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Mitochondria-targeted ROS burst synergistically induces pyroptosis and remodels the immune microenvironment to enhance photodynamic efficacy against colorectal cancer

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Abstract

Over the past decades, the prognosis for patients with advanced colorectal cancer (CRC) remains unsatisfactory, indicating persistent challenges in current treatment modalities. Photodynamic therapy (PDT) has emerged as a promising local treatment option for CRC. However, the efficacy of PDT is severely constrained by tumor hypoxia and an over-reliance on apoptotic pathways, which fail to induce robust immunogenic cell death (ICD). Pyroptosis, a pro-inflammatory form of regulated cell death (RCD) characterized by the release of abundant damage-associated molecular patterns (DAMPs), holds the potential to enhance the ICD effect of PDT and thereby improve its anti-tumor immune efficacy. Notably, emerging evidence suggests that mitochondrial dysfunction may play a key role in enhancing reactive oxygen species (ROS) generation and triggering pyroptosis. In this study, we combined a mitochondria-

targeting strategy with PDT to induce a burst of ROS and effectively trigger pyroptosis. Furthermore, we preliminarily identified the potential involvement of the NF κ B signaling cascade in pyroptosis initiation. In conclusion, these findings provide new theoretical evidence for the role of mitochondria in orchestrating pyroptosis and offer a novel paradigm for precision photo-immunotherapy in CRC.

Keywords: Tumor microenvironment, Photodynamic therapy, Mitochondrial dysfunction, Reactive oxygen species, Pyroptosis

Highlights

- Systemic therapies for CRC cause severe off-target toxicity. The colorectum's endoluminal accessibility makes local PDT an attractive strategy.
- A hybrid organic–inorganic nanoplatform (ZMRT NPs) was engineered to both supply oxygen and target mitochondria, maximizing the PDT-induced ROS burst.
- Photosensitizers capable of inducing pyroptosis are rarely reported. This study integrates a mitochondria-targeting strategy with PDT to achieve pyroptosis via the NF- κ B/NLRP3/GSDMD canonical pathway, thereby amplifying the immunogenic cell death (ICD) response.
- Mitochondrial dysfunction releases mtDNA, activating the STING pathway and reprogramming M2 tumor-associated macrophages toward an M1 phenotype.
- This study demonstrates that ZMRT-based PDT generates systemic antitumor immunity, inhibits metastasis, and establishes long-term immune memory, providing a reference for local PDT or its combination with immunotherapy as an adjuvant strategy for CRC treatment.

1. Introduction

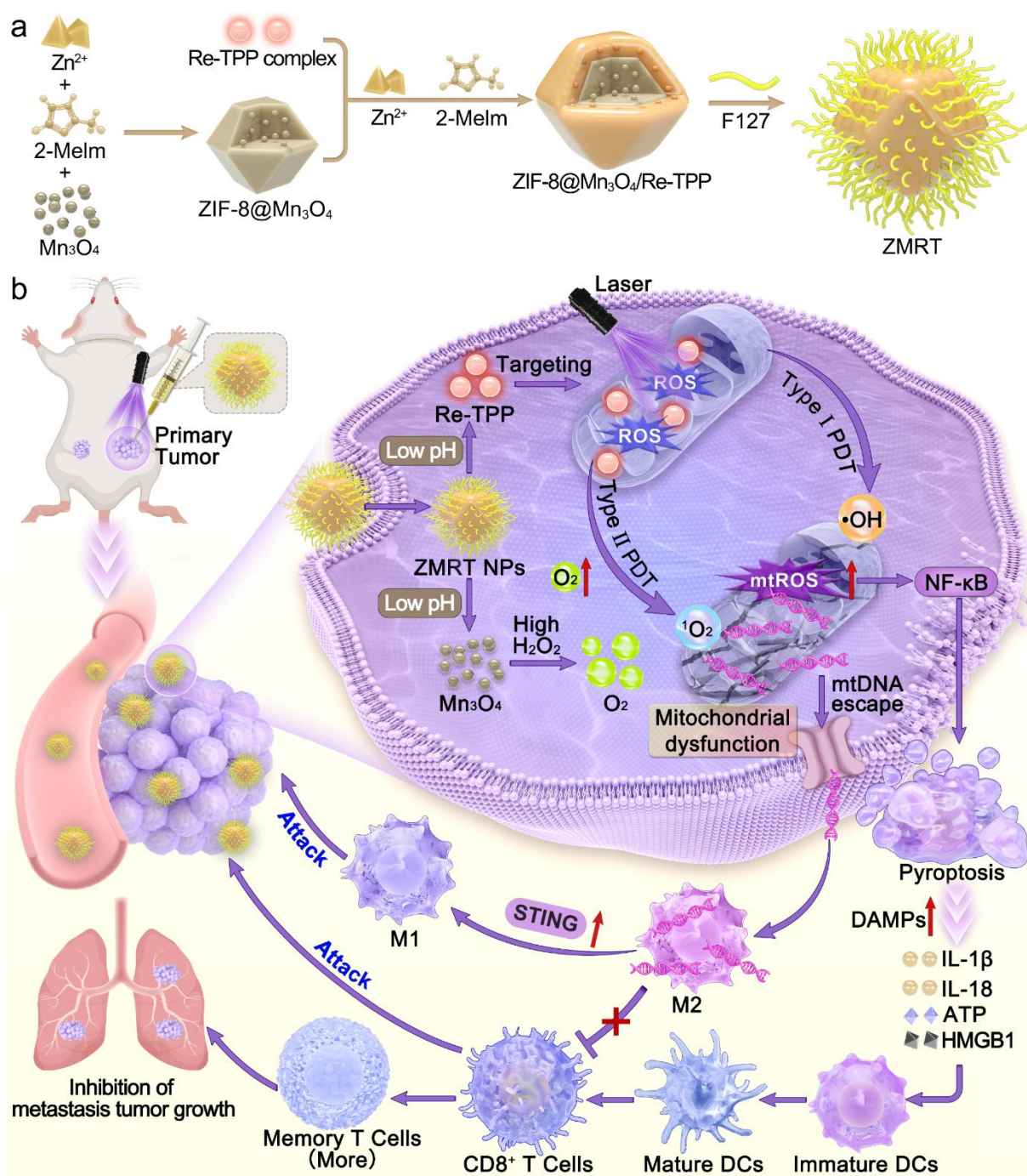
Colorectal cancer (CRC) ranks among the most prevalent and lethal malignancies worldwide, with patients diagnosed at advanced stages facing dismal 5-year survival rates.^{1, 2} Current standard treatments, including surgical resection, chemotherapy, radiotherapy, and targeted therapy, are often accompanied by severe systemic toxicity. Moreover, tumor heterogeneity and drug resistance frequently lead to disease recurrence and metastasis.^{3, 4} For patients with low-lying rectal cancer, the major clinical challenge is to achieve complete tumor eradication while preserving anal function and avoiding a permanent colostomy.⁵ Given the unique endoluminal accessibility of the colorectum—particularly the rectum—developing novel therapeutic strategies that enable precise local tumor ablation, reduce systemic toxicity, and provoke systemic antitumor immunity is of great importance for improving patient outcomes.

In recent years, photodynamic therapy (PDT) has emerged as a promising modality for the local treatment of gastrointestinal tumors, owing to its non-invasive nature, spatiotemporal controllability, and low systemic toxicity.⁶⁻⁸ Clinically, endoscopic PDT has proven to be an effective and tolerable option for recanalizing obstructing, inoperable esophageal cancers.⁹⁻¹¹ Zhu et al. first developed an endoscopic device for local PDT that effectively suppressed the growth of orthotopic colon cancer in mice.¹² Unlike conventional radiotherapy, PDT utilizes a photosensitizer activated by illumination to generate reactive oxygen species (ROS), directly inducing tumor cell death while minimizing damage to surrounding healthy tissues, thus representing a promising alternative for cancer therapy.¹³⁻¹⁵ However, the clinical efficacy of PDT is severely constrained by the hypoxic tumor microenvironment characteristic of solid tumors, which limits ROS production efficiency.^{16, 17} Furthermore, conventional PDT primarily relies on the apoptotic pathway and fails to induce robust immunogenic cell death (ICD), thereby limiting its anti-tumor immune effects.^{13, 18} This dilemma suggests that activating the apoptotic pathway alone is insufficient to awaken systemic anti-tumor immunity, and thus there is an urgent need to develop new PDT modalities that can overcome the hypoxia barrier and trigger stronger inflammatory forms of cell death.

Pyroptosis is a distinct form of regulated cell death (RCD) characterized by the release of abundant damage-associated molecular patterns (DAMPs), endowing it with a significantly stronger immune activation capacity than apoptosis.^{13, 19, 20} However, photosensitizers capable of initiating pyroptosis remain rarely reported, with pyroptosis induction being largely limited to a few chemotherapeutic agents.²¹⁻²³ Recent studies have highlighted mitochondrial

dysfunction as a key event in enhancing ROS generation and triggering inflammatory cell death.^{24, 25} A mitochondria-targeted photodynamic strategy can induce a burst of mitochondrial ROS, potentially triggering non-apoptotic RCD pathways, thereby activating antitumor immunity and converting "cold" tumors into "hot" ones.^{13, 26, 27} Based on these observations, we hypothesized that integrating a photosensitizer with a mitochondria-targeting strategy could overcome the limitation that photosensitizers alone rarely induce pyroptosis, and that inducing mitochondrial dysfunction might enable pyroptosis initiation, thereby enhancing the anti-tumor immune efficacy of PDT.

In this study, we demonstrate that a mitochondria-targeted ROS-generating strategy effectively overcomes intratumoral hypoxia and induces pyroptosis, significantly enhancing both the local ablation and anti-tumor immune efficacy of PDT. In our system (**Scheme 1a**), Mn_3O_4 acts as a nanozyme that catalyzes the conversion of endogenous H_2O_2 into O_2 to alleviate tumor hypoxia and generates hydroxyl radicals ($\bullet\text{OH}$) through a Fenton-like reaction.^{28, 29} Simultaneously, the triphenylphosphine (TPP) moiety directs a rhenium(I) complex to the mitochondria, where laser irradiation triggers synergistic Type I and Type II ROS generation, amplifying intracellular oxidative stress and promoting pyroptosis (**Scheme 1b**). Furthermore, we preliminarily explored the potential regulatory mechanism of the NF κ B signaling cascade in pyroptosis initiation. Finally, through a series of in vitro and in vivo experiments, this study elucidates the enhanced potential of a mitochondria-targeted ROS-generating strategy in PDT-induced pyroptosis, offering a new paradigm for precision photo-immunotherapy for colorectal cancer.



Scheme 1. Mitochondria-targeted photodynamic therapy induces pyroptosis and remodels the immune microenvironment to enhance antitumor immunity against colorectal cancer. (a) Synthetic procedure for the preparation of the mitochondria-targeted photodynamic nano-system incorporating Mn_3O_4 nanozyme and TPP-modified rhenium complex. (b) Proposed mechanism: laser irradiation induces mitochondrial ROS burst, triggering pyroptosis and ICD, which remodels the immune microenvironment to suppress metastasis and establish long-term immune memory.

2. Materials and methods

2.1. Materials

Rhenium(I) pentacarbonyl chloride ($[\text{Re}(\text{CO})_5\text{Cl}]$), Triphenyl (2-pyridylmethyl) phosphonium chloride hydrochloride, sodium carbonate, Zinc nitrate hexahydrate ($[\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}]$) and Polyvinylpyrrolidone (PVP) were purchased from Aladdin (China). 2,2'-bipyridine-5,5'-dicarboxylic acid was purchased from Meryer (China). Silver nitrate was purchased from Xihua (China). Manganese acetate tetrahydrate ($[\text{Mn}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}]$) and 2-methylimidazole were purchased from Bidepharm (China). Indocyanine Green (ICG) and Polyethylene-polypropylene glycol F127 were purchased from Macklin (China). The light source was UVLED surface light source irradiation under 425 nm (HTLD-4II-FB130100-NL, Shenzhen Hainedie). RPMI-1640 medium, 0.25% trypsin-EDTA, and fetal bovine serum (FBS) were purchased from Gibco (USA). 2-(4-Amidinophenyl)-6-indolecarbamide dihydrochloride (DAPI), mitochondrial membrane potential assay kit (JC-1), Enhanced ATP Assay Kit, TUNEL Apoptosis Assay Kit, LDH Cytotoxicity Assay Kit and Reactive Oxygen Species Assay Kit were obtained from Beyotime Inst. Biotech (Shanghai, China). Calcein-AM/PI Assay Kit was obtained from Solarbio (Beijing, China). 1,3-diphenylisobenzofuran (DPBF) was obtained from MCE (Shanghai, China). MitoSOXTM Red Mitochondrial Superoxide Indicator, Mito Tracker Green FM and Mito Tracker Deep Red FM were purchased from Invitrogen (USA). All chemical agents were of analytical grade and were used directly with no further purification.

2.2. Methods

2.2.1. Original Re complex

$\text{Re}(\text{CO})_5\text{Cl}$ (200 mg, 0.55 mmol) and 2,2'-bipyridine-5,5'-dicarboxylic acid (130 mg, 0.53 mmol) were dissolved in a mixture of toluene (50 mL) and methanol (20 mL) in a 100 mL round-bottom flask. The reaction was carried out at 85 °C under reflux for 3 h with stirring, yielding a deep orange-red solution. After cooling and standing at 4 °C overnight, the mixture was centrifuged to remove insoluble solids. The solvent then was removed, and the product was dried under vacuum at 60 °C to obtain the orange-red powder. ¹H NMR (400 MHz, *d*₆-DMSO): δ 9.34 (s, 2H), 8.99 (d, 2H, *J* = 8.8 Hz), 8.76 (d, 2H, *J* = 8.5 Hz).

2.2.2. TPP Ligand

The compound was synthesized following a literature method with modifications. Triphenyl (2-pyridylmethyl) phosphonium chloride hydrochloride (159 mg, 0.37 mmol) and sodium carbonate (19.9 mg, 0.19 mmol) were added into a 25 mL vial and stirred in 2 mL of pure water

for 1 h at room temperature. After removing water by vacuum drying, the residue was dissolved in 5 mL of ethanol and filtered. The filtrate was concentrated under reduced pressure to remove ethanol and further dried under vacuum at 60 °C overnight, finally obtaining the white powder. ^1H NMR (400 MHz, d_6 -DMSO): δ 8.34 (d, 1H, J = 5.1 Hz), 7.88–7.68 (m, 16H), 7.34 (d, 1H, J = 7.9 Hz), 7.25 (t, 1H, J = 6.0 Hz), 5.48 (d, 2H, J = 15.6 Hz).

2.2.3. Re-TPP complex

The Re-TPP complex was synthesized based on a modified literature method. Original Re complex (33.8 mg, 0.0615 mmol) and silver nitrate (10.4 mg, 0.0615 mmol) were dissolved in 3 mL of DMF and kept stirring at 80 °C for 4 h in the dark. After cooling, the mixture was centrifuged to remove insoluble solids, and the yielding filtrate a. Separately, TPP ligand (23.5 mg, 0.0615 mmol) and silver nitrate (10.4 mg, 0.0615 mmol) were dissolved in 2 mL of DMF and stirred at room temperature for 2 h in the dark. After centrifugation, the filtrate b was collected. Then, filtrate a and b were mixed and kept stirring at 65 °C for 24 h in the dark. The solution was later cooled, centrifuged, and the filtrate was concentrated to an oily residue. Approximately 10 mL of water was then added to precipitate the orange-yellow solid, which was collected by centrifugation, washed twice with an ethanol-water mixture (v/v = 1:5), and dried under vacuum at 60 °C overnight to obtain the orange-yellow powder. ^1H NMR (400 MHz, d_6 -DMSO): δ 9.33 (m, 2H), 8.75 (m, 2H), 8.62 (m, 2H), 8.33 (d, 1H, J = 4.8 Hz), 7.89–7.66 (m, 16H), 7.32 (d, 1H, J = 8.0 Hz), 7.24 (t, 1H, J = 6.2 Hz), 5.45 (d, 2H, J = 15.6 Hz).

2.2.4. Mn_3O_4 nanoparticles

The synthesis of Mn_3O_4 nanoparticles was adapted from a reported method. Briefly, $\text{Mn}(\text{OAc})_2 \cdot 4\text{H}_2\text{O}$ (409 mg, 1.67 mmol) was dissolved in 14 mL of anhydrous ethanol under ultrasonication and stirred for 1 h at room temperature. The mixture was then transferred to a 50 mL Teflon-lined autoclave and heated at 120 °C for 24 h. After cooling to room temperature, the precipitate was collected by centrifugation and washed once each with 5 mL of ethanol, water, and ethanol, respectively. The resulting precipitate was redispersed in 2 mL of methanol. Subsequently, polyvinylpyrrolidone (160 mg) was dissolved in 16 mL of methanol, mixed with the 2 mL of Mn_3O_4 methanol dispersion, and stirred overnight at room temperature. The final product was collected by centrifugation, redispersed in 2 mL of methanol, and stored as the Mn_3O_4 nanoparticle methanol solution.

2.2.5. ZIF-8@ Mn_3O_4

$\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (150 mg, 0.50 mmol) was dissolved in 3 mL of methanol and mixed with 250 μL of the as-prepared Mn_3O_4 methanol solution under stirring at room temperature for 0.5 h. Then, a solution of 2-methylimidazole (330 mg, 4.02 mmol) in 5 mL of methanol was added dropwise to the mixture and stirred for another 1.5 h at room temperature. The precipitate was collected by centrifugation, washed twice with 3 mL of methanol, and dried under vacuum at 60 °C to obtain the brown powder.

2.2.6. ZIF-8@ Mn_3O_4 /Re-TPP

ZIF-8@ Mn_3O_4 (18 mg) was dispersed in 1 mL of methanol by ultrasonication. Separately, 1.5 mg of the Re-TPP complex was dissolved in 2 mL of a DMSO-methanol mixture (v/v = 1:3) under sonication. This solution was then added dropwise to the ZIF-8@ Mn_3O_4 dispersion and stirred at room temperature for 20 minutes to obtain the solution a. Subsequently, $\text{Zn}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (50 mg) dissolved in 1 mL of methanol was added dropwise to the solution a and stirred for another 20 minutes, yielding the solution b. 2-methylimidazole (400 mg in 3 mL of water) was then introduced into the solution b and stirred for 1.5 h at room temperature. The resulting precipitate was collected by centrifugation, washed twice with 4 mL of methanol, and dried under vacuum at 60 °C to obtain the light-yellow powder. For ICG-loaded samples, 0.3 mg of ICG was co-dissolved with 1.5 mg of Re-TPP in the DMSO-methanol mixture (v/v = 1:3), and the subsequent steps remained identical, except that the final product was dried at room temperature in the dark.

2.2.7. ZIF-8@ Mn_3O_4 /Re

The procedure was identical to that described above, except that the Re-TPP complex was replaced with the original Re complex (4.5 mg, dissolved in 2 mL of methanol under ultrasonication). The light-yellow powder was obtained finally.

2.2.8. ZIF-8^{F127}@ Mn_3O_4 /Re-TPP (ZMRT)

The synthesis of ZMRT was adapted from a literature method with modifications. Briefly, 30 mg of ZIF-8@ Mn_3O_4 /Re-TPP was dispersed in 10 mL of water by ultrasonication. Separately, 50 mg of Pluronic F127 was dissolved in 10 mL of water under sonication and then added dropwise to the above dispersion. The mixture was stirred at room temperature for 12 h. The resulting precipitate was collected by centrifugation, washed twice with 5 mL of water, and dried under vacuum at 60 °C to obtain ZMRT as the light-yellow powder.

2.2.9. ZIF-8^{F127}@Mn₃O₄/Re (ZMR)

The procedure was identical to that described above, except that 30 mg of ZIF-8@Mn₃O₄/Re-TPP was replaced with 30 mg of ZIF-8@Mn₃O₄/Re. The light-yellow powder, denoted as ZMR, was obtained.

2.2.10. Cell lines and animals

Human umbilical vein endothelial cells (HUVECs, Cat. No.: FH1122) were purchased from Shanghai FuHeng Biological Co., LTD. Mouse colon carcinoma cells (CT26, Cat. No.: CL-0071) were purchased from Procell Life Science & Technology Co., LTD. These cells were authenticated using short tandem repeat (STR) profiling and tested negative for mycoplasma contamination. All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin, and incubated at 37 °C in a humidified atmosphere containing 5% CO₂. Female BALB/c mice (6–8 weeks old, 18–20 g) were purchased from GemPharmatech Co., Ltd. (Jiangsu, China). The animals were housed under specific pathogen-free (SPF) conditions at 21 ± 2 °C and 40%–70% relative humidity, with a 12 h light/dark cycle. This study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University.

2.2.11. ROS Detection

ROS generation in CT26 cells was evaluated using DCFH-DA (Beyotime, S0033M) and DPBF (MCE, 5471-63-6) probes. Cells were seeded in 24-well plates and cultured to ≈70% confluency before 24-hour treatment with PBS, PBS+L, ZMR, ZMR+L, ZMRT, or ZMRT+L (all at 30 µg/mL). After staining according to manufacturer instructions, ROS levels were assessed by fluorescence imaging and flow cytometry.

2.2.12. Detection of mitochondrial superoxide (mtROS)

CT26 cells were seeded and cultured at 37 °C for 24 h, followed by treatment with PBS, ZMR, or ZMRT solutions under both irradiated and non-irradiated conditions. Firstly, the 5 mM MitoSOX™ (Thermo Fisher Scientific, M36008) Red stock solution was diluted with culture medium to prepare a 5 µM working solution. One milliliter of the diluted working solution was added to each confocal dish per group, followed by incubation at 37 °C for 10 minutes. After incubation, the cells were washed three times with pre-warmed PBS (37 °C) to remove extracellular MitoSOX™ Red. An appropriate amount of pre-warmed culture medium (37 °C) containing MitoTracker probe (Thermo Fisher Scientific, M7514-1) was then added to fully

cover the cells, and incubation was continued for 30 minutes under specific growth conditions. The probe-containing supernatant was aspirated, and without further washing, fresh culture medium was directly added. The cells were immediately observed under CLSM.

2.2.13. Pyroptosis Assays

CT26 cells were seeded on glass slides in 6-well plates and cultured to $\approx 70\%$ confluency. After 24-hour treatment with PBS, PBS+L, ZMR, ZMR+L, ZMRT, or ZMRT+L (all at 30 $\mu\text{g/mL}$), cellular morphology was initially examined by phase-contrast microscopy. Upon observation of pyroptotic features in experimental groups, cells were harvested for molecular analysis. Total RNA and protein were extracted to assess expression of pyroptosis-related markers via RT-qPCR and Western blot. Culture supernatants were collected to measure IL-1 β and IL-18 levels by ELISA. Ultrastructural changes indicative of pyroptosis were further examined by TEM.

2.2.14. Detection of immunogenic cell death

CT26 cells were cultured at 37 °C for 24 h and then treated with PBS, ZMR, or ZMRT under light and dark conditions. After 24 hours, cells were collected for analysis. Expression of CRT, HMGB1, and HSP70 was assessed by Western blot, flow cytometry (Fc), and confocal microscopy (CLSM). Extracellular HMGB1 and ATP levels were quantified using ELISA and an Enhanced ATP Assay Kit (Beyotime, S0027), respectively.

2.2.15. In Vivo Anti-Tumor Effects

Antitumor efficacy and safety were evaluated in a CT26 subcutaneous tumor model. Female BALB/c mice were inoculated with CT26 cells on the right dorsal flank to establish primary tumors, followed by inoculation of the same number of cells on the left flank three days later to simulate distant tumors. The right-flank tumors were used to evaluate photodynamic therapy efficacy, while the left-flank tumors assessed systemic immune responses. When right-flank tumor volumes reached $\approx 100 \text{ mm}^3$ on day 7, mice were randomized into five groups: PBS, ZMR, ZMR+L, ZMRT, or ZMRT+L. Intratumoral injections (5 mg/kg in 50 μL) were administered on days 1, 3, and 5. Six hours post-injection, tumors were irradiated with a 425 nm laser (20 mW/cm ^{2}) for 60 min. Body weight and tumor volume ($V = \text{width}^2 \times \text{length} / 2$) were measured every two days. On day 12, all mice were euthanized, and tumors and major organs (heart, liver, spleen, lungs, kidneys) were collected for H&E staining and further analysis. All animal experimental protocols were approved by the Laboratory Animal

Welfare and Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (Approval No. SYSU-2023-334).

2.2.16. Antitumor immunity in vivo

To evaluate antitumor immune responses in vivo, immune cell populations were analyzed via flow cytometry. Primary and distant tumors were harvested from mice ($n = 3$), digested into single cells using collagenase IV (1 mg/mL) and DNase I (0.1 mg/mL) for 1 h at 37 °C, filtered through a 70 μ m strainer, and treated with erythrocyte lysis buffer. For macrophage and T cell analysis, cells were stained with anti-CD45-V500 (BD, 561487), anti-CD3-FITC (BD, 553061), anti-CD4-APC-Cy7 (Biolegend, 100526), anti-CD8-APC (Biolegend, 100712), anti-CD11b-BV421 (Biolegend, 101236), anti-F4/80-APC (Biolegend, 123116), anti-CD86-PC7 (Biolegend, 159208), and anti-CD206-PE (Biolegend, 141706). Single-cell suspensions from lymph nodes and spleens were stained with anti-CD45-V500 (BD, 561487), anti-CD3-FITC (BD, 553061), anti-CD4-APC-Cy7 (Biolegend, 100526), anti-CD8-APC (Biolegend, 100712), anti-CD11c-PC5.5 (BD, 560584), anti-CD80-PE (BD, 553769), and anti-CD86-PC7 (Biolegend, 159208).

2.2.17. Statistical Analysis

Data are presented as mean $\pm$ standard deviation (SD). Statistical differences between two groups were analyzed using two-tailed unpaired Student's t-tests. For comparisons involving more than two groups, one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was applied. All statistical analyses were performed using GraphPad Prism version 9. A p-value less than 0.05 was considered statistically significant, with significance levels denoted as follows: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

3. Results and Discussion

3.1. Synthesis and Characterization of ZMRT NPs

ZMRT NPs were constructed by integrating the metal–organic framework ZIF-8, Mn_3O_4 nanoparticles, a rhenium (Re)-based photosensitizer, and the mitochondrial targeting ligand triphenylphosphonium (TPP). A pyridine-based ligand bearing a TPP cation was introduced to modify the parent Re complex, yielding the mitochondria-targeted Re-TPP conjugate (**Figure S1**).³⁰ Fourier transform infrared (FTIR) spectroscopy confirmed the successful synthesis of Re-TPP. The spectrum of ZMRT NPs displayed characteristic P–C stretching vibrations corresponding to the TPP moiety at 1471 and 1437 cm^{-1} , along with hallmark TPP absorptions in the 2800–3700 cm^{-1} region (orange region).³¹ Additionally, the signature C=O stretching vibrations corresponding to the original Re carbonyl complex persisted (red region) (**Figure S2**).³² Photoluminescence spectra of Re-TPP and Re in aqueous solution (0.2 mM) showed maximum emission peaks at 662 and 722 nm, respectively (**Figure S3**). The observed red shift is likely attributable to coordination between the TPP group and the Re center.

Following the successful synthesis of Re-TPP, ZIF-8-based composites were fabricated by integrating Mn_3O_4 nanoparticles and Re complexes using a coordination-assisted secondary assembly strategy previously established by our group.^{33–35} Mn_3O_4 was first encapsulated in situ within ZIF-8 to obtain ZIF-8@ Mn_3O_4 . Re or Re-TPP complexes were subsequently loaded onto the external surface, followed by in situ growth of a secondary ZIF-8 shell, yielding core–shell structured ZIF-8@ Mn_3O_4 /Re and ZIF-8@ Mn_3O_4 /Re-TPP. Surface modification with Pluronic F127 enhanced aqueous dispersibility and biocompatibility,³⁶ affording the final ZIF-8^{F127}@ Mn_3O_4 /Re (ZMR) and ZIF-8^{F127}@ Mn_3O_4 /Re-TPP (ZMRT) composites (**Figure 1a**). X-ray diffraction (XRD) patterns of ZMR and ZMRT exhibited diffraction peaks consistent with pristine ZIF-8 (**Figure 1b**), while FTIR spectra confirmed the presence of C=O stretching vibrations corresponding to Re carbonyl complexes (orange region) (**Figure 1c**). UV-vis diffuse reflectance spectroscopy revealed Re-based metal–ligand charge transfer absorption at approximately 400 nm (orange region) and the absorption edge of Mn_3O_4 (red region),³⁷ confirming the successful integration of both components (**Figure 1d**). Transmission electron microscopy (TEM) revealed that ZMRT possessed a size of approximately 200 nm with visible Mn_3O_4 encapsulation (**Figure 1e**). Energy-dispersive X-ray spectroscopy (EDS) element mapping indicated uniform distribution of Zn and Re, while Mn was localized within Mn_3O_4 domains (**Figure 1f**). X-ray photoelectron spectroscopy (XPS) confirmed the presence of Mn and Re in ZMRT, with Mn exhibiting mixed +2/+3 oxidation states consistent with Mn_3O_4

(Figure 1g-i).³⁸ Inductively coupled plasma optical emission spectroscopy (ICP-OES) revealed Re and Mn loadings of 0.39 wt% and 0.96 wt%, respectively, in ZMR, and 0.41 wt% and 0.90 wt%, respectively, in ZMRT, indicating comparable elemental compositions between the two composites (Figure 1j).

The acidic tumor microenvironment (TME) triggers degradation of ZIF-8 nanoparticles, enabling controlled release of therapeutic payloads for enhanced antitumor efficacy.³⁹ As shown in Figure 1k, Re release was progressively enhanced under acidic conditions (pH 6.0), with >80% of the payload released within 24 h. These results demonstrate that Re release is governed by environmental acidity, confirming the pH-responsive behavior of ZMRT NPs. Both free Mn₃O₄ NPs and ZMRT NPs exhibited potent H₂O₂ decomposition activity. Using a portable dissolved oxygen meter, the generated bubbles were confirmed to be O₂. Real-time O₂ concentration increased rapidly upon addition of Mn₃O₄ or ZIF-8@Mn₃O₄/Re-TPP to H₂O₂ solution, whereas ZIF-8@Re-TPP showed negligible O₂ production (Figure 1l). As shown in Figure S4, ZIF-8@Mn₃O₄ (1 mg/mL) generated O₂ bubbles in 0.5% H₂O₂ aqueous solution, demonstrating that Mn₃O₄ NPs possess catalase-like activity, efficiently decomposing H₂O₂ to generate O₂, consistent with previous reports.^{40, 41}

The photodynamic performance of ZMRT NPs was evaluated by quantifying ROS generation under 425 nm laser irradiation using the ROS probe 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA).⁴² As shown in Figures 1m and S5, the irradiated groups (ZMR+L and ZMRT+L) exhibited significantly enhanced DCF fluorescence intensity at 524 nm, with an approximately 87-fold increase after 180s of irradiation, whereas non-irradiated groups showed minimal fluorescence. These findings confirm their exceptional ROS generation capability and indicate that the TPP moiety does not compromise the photochemical performance of Re. The types of ROS generated were further identified using the hydroxyl radical (•OH) fluorescent probe HPF and the singlet oxygen (¹O₂) absorption probe ABDA.⁴³⁻⁴⁵ As shown in Figures 1n and S6, both ZMR+L and ZMRT+L groups exhibited approximately 10-fold enhancement in HPF fluorescence intensity at 515 nm following 180s of laser irradiation, confirming Type I ROS generation. Meanwhile, ABDA absorbance at 402 nm decreased by approximately 60% after 180s of irradiation, confirming Type II ROS generation (Figures 1o and S7).⁴⁶ Collectively, these findings establish ZMRT NPs as a dual-type nano-photosensitizer with exceptional photodynamic performance, capable of effectively mitigating tumor hypoxia and enhancing therapeutic outcomes.

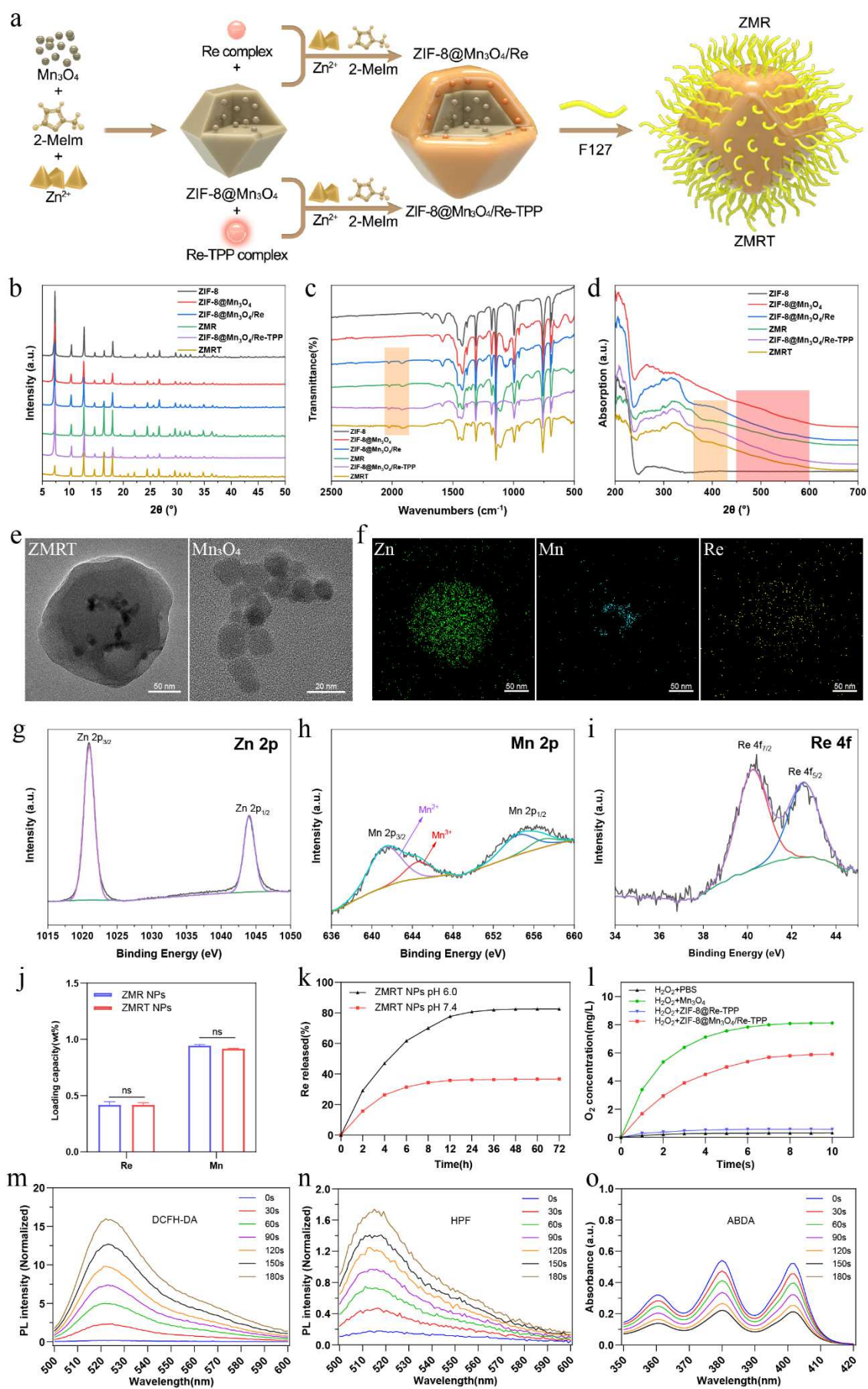


Figure 1. Preparation and characterizations of ZMRT NPs. (a) Schematic illustration of the assembly of ZMR and ZMRT. (b) X-ray diffraction (XRD) pattern, (c) Fourier transform infrared spectra, and (d) UV-vis absorption spectra of ZIF-8 composites. (e) Transmission electron microscopy (TEM) images of ZMRT NPs and Mn_3O_4 NPs. (f) Energy spectrum analysis of ZMRT NPs. High-resolution X-ray photoelectron spectroscopy (XPS) scans of (g) Zn 2p, (h) Mn 2p, and (i) Re 4f in ZMRT NPs. (j) Loading capacity of Mn and Re in ZMR and ZMRT. (k) Release rates of Re under different pH conditions (6.0 vs. 7.4). (l) Generation of O_2 after reaction with different NPs in H_2O_2 solution. Total ROS, $\cdot\text{OH}$, and $^1\text{O}_2$ produced by ZMRT NPs (30 $\mu\text{g/mL}$) were detected using (m) DCFH-DA, (n) HPF, and (o) ABDA sensors, respectively, under irradiation conditions at 425 nm (20 mW cm^{-2}).

3.2. *In Vitro* Photo-Controlled Activation and Cytotoxicity of ZMRT NPs

Efficient cellular uptake is critical for photodynamic efficacy. Confocal laser scanning microscopy (CLSM) revealed time-dependent internalization of ICG-labeled ZMRT NPs in CT26 cells, reaching saturation at 6-8 h of incubation (**Figure 2a**). Flow cytometric (Fc) analysis confirmed a 90% uptake rate at 6 h (Figure S8). Accordingly, a 6-hour incubation period was adopted for all subsequent *in vitro* PDT studies.

The pH-responsive property of nanomaterials was strategically exploited for tumor-selective targeting to minimize off-target effects and damage to healthy tissues.⁴⁷ Dark toxicity assays showed >70% viability in HUVECs even at 50 $\mu\text{g/mL}$ after 24 h (Figure 2b), whereas CT26 cell viability decreased to ~50% under the same conditions (Figure 2c), likely due to enhanced payload release in the acidic tumor microenvironment, confirming the favorable biosafety profile of ZMRT. Upon 425 nm laser irradiation, PBS+L caused negligible cytotoxicity, while ZMR+L and ZMRT+L induced concentration-dependent killing of CT26 cells. Notably, ZMRT+L (30 $\mu\text{g/mL}$) reduced viability to 40%, outperforming ZMR+L (60%), underscoring the therapeutic benefit of mitochondrial targeting (Figure 2d). Live/dead staining revealed the highest cell death in the ZMRT+L group (Figure 2e), and Fc analysis showed a 60.1% apoptosis rate (Figure 2h,i), consistent with Cell Counting Kit-8 results. Collectively, these findings demonstrate the low off-target toxicity and potent antitumor efficacy of ZMRT NPs against CT26 cells.

The capacity of ZMRT NPs to generate total ROS and $^1\text{O}_2$ at the cellular level was further validated using DCFH-DA and $^1\text{O}_2$ -specific probes. As shown in Figures 2f and S9, CT26 cells

treated with ZMRT+L exhibited significantly stronger fluorescence signals than ZMR+L-treated cells, with negligible signals in control groups. This enhancement is attributed to the mitochondria-targeting ability of ZMRT, which facilitates ROS generation at the primary organelle site.^{48, 49} Fc quantification of total ROS and $^1\text{O}_2$ levels corroborated the fluorescence imaging data (Figures 2g and S10). Overall, ZMRT NPs leverage mitochondria-targeted ROS generation to achieve significantly enhanced PDT efficacy.

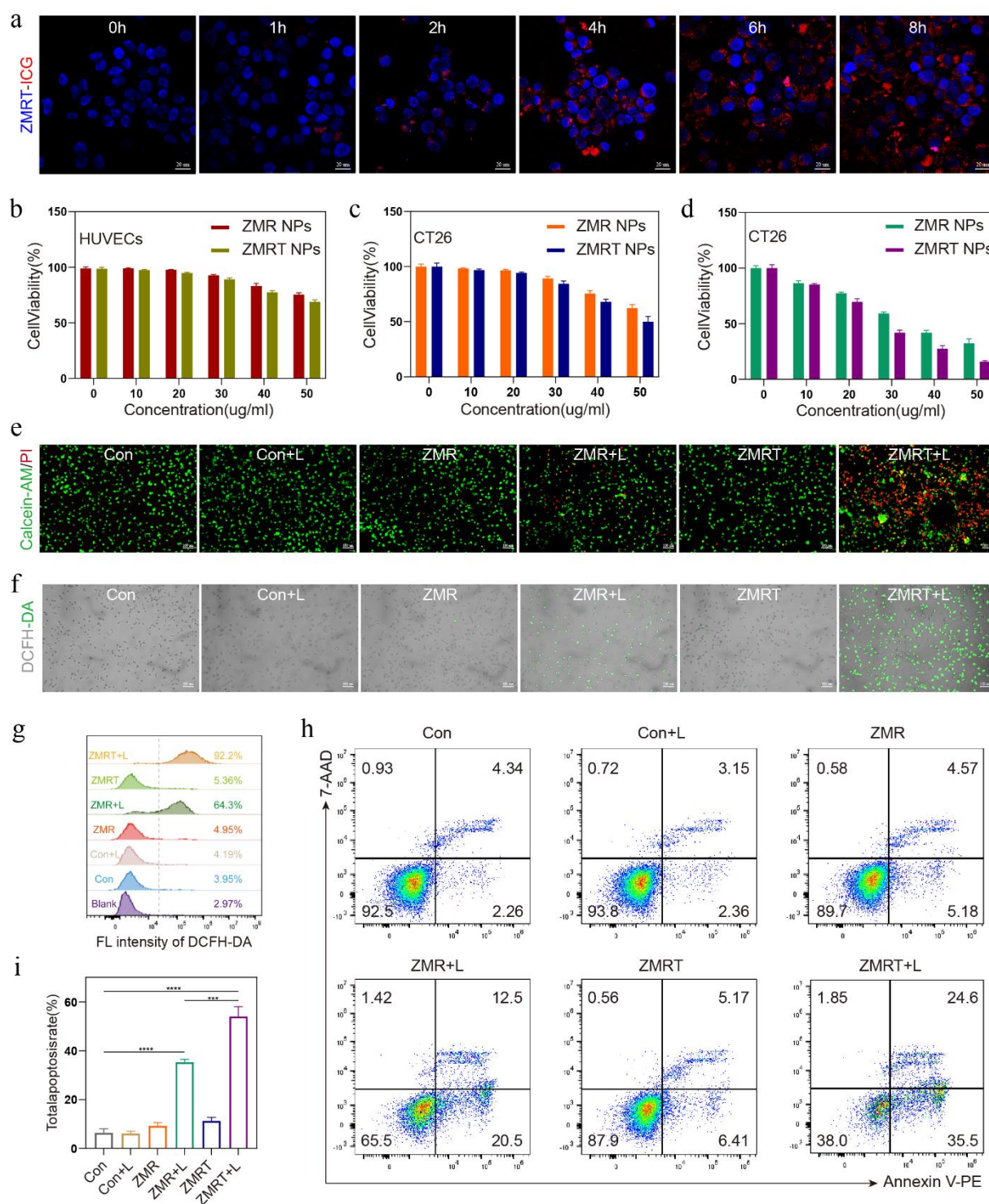


Figure 2. *In vitro* biocompatibility and antitumor efficacy of ZMRT NPs. (a) Cellular uptake of indocyanine green (ICG)-labeled ZMRT NPs in CT26 cells at various incubation time points. Scale bar: 20 μm . Viability of (b) human umbilical vein endothelial cells (HUVECs) and (c) CT26 cells following treatment with ZMR or ZMRT at varying concentrations ($n = 6$). (d) Photodynamic cytotoxicity of ZMR and ZMRT toward CT26 cells under 425 nm irradiation (20 mW cm^{-2} , 10 min) ($n = 6$). (e) Live/dead staining of CT26 cells following treatments with PBS, ZMR, or ZMRT in the presence or absence of 425 nm laser irradiation (20 mW cm^{-2}). Scale bar: 100 μm . (f) Fluorescence images and (g) flow cytometry histograms of total reactive oxygen species (ROS) levels detected by fluorescent probes. Scale bar: 100 μm . (h) Representative flow cytometry plots and (i) quantitative analysis of total apoptotic rates across treatment groups ($n = 6$). Statistical analysis was performed using one-way analysis of variance ($***p < 0.001$ and $****p < 0.0001$).

3.3. Mitochondrial Dysfunction-Mediated mtDNA Release and STING Pathway Activation

Mitochondria are the primary sites of intracellular ROS production, and their dysfunction directly disrupts ROS homeostasis.^{24, 49, 50} The mitochondria-targeting efficiency of ZMRT in CT26 cells was assessed by confocal microscopy-based co-localization analysis. ICG-labeled ZMRT exhibited significant overlap with MitoTracker-stained mitochondria, yielding prominent yellow fluorescence in merged images, whereas ZMR showed minimal co-localization (**Figure 3a**). Pearson's correlation coefficients quantified by ImageJ confirmed superior mitochondrial targeting of ZMRT ($R = 0.923$) compared to ZMR ($R = 0.007$) (Figure 3b), validating the successful synthesis of a mitochondria-targeted nano-photosensitizer.³²

We next assessed whether ZMRT NPs induce mitochondrial ROS (mtROS) production and subsequent mitochondrial dysfunction. Mitochondrial superoxide generation was detected using MitoSOX Red, with mitochondria labeled by MitoTracker Green FM. Confocal imaging revealed bright red fluorescence within mitochondria of ZMRT+L-treated CT26 cells (Figure S11),⁵¹ and flow cytometry quantified a significantly increased fluorescence intensity ratio (55.8%) in the ZMRT+L group, substantially exceeding that of ZMR+L and all other treatment groups (Figure 3c). These results confirm that ZMRT+L treatment induces substantial mtROS production via mitochondrial oxidative stress. Mitochondrial membrane potential (MMP), a critical indicator of mitochondrial function, was assessed using JC-1 staining. The fluorescence transition from red to green indicates MMP dissipation.⁵² As shown in Figure 3d, both ZMR+L

and ZMRT+L groups exhibited reduced MMP due to PDT effects, with the ZMRT+L group displaying predominantly green fluorescence and minimal red signal, indicating severe mitochondrial dysfunction attributable to TPP-mediated targeting.

Mitochondrial oxidative stress has been shown to remodel the phenotype of TAMs through the release of damaged mtDNA, thereby enhancing antitumor immune responses.⁵³ The oxidative DNA lesion marker 8-hydroxy-2'-deoxyguanosine (8-OH-dG) was visualized by confocal microscopy,⁵⁴ revealing the strongest fluorescence signal in the ZMRT+L group (Figure S12). Extracellular 8-OH-dG detection confirmed ZMRT+L-induced ox-mtDNA release from mitochondria into the extracellular space (Figure 3e). To visualize mtDNA release, mitochondrial transcription factor A staining was performed. CLSM images showed tight perinuclear clustering of mtDNA in control cells, whereas ZMRT+L treatment triggered mtDNA dispersion and translocation away from the nucleus and into the extracellular space (Figure S13), demonstrating the capacity of ZMRT to induce mtDNA escape and release.

A tumor cell/M2 macrophage co-culture system was established to investigate whether mtDNA release induces M2-to-M1 TAM repolarization (Figure 3f). Bone marrow-derived macrophages (BMDMs) were isolated, polarized to M2 phenotype, and co-cultured with differentially treated CT26 cells in a Transwell system for 48 h. The BMDMs were stained with antibodies against F4/80, CD86, and CD206. As shown in Figures 3g, S14, and S15, the proportion of M1 macrophages significantly increased in the ZMR+L and ZMRT+L groups, while that of M2 macrophages markedly decreased. Cytokine analysis^{55, 56} of the supernatants revealed significantly elevated TNF- α and reduced IL-10 levels in both ZMR+L and ZMRT+L groups (Figure 3h, i). Given that cytosolic DNA, particularly mtDNA released from damaged organelles, can be sensed by the cGAS-STING pathway to initiate innate immune responses⁵⁷, we next investigated whether ZMRT-mediated mtDNA release activates this critical axis in macrophages. As shown by immunoblotting in Figure 3j and S16, the ZMRT+L group exhibited the most pronounced activation of the STING signaling pathway, as evidenced by markedly increased phosphorylation of STING, IRF3, and TBK1 compared to other groups (Quantitative densitometric analysis of protein expression levels is presented in **Table S1**). Notably, the mitochondria-targeted PDT group exhibited the most pronounced effects, demonstrating that mitochondrial targeting-mediated mtDNA release activates the STING pathway, thereby enhancing M2-to-M1 macrophage repolarization by ZMRT NPs.

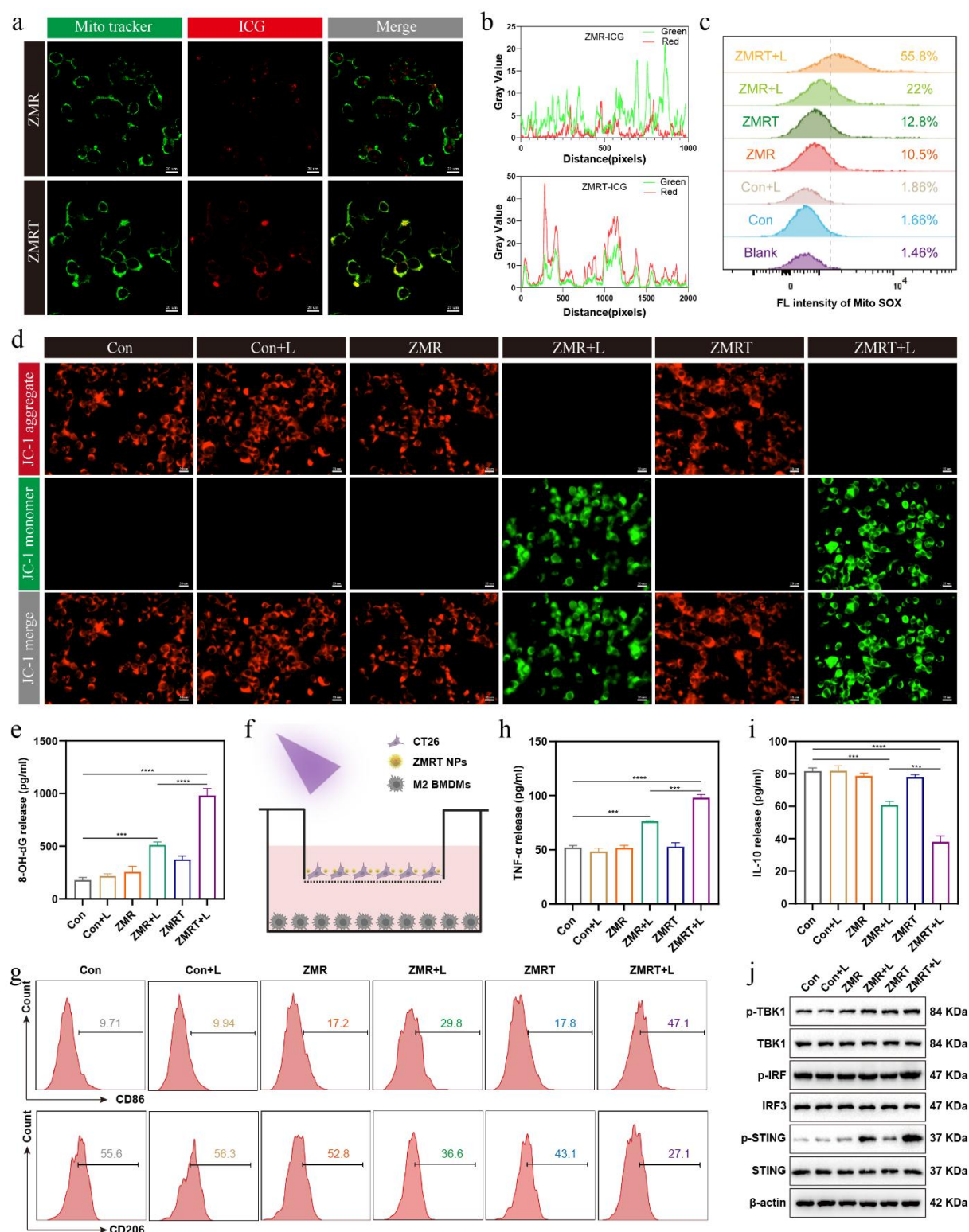


Figure 3. ZMRT NPs promote M1 macrophage polarization by inducing mitochondrial dysfunction-mediated mtDNA release. (a) Representative CLSM images of CT26 cells incubated with indocyanine green (ICG)-labeled ZMR or ZMRT NPs. Mitochondria were stained with MitoTracker Green FM. Scale bars: 20 μ m. (b) The overlap coefficient scatter plot of red and green pixel co-localization in the merged channel (generated by ImageJ). (c) Flow

cytometry analysis of mtROS in CT26 cells across treatment groups. (d) Analysis of MMP (JC-1 staining) in CT26 cells after the indicated treatments. Scale bar: 20 μ m. (e) Quantification of extracellular 8-OH-dG in CT26 cell supernatants post-treatment (n = 6). (f) Schematic depicting the Transwell co-culture setup. (g) Macrophage polarization analysis. Surface expression of M1 (CD86) and M2 (CD206) markers on BMDMs following co-culture with differentially treated CT26 cells (n=3). (h) TNF- α and (i) IL-10 levels measured by ELISA across treatment groups (n=3). (j) Western blot analysis of STING pathway activation in macrophages co-cultured with differently treated CT26 cells. Statistical analyses were performed using one-way analysis of variance (** $p < 0.001$ and **** $p < 0.0001$).

CLSM, confocal laser scanning microscopy; BMDMs, bone marrow-derived macrophages; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; TNF- α , tumor necrosis factor- α ; IL-10, interleukin-10; mtDNA, mitochondrial DNA; mtROS, mitochondrial reactive oxygen species; MMP, mitochondrial membrane potential; ELISA, enzyme-linked immunosorbent assay.

3.4. Induced Pyroptosis and Amplified Immunogenic Cell Death Response

Notably, ZMRT+L-treated CT26 cells exhibited characteristic pyroptotic morphology under light microscopy, including pronounced cellular swelling and bubble-like membrane protrusions (Figure S17). These features were most prominent in the ZMRT+L group, where nearly all cells displayed such structures, but were less frequent in the ZMR+L group and absent elsewhere, suggesting pyroptosis.¹⁹ Studies have shown that PDT can trigger pyroptosis through massive ROS generation, primarily via canonical (Caspase-1/GSDMD) or non-canonical (Caspase-3/GSDME) pathways.⁵⁸ To verify the pathway involved, mRNA expression levels of pyroptosis-related markers were measured by RT-qPCR (primer sequences listed in **Table S2**). In the ZMRT+L group, canonical pathway molecules (NLRP3, Caspase-1, GSDMD, IL-1 β and IL-18) were significantly upregulated, while non-canonical markers (Caspase-3/GSDME) showed minimal changes (Figures S18–S24), suggesting that ZMRT NPs induce pyroptosis via the canonical pathway.

Protein expression of canonical pyroptosis markers was subsequently examined.⁵⁹ Western blot confirmed NLRP3 upregulation in the ZMRT+L group. Both ZMR+L and ZMRT+L groups exhibited decreased full-length GSDMD and Caspase-1 with elevated cleaved forms, most pronounced in ZMRT+L (**Figure 4a** and S25). Since pyroptosis is typically accompanied by release of IL-1 β , IL-18, and LDH,⁶⁰ supernatants from treated CT26 cells were analyzed by ELISA and LDH release assays. ZMRT+L treatment significantly enhanced release of all three

markers compared with other groups (Figure 4b-d). Although ZMR+L induced moderate LDH release, IL-1 β and IL-18 levels remained unchanged. These findings confirm that ZMRT NPs induce pyroptosis in CT26 cells via the canonical pathway, likely attributable to mitochondrial targeting-enhanced ROS generation.¹⁹ TEM examination of treated cells revealed that ZMRT+L-treated cells lost normal morphology, exhibiting pyknotic nuclei and ruptured membranes with a "fried egg" appearance. At higher magnification, virtually no normal organelles remained, and significant cellular edema was observed. In contrast, ZMR+L and control groups maintained relatively normal cellular and organellar structures, further confirming the potent mitochondrial targeting effect mediated by TPP (Figure 4e).

Beyond direct tumoricidal effects, PDT can trigger ICD, leading to release of immunogenic DAMPs that stimulate antitumor immunity.⁶¹ ICD is defined by its ability to elicit potent antitumor immunity and convert immunologically "cold" tumors into "hot" phenotypes.⁶² Pyroptosis-driven ICD presents a promising strategy to overcome TME heterogeneity and enhance antitumor efficacy.⁶³ Having demonstrated pyroptosis induction by ZMRT NPs, we next assessed ICD markers, including CRT, HMGB1, ATP, and HSPs.⁶² CRT surface exposure and nuclear HMGB1 expression were assessed by CLSM, flow cytometry, and western blot. Both ZMR+L and ZMRT+L groups showed significantly increased CRT-positive rates compared with other groups, with the highest level observed in ZMRT+L-treated cells (Figures 4f,g and S26-27). Conversely, nuclear HMGB1 expression decreased markedly (Figures 4f, S28, and S29), which correlated with a significant increase in its extracellular release. ELISA quantification confirmed that secretion of both HMGB1 and ATP was substantially elevated in the ZMR+L and ZMRT+L groups compared with other treatments, with the ZMRT+L group exhibiting the highest levels (Figure 4h,i). HSP70 was notably upregulated in the ZMRT+L group (Figure 4f). Collectively, these findings demonstrate that targeting pyroptosis serves as an effective strategy to amplify ICD.

DAMPs function as "danger signals" that stimulate DC maturation and prime T cell-dependent antitumor immunity.⁶⁴ A Transwell co-culture system was employed to investigate whether ZMRT-based mitochondrial targeting induces pyroptosis in CT26 cells and releases sufficient DAMPs to promote DC maturation (indicated by CD80⁺CD86⁺) (Figure 4k). Bone marrow-derived DCs were co-cultured with differentially treated CT26 cells. DAMPs released from ZMRT+L-treated cells significantly increased the proportion of mature DCs, exhibiting a more pronounced effect than ZMR+L and control groups (Figure 4j, l). Collectively, these findings

demonstrate that by inducing pyroptosis and promoting DAMP release, ZMRT amplifies ICD and facilitates DC maturation, positioning pyroptosis induction as a promising strategy to enhance antitumor immunotherapy.

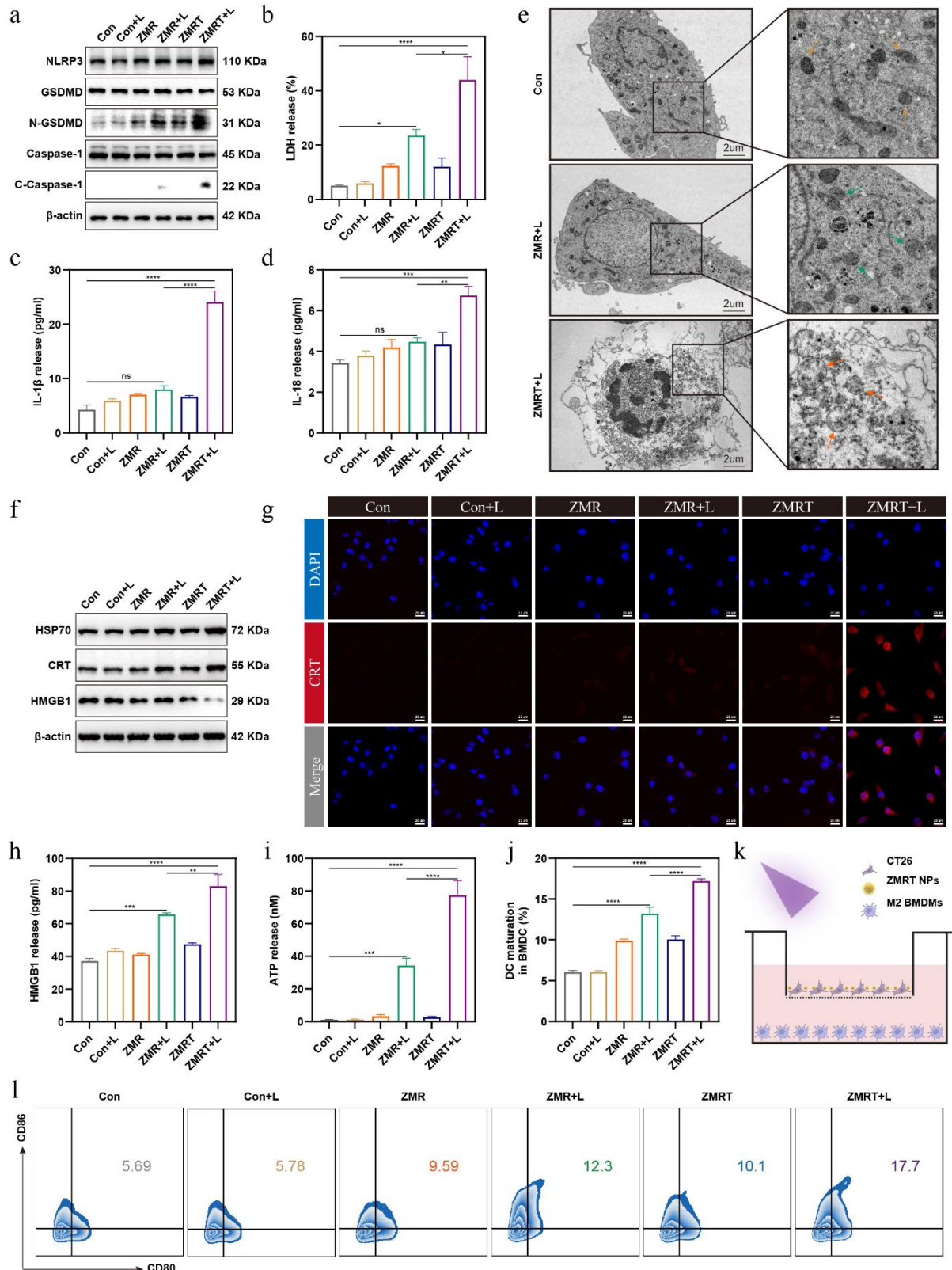


Figure 4. ZMRT NPs induce pyroptosis and immunogenic cell death. (a) Western blot detection of pyroptosis-associated proteins in differently treated CT26 cells. Release of (b) LDH, (c) IL-1 β , and (d) IL-18 in CT26 cell supernatant after various treatments (n = 3). (e) TEM images of CT26 cells following the indicated treatments. Scale bar: 2 μ m. (f) Western blot detection of ICD-associated proteins HSP70, CRT, and HMGB1 in differently treated CT26 cells. (g) CRT surface exposure in CT26 cells. Representative CLSM images post-treatment. Scale bar: 20 μ m. (h) HMGB1 and (i) ATP levels in cell supernatants across treatment groups (n=3). (j) Statistical graph of DC maturation frequency for different treatments (n = 3). (k) Schematic of the Transwell co-culture system. (l) DC maturation following co-culture with differentially treated CT26 cells. Statistical significance was obtained using a one-way analysis of variance (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001).

3.5. Mechanism Exploration of Pyroptosis Occurrence via Transcriptome Sequencing

To elucidate the molecular mechanism underlying pyroptosis induction, transcriptomic analysis was performed on ZMRT+L-treated and untreated CT26 cells. RNA sequencing identified 5,028 differentially expressed genes (DEGs) (3,746 upregulated and 1,282 downregulated) from 32,354 cellular genes (**Figure 5a**). Functional enrichment analysis revealed that these DEGs were predominantly involved in pathways related to the immune system, inflammatory response, mitochondrial, ATP, ROS, etc (Figure 5b, c). Notably, pathway analysis highlighted significant enrichment in the TNF and nuclear factor kappa (NF- κ B) pathways (Figure 5d). Given that ROS can act as upstream signals regulating the NF- κ B–NLRP3 axis to promote pyroptosis,⁶⁵ we hypothesized that ZMRT NP-induced pyroptosis in CT26 cells is mediated through the ROS–NF- κ B–NLRP3 signaling cascade. Quantitative western blot analysis confirmed ZMRT+L-induced phosphorylation of NF- κ B pathway components, including p65 and I κ B α , verifying pathway activation (Figure 5e, f). Collectively, these findings elucidate the complex mechanisms through which ZMRT NPs induce pyroptosis in CT26 cells.

Figure 5g presents the top 30 gene set enrichment analysis (GSEA) results. Among these 30 enriched terms, four were closely associated with mitochondrial dysfunction. Figure 5h-l displays five representative GSEA results related to mitochondrial dysfunction: electron transfer activity, electron transport chain, mitochondrial electron transport, mitochondrial respiratory chain complex I, and proton motive force-driven mitochondrial ATP synthesis. These findings further confirm the mechanism by which ZMRT NPs target mitochondria and induce mitochondrial dysfunction.

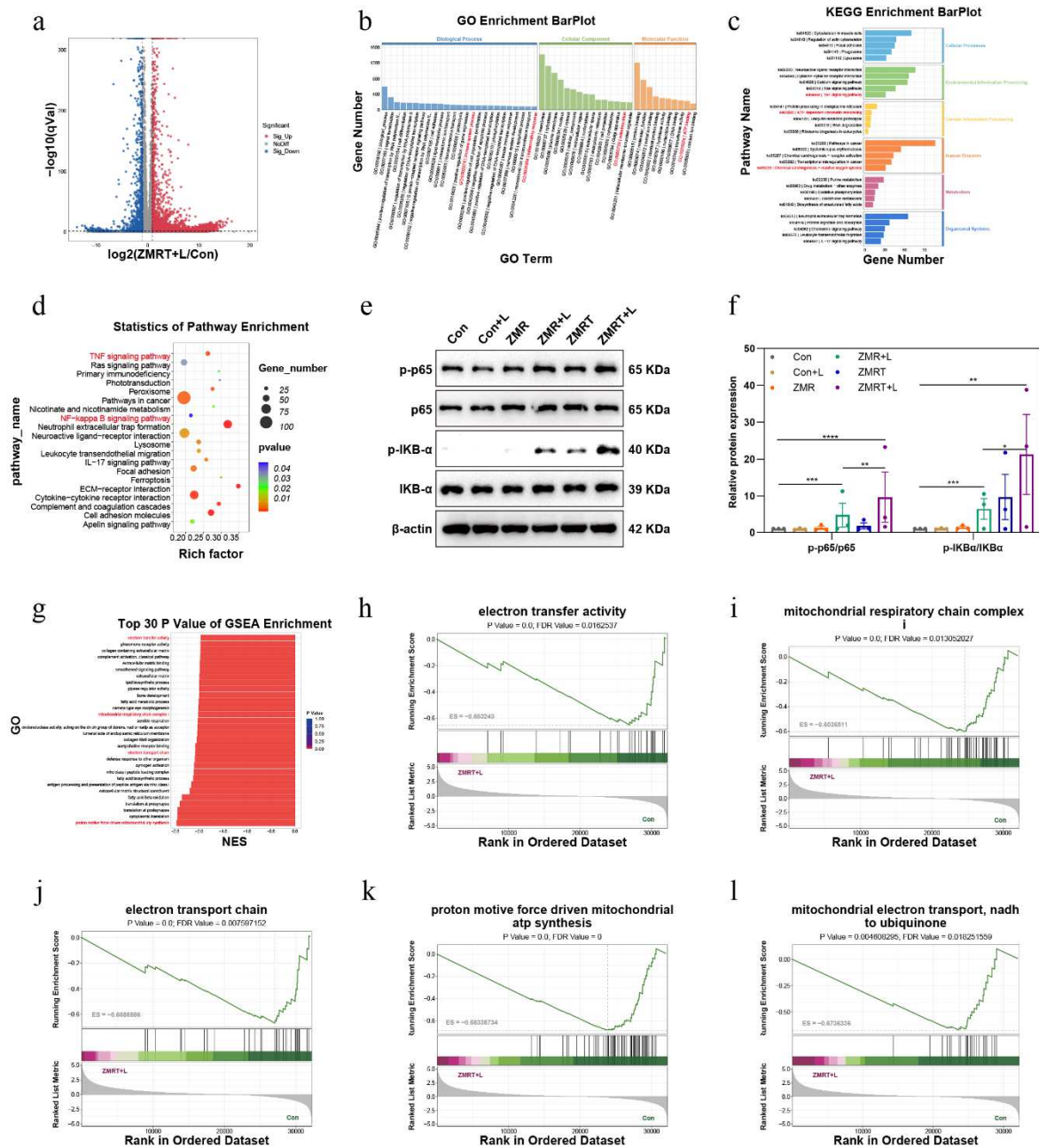


Figure 5. Transcriptome sequencing revealed that ZMRT NPs significantly activate the NF- κ B signaling pathway and induce mitochondrial dysfunction. (a) Differential gene expression profile. Red and blue points denote up- and down-regulated genes, respectively. (b) Significantly enriched GO terms and (c) KEGG pathways for the identified DEGs. (d) Statistics of pathway enrichment analysis. (e) Western blot detection of NF- κ B signaling pathway proteins (p-p65, p65, p-IkB- α , and IkB- α) in differently treated CT26 cells. (f) Quantitative analysis of the relative protein expression levels presented in (e). The ratios of p-p65/p65 and p-IkB- α /IkB- α were calculated and are displayed as mean $\pm$ SEM (n=3). (g) The top 30 pathways ranked in the GSEA. GSEA revealed five pathways closely associated with mitochondrial dysfunction: (h) electron transfer activity, (i) mitochondrial respiratory chain

complex I, (j) electron transport chain, (k) proton motive force-driven mitochondrial atp synthesis, and (l) mitochondrial electron transport, nadh to ubiquinone. Data were analyzed using one-way analysis of variance. (* $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$).

DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; NF- κ B, nuclear factor kappa B; p-p65, phosphorylated p65; p-I κ B- α , phosphorylated inhibitor kappa B alpha; GSEA, gene set enrichment analysis.

3.6. *In Vivo* Antitumor Effect and Pyroptosis Induction via the Mitochondria-Targeting Strategy of ZMRT NPs

Building on the potent pyroptosis induction and immune activation observed *in vitro*, the *in vivo* photodynamic efficacy and systemic immunostimulatory effects of ZMRT NPs were subsequently evaluated using an intratumoral injection strategy. CT26 colon cancer xenografts were established in immunocompetent BALB/c mice. When tumors reached approximately 100 mm³, mice were randomized into five groups: Con, ZMR, ZMR+L, ZMRT, and ZMRT+L (**Figure 6a, b**). Groups ZMR+L and ZMRT+L received 425 nm laser irradiation (20 mW cm⁻², 1 h) six hours post-injection. Tumor volume analysis revealed that ZMR or ZMRT alone did not significantly inhibit tumor growth, whereas both ZMR+L and ZMRT+L treatments substantially suppressed tumor progression, with ZMRT+L exhibiting the most potent antitumor effect—attributable to mitochondrial targeting-induced pyroptosis and enhanced immune activation (**Figure 6c, d**).¹⁹ Tumor weights measured on day 12 confirmed significant reduction in both irradiated groups (**Figure 6e**). Notably, all groups maintained stable body weights, suggesting favorable biosafety profiles (**Figure S30**). Histopathological analysis (H&E staining, TUNEL, and Ki-67 immunohistochemistry) demonstrated that ZMRT+L induced the most substantial apoptosis and suppression of proliferative activity (**Figures 6l and S31**).

Pyroptosis and ICD markers in tumor tissues were subsequently analyzed. Immunoblotting revealed significant upregulation of NLRP3, N-GSDMD, and HSP70 in ZMRT+L-treated tumors (**Figure 6f and S32**). Quantitative densitometric analysis of protein expression levels is presented in **Table S3**. Consistently, IL-1 β and IL-18 levels were significantly elevated in the ZMRT+L group (**Figure 6g, h**), confirming *in vivo* pyroptosis induction. Analysis of IL-6, IFN- γ , and TNF- α showed significant upregulation in both irradiated groups, indicating that ZMRT NPs remodel the tumor immune microenvironment by promoting cytokine secretion (**Figure 6i-k**).⁵³ Immunofluorescence staining demonstrated that ZMRT+L treatment enhanced CRT expression, increased 8-OH-dG-positive cells (oxidative damage marker), promoted CD8⁺ T-

cell infiltration, and elevated iNOS-positive cells (M1 macrophage marker) (Figure S33). Collectively, these findings demonstrate that ZMRT NPs alleviate the immunosuppressive TME in CRC through pyroptosis and ICD activation *in vivo*.

Immune cell profiling by flow cytometry revealed that the proportion of CD3⁺ T cells in tumors was substantially higher in PDT groups (ZMR+L: 24.3%; ZMRT+L: 30%) compared to non-PDT groups (<17%) (Figure 6m, n). Notably, ZMRT+L yielded the highest CD8⁺CD3⁺ T-cell percentage (75.9%), 1.5-fold higher than Con group (Figure 6o, p), consistent with immunofluorescence findings. Analysis of tumor-associated macrophages showed that ZMRT+L significantly increased M1-type and decreased M2-type populations (Figures 6q and S34), confirming *in vivo* M1 polarization. In tumor-draining lymph nodes (TDLNs), ZMR+L and ZMRT+L groups, owing to their robust DAMP release capacity, significantly promoted DC maturation, with the frequency of mature DCs increasing by 1.2- and 1.6-fold, respectively, compared with the control group (Figures 6r and S35). Furthermore, ZMRT+L treatment significantly increased the splenic CD8⁺ T-cell population by 1.6-fold compared to the control, whereas minimal changes were observed in other groups (Figures 6s and S36). Serum cytokine analysis confirmed systemic immune activation, with IFN- γ and TNF- α significantly elevated in both irradiated groups, most prominently in ZMRT+L (Figure 6t, u).

Finally, the biocompatibility of the as-prepared ZMRT NPs was evaluated. H&E-stained sections of major organs from different treatment groups revealed no signs of inflammation or tissue damage, indicating favorable biosafety profiles (Figure S37). Body weights of mice across all five groups remained stable throughout the 12-day treatment period (Figure S30). Routine blood tests (including white blood cells, red blood cells, and platelets) and serum biochemical analysis (alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, and creatinine) revealed no notable abnormalities, demonstrating the excellent biocompatibility and biosafety of ZMRT NPs (Figure S38).⁵¹

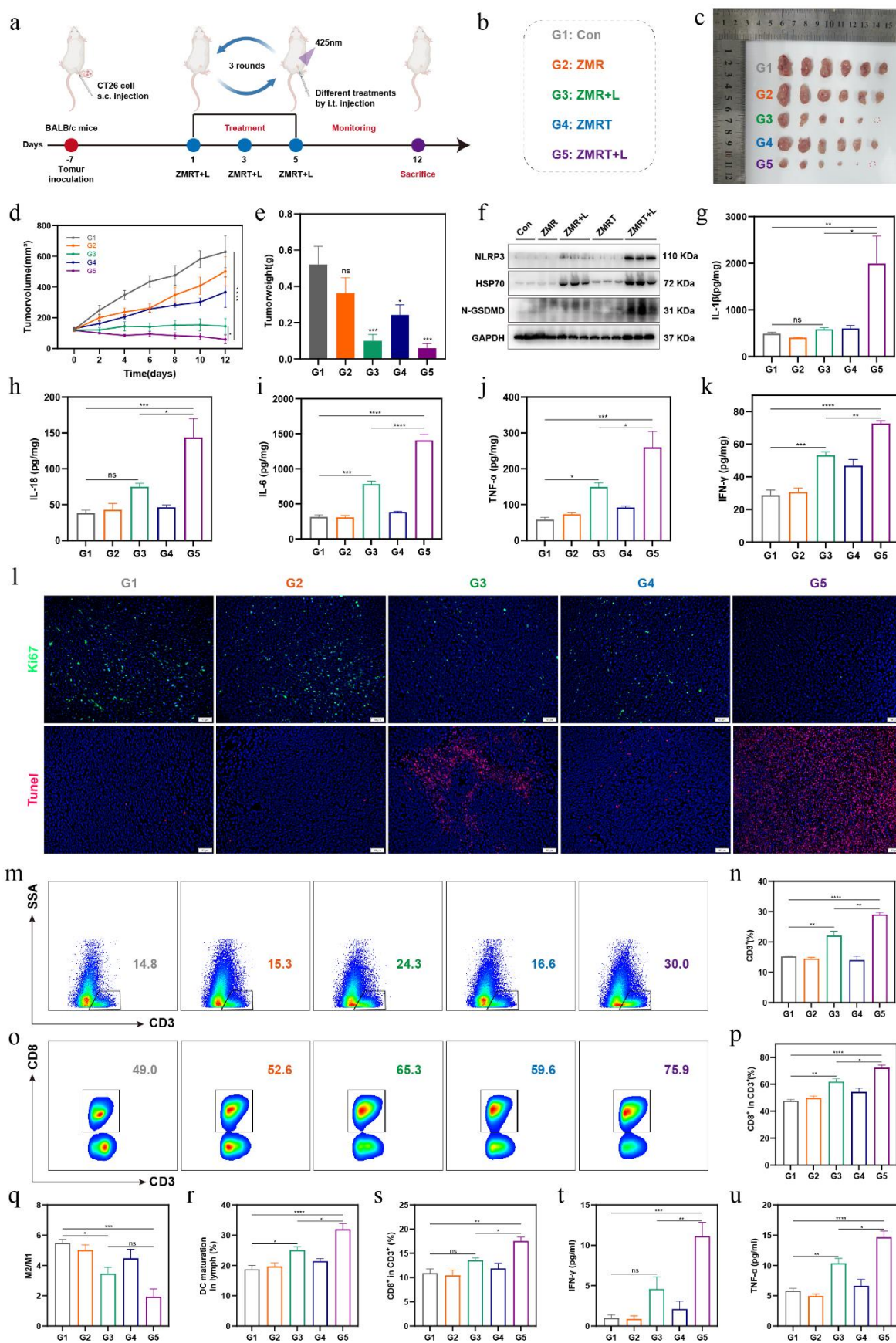


Figure 6. *In vivo* antitumor efficacy across treatment groups. (a) Schematic illustration of the administration design. (b) Experimental group assignment. (c) Representative tumor images. (d) Tumor growth curves and (e) final tumor weights across treatment groups (n = 6). (f) Western blot detection of pyroptosis and ICD-associated proteins (NLRP3, HSP70, and N-GSDMD) in differentially treated CT26-derived tumor tissue. The levels of (g) IL-1 β , (h) IL-18, (i) IL-6, (j) TNF- α , and (k) IFN- γ measured using enzyme-linked immunosorbent assay (ELISA) across treatment groups (n=6). (l) Representative images of Ki-67 and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining in tumor sections from different treatment groups. Scale bars: 50 μ m. (m) Flow cytometry analysis and (n) quantification results of CD3⁺ T-cells in tumors (n = 3). (o) Flow cytometry plots and (p) quantification of CD3⁺CD8⁺ T-cells (n = 6). (q) M2/M1 macrophage ratio in tumor tissue (n = 6). (r) DC maturation in tumor-draining lymph nodes (n = 6). (s) Splenic CD8⁺ T-cell proportion (n = 6). (t) IFN- γ and (u) TNF- α concentrations (n = 6). Data were analyzed using one-way analysis of variance. (* p < 0.05, ** p < 0.01, *** p < 0.001, and **** p < 0.0001).

ICD, immunogenic cell death; NLRP3, NOD-like receptor protein 3; HSP70, heat shock protein 70; GSDMD, gasdermin D; N-GSDMD, N-terminal gasdermin D; IL-1 β , interleukin-1 beta; IL-18, interleukin-18; IL-6, interleukin-6; TNF- α , tumor necrosis factor-alpha; IFN- γ , interferon-gamma; DCs, dendritic cells; PDT, photodynamic therapy.

3.7. *In Vivo* Anti-Distant Metastasis and Immunostimulatory Effects of ZMRT NPs

Given the mitochondrial targeting effects of ZMRT and its ability to induce pyroptosis-mediated ICD, its capacity to elicit systemic antitumor immunity was subsequently evaluated. Bilateral CT26 colon cancer xenografts were established in BALB/c mice to simulate metastatic disease.^{32, 66} **Figure 7a** illustrates the experimental timeline. Primary tumors (right dorsal flank) received local treatment, while distant tumors (left dorsal flank) remained untreated to assess abscopal effects. Mice were randomized into five groups: Con, ZMR, ZMR+L, ZMRT, and ZMRT+L (Figure 7b). Six hours post-intratumoral injection, primary tumors in the irradiated groups (ZMR+L and ZMRT+L) were exposed to 425 nm laser (20 mW cm⁻², 1 h), with distant tumors shielded. The ZMRT+L treatment demonstrated the most pronounced suppression of both primary and distant tumors, while the ZMR+L treatment showed significant inhibitory effects (Figures 7d-g, S39, and S40). Histopathological analysis by H&E staining corroborated these findings, revealing extensive tissue damage in both primary and distant tumors from ZMRT+L-treated mice (Figure S41). All groups maintained stable body weight without signs

of major organ damage, confirming favorable biosafety (Figure 7c). Collectively, these findings demonstrate that ZMRT NPs effectively suppress both primary and distant colon tumor growth.

Given the established capacity of ZMRT to enhance CD8⁺ T-cell and M1 macrophage infiltration in primary tumors, the potential involvement of immune microenvironment remodeling in its abscopal effect on distant tumors was investigated. In the control group, CD4⁺ and CD8⁺ T-cell proportions in primary and distant tumors were 36.7%/29.7% and 28.4%/18.4%, respectively (Figure 7h, j), with notable baseline variation between bilateral tumors attributable to tumor size or sample processing differences. Control tumors displayed a CD4⁺/CD8⁺ T-cell ratio skewed toward CD4⁺ populations, consistent with immunosuppression. All treatments increased CD8⁺ T-cell infiltration, with ZMR+L and ZMRT+L increasing their proportions in primary tumors to 48.6% and 65.9% (1.7- and 2.3-fold vs. control, respectively), levels critical for antitumor immunity. The ZMRT+L group had 1.3 times more CD8⁺ T-cells than the ZMR+L group, indicating that mitochondrial targeting enhances ICD induction and CD8⁺ T-cell recruitment (Figure 7h, i). A similar trend was observed in distant tumors (Figure 7j, k), although CD8⁺ T-cell levels were moderately lower, likely due to the localized effect of intratumoral injection. Both primary and distant tumors also displayed higher M1 and lower M2 macrophage proportions in the ZMR+L and ZMRT+L groups than in the other treatment groups, with the most significant change in ZMRT+L (Figure 7l-o). These findings align with the *in vitro* co-culture results and affirms that ZMRT NPs reverse the immunosuppressive TME in colon cancer via M1 macrophage polarization.

DC maturation status in TDLNs was also examined. The proportion of mature DCs was 11.0% in the control group, with comparable levels in ZMR (11.5%) and ZMRT (14.3%) groups. In contrast, ZMR+L and ZMRT+L groups exhibited significant increases, reaching 2.1- and 2.9-fold that of Con group (Figure 7p, q), consistent with *in vitro* observations. The spleen, as the largest immune organ, serves as a reservoir for antitumor immunity. Beyond tumor-resident and lymph node-derived CD8⁺ T-cells, splenic intermediate-exhausted CD8⁺ T-cells represent a key population capable of responding to immunotherapy and infiltrating tumors.⁶⁷ splenic CD8⁺ T-cell analysis revealed percentages of 12.2% (Con), 12.6% (ZMR), 16.3% (ZMR+L), and 13.8% (ZMRT), with no statistically significant differences. However, ZMRT+L treatment significantly increased the CD8⁺ T-cell proportion compared with all other treatment groups (Figures S42 and S43), consistent with previous findings. These results suggest that the mitochondrial targeting strategy enhances the ICD effect through pyroptosis, thereby eliciting

a systemic antitumor immune response. Similarly, H&E staining of major organs from all treatment groups confirmed the favorable biosafety profile of ZMRT NPs (Figure S44).

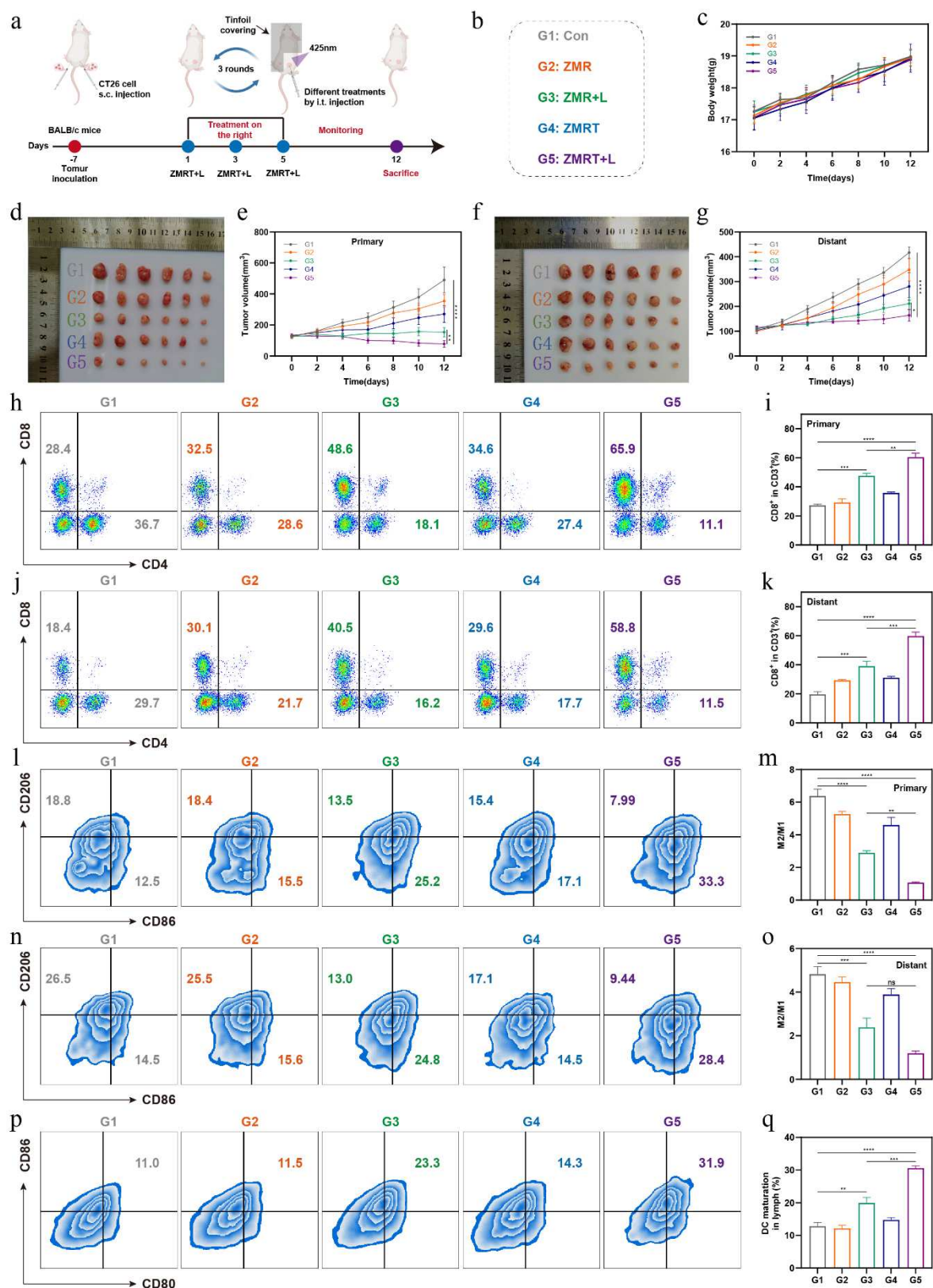


Figure 7. Assessment of systemic antitumor efficacy and immune activation after different treatments. (a) Schematic illustration of the *in vivo* treatment procedure in mice. (b) Experimental group assignment. (c) Body weight changes after different treatments (n = 6). (d) Primary tumor images and (e) tumor volume changes after different treatments. (f) Distant tumor images and (g) tumor volume changes after different treatments. Flow cytometry analysis and quantification results of immune cells (n = 6), including (h) CD4⁺ T-cell and CD8⁺ T in primary tumors, (i) CD3⁺CD8⁺ T-cells in primary tumors, (j) CD4⁺ T-cell and CD8⁺ T in distant tumors, (k) CD3⁺CD8⁺ T-cells in distant tumors, (l) M1 and M2 macrophages in primary tumors, (m) M2/M1 in primary tumors, (n) M1 and M2 macrophages in distant tumors, and (o) M2/M1 in distant tumors. (p) Flow cytometry analysis and (q) quantification results of dendritic cells (DCs) in tumor-draining lymph nodes (n = 6). Statistical significance was obtained using a one-way analysis of variance (**p* < 0.05, ***p* < 0.01, ****p* < 0.001, and *****p* < 0.0001).

3.8. Long-Term Protective Immunity and Antigen-Specific Immune Memory Induced by ZMRT NPs

Given the established efficacy of ZMRT NPs in suppressing primary and metastatic tumors via immune microenvironment remodeling—and the observed expansion of peripheral CD8⁺ T-cells specifically in the ZMRT+L group—its mitochondrial targeting strategy was hypothesized to enhance pyroptosis and DAMP release to promote immune memory. To this end, surviving mice from the initial CT26 tumor challenge were rechallenged with CT26-Luc cells. Lung metastasis was monitored via *in vivo* bioluminescence imaging on day 30 (**Figure 8a**). The ZMRT+L group exhibited significantly reduced lung metastatic burden, as evidenced by markedly lower bioluminescence signal intensity compared to the strong signal observed in the control group (Figure 8b). Histological analysis and metastatic nodule quantification confirmed the therapeutic advantage of ZMRT NPs, revealing markedly reduced tumor infiltration and fewer metastatic foci than controls (Figure 8c, d).

To validate the immune memory response triggered by ZMRT NPs, peripheral blood mononuclear cells were isolated to assess memory CD8⁺ T-cell populations.⁶⁸ Consistent with our previous observations, ZMRT treatment significantly elevated the splenic CD8⁺ T-cell population (Figures 8h and S45). Moreover, ZMRT+L treatment markedly increased the proportions of both central (CD44⁺CD62L⁺) and effector (CD3⁺CD8⁺CD44⁺CD62L⁻) memory T-cell subsets relative to the control group (Figure 8e, i). In parallel, serum levels of IFN-γ and TNF-α were significantly elevated in ZMRT-treated mice compared to controls, confirming the

induction of a robust pro-inflammatory immune response (Figure 8f, g). Collectively, these findings demonstrate that ZMRT induces durable antitumor immunity by promoting CD8⁺ T-cell activation and infiltration, enhancing immune memory, and suppressing tumor metastasis, highlighting its potential for preventing tumor recurrence.

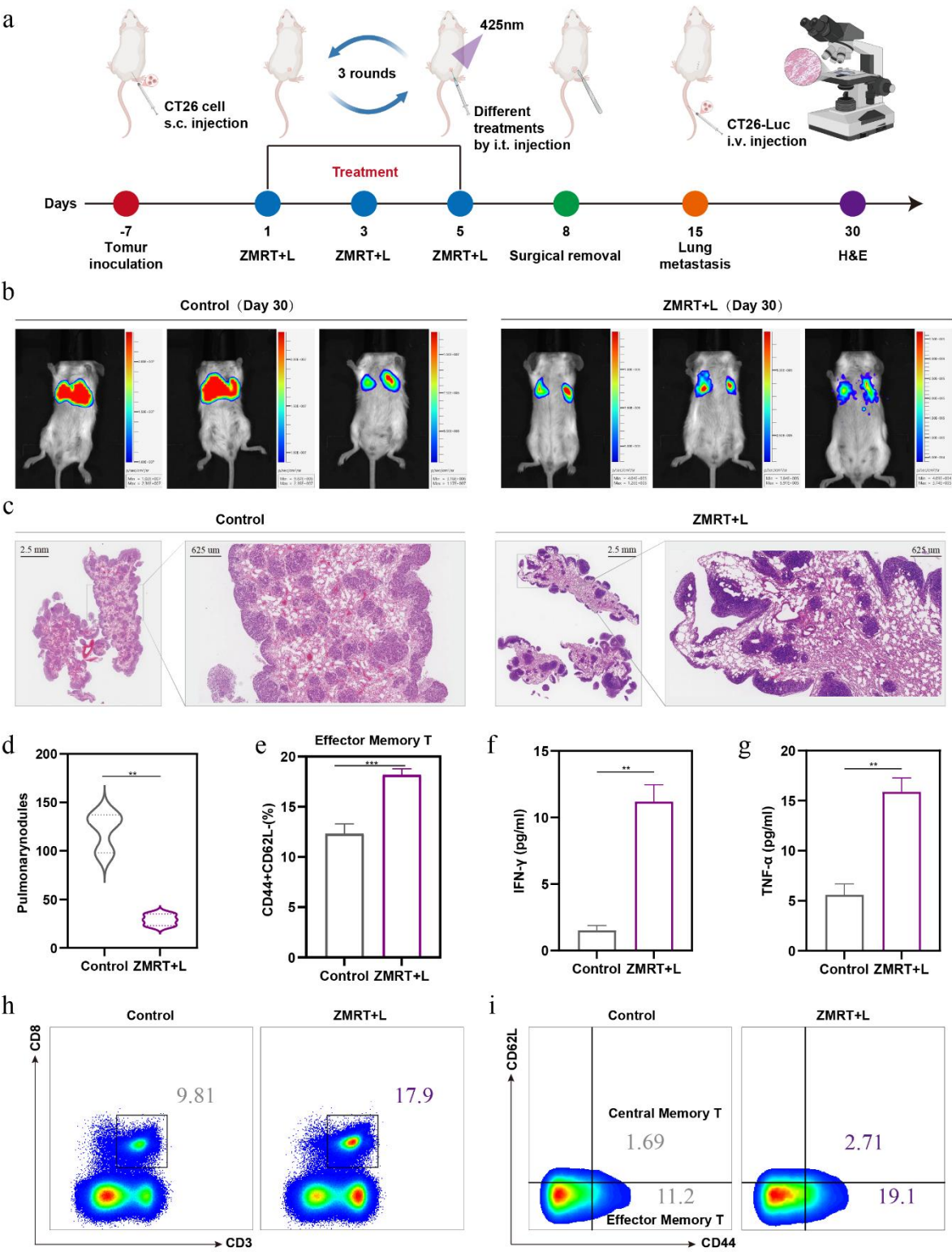


Figure 8. Immune memory effect of ZMRT NPs against lung metastasis. (a) Treatment schedule of ZMRT NPs-mediated therapy against lung metastasis. (b) The luciferase signal of CT26-Luc cells in mice monitored to track the growth of colon lung metastasis on day 30 (n = 3). (c) Representative images of the tumor lung metastasis sections (hematoxylin and eosin (H&E) staining) (n = 3). Scale bars, 625 μ m. (d) Quantification of pulmonary nodules from Figure 8c. (e) Proportions of memory T-cell subsets in peripheral blood mononuclear cells following ZMRT+L treatment (n=3). Detection of serum cytokines: (f) IFN- γ and (g) TNF- α (n=3). (h) Alterations in peripheral blood CD8⁺ T-cell proportion following ZMRT+L treatment. (i) Representative flow cytometry images of central and memory T-cells in peripheral blood mononuclear cells following ZMRT+L treatment.

IFN- γ , interferon-gamma; TNF- α , tumor necrosis factor-alpha.

4. Conclusions

In summary, given the endoluminal accessibility of colorectal cancer and the significant systemic toxicity associated with current treatments, local photodynamic therapy represents an attractive therapeutic option. This study addresses the major limitations of conventional PDT—namely tumor hypoxia and an overreliance on apoptotic pathways that lead to insufficient immunogenic cell death—by developing a mitochondria-targeted ROS burst nanoplatfrom (ZMRT NPs) for CRC treatment. This platform integrates an oxygen-supplying Mn₃O₄ nanozyme with a TPP-conjugated rhenium photosensitizer to achieve mitochondrial localization, thereby overcoming tumor hypoxia and generating a potent mitochondrial ROS burst upon laser irradiation. Mechanistically, this ROS overload triggers canonical pyroptosis via the NF- κ B/NLRP3/GSDMD pathway and releases mitochondrial DNA to activate the STING pathway, which reprograms M2 tumor-associated macrophages toward an M1 phenotype and remodels the immunosuppressive tumor microenvironment. More importantly, local ZMRT-based PDT not only suppresses primary tumor growth but also induces systemic antitumor immunity, inhibits distant metastasis, and establishes long-term immune memory. Collectively, this study integrates bio-targeting strategies and nanodelivery approaches to substantially enhance the antitumor immune efficacy of PDT, offering new perspectives for overcoming the traditional limitations of PDT and for the local treatment of colorectal cancer.

Abbreviations

CRC	Colorectal cancer
PDT	Photodynamic therapy

ICD	Immunogenic cell death
RCD	Regulated cell death
DAMPs	Damage-associated molecular patterns
ROS	Reactive oxygen species
TPP	Triphenylphosphine
TME	Tumor microenvironment
CLSM	Confocal laser scanning microscopy
8-OH-dG	8-hydroxy-2'-deoxyguanosine
mtDNA	Mitochondrial DNA
TAM	Tumor-associated macrophage
DC	Dendritic cell
BMDMs	Bone Marrow-Derived Macrophages
BMDCs	Bone Marrow-Derived Dendritic Cells
GSDMD	Gasdermin D
CRT	Calreticulin
HMGB1	High mobility group protein 1
TDLNs	Tumor-draining lymph nodes

Declarations

Ethics approval and consent to participate

All animal experimental protocols were approved by the Laboratory Animal Welfare and Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University (Approval No. SYSU-2023-334; 11 December 2023). All animal experiments were carried out in strict compliance with the guidelines of the International Council for Laboratory Animal Science (ICLAS) and in accordance with ICLAS ethical principles.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequencing data generated in this study have been deposited in the NCBI Sequence Read Archive (SRA) under BioProject accession number PRJNA1503378. The data are currently under a temporary embargo and will be publicly released on August 1, 2027. For peer review purposes, the datasets can be accessed via the following reviewer link:

<https://dataview.ncbi.nlm.nih.gov/object/PRJNA1503378?reviewer=mfjo0ob7latqkibgu91kgh1akd> .

Competing interests

The authors declare no competing interests.

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Authors' contributions

Q.T., J.F., T.L., and L.T. contributed equally to this work. Q.T. participated in study conception and design, performed most of the material characterization and biological experiments, contributed to data analysis and figure preparation, and drafted the manuscript. J.F. synthesized and characterized the ZMRT nanoparticles. T.L., and L.T. assisted with data processing and figure preparation. Y.Z., Z.L., W.L., T.Y., H.W., Y.Z. and Y.W. provided technical assistance and contributed to data collection. X.W. provided experimental guidance. J.S. provided nanotechnology support and design. W.S. provided funding acquisition, resources, and study supervision. All authors reviewed and approved the final manuscript.

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Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Supplementary Tables

Table S1. Densitometric quantification of ICD, NF- κ B, STING, and pyroptosis marker expression in treated cells (n = 3).

Table S2. Primer sequences used for qPCR.

Table S3. Densitometric quantification of protein expression in mouse tumor tissues (n = 3).

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