mL of oleylamine containing 1 mmol of Cu(DEDTC)₂ was rapidly injected into the above hot oleylamine, forming dark green solution immediately. After maintaining the temperature for 10 min, the solution was cooled to room temperature naturally. The resultant mixture was centrifugally separated with a centrifugal force of 12,000 rpm for 10 min and then was washed with hexane and ethanol twice. Finally, Cu₉S₅ nanocrystals were dispersed in 10 mL of chloroform for later use.

Synthesis of CTAB modification of Cu₉S₅ nanocrystals. Briefly, the above 10 mL of chloroform solution containing the as-prepared Cu₉S₅ nanocrystals was added dropwise into 80 mL, 25 mg mL⁻¹ of CTAB aqueous solution under stirring at 40 °C, forming a brown oil-in-water microemulsion. Subsequently, the microemulsion was stirred vigorously at 40 °C for 48 h, and then chloroform was completely evaporated from the mixture under rotary evaporation. Finally, the as-formed solution was diluted to 100 mL with distilled water and kept in the oven at 36 °C for further uses.

Synthesis of Cu₉S₅@mSiO₂–PpIX (CSP). To synthesize CSP, PpIX was first modified with APTES by adding 6 mg of 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC•HCl), 4 mg of N-hydroxsuccinimide (NHS), and...
12 μL of APTES into 2.0 mg of PpIX predisolved in 500 μL of dimethylsulfoxide (DMSO). Subsequently, the mixture was stirred at room temperature for 2 h at 800 rpm, then silica shell was coated to form CS nanospheres. In a typical procedure, 3 mL of ethanol was added to 50 mL of the above Cu9S5/CTAB solution, followed by continuous stirring for 1 h at 60 °C. Then 100 μL of NaOH (30 mg mL⁻¹), subsequently, 100 μL of TEOS was immediately dropped into the solution, the reaction system stirring for another 1 h, obtaining a solution containing Cu9S5@mSiO2 core–shell nanocomposites. For coating PpIX conjugated mesoporous silica onto Cu9S5, 200 μL of PpIX modified with APTES was added to as-formed solution upon another 3 h of stirring, leading to a solution of PpIX modified Cu9S5@mSiO2 core–shell nanocomposites. The loading efficiency (LE) and the encapsulating efficiency (EE) of PpIX were calculated according to the following formula:

\[
\text{Loading efficiency (\%)} = \frac{\text{Weight of loaded PpIX}}{\text{Weight of CSP NPs}} \times 100\% \\
\text{Encapsulating efficiency (\%)} = \frac{\text{Weight of loaded PpIX}}{\text{Weight of devoted PpIX}} \times 100\%
\]

After that, the mixture was centrifuged and washed with ethanol to remove the impurities. Finally, the obtained product was redispersed in 60 mL acetone and refluxed at 80 °C for 48 h to remove the template CTAB according to the method reported by Zhao et al.41.

**Synthesis of Cu9S5@mSiO2-PpIX@MnO2 (CSPM).** In total, 5 mL of KMnO4 solution (50 μg mL⁻¹) was added into 2 mL of CSP suspension (1 mg mL⁻¹) under ultrasonication drop by drop. After 5 min, CSPM was obtained by centrifugation at 10,000 rpm and washed with water for three times.

**Synthesis of Cu9S5@mSiO2-PpIX@MnO2@CpG (CSPM@CpG) nanocomposite.** In all, 5 mL, 4 mg mL⁻¹ of CSPM solution was added to 10 mL, 5 mg mL⁻¹ of PAH solution under ultrasonication and stirred continuously for 2 h. The resultant solution was centrifuged and washed with water for three times. After that, the solution was added into CpG solution under magnetic stirring at ice-bath for 2 h. The solution was centrifuged, washed with water for three times and then freeze-dried in vacuum for further experiments.

**Characterizations.** Powder X-ray diffraction (XRD) patterns were conducted by a D8 advance (Bruker, Germany). The sizes and morphologies of samples were determined by a transmission electron microscope (TEM) at an accelerating voltage of 200 KV (JEM-2100, JEOL, Japan). The specific surface area and pore

**Fig. 6 Immunological responses after combined PTT-PDT-immunotherapy with CSPM@CpG.** a Schematic illustration of CSPM@CpG and aPD-L1 combination therapy. b, c Representative flow cytometry data (b) and proportion of CTLs infiltration in tumors (c) (CD8⁺ Granzyme B⁺ cells were defined as CTLs). d, e The production of IL-12 (d), TNF-α (e), and IFN-γ (f) in serum of mice with different treatments. g Representative immunofluorescence images of tumor tissues from PBS and aPD-L1 + PDT + PTT group. 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI)-labeled nuclei (blue); anti-CD8 + antibody-labeled T cells (green); anti-Granzyme B antibody-labeled T cells (pink). Scale bar: 100 μm. Data are presented as means ± standard deviation (s.d.; n = 5). p values *<0.05, **<0.01, ***<0.001, and ****<0.0001.
Catalytic activity of CSPM@CpG. The catalytic-like activity of CSPM@CpG was evaluated by observing the decomposition of hydrogen peroxide. The tubes containing (1) H₂O₂ (10 mM), (2) CSPM@CpG (100 μg mL⁻¹), and (3) H₂O₂ (10 mM) respectively, were reacted at room temperature for 30 min, and UV-Vis spectra were recorded at intervals of 10 min. Besides, to investigate the influence of pH on the H₂O₂ decomposition, the experiment was carried out by adding CSPM@CpG (100 μg mL⁻¹) in PBS (pH 6.5) containing H₂O₂ (10 mM) at room temperature. H₂O₂ (10 mM) in PBS (pH 6.5) was performed as comparison.

In vitro cell experiments. 4T1 murine breast cancer cell was obtained from ATCC (ATCC® CRL-2539™) and not listed in the database of commonly mis-identified cell lines, ICLAC (the cell lines were not tested for mycoplasma contamination). Then 4T1 cells were cultured in a high glucose DMEM medium supplemented with 10% FBS and 1% PS at 37 °C in a fully humidified atmosphere with 5% CO₂. The cells were seeded in 96-well at 8 x 10⁵ cells per well with 100 μl high glucose DMEM medium. After incubated for 24h, 4T1 cells were treated by different concentrations of CSPM@CpG (ranging from 2.5 to 1000 μg mL⁻¹) in PBS (pH 6.5) for another 24 h. In all, 100 μl of DMEM (without FBS) containing 10% CCK8 was added into each well. After incubation for 4 h at 37 °C, the absorbance at 450 nm was measured using a standard enzyme linked immunosorbent assay (ELISA) format spectrophotometer. Experiment was repeated three times and the cell viability percentage was calculated based on the average of each experiment.

To quantify the cellular uptake efficiencies of CSPM@CpG, 4T1 cells were seeded in a 24-well plate at a density of 2 x 10⁶ cells per well and incubated for 24 h, then 800 μl of different concentrations of CSPM@CpG (0, 25, 50, and 100 μg mL⁻¹) was added into well. After incubation for different time periods, the cells were collected and washed three times with PBS. Then 4T1 cells were resuspended into 500 μl PBS and digested with aqua regia to quantify the content of Cu with ICP. Additionally, 4T1 cells were incubated with CSPM@CpG (100 μg mL⁻¹) for different time periods, then harvested by trypsinization, and were visualized under a confocal laser scanning microscope (CLSM, Leica TCS SP5 II).

The intracellular reactive oxygen species generation of CSPM@CpG NPs with 650 nm laser irradiation was also detected using DCFH-DA Reactive Oxygen Species Assay Kit. 4T1 cells were plated at 5 x 10⁵ cells per well on confocal culture dish until cell attachment. Following incubation with different concentration of CSPM@CpG NPs (25, 50, 100, and 200 μg mL⁻¹) for 4 h at 37 °C, the cells were washed with PBS for three times and then proper amount of DCFH-DA was added into the wells. After incubation for another 0.5 h, the cells were irradiated under 650 nm laser with the power intensity of 1 W cm⁻² for 5 min. Finally, the cell imaging was captured by using CLSM with an excitation of 488 nm and emission of 530 nm. The generation of ROS in 4T1 cells without adding nanocomposite under 655 nm laser irradiation for 5 min was taken as control.

The production of IL-12 by the dendritic cells 2 (DCs 2.4) under different treatments (CpG, CSP, CSPM, and CSP@CpG) was quantified by mouse IL-12 precoated ELISA kit and mouse IL-12 precoated ELISA kit (Lianke Bio). DCs 2.4 cells were seeded in transwell plates at a density of 1 x 10⁶ cells per well overnight and then incubated with free CpG, CSP, CSPM, and CSP@CpG for 12 h, respectively. Then lower supernatant solution was collected and detected by ELISA, using antibody pair specific to these cytokines following the protocol recommended by the manufacturer. Additionally, the synergistic therapeutic efficacy of the combination of PTT/immunotherapy agent, the cell cytotoxicity was measured with CCK-8 assay. Briefly, 4T1 cells seeded in 96-well plates were incubated with different concentration of NPs (0, 25, 50, 100, and 200 μg mL⁻¹) for 12 h. For PDT, PTT, and PDT + PTT groups, 4T1 cells were treated with CSPM NPs. After they were washed three times with PBS to remove the excess material, 4T1 cells were incubated with an excitation of 655 nm laser at 1 W cm⁻² for 5 min for PDT and irradiated with 808 nm (2 W cm⁻²) laser for PTT, respectively. PDT + PTT group was conducted under 808 nm and 655 nm irradiation for 5 min simultaneously. Immunotherapy group was performed through adding CpG into wells. PDT + PTT + CpG group was treated with CSPM@CpG and irradiated under 650 nm and 808 nm irradiation for 5 min at 37 °C. Then 100 μl of DMEM (without FBS) containing 10% CCK8 was added into each well. To quantitatively measure the number of 4T1 cells survived after various treatments, the cell viability was also measured by CCK8 assay as described above. Cells without treatment were used as control groups for comparison. The synergistic effect of PTT-PDT-immunotherapy was evaluated by combination index (CI) analysis. CI > 1 denotes antagonism; CI ≤ 1 additivity, and CI < 1 synergism ⁸⁹.

Cells apoptosis was detected by using an Annexin-V-FITC Staining kit. 4T1 cells were placed into 24-well plates at a density of 2 x 10⁶ cells per well and allowed to adhere prior to addition of CSPM@CpG. After 4 h incubation, cells were irradiated under different treatments: no treatment; 650 nm laser irradiation at a power density of 1 W cm⁻² for 5 min; 808 nm irradiation at 2 W cm⁻² for 5 min; 650 nm and 808 nm simultaneous irradiation at 1 W cm⁻² and 2 W cm⁻², respectively for 5 min and then incubated for a further 12 h. Cells were digested, collected, and stained with 5 μL, 5μl recombinant human anti-Annexin-V-FITC and 5 μl of propidium iodide (PI). After reaction at room temperature in the dark for 10 min, all the samples were immediately detected by using flow cytometry (BD FACSCalibur).

Animal models. Female Balb/c mice (4~5 weeks) were purchased from Shanghai Laboratory Animal Center (SLAC, Shanghai, China) and bred in a sterilized, specific pathogen-free (SPF) Lab of Tongji University. Then the 5 x 10⁵ 4T1 cells...
were injected into the left mammary fat pad of 5-week-old female BALB/c mice. All animal procedures were conformed to the Guide for the Care and Use of Laboratory Animals and animal study protocols were approved by Tongji University Experimental Animal Center.

In vivo photothermal imaging and biodistribution. For in vivo photothermal effect evaluation, 4T1 tumor-bearing BALB/c mice were injected with 100 μL of CSPM@CpG suspension (100 and 200 μg mL⁻¹, respectively) and irradiated with 808 nm NIR laser at the power density of 2 W cm⁻² for 5 min after 12 h post-intravenous injection. The infrared thermal images of mice were obtained by the LTX3-P infrared Imaging for a designated time period. The tumor-bearing mice injected with PBS were selected as the control.

In order to study the biodistribution of CSPM@CpG, 4T1 tumor-bearing mice were injected intravenously with CSPM@CpG (450 μg mL⁻¹, 0.15 mL) and killed after 2 h, 6 h, 12 h, and 24 h. The heart, liver, spleen, lung, kidney, and tumor were weighed and digested in aqua regia. Finally, the Cu content per gram of each organ was measured by ICP.

In vivo animal experiments. After the tumors grew to a size of 80 mm³, the tumor-bearing mice were randomly assigned to six groups (N = 4 per group) and treated differently: (1) PBS; (2) CpG and PD-L1 antibody (1000 μg/kg) (InvivoMab anti-mouse PD-L1 (B7-H1), catalog number: BE0101; concentration: 5.8 mg/mL) injected percutaneously in advance; (3) CSPM exposed to 650 nm and 808 nm laser; (4) CSPM@CpG exposed to 650 nm laser with the power intensity of 1 W cm⁻² for 5 min (PDT); (5) CSPM@CpG irradiated by 808 nm laser at a power intensity of 2 W cm⁻² for 5 min (PDT); (6) CSPM@CpG irradiated by 650 nm laser (1 W cm⁻², 5 min) and 808 nm laser (2 W cm⁻², 5 min) simultaneously (PDT + PTT); (7) CSPM@CpG with 650 nm laser and 808 nm laser irradiation (the power intensity and irradiation time is the same as that mentioned above) and PD-L1 antibody (1000 μg/kg) injected percutaneously in advance (aPDT-L1 + PDT + PTT); briefly, CSPM@CpG dissolved in 0.15 mL of PBS buffer solution at a concentration of 450 μg mL⁻¹, were injected via the tail vein every other day for 2 weeks. Mice weight and tumor sizes were measured every 2 days. Tumor volume was calculated as tumor length × (tumor width)² × 1/2 and tissue samples were collected for H&E staining.

Tumors were collected and fixed with 4% paraformaldehyde after 14 days treatment. Subsequently, tumor sections were dehydrated with different solutions. The single-cell suspension was incubated with anti-CD16/32 (Anti-mouse CD16/32; catalog number: 08212-20-100; concentration: 0.5 mg/mL) overnight at 4 °C, followed by incubation with dye-conjugated secondary antibodies with anti-CD8 antibody and anti-Granzyme B on the end of a syringe and filtered through nylon mesh filters to acquire single-cell suspensions. Further information on research design is available in Supplementary Data 1. All findings in the manuscript are expressed as mean ± standard deviation. One-way ANOVA was used to calculate the statistical significance of the differences was expressed as p < 0.05, **p < 0.01, ***p < 0.001, and ****p < 0.0001. All experiments have been reproduced and results confirmed.

References

1. Chen, G., Roy, I., Yang, C. & Prasad, P. N. Nanochemistry and nanomedicine for nanoparticle-based diagnostics and therapy. *Chem. Rev.* 116, 2826–2885 (2016).
2. Yang, J., Chen, Y., Li, Y. & Yin, X. Magnetic resonance imaging-guided multi-drug chemotherapy and photothermal synergistic therapy with pH and NIR-stimulation release. *Adv. Appl. Mater. Inter.* 9, 22278–22289 (2017).
3. Lv, G. et al. Near-infrared emission Cuâ±Snâ±Znâ quantum dots: all-in-one theranostic nanomedicines with intrinsic fluorescence/photoacoustic imaging for tumor phototherapy. *ACS Nano* 10, 9637–9645 (2016).
4. Li, Y. et al. A smart and versatile theranostic nanomedicine platform based on nanoporphyrin. *Nat. Commun.* 10, 106–110 (2019).
5. Ma, Z. et al. Fe(III)–doped two-dimensional Câ±Nâ±O nanosheet: a new Oâ±2 evolving and mitochondria-targeting photodynamic agent for MRI and enhanced anticancer tumor. *Small* 12, 5477–5487 (2016).
6. Bai, J., Jia, X., Zhen, W., Cheng, W. & Jiang, X. A facile ion-doping strategy to regulate tumor microenvironments for enhanced multimodal tumor theranostics. *J. Am. Chem. Soc.* 140, 106–110 (2018).
7. Liang, C., Xu, L., Song, G. & Liu, Z. Emerging nanomedicine approaches fighting tumor metastasis: animal models, metastasis-targeted drug delivery, phototherapy, and immunotherapy. *Chem. Soc. Rev.* 45, 6250–6269 (2016).
8. Jaqué, D. et al. Nanoparticles for photothermal therapies. *Nanoscale* 6, 9494–9530 (2014).
9. Luther, J. M., Jain, P. K., Ewers, T. & Alvisatos, A. P. Localized surface plasmon resonances arising from free carriers in doped quantum dots. *Nat. Mater.* 10, 361–366 (2011).
10. Zhang, M. et al. Tumor environment responsive degradable Cuâ±Znâ±Oâ±Nâ±DOX for MRI guided synergetic chemo-photothermal therapy and chemodynamic therapy. *Chem. Eng. J.* 389, 124450 (2020).
11. Chen, L., Sun, S., Wang, Y., Yang, C., Wu, S. & Yan, X. Activatable multifunctional persistent luminescence nanoparticle/copper sulfide nanoprobe for in vivo luminescence imaging guided photothermal therapy. *ACS Appl. Mater. Interfaces* 8, 32667–32674 (2016).
12. Guo, S. et al. Oxygen-triggered hybrid nanoparticles to enhance fluorescence/photoacoustic/ultrasound imaging guided tumor photodynamic therapy. *Biomaterials* 112, 324–335 (2017).
13. Deng, K. et al. Recent progress in near infrared light triggered photodynamic therapy. *Small* 13, 1702297 (2017).
14. Susan, M. et al. Transition metal complexes and photodynamic therapy from a tumor-centered approach: challenges, opportunities, and highlights from the development of TDL1433. *Chem. Rev.* 119, 797–828 (2019).
15. Huang, Z. et al. Photodynamic therapy for treatment of solid tumors - potential and technical challenges. *Technol. Cancer Res. Treat.* 7, 309–320 (2008).
16. Quan, C. et al. Light-activated hypoxia-responsive nanocarriers for enhanced anticancer therapy. *Adv. Mater.* 28, 3313–3320 (2016).
17. Tong, X. et al. Monitoring tumor hypoxia using [18F]-FMISO PET and pharmacokinetics modeling after photodynamic therapy. *Sci. Rep.* 6, 31511 (2016).
18. Prasad, P. et al. Multifunctional albumin-MnO₂ nanoparticles modulate solid tumor microenvironment by attenuating hypoxia, acidosis, vascular endothelial growth factor and enhance radiation response. *ACS Nano* 8, 3202–3212 (2014).
19. Cheng, H. et al. An O₂ self-sufficient biomimetic nanoplanform for highly specific and efficient photodynamic therapy. *Adv. Funct. Mater.* 26, 7767–7780 (2016).
20. Chen, H., Tian, J., He, W. & Guo, Z. H₂O₂-activatable and O₂ envolving nanoparticles for highly efficient and selective photodynamic therapy against hypoxic tumor Cells. *J. Am. Chem. Soc.* 137, 1539–1547 (2015).
21. Yang, J., Shang, Y., Li, Y., Cui, Y. & Yin, X. An “all-in-one” antitumor and anti-recurrence/metastasis nanomedicine with multi-drug co-loading and burst drug release for multi-modality therapy. *Chem. Sci.* 9, 7210–7217 (2018).
22. Hahn, A. W., Gill, D. M., Pal, S. K. & Agarwal, N. The future of immune checkpoint cancer therapy after PD-1 and CTLA-4. *Immunotherapy* 9, 681–692 (2017).
23. Sharma, P. & Allison, J. P. The future of immune checkpoint therapy. *Science* 348, 56–61 (2015).
24. Sharma, P. & Allison, J. Immune checkpoint targeting in cancer therapy: toward combination strategies with curative potential. *Cell* 161, 205–214 (2015).
25. Wang, C., Sun, W., Wright, G., Wang, A. & Gu, Z. Inflammation-triggered cancer immunotherapy by programmed delivery of CpG and anti-PD1 antibody. *Adv. Mater.* 28, 8912–8920 (2016).
26. Krieg, A. M. Therapeutic potential of Toll-like receptor 9 activation. *Adv. Drug Deliv. Rev.* 5, 471–484 (2006).
27. Krieg, A. M. Toll-like receptor 9 (TLR9) agonists in the treatment of cancer. *Oncogene* 27, 161–167 (2008).
28. Vormer, J. & Krieg, A. M. Immunotherapeutic applications of CpG oligodeoxynucleotide TLR9 agonists. *Adv. Drug Deliv. Rev.* 61, 195–204 (2009).
29. Kun, W., Shuman, W. & Lianghua, H. et al. 'Minimalist' nanovaccine constituted from near whole antigen for cancer immunotherapy. ACS Nano 2, 6398–6409 (2018).

30. Kroll, A. V., Fang, R. H. & Jiang, Y. et al. Nanoparticulate delivery of cancer cell membrane elicits multiantigenic antitumor immunity. Adv. Mater. 29, 1018–1026 (2017).

31. Pardoll, D. M. The blockade of immune checkpoints in cancer immunotherapy. Nat. Rev. Cancer 12, 252–264 (2012).

32. Yang, G., Xu, L. & Chao, Y. et al. Hollow MnO2 as a tumor-microenvironment-responsive biodegradable nano-platform for combination therapy favoring antitumor immune responses. Nat. Commun. 8, 902 (2017).

33. Han, Q. et al. CpG loaded MoS 2 nanosheets as multifunctional agents for photothermal enhanced cancer immunotherapy. Nanoscale 9, 5927–5934 (2017).

34. Wilson, K. D., de Jong, S. D. & Tam, Y. K. Lipid-based delivery of CpG oligonucleotides enhances immunotherapeutic efficacy. Adv. Drug Deliv. Rev. 61, 233–242 (2009).

35. Xu, L. et al. Morphologically virus-like fullerenol nanoparticles act as the dual-functional nanoadjuvant for HIV-1 vaccine. Adv. Mater. 25, 5928–5936 (2013).

36. Guo, L. et al. Combinatorial photothermal and immuno cancer therapy using chitosan-coated hollow copper sulfide nanoparticles. ACS Nano 8, 5670–5681 (2014).

37. Shanmugam, V., Selvakumar, S. & Yeh, C. S. Near-infrared light-photothermal enhanced cancer immunotherapy. Nanoscale 8, 6128–6139 (2016).

38. Li, H. et al. Dual-function nanostructured lipid carriers to deliver IR780 for photothermal enhanced cancer immunotherapy. Mol. Pharm. 16, 3430–3440 (2019).

39. Zou, L. et al. Current approaches of photothermal therapy in treating cancer metastasis. Adv. Mater. 29, 402–413 (2017).

40. Cheng, L., Wang, C., Feng, L., Yang, K. & Liu, Z. Functional nanomaterials for phototherapies of cancer. Chem. Rev. 114, 10869–10939 (2014).

41. Deng, Y., Qi, D., Deng, C., Zhang, X. & Zhao, D. Superparamagnetic high-magnetization microspheres with an Fe3O4@SiO2 core and perpendicularly aligned mesoporous SiO2 shell for removal of microcysts. J. Am. Chem. Soc. 130, 28–39 (2008).

42. Song, G. et al. A low-toxic multifunctional nanoplatform based on Cu9S5@mSiO2 core-shell nanocomposites: combining photothermal- and chemotherapies with infrared thermal imaging for cancer treatment. Adv. Funct. Mater. 23, 4281–4292 (2013).

43. Xu, J. et al. Tumor microenvironment-responsive mesoporous MnO2-coated upconversion nanoplatform for self-enhanced tumor theranostics. Adv. Funct. Mater. 28, 1803804 (2018).

44. Deng, X. et al. A hollow-structured CuS@CuS@Au nanohybrid: synergistically enhanced photothermal efficiency and photoswitchable targeting effect for cancer theranostics. Adv. Mater. 29, 36 (2017).

45. Zhang, W. et al. Oxygen-generating MnO2 nanodots-anchored versatile nanoplatform for combined chemo-photodynamic therapy in hypoxic cancer. Adv. Funct. Mater. 28, 1706375 (2018).

46. Feng, Q. et al. Programmed near-infrared light-responsive drug delivery system for combined magnetic tumor-targeting magnetic resonance imaging and chemo-phototherapy. Acta Biomater. 49, 402–413 (2017).

47. Lin, L. et al. Graphitic-phase C3N4 nanosheets as efficient photosensitizers and pH-responsive drug nanocarriers for cancer imaging and therapy. J. Mater. Chem. B. 2, 1031–1037 (2014).

48. Yu, Y., Yan, C., Hong, W. & Dong, S. Type I photodynamic therapy by organic–inorganic hybrid materials: From strategies to applications. Coord. Chem. Rev. 395, 46–62 (2019).

49. Lan, Y. et al. Co-Delivery of paclitaxel by a capsaicin prodrug micelle facilitating for combination therapy on breast cancer. Mol. Pharm. 16, 3430–3440 (2019).

Acknowledgements
This work was supported by the National Natural Science Foundation of China (nos. 1207232, 8186110475, 81573008, and 81606547), the Science and Technology Commission of Shanghai Municipal Government (No. 14DZ2261100) and the Fundamental Research Funds for the Central Universities, Key Cross-cutting Projects of Central Universities (107219057), and the Project of Key Disciplines Group Construction of Pudong Health Bureau of Shanghai (PWZaq2017D-10), and the Science and Technology Commission of Shanghai Municipal Government, Innovation Fund (No. 16DZ1930509), and the Shanghai Pudong New Area Science and Technology Development Fund, Innovation Fund (No. PKJ2017-Y18).

Author contributions
S.S. and C.D. conceived this project. S.S., C.D., L.Z., and L.C. designed, supervised the project and commented on the project. L.Z., L.C., X.H., Y.L., W.L., Y.S., and T.Y. carried out the experiments and analyzed the results. L.Z., L.C., C.D., and S.S. wrote the manuscript. All the authors contributed to the discussion during the whole project.

Competing interests
The authors declare no competing interests.

Additional information
Supplementary information is available for this paper at https://doi.org/10.1038/s42003-020-1070-6.

Correspondence and requests for materials should be addressed to C.D. or S.S.

Reprints and permission information is available at http://www.nature.com/reprints

Publisher’s note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.