

Cu₃P/I-MT Nanocomposites Potentiated Photothermal-Immunotherapy

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Purpose: Photothermal therapy (PTT) is a promising anticancer treatment that involves inducing thermal ablation and enhancing antitumor immune responses. However, it is difficult to completely eradicate tumor foci through thermal ablation alone. Additionally, the PTT elicited antitumor immune responses are often insufficient to prevent tumor recurrence or metastasis, due to the presence of an immunosuppressive microenvironment. Therefore, combining photothermal and immunotherapy is believed to be a more effective treatment approach as it can modulate the immune microenvironment and amplify the post-ablation immune response.

Methods: Herein, the indoleamine 2, 3-dioxygenase-1 inhibitors (I-MT) loaded copper (I) phosphide nanocomposites (Cu₃P/I-MT NPs) are prepared for PTT and immunotherapy. The thermal variations of the Cu₃P/I-MT NPs solution under different conditions were measured. The cellular cytotoxicity and immunogenic cell death (ICD) induction efficiency of Cu₃P/I-MT NPs were analyzed by cell counting kit-8 assay and flow cytometry in 4T1 cells. And the immune response and antitumor therapeutic efficacy of Cu₃P/I-MT NPs were evaluated in 4T1-tumor bearing mice.

Results: Even at low energy of laser irradiation, Cu₃P/I-MT NPs remarkably enhanced PTT efficacy and induced immunogenic tumor cell death. Particularly, the tumor-associated antigens (TAAs) could help promote the maturation of dendritic cells (DCs) and antigen presentation, which further activates infiltration of CD8⁺ T cells through synergistically inhibiting the indoleamine 2, 3-dioxygenase-1. Additionally, Cu₃P/I-MT NPs decreased the suppressive immune cells such as regulatory T cells (Tregs) and M2 macrophages, indicating an immune suppression modulation effect.

Conclusion: Cu₃P/I-MT nanocomposites with excellent photothermal conversion efficiency and immunomodulatory properties were prepared. In addition to enhanced the PTT efficacy and induced immunogenic tumor cell death, it also modulated the immunosuppressive microenvironment. Thereby, this study is expected to offer a practical and convenient approach to amplify the antitumor therapeutic efficiency with photothermal-immunotherapy.

Keywords: copper (I) phosphide (Cu₃P), photothermal therapy, IDO-1 inhibitors, immunotherapy

Introduction

Photothermal therapy (PTT) is rapidly emerging as a highly effective and significant strategy for tumor therapy. Due to its high efficiency and non-invasive nature, it has been widely applied for treatment of malignant tumors such as melanoma, breast cancer, and pancreatic cancer.¹⁻⁵ Particularly, with the development of nanoparticles with better light absorption properties, it highly efficiently absorbs near-infrared (NIR) light and converts it into heat (>42 °C), thereby causing cell death.⁶ PTT can well control the hyperthermia damage at the site of the tumor, owing to the elaborate and convenient adjustment of NIR light, which endows PTT with the characteristics of low invasiveness and high

specificity.^{7–9} Moreover, tumors are more susceptible to heat damage than normal cells, because of the unique biological characteristics such as the abnormal blood flow and reduced heat dissipating ability in solid tumors.¹⁰ This specificity confers PTT with highly advantageous therapeutic properties, making it gradually become an alternative strategy for certain tumor therapy, especially those that are not suitable for traditional surgical treatment.^{11–13} In addition, a growing number of studies have indicated that PTT can not only effectively ablate local tumor lesions, but also inhibit metastasis by activating anti-tumor immune effects.^{14–16} In clinical trials, PTT showed significant therapeutic effects in 603 patients with metastatic liver from colorectal carcinoma and 500 patients with hepatocellular carcinoma, respectively.^{15,17}

To improve the performance of PTT, researchers have employed nanoparticles with exceptional photothermal conversion efficiency, including noble metal nanostructures such as gold nanospheres, gold nanorods, and multifunctional gold nanoshells.^{18–25} However, the price of noble metals restricts their widespread use. Copper materials, on the other hand, have garnered the interest of researchers due to their low cost, biocompatibility, biodegradability, and multifunctional properties.²⁶ Copper-based nanomaterials have shown great promise in PTT due to their exceptional light absorption properties in the NIR region, which is enabled by the local surface plasmon resonance (LSPR).²⁷ Copper-based nanomaterials, such as copper sulfide (CuS), copper selenide (CuSe), and copper (CuTe) nanomaterials are examples of copper-based nanomaterials with excellent NIR light-activated photothermal characteristics resulting from copper vacancy in the crystal lattice.^{28–30} Optics and electronics have been extensively studied in copper (I) phosphide (Cu₃P) nanocomposites (NPs), which are among copper-based nanomaterials.^{31,32} Cu₃P NPs, a p-type semiconductor, display remarkable LSPR absorption in the NIR region.³³ Recent studies have shown that Cu₃P NPs additionally exhibit tunable absorption in the mid-IR region and Cu vacancy regulated properties, which indicates that they have the potential to be used as a photothermal conversion medium for PTT.³³

High levels of tumor cell death, exposure of tumor-associated antigens (TAAs), and release of damage-associated molecular patterns (DAMPs), resulting in immunogenic cell death (ICD), are caused by the intense heat produced by PTT.³⁴ This process recruits immune cells and induces immune responses.³⁵ Despite the efficacy of PTT in destroying local tumors, it is difficult to completely eradicate tumor foci.³⁶ Due to the immunosuppressive tumor microenvironment, the immune responses elicited by ICD-based PTT are insufficient to inhibit tumor recurrence and metastasis.³⁷ Combining PTT with immunotherapy can increase the therapeutic efficacy against both primary tumors and distant metastatic cancer cells.^{38,39} This combination enhances the immune responses induced by ICD to track and eliminate tumor cells.^{40,41} PTT and several immunosuppressants, such as checkpoint inhibitors, indoleamine 2,3-dioxygenase (IDO-1) inhibitors, immune adjuvants, CAR-T therapy, and cytokine therapy, have exhibited significant therapeutic effects to date.^{42–46} IDO inhibitors, in particular, have received considerable attention.⁴⁷ IDO-1 catalyzes the oxidative catabolism of tryptophan (Trp) to kynurenine (Kyn) in order to inhibit the proliferation of CD8⁺ T cells and induce intra-tumoral recruitment of regulatory T cells (Tregs), which impedes the ICD-induced immunoreaction and enables cancer progression.^{48–50} Therefore, inhibiting IDO-1 can potentially activate the ICD-mediated immune response and enhance antitumor efficacy. However, current IDO inhibitors such as Epacadostat and NLG919 have insufficient therapeutic effects due to their limited bioavailability and high lipophilicity.^{51,52} Consequently, IDO inhibitor-loaded photothermal agents are anticipated to overcome these limitations of PTT or immunotherapy alone and enhance therapeutic efficacy by modulating the immune microenvironment.

In this study, Cu₃P nanoparticles are prepared to serve as photothermal agents owing to their ideal optical characteristics and biocompatibility. Then, the IDO inhibitor (1-MT) is loaded into the Cu₃P NPs to form a nanocomposite (Cu₃P/1-MT) for PTT and immunotherapy. Benefiting from the excellent photothermal properties, even under low energy of laser irradiation, Cu₃P/1-MT could remarkably enhance PTT efficacy. Meanwhile, it also induces the immunogenic cell death and triggers the release of TAAs, which will promote the dendritic cell (DC) maturation and antigen presentation. Furthermore, the immunosuppressive microenvironment could be comprehensively reprogrammed with the IDO-1 pathway blocker 1-MT, which allows the infiltration of CD8⁺ T cells while inhibiting suppressive immune cells, such as Tregs and M2 macrophages at the tumor lesions (Figure 1). Therefore, the Cu₃P/1-MT nanocomposites are expected to provide an easily accessible, safe and efficient agent for synergistically improving the efficacy of PTT-immunotherapy.

Materials and Methods

Reagents

$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, 1-Hexadecylamine (HDA), Trioctylphosphine oxide (TPOP), 2,7-dichlorofluorescein diacetate (DCFH-DA), 1-methyl tryptophan (1-MT), and 1-octadecene (ODE) were purchased from Sigma-Aldrich. Hyaluronic acid sodium (HA), cyclohexane, and ethanol were purchased from Aladdin Reagents. All chemicals were used as received without any additional purification unless otherwise stated.

Cells

The 4T1 breast cancer cells obtained from ATCC were grown in Gibco RPMI 1640 Medium supplemented with 10% fetal bovine serum (FBS; Gibco), 100 $\mu\text{g}/\text{mL}$ penicillin (Gibco), and 100 $\mu\text{g}/\text{mL}$ streptomycin (Gibco). The cells were routinely tested for the presence of Mycoplasma and maintained in a 37 °C humidified atmosphere containing 5% CO_2 . Using standard techniques, bone marrow-derived macrophages and dendritic cells (BMDMs & BMDCs) were isolated from BALB/c mice. The mice were euthanized, and bone marrow cells were extracted from their leg bones. The extracted cells were then maintained in an RPMI medium supplemented with 10% FBS. Monocytes were differentiated for seven days with GM-CSF and IL-4 in order to collect BMDMs.

Xenograft Tumor Model

Female BALB/c mice (6–8 weeks) were purchased from Guangdong Medical Laboratory Animal Center (Guangzhou, China). The 4T1 cells (5×10^5) were subcutaneously injected into the back of BALB/c mice. Once the tumor volume reached between 100 and 150 mm^3 , the mice were randomly assigned to our experiments. All the experiments were performed under protocols approved by the Animal Research Ethics Committee of Hainan university (ethics approval number: HNUAUCC-2021-00025).

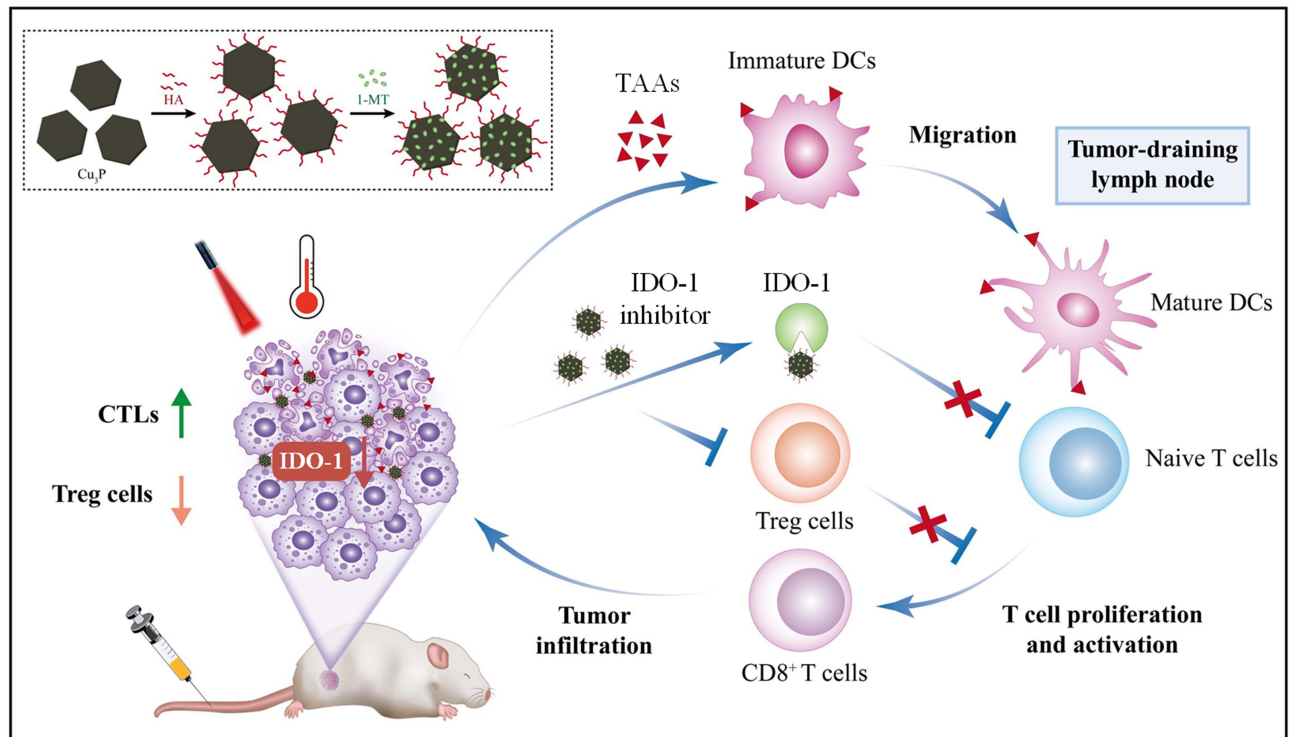


Figure 1 Schematic illustration of PTT-immunotherapy potentiated by $\text{Cu}_3\text{P}/1\text{-MT}$ nanocomposites.

Synthesis of Cu₃P

In accordance with a standard method for synthesizing Cu₃P NPs, the following were added to a 50 mL three-neck flask: 0.5 mmol CuCl₂·2H₂O, 1.209 g of HAD, 1.551 g of TPOP, and 12 mL of ODE. Under a N₂ atmosphere, the solution was dehydrated by heating it for 30 minutes at 150 °C. Subsequently, the temperature was raised to 300 °C for 1.5 hours in an atmosphere of N₂ before being cooled to 25 °C. The resultant NPs were combined with ethanol to create nanoparticles, then centrifuged at 8000 rpm for 5 minutes and washed three times with cyclohexane and ethanol.

Synthesis of HA-Capped Cu₃P/I-MT

Cu₃P NPs were dispersed in an ethanol solution of HA and sonicated for one hour. The resulting mixture was stirred for 12 hours before being centrifuged at 10,000 rpm for 5 minutes to collect the HA-capped Cu₃P NPs. Next, they were washed three times with water before being redistributed in PBS. Approximately 8 mg 1-MT was added to the HA-capped Cu₃P NPs solution (20 mL, 1 mg/mL in PBS for 1-MT loading). To collect Cu₃P/I-MT, the mixture was stirred overnight at 25 °C and centrifuged at 13,000 rpm for 10 minutes. Using fluorescence spectrophotometry with an excitation wavelength of 285 nm and an emission wavelength of 355 nm, the amount of 1-MT that was loaded was determined. The following equation was used to determine the loading efficiency of 1-MT:

$$\text{loading capacity of 1 - MT (\%)} = \frac{(\text{weight of 1 - MT}) - (\text{weight of unloaded 1 - MT})}{(\text{weight of 1 - MT})} \times 100\%$$

Characterization of NPs

Powder X-ray diffraction patterns for samples were acquired using an X-ray diffractometer (MiniFlex2, Rigaku, Japan) with Cu K α 1 radiation ($\lambda = 0.154187$ nm). Additionally, a JEOL-2010 transmission electron microscope (TEM, JEOL, Japan) was utilized to carry out the TEM measurements. Moreover, a UV-vis-NIR spectrophotometer (Cary 50 Bio, USA) was utilized to determine the amounts of loaded 1-MT. In addition, the FTIR spectra were obtained by compressing thin films composed of samples and potassium bromide (KBr) powder. Subsequently, a Fourier Transform Infrared spectrometer (FTIR, Frontier, PerkinElmer, USA) was used for acquiring the FTIR spectra of the samples.

In vitro Phototoxicity of Cu₃P/I-MT NPs

The phototoxicity of Cu₃P/I-MT NPs was evaluated using the CCK-8 assay. 4T1 cells were seeded at a density of 5×10^3 cells per well in a 96-well plate and incubated for 12 hours. Subsequently, the cells were exposed to Cu₃P/I-MT NPs at varying concentrations. The cells were then exposed to 808 nm laser irradiation with an intensity of 0.8 W/cm² for 5 minutes, followed by 24 hours of incubation for CCK-8 cell viability assay.

Cell Staining Assays

For the cell death assay, the cells were stained with calcein acetoxymethyl ester (calcein-AM)/PI, which were then detected using a fluorescence microscope (MD43-N, Mshot, Guangzhou, China). To indicate calreticulin (CRT) expression, the cells were stained with anti-rabbit CRT polyclonal antibody (Immunoway, Beijing, China) and Alexa Fluor Plus 488-Goat anti-rabbit IgG (Invitrogen) at 1 hour after laser irradiation.

Maturation of DCs

To conduct DC stimulation experiments in vitro, each 1×10^6 DCs were placed in 12-well plates. After an incubation period of approximately 24 hours, the cells were co-incubated with tumor cells that had been treated with Cu₃P/I-MT NPs, PBS + L, or Cu₃P/I-MT NPs + L. The BMDCs were then stained with anti-CD86-PE antibodies and analyzed with a Stratigigm S1200Ex flow cytometer (Stratigigm, USA).

Anticancer Efficacy in vivo

The tumor-bearing mice were randomly divided into four groups of five mice each: the PBS group, the Cu₃P/I-MT NPs group, the Cu₃P NPs + L group, and the Cu₃P/I-MT NPs + L group. Following intravenous administration of either PBS

or related NPs, PTT was performed using an 808 nm laser for 10 minutes with a power density of 0.8 W/cm². The optical fiber used to provide uniform light distribution on the treatment surface was manufactured by Pioneer Optics of Bloomfield, Connecticut. The temperature of the surface of the tumor was measured using an infrared thermal camera E80 (FLIR, Boston, USA). Using a digital caliper, tumor size was measured every two days, and the ellipsoidal formula $V = (\text{width})^2 \times \text{length} \times 2^{-1}$ was used to calculate the tumor volume. When tumors reached a maximum size of 2.0 cm in any dimension or when ulcerations appeared, the mice were euthanized.

Flow Cytometry

The tumor tissues were harvested, minced, and then digested at 37 °C for 20–30 minutes with Collagenase IV (1 mg/mL) and DNase I (20 µg/mL) in RPMI. In addition, the spleen was gathered, ground, and treated with ACK lysis buffer to eliminate red blood cells. To obtain a single-cell solution, the remaining cells were filtered and washed in PBS. To minimize non-specific bindings to Fc receptors, anti-CD16/32 (clone 93; eBioscience, USA) was used. The cells were then stained with the following antitumor fluorescent antibodies in order to evaluate active T cells: Live/Dead-Zombie 405, CD45-eFluor 450, CD3-APC-eFluor 780, CD8a-FITC, CD4-PE-CY7, CD25-APC, and Foxp3-PE. Cells were stained with Live/Dead-Zombie 405, CD45-eFluor 450, CD11b-PE, F4/80-APC, and CD206-FITC in order to evaluate tumor-associated macrophages (TAM). Stratified S1200Ex flow cytometer (Stratified, USA) and FlowJo software were used to analyze the procedure.

Statistical Analysis

Statistical comparisons were carried out using SPSS 23.0 (IBM Corp., Armonk, NY, USA) and described as mean ± standard deviation. Figures were performed using GraphPad Prism 8.1 (GraphPad Software, Inc., La Jolla, CA, USA). The Shapiro–Wilk test was used for normal distribution. The data were normally distributed. The Student's *t*-test was used for comparing two groups. The difference between multiple groups was analyzed by one-way analysis of variance followed by Tukey's test. The difference was considered statistically significant at $P < 0.05$.

Results and Discussion

Synthesis and Characterization of Cu₃P/I-MT

Cu₃P NPs were obtained as described previously.⁵³ The morphology of the resulting NPs was confirmed by TEM, as depicted in Figure 2a. The images displayed the platelet-like hexagonal shape of Cu₃P NPs, with a size of approximately 97.9 nm (Figure 2b). The previous study demonstrates that platelet-like hexagonal NPs, also known as nanoplatelets, are more cellular uptake-friendly than nanorods.⁵⁴ X-ray diffraction (XRD) and X-ray photoelectron spectroscopy (XPS) were used to characterize Cu₃P NPs, as shown in Figure 2c and d. The XRD pattern of Cu₃P NPs was in accordance with the hexagonal phase of Cu₃P. There are several diffraction peaks at 36.1°, 39.3°, 41.7°, 45.1°, and 46.5°, corresponding to (112), (202), (211), (300), and (113) planes of hexagonal Cu₃P, respectively.^{33,55} Two prominent peaks in the XPS spectrum of Cu 2p at 932.2 eV (Cu 2p_{3/2}) and 952.1 eV (Cu 2p_{1/2}) indicate that Cu is present in the form of Cu 2p (I). Figure 2e depicts an optical absorption of these platelets in the NIR region, supporting their viability as a candidate photothermal agent. The outer surface of Cu₃P NPs was coated with hyaluronic acid to improve their dispersibility and hydrophilicity. The I-MT was then capped to Cu₃P@HA to produce Cu₃P/I-MT. Figure 2f displays the Fourier transform infrared (FTIR) spectra of Cu₃P, Cu₃P@HA, and Cu₃P/I-MT components. After HA was capped, the peak around the 1203 cm⁻¹ fingerprint region corresponds to the -C-O-C- stretching vibration, the band at 2903 cm⁻¹ corresponds to the characteristic vibrations of C-H stretching of HA, and the broad band at 3411 cm⁻¹ indicates the O-H stretching of HA.⁵³ In addition, the stretching vibrations of benzene and heteroaromatic rings at 1540 cm⁻¹ and 1400 cm⁻¹ were observed in Cu₃P/I-MT, confirming the successful loading of Cu₃P/I-MT. The amount of loaded I-MT was quantified using a fluorescence spectrophotometer, and the final drug load rate was determined to be 68%.

The photothermal properties of Cu₃P/I-MT NPs were further investigated by laser-induced temperature monitoring. Different concentrations (0.25, 0.5, 1, and 1.5 mg/mL) of Cu₃P/I-MT NPs were exposed to 808 nm NIR light (0.8 W/cm²) for 8 minutes, and temperature changes were monitored until the 14th minute. As depicted in Figure 2g, the temperature of a solution of Cu₃P/I-MT NPs increased with increasing irradiation intensity and concentration. Within an 8-minute timeframe, laser irradiation at a power density of 0.8 W/cm² can elevate the temperature of 1.5 mg/mL Cu₃P/I-MT NPs solution by nearly 30 °C (Figure 2h).

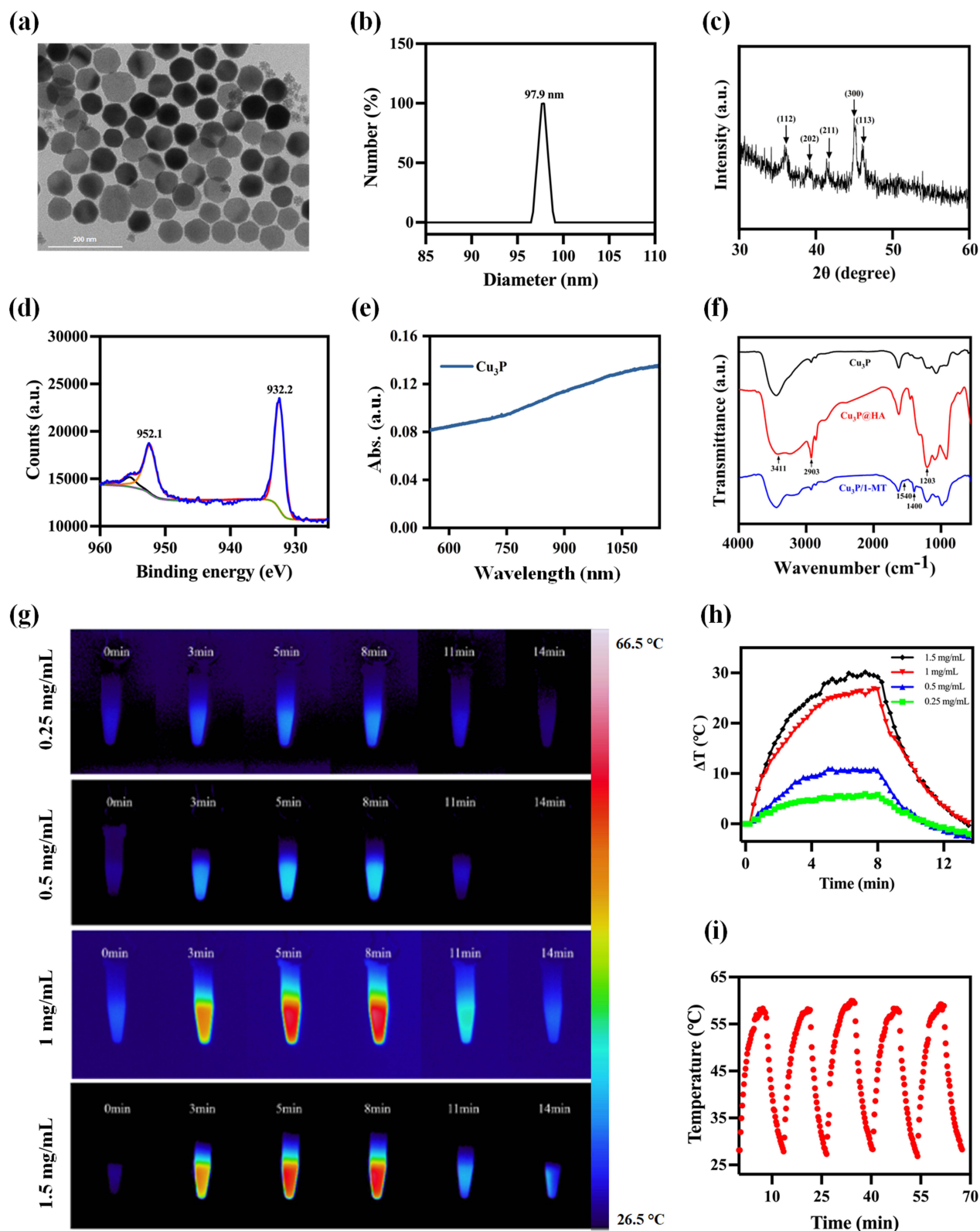


Figure 2 Synthesis and characterization of $\text{Cu}_3\text{P}/\text{I-MT}$ NPs. (a) TEM image of Cu_3P NPs. Scale bar = 200 nm. (b) Size distribution of Cu_3P NPs. (c) XRD of Cu_3P NPs. (d) XPS spectra of Cu_3P NPs. (e) Vis-NIR absorbance spectrum of Cu_3P NPs. (f) FTIR spectra of Cu_3P , $\text{Cu}_3\text{P}@HA$, $\text{Cu}_3\text{P}/\text{I-MT}$ NPs. (g) Near-infrared thermal images for $\text{Cu}_3\text{P}/\text{I-MT}$ NPs solution of different concentrations (0.25, 0.5, 1 and 1.5 mg/mL) under 808 nm laser irradiation at $0.8 \text{ W}/\text{cm}^2$. (h) Infrared thermal imaging-based temperature elevation records. (i) Temperature curves of $\text{Cu}_3\text{P}/\text{I-MT}$ NPs during five times "on/off" irradiation with 808 nm laser at $0.8 \text{ W}/\text{cm}^2$.

The photothermal stability of Cu₃P/1-MT NPs power density was set as 0.8w/cm², the laser irradiation was cycled five times, the interval time for per cycle is 2 minutes and no discernible degradation was observed (Figure 2i). These results demonstrated that the Cu₃P/1-MT NPs could be an effective photothermal agent for PTT.

Cu₃P/1-MT Enhanced PTT in vitro

To examine the biocompatibility of Cu₃P/1-MT NPs, the cytotoxicity of 4T1 tumor cells treated with Cu₃P/1-MT NPs (0, 25, 50, 100, and 200 µg/mL) was examined using the standard cell counting kit-8 (CCK-8) assay (Figure 3a). 4T1 cells were incubated with Cu₃P/1-MT NPs for 12 hours, followed with or without laser irradiation. More than 95% of 4T1 cells exposed to 200 µg/mL of Cu₃P/1-MT NPs survived in the absence of laser irradiation, indicating excellent biocompatibility. Conversely, with 50 µg/mL of Cu₃P/1-MT NPs, nearly 50% of laser-irradiated cells were killed, and the cytotoxicity was concentration-dependent. This provided support for the viability of Cu₃P/1-MT NPs in PTT. Afterward, 4T1 cells treated with 200 µg/mL Cu₃P/1-MT NPs were examined for the presence of live (calcein AM-stained, green) and dead (PI-stained, red) cells. Overall, the results indicated that Cu₃P/1-MT might have enormous potential for achieving photothermal ablation of cancer cells.

By exposing DAMPs on the cell surface, PTT has been shown to induce synergistic ICD.⁵⁶ These DAMPs, such as CRT, HMGB1, and ATP, could act as “eat-me” signals to activate DCs.⁵⁷⁻⁵⁸ Cu₃P NPs-based PTT induced CRT exposure was, therefore, tested as an indicator of ICD induction. Flow cytometry demonstrated that laser irradiation of Cu₃P/1-MT-treated cells resulted in a 7-fold higher CRT level than in the Cu₃P/1-MT group (Figure 3b). Therefore, Cu₃P/1-MT-based PTT exhibited exceptional ICD-induced efficiency. DCs are the most formidable antigen-presenting cells. DCs presented antigen potently in order to stimulate naive T cell activation and proliferation.⁵⁹ The levels of CD80⁺ and CD86⁺ were utilized to denote DCs' maturation.⁶⁰ To evaluate the antitumor immune response, the maturation of DCs stimulated by treated tumor cells with PBS, Cu₃P/1-MT, or Cu₃P/1-MT + NIR laser was evaluated using flow cytometry (Figure 3c). In the group of 4T1 cells treated with Cu₃P/1-MT, the expression of CD86⁺ in DCs was found to be only 1.3% higher compared to the 4T1 cells treated with PBS group. This suggests that Cu₃P/1-MT treatment without PTT has limited capacity to induce DC maturation. When 4T1 cells were treated with Cu₃P/1-MT and NIR laser irradiation, a 3.1-fold increase in CD86⁺ expression was observed compared to Cu₃P/1-MT alone, demonstrating the critical role of laser irradiation in Cu₃P/1-MT NPs-induced DCs maturation. The findings suggested that Cu₃P/1-MT with laser irradiation can significantly promote DC maturation by inducing TAAs and inhibiting the IDO pathway with 1-MT. Overall, these in vitro results confirmed that Cu₃P/1-MT plus laser-induced ICD, with enhanced maturation of DCs and mass 4T1 cell death.

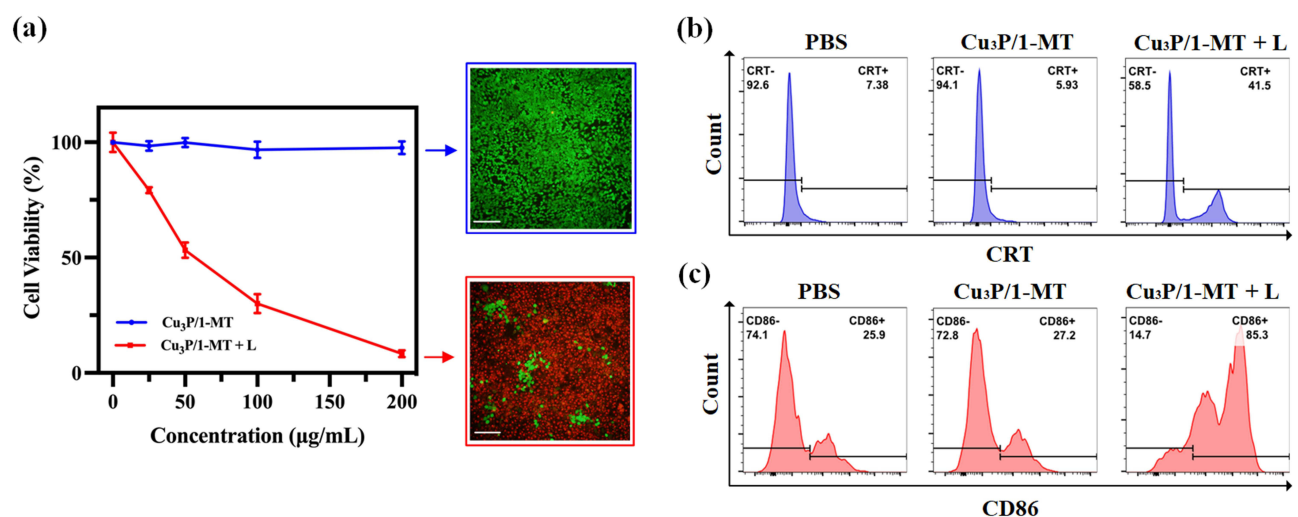


Figure 3 Cu₃P/1-MT NPs enhanced PTT in vitro. (a) Cell viability and fluorescence images with or without laser irradiation (808 nm, 0.8 W/cm²) for different concentrations of Cu₃P/1-MT NPs (n = 4). Scale bar = 100 µm. (b) Flow cytometry analysis of CRT in differently treated with 4T1 cells. (c) Flow cytometry indicated CD86 positive DCs induced by Cu₃P/1-MT NPs treated 4T1 cells.

In vivo PTT Efficacy

To assess the photothermal effects of Cu₃P/1-MT NPs in vivo, we conducted experiments on 4T1 tumor-bearing mice (Figure 4a). An 808 nm laser was used to measure the photothermal effect of Cu₃P/1-MT NPs. Accordingly, in vivo photothermal imaging was performed on tumor-bearing mice administered either PBS or Cu₃P/1-MT NPs for 24 hours. As measured in vivo with an infrared thermal camera, laser irradiation of mice treated with Cu₃P/1-MT NPs caused a rapid 25 °C-temperature increase on the tumor surface. Compared to the Cu₃P/1-MT NPs group, the increase in temperature in the tumor regions of the PBS + NIR laser group was only approximately 10 °C, indicating a lower effect. The results support the high absorption and photothermal conversion efficiency of Cu₃P/1-MT nanoparticles at 808 nm in vivo.

To examine the killing effect of Cu₃P/1-MT NPs in vivo, 4T1 tumor-bearing mice were randomly assigned to PBS + L, Cu₃P/1-MT NPs, and Cu₃P/1-MT NPs + L treatment groups. The harvested tumor section was stained with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and hematoxylin and eosin (H&E) seven days after laser treatment (Figure 4b). Compared to the PBS + L or Cu₃P/1-MT NPs group, the Cu₃P/1-MT NPs + NIR laser group induced a significant amount of apoptosis in the tumors, as shown by TUNEL staining. Similar trends were observed in the H&E staining, with the Cu₃P/1-MT NPs + NIR laser group exhibiting significantly more cancer cells with deformed nuclei (karyopyknosis, karyorrhexis, and karyolysis) than the other groups. TUNEL and H&E tests revealed that synergistic treatment with Cu₃P/1-MT NPs + NIR laser was more effective than immunotherapy alone (Cu₃P/1-MT NPs group).

Cu₃P/1-MT-Induced Immune Response

The DAMPs and antigens released by dying tumor cells after PTT can be transported to the spleen, thereby promoting DC recruitment and maturation, which results in a robust immune response from the host.⁶¹ CD8⁺ cytotoxic T lymphocytes and CD4⁺ helper T lymphocytes play a vital role in this systemic host immune response. Compared to PBS + L group, increased CD8⁺ T infiltration in the spleen was observed with the treatment of Cu₃P + L (1.4-fold) and Cu₃P/1-MT + L (1.6-fold), demonstrated in Figure 5a. CD4⁺ T cells in the spleen were increased in Cu₃P/1-MT + L group higher than in any other group (Figure 5b). The Cu₃P + L and Cu₃P/1-MT + L groups had proportions of CD8⁺ T cells in the primary tumor that was 1.6- and 2.2-fold greater than the PBS + L group, respectively (Figure 5c). As shown in Figure 5d, the Cu₃P/1-MT + L group had a 1.4-fold increase in CD4⁺ T cells at tumor sites compared to the PBS + L group. These results indicated that the synergistic combination of Cu₃P/1-MT NPs and NIR laser could increase the infiltration of T cells systemically. Previous research also supported the inhibition of Treg cells by IDO.⁴⁸ We further explored the population of Treg cells in the tumors post-treatment by flow cytometry (gated on CD4⁺ CD25⁺ Foxp3⁺ T cells, Figure 5e). The Treg population in tumors decreased from ≈29% to ≈25% in Cu₃P/1-MT NPs treated mice. In mice synergistically treated with Cu₃P/1-MT + L, the population of Treg cells decreased to ≈20% (Figure 5f). To investigate further whether immunosuppression in the tumor microenvironment had been reversed in tumor-bearing mice, alternatively

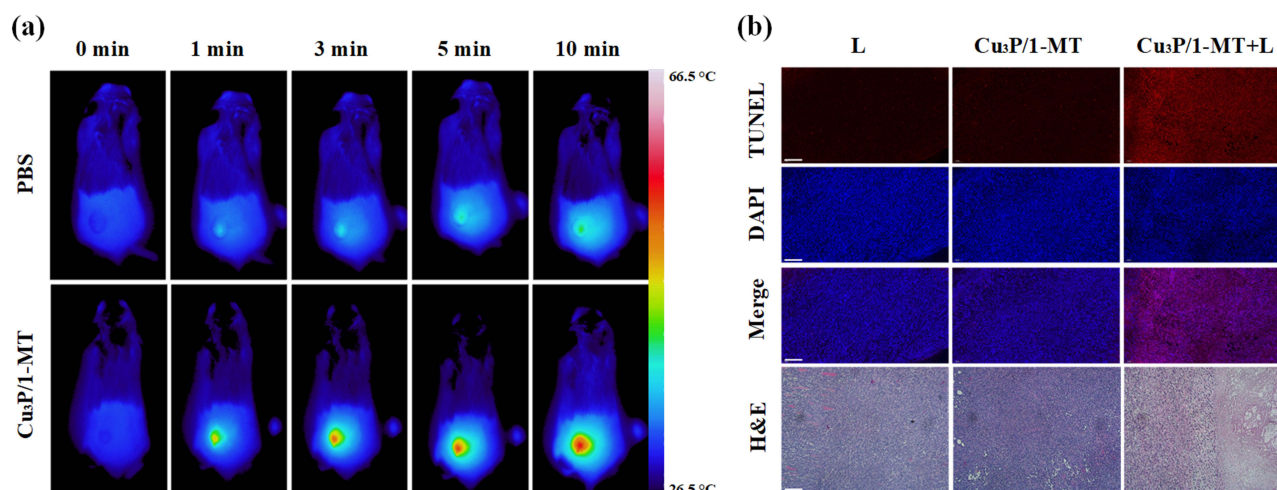


Figure 4 In vivo PTT efficacy. (a) Infrared thermal images of 4T1 tumor-bearing mice under Cu₃P/1-MT NPs and laser irradiation. (b) Representative TUNEL and H&E staining images for the primary tumors. Scale bar = 200 μm.

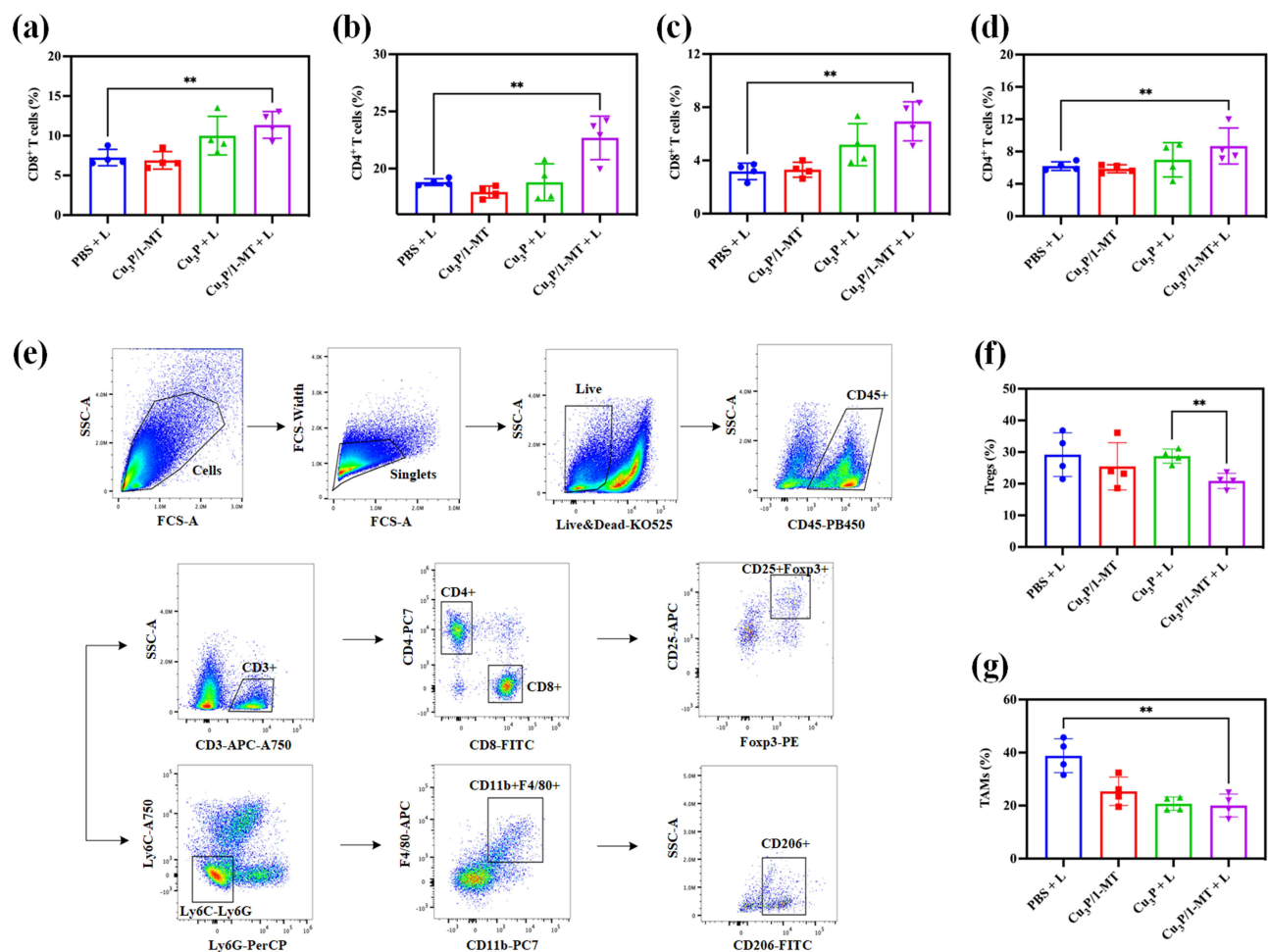


Figure 5 Cu₃P/1-MT NPs-induced immune response. Flow cytometry analyses of spleen CD8⁺ (a) and CD4⁺ (b) T cell subsets (n = 4, **P < 0.01). Intratumoral infiltration of CD8⁺ (c) and CD4⁺ (d) T cells in the primary tumor (n = 4, **P < 0.01). (e) Gating strategy showing the analysis of Treg cells and TAMs subsets from mouse tumors. (f) The level of tumor-infiltrating Treg cells was quantified by gating for CD4⁺CD25⁺Foxp3⁺ cells (n = 4, **P < 0.01). (g) The level of tumor-infiltrating TAMs was quantified by gating for CD11b⁺F4/80⁺CD206⁺ cells (n = 4, **P < 0.01).

Abbreviations: n.s., no significant difference.

activated myeloid (M2) cells (identified by flow cytometry, gated on CD11b⁺ F4/80⁺ CD206⁺ Myeloid-derived suppressor cells, Figure 5e) were isolated from the primary tumor. The decrease of M2 macrophage was found in Cu₃P/1-MT, Cu₃P + L, and Cu₃P/1-MT + L groups (Figure 5g). Cu₃P/1-MT NPs decreased the proportion of M2 macrophages because 1-MT inhibited the recruitment and phagocytic function of macrophages and stimulated the polarization of macrophages towards the M1 state.⁶² The reduction of M2 macrophages in the Cu₃P NPs-based PTT group revealed the transformation of a “cold tumor” into a “hot tumor”. Taken together, these findings demonstrated that combining PTT with an IDO-1 inhibitor could effectively reverse the immunosuppressive microenvironment and enhance the post-ablation immune response, which explains its superior efficacy against tumors. Future research is warranted on the immune system-regulatory effect of photothermal agents with multiple functions.

Antitumor Effect and Side Effects

In light of the promising *in vitro* therapeutic effect of Cu₃P/1-MT NPs, the therapeutic efficacy of this system was evaluated in mice bearing the 4T1 tumor. When the volume of the tumors reached 100 mm³, the mice received intravenous injections of the corresponding drugs. The mice were exposed to the 808 nm NIR laser for 10 minutes at a power density of 0.8 W/cm² for laser irradiation. The volumetric changes of a tumor are a direct indicator of therapeutic efficacy. Increasing tumor volume was observed in the PBS+NIR and Cu₃P/1-MT groups (Figure 6a). In contrast, the tumor in the laser irradiation co-treated mice (Cu₃P + L and Cu₃P/1-MT+L groups) seemed completely eliminated on day 25. Compared to the Cu₃P + L group, mice in the Cu₃P/

1-MT + L group had significantly longer survival times (Figure 6b). It is possible that the combination of PTT and IDO inhibitor (1-MT) could stimulate the body's natural long-term immunity, prevent tumor recurrence, and ultimately increase the individual survival rate. Throughout the 34-day therapeutic period, there were no measurable variations in the mice's body weights

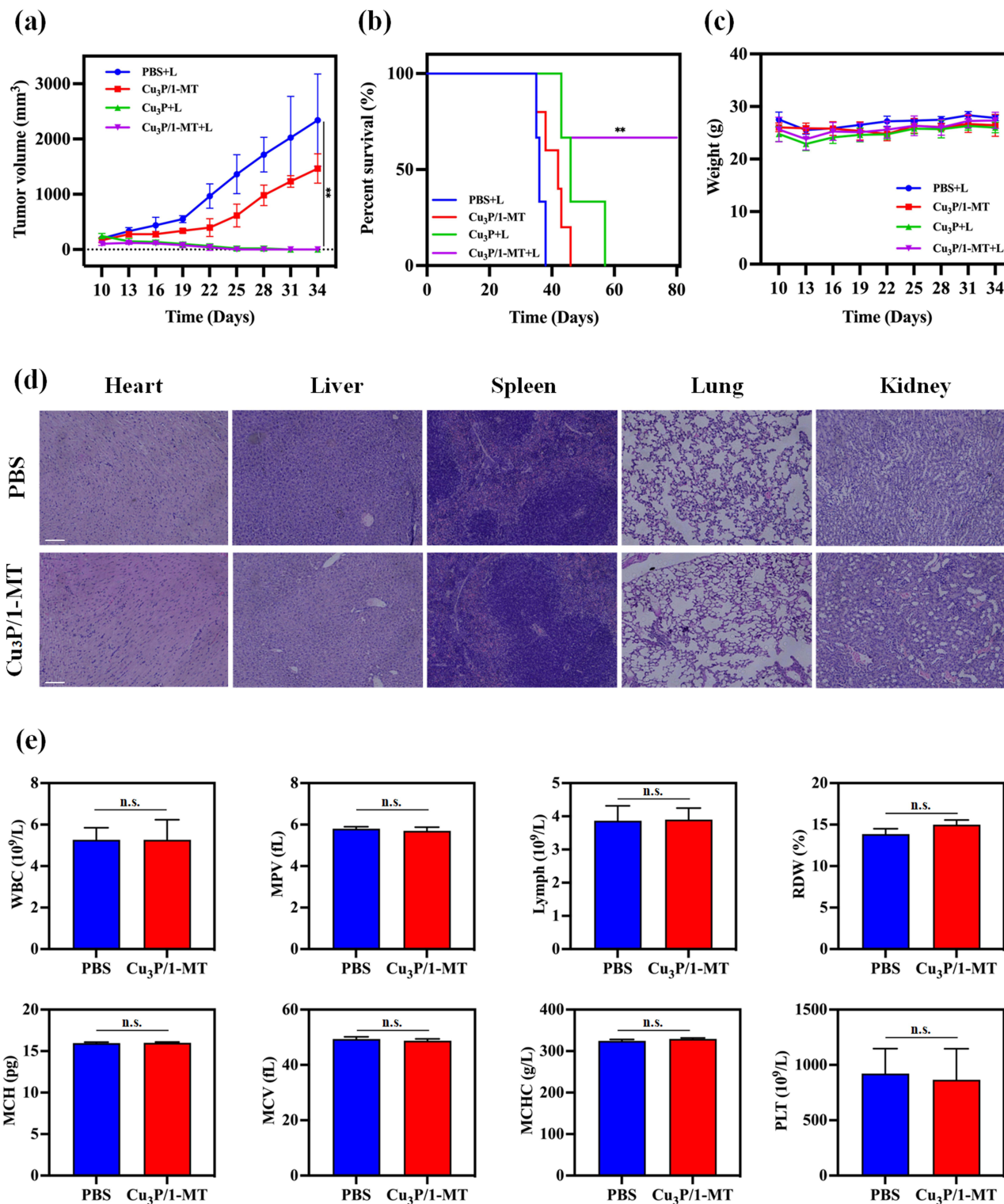


Figure 6 Antitumor effect and side effects. (a) Tumor growth curves of the primary tumor from treated mice (n = 5, **P < 0.01). (b) Survival percentage of mice after modeling (n=5, **P < 0.01). (c) Body weight changes of mice under treatment (n = 5). (d) Histopathological of main organs and (e) Hematological analysis evaluated of systemic toxicity. Scale bar = 200 μm.

Abbreviations: n.s., no significant difference.

(Figure 6c). No obvious damage was observed in the major organs (heart, liver, spleen, lung, and kidney) of mice treated with or without Cu₃P/1-MT NPs (Figure 6d), as determined by H&E staining. Body weight and H&E staining in the treated mice further demonstrated the biosecurity of our therapeutic approach. Blood samples were obtained from the mice to conduct hematological analysis. The analysis of standard hematology markers revealed no statistically significant differences among the various groups (Figure 6e). These findings indicate that the administration of Cu₃P/1-MT NPs and the therapeutic procedure did not result in noticeable infection or inflammation in the mice.

Conclusion

In this study, the IDO-1 pathway inhibitor 1-MT was utilized to develop an NP-containing NIR photothermal agent (Cu₃P/1-MT NPs) so that photothermal therapy and immunotherapy could be implemented synergistically to enhance therapeutic efficacy. In vitro and in vivo, the post-ablation immune response was amplified when treated with Cu₃P NPs and laser irradiation. Specifically, Cu₃P/1-MT NPs significantly enhanced the maturation of DCs and immune cell infiltration at tumor sites while alleviating immunosuppression. Our research suggests a promising cancer treatment strategy to inhibit the growth of primary tumors and prolong individual survival, which will pave the way for the future application of potentiating PTT against various types of solid tumors.

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Disclosure

The authors report no conflicts of interest in this work.

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