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Article

pH-Responsive Metal-Organic Framework Nanoparticles Loaded with Doxorubicin and CpG Oligodeoxynucleotides for Synergistic Chemo-Immunotherapy of Melanoma

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ABSTRACT

Melanoma, the most aggressive form of skin cancer, is characterized by high metastatic potential and resistance to conventional therapies. Chemotherapy (e.g., doxorubicin, DOX) can kill tumor cells but often induces immunosuppression, while immunotherapy (e.g., CpG oligodeoxynucleotides, CpG ODNs) activates anti-tumor immunity but has limited efficacy in poorly immunogenic tumors. Herein, we developed a pH-responsive metal-organic framework (MOF) nanoparticle system (ZIF-8) loaded with DOX (chemotherapeutic) and CpG ODNs (TLR9 agonist) for synergistic chemo-immunotherapy of melanoma. The ZIF-8 MOFs were synthesized via a solvothermal method, surface-modified with polyethylene glycol (PEG) to enhance biocompatibility, and co-loaded with DOX (hydrophobic, loaded in MOF pores) and CpG ODNs (hydrophilic, adsorbed on MOF surface via electrostatic interaction). The resulting DOX/CpG@ZIF-8-PEG nanoparticles exhibit uniform size (~120 nm), high drug loading efficiency (DOX: 28.5 ± 2.3%, CpG ODNs: 15.2 ± 1.8%), and pH-responsive release (92.3% DOX and 87.6% CpG ODNs released at pH 5.0 vs. 18.5% DOX and 12.8% CpG ODNs at pH 7.4 after 48 h). In vitro studies show that DOX/CpG@ZIF-8-PEG induces 82.3% apoptosis in B16-F10 melanoma cells, promotes dendritic cell (DC) maturation ($CD80^+CD86^+$ DCs: 68.5% vs. 21.3% for saline), and enhances cytotoxic T cell activation (IFN-γ^+ $CD8^+$ T cells: 45.6% vs. 8.7% for saline). In vivo, the nanoparticles significantly inhibit B16-F10 melanoma growth (tumor volume reduction: 81.2% vs. saline) and lung metastasis (metastatic nodules: 6.3 vs. 45.7 for saline), while prolonging mouse survival (median survival: 56 days vs. 23 days for saline). This work demonstrates the potential of MOF-based nanocarriers for co-delivery of chemotherapeutics and immunostimulants, bridging nanomaterial engineering, chemotherapy, and immunology for precision cancer therapy.

Keywords: Metal-Organic Frameworks; ZIF-8; Chemo-Immunotherapy; Melanoma; Doxorubicin; CpG Oligodeoxynucleotides; pH-Responsive Release; Anti-Tumor Immunity

1. Introduction

1.1 Background

Melanoma accounts for only 1% of skin cancer cases but causes 80% of skin cancer-related deaths, with over 325,000 new cases diagnosed annually worldwide. Advanced melanoma is highly metastatic, with a 5-year survival rate of only 27% for stage IV disease. Conventional therapies, such as chemotherapy and targeted therapy, have limited efficacy due to acquired resistance and systemic toxicity. Doxorubicin (DOX), a widely used chemotherapeutic, kills tumor cells via DNA intercalation and topoisomerase II inhibition but induces severe side effects (e.g., cardiotoxicity) and immunosuppression by depleting immune cells.

Immunotherapy has revolutionized melanoma treatment by activating the host immune system to recognize and eliminate tumor cells. CpG oligodeoxynucleotides (CpG ODNs), synthetic agonists of toll-like receptor 9 (TLR9), activate dendritic cells (DCs) and natural killer (NK) cells, promoting the generation of cytotoxic $CD8^+$ T cells. However, CpG ODNs have poor stability in vivo (degraded by nucleases) and low tumor accumulation, limiting their efficacy as monotherapy—especially in poorly immunogenic tumors like melanoma.

Synergistic chemo-immunotherapy combines chemotherapy's direct tumor-killing effect with immunotherapy's anti-tumor immunity activation, addressing the limitations of single-modal therapies. Chemotherapy can induce immunogenic cell death (ICD) of tumor cells, releasing tumor-associated antigens (TAAs) that enhance the efficacy of immunostimulants like CpG ODNs. However, co-delivery of chemotherapeutics and immunostimulants remains challenging due to their distinct physicochemical properties (hydrophobic vs. hydrophilic) and incompatible pharmacokinetics.

1.2 Research Gaps

Existing co-delivery systems for chemo-immunotherapy face several limitations. Liposomes and polymersomes have low loading capacity for hydrophobic drugs like DOX and cannot protect CpG ODNs from nuclease degradation. Mesoporous silica nanoparticles (MSNs) have high drug loading but lack stimuli-responsive release, leading to premature drug leakage in normal tissues. Metal-organic frameworks (MOFs), porous crystalline materials composed of metal ions and organic ligands, offer a promising solution due to their high surface area, tunable pore size, and stimuli-responsive degradation. However, few MOF-based systems have been developed for co-delivery of DOX and CpG ODNs, and even fewer have been validated in melanoma models with metastatic potential.

1.3 Research Objectives and Contributions

The primary objective of this study is to develop a pH-responsive ZIF-8 MOF nanoparticle system for co-delivery of DOX and CpG ODNs to achieve synergistic chemo-immunotherapy of melanoma. Specific objectives include:

(1) Synthesize and characterize DOX/CpG@ZIF-8-PEG nanoparticles with high loading efficiency and pH-responsive release.

(2) Evaluate in vitro tumor cell apoptosis, DC maturation, and cytotoxic T cell activation induced by the nanoparticles.

(3) Validate in vivo anti-tumor efficacy, anti-metastatic activity, and survival improvement in B16-F10 melanoma xenograft and metastasis models.

The key contributions of this research are:

•**Nanomaterial Engineering Contribution**: ZIF-8 MOFs enable co-loading of hydrophobic DOX and hydrophilic CpG ODNs, with pH-responsive degradation ensuring targeted drug release in acidic tumor microenvironments (TME, pH 6.5–6.8) and endosomes (pH 5.0–5.5).

•**Therapeutic Synergy Contribution**: The nanoparticles combine DOX-induced ICD (releasing TAAs) with CpG ODN-mediated DC activation, enhancing anti-tumor immunity and overcoming melanoma's poor immunogenicity.

•**Translational Contribution**: The system inhibits both primary tumor growth and lung metastasis in clinically relevant melanoma models, providing a translatable strategy for advanced melanoma therapy.

2. Literature Review

2.1 Metal-Organic Frameworks in Drug Delivery

Metal-organic frameworks are porous materials with a crystalline structure formed by coordination bonds between metal ions (e.g., Zn^{2+}, Fe^{3+}, Cu^{2+}) and organic ligands (e.g., imidazoles, carboxylates). MOFs have unique properties for drug delivery: (1) high surface area (500–4000 m^2/g) and pore volume (0.5–4.0 cm^3/g) enabling high drug loading; (2) tunable pore size (1–50 nm) for loading drugs of different sizes; (3) stimuli-responsive degradation (pH, temperature, redox) for controlled drug release.

ZIF-8, composed of Zn^{2+} and 2-methylimidazole, is one of the most widely used MOFs for drug delivery due to its low toxicity (Zn^{2+} is biodegradable), high stability in neutral conditions, and rapid degradation in acidic environments. ZIF-8 has been used to deliver chemotherapeutics (e.g., DOX, cisplatin) and nucleic acids (e.g., siRNA, mRNA), but its application for co-delivery of chemotherapeutics and immunostimulants remains underexplored.

2.2 Chemo-Immunotherapy for Melanoma

Chemotherapy-induced immunogenic cell death (ICD) is a key mechanism for synergizing chemo- and immunotherapy. ICD triggers the release of damage-associated molecular patterns (DAMPs), such as calreticulin (CRT) on the cell surface, ATP in the extracellular space, and high-mobility group box 1 (HMGB1) in the cytoplasm. These DAMPs recruit and activate DCs, which present TAAs to T cells, initiating anti-tumor immunity.

DOX is a potent inducer of ICD, but its immunosuppressive effects (e.g., depletion of T cells) limit its ability to activate long-term anti-tumor immunity. CpG ODNs, by activating TLR9 on DCs and NK cells, can reverse chemotherapy-induced immunosuppression and enhance T cell activation. Co-delivery of DOX and CpG ODNs can amplify ICD-induced anti-tumor immunity, but existing co-delivery systems lack targeted release and high loading efficiency for both drugs.

2.3 Stimuli-Responsive Nanoparticles in Cancer Therapy

Stimuli-responsive nanoparticles release drugs in response to tumor-specific cues, such as acidic pH, high redox potential, or overexpressed enzymes. Acidic pH is a key TME feature, with tumor extracellular pH (6.5–6.8) and endosomal/lysosomal pH (5.0–5.5) significantly lower than normal tissue pH (7.4). pH-responsive nanoparticles, such as ZIF-8 MOFs and pH-sensitive polymers, ensure drug release at the tumor site, reducing off-target toxicity.

ZIF-8 MOFs degrade in acidic conditions due to protonation of imidazole ligands, which weakens coordination bonds with Zn^{2+}. This pH-responsive degradation enables controlled release of encapsulated

drugs, making ZIF-8 an ideal carrier for co-delivery of DOX and CpG ODNs. However, few studies have evaluated ZIF-8-based co-delivery systems in melanoma models, especially for metastasis inhibition.

3. Methodology

3.1 Synthesis and Characterization of DOX/CpG@ZIF-8-PEG Nanoparticles

3.1.1 Synthesis of ZIF-8 MOFs

ZIF-8 MOFs were synthesized via a solvothermal method. Briefly, 0.61 g of zinc nitrate hexahydrate ($Zn(NO_3)_2 \cdot 6H_2O$) was dissolved in 20 mL of methanol, and 1.64 g of 2-methylimidazole was dissolved in 20 mL of methanol. The two solutions were mixed under vigorous stirring at room temperature, and the mixture was incubated for 24 h. The resulting white ZIF-8 precipitate was collected by centrifugation (8,000 rpm, 15 min), washed with methanol three times, and dried at 60°C overnight.

3.1.2 Co-Loading of DOX and CpG ODNs

DOX (hydrophobic) was loaded into ZIF-8 pores via diffusion. 100 mg of ZIF-8 was dispersed in 10 mL of DOX methanol solution (1 mg/mL), and the mixture was stirred at room temperature for 24 h. The DOX@ ZIF-8 nanoparticles were collected by centrifugation, washed with methanol to remove free DOX, and dried.

CpG ODNs (sequence: 5'-TCC ATG ACG TTC CTG ATG CT-3', phosphorothioate-modified) were purchased from Integrated DNA Technologies (Coralville, IA, USA). CpG ODNs (hydrophilic) were adsorbed on the surface of DOX@ZIF-8 via electrostatic interaction. 50 mg of DOX@ZIF-8 was dispersed in 5 mL of CpG ODNs aqueous solution (0.5 mg/mL), and the mixture was stirred at 4°C for 12 h. The DOX/CpG@ZIF-8 nanoparticles were collected by centrifugation, washed with deionized water (DI) to remove free CpG ODNs, and resuspended in DI water.

3.1.3 Surface Modification with PEG

To enhance biocompatibility and reduce aggregation, DOX/CpG@ZIF-8 was modified with PEG. 50 mg of DOX/CpG@ZIF-8 was dispersed in 10 mL of DI water, and 25 mg of NH_2-PEG-COOH (molecular weight: 5 kDa) was added. The mixture was stirred at room temperature for 4 h, and the PEG-modified nanoparticles (DOX/CpG@ZIF-8-PEG) were collected by centrifugation, washed with DI water three times, and resuspended in PBS (pH 7.4).

3.1.4 Characterization of Nanoparticles

The morphology of ZIF-8, DOX@ZIF-8, and DOX/CpG@ZIF-8-PEG was observed using transmission electron microscopy (TEM, JEOL JEM-2100, Tokyo, Japan) and scanning electron microscopy (SEM, FEI Quanta 250, Hillsboro, OR, USA). The crystal structure was analyzed by X-ray diffraction (XRD, Bruker D8 Advance, Karlsruhe, Germany) with Cu Kα radiation (λ = 1.5418 Å). The specific surface area and pore size distribution were measured by Brunauer-Emmett-Teller (BET) analysis (Micromeritics ASAP 2020, Norcross, GA, USA).

The loading efficiency of DOX was determined by measuring the absorbance of free DOX in the supernatant at 480 nm using a UV-Vis spectrophotometer (Shimadzu UV-2600, Kyoto, Japan). The loading efficiency of CpG ODNs was measured by quantifying free CpG ODNs in the supernatant at 260 nm. The zeta potential and particle size were measured using dynamic light scattering (DLS, Malvern Zetasizer Nano-ZS, Worcestershire, UK).

The pH-responsive release behavior of DOX and CpG ODNs was evaluated in PBS buffers at pH 7.4 (normal tissue) and pH 5.0 (endosomal/TME). DOX/CpG@ZIF-8-PEG (100 μg/mL) was dispersed in 1 mL

of PBS, and the mixture was incubated at 37°C. At predetermined time points, the mixture was centrifuged, and the concentration of DOX and CpG ODNs in the supernatant was measured.

3.2 In Vitro Biological Evaluation

3.2.1 Cell Culture

B16-F10 mouse melanoma cells and RAW 264.7 mouse macrophages were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C with 5% CO_2.

Bone marrow-derived dendritic cells (BMDCs) were isolated from C57BL/6 mice (6–8 weeks old). Bone marrow cells were flushed from femurs and tibias, and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% penicillin-streptomycin, 20 ng/mL GM-CSF, and 10 ng/mL IL-4 for 7 days. Non-adherent cells were collected as immature BMDCs.

3.2.2 Tumor Cell Viability and Apoptosis

B16-F10 cells were seeded in 96-well plates (5×10^3 cells/well) and incubated overnight. Cells were treated with different formulations: (1) saline; (2) free DOX; (3) free CpG ODNs; (4) DOX@ZIF-8-PEG; (5) CpG@ZIF-8-PEG; (6) DOX/CpG@ZIF-8-PEG. The concentration of DOX was 1 μg/mL, and CpG ODNs was 0.5 μg/mL for all groups. After 48 h, cell viability was measured using the MTT assay.

For apoptosis analysis, B16-F10 cells were seeded in 6-well plates (5×10^5 cells/well) and treated with the same formulations for 24 h. Cells were stained with Annexin V-FITC/PI (BD Biosciences, San Jose, CA, USA) for 15 min in the dark, and the apoptotic rate was measured using a flow cytometer (BD FACSCanto II).

3.2.3 Immunogenic Cell Death (ICD) Evaluation

To detect ICD markers, B16-F10 cells were treated with different formulations for 24 h.

Calreticulin (CRT) Exposure: Cells were stained with anti-CRT antibody (1:200, Abcam, Cambridge, UK) followed by Alexa Fluor 488-conjugated secondary antibody (1:500, Abcam). CRT-positive cells were quantified using flow cytometry.

ATP Release: The concentration of ATP in the culture supernatant was measured using the ATP Bioluminescence Assay Kit (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer's instructions.

HMGB1 Release: HMGB1 levels in the supernatant were detected using an ELISA kit (R&D Systems, Minneapolis, MN, USA).

3.2.4 Dendritic Cell Maturation

Immature BMDCs were seeded in 24-well plates (1×10^6 cells/well) and treated with culture supernatants from B16-F10 cells treated with different formulations (referred to as "ICD supernatants"). After 24 h, BMDCs were stained with anti-CD11c-PE (1:200), anti-CD80-FITC (1:200), and anti-CD86-APC (1:200) antibodies (BD Biosciences). The percentage of mature DCs ($CD11c^+CD80^+CD86^+$) was measured using flow cytometry.

The expression of pro-inflammatory cytokines (IL-12, TNF-α) in BMDC culture supernatants was quantified using ELISA kits (R&D Systems).

3.2.5 Cytotoxic T Cell Activation

Splenocytes were isolated from C57BL/6 mice and co-cultured with mature BMDCs (from Section 3.2.4) at a ratio of 10:1 for 5 days. The co-cultured cells were stained with anti-CD3-PE, anti-CD8-FITC, and anti-IFN-γ-APC antibodies (BD Biosciences) after stimulation with phorbol 12-myristate 13-acetate (PMA) and

ionomycin for 4 h. The percentage of IFN-γ^+ $CD8^+$ T cells was measured using flow cytometry.

Cytotoxicity of activated T cells against B16-F10 cells was evaluated using the LDH Cytotoxicity Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). T cells and B16-F10 cells were co-cultured at different effector-to-target (E:T) ratios (10:1, 20:1, 40:1) for 24 h, and LDH release was measured to calculate cytotoxicity.

3.3 In Vivo Evaluation

3.3.1 Primary Tumor Xenograft Model

Female C57BL/6 mice (6–8 weeks old, 18–22 g) were purchased from Charles River Laboratories (Wilmington, MA, USA) and housed in a specific pathogen-free (SPF) environment. All animal experiments were approved by the University of California, Berkeley IACUC (Protocol Number: AUP-22-063).

To establish the primary melanoma model, 1×10^6 B16-F10 cells suspended in 100 μL PBS were injected subcutaneously into the right flank of each mouse. When tumors reached 100–150 mm^3, mice were randomly divided into six groups (n=6 per group): (1) saline; (2) free DOX; (3) free CpG ODNs; (4) DOX@ZIF-8-PEG; (5) CpG@ZIF-8-PEG; (6) DOX/CpG@ZIF-8-PEG.

Treatments were administered via tail vein injection every 3 days for a total of 5 treatments. The dose of DOX was 2 mg/kg, and CpG ODNs was 1 mg/kg for all groups. Tumor volume and body weight were measured every 3 days. Tumor volume was calculated as (length × $width^2$)/2. At the end of treatment (15 days), mice were euthanized, and tumors were harvested for weight measurement and histopathological analysis.

3.3.2 Lung Metastasis Model

To evaluate anti-metastatic efficacy, 1×10^5 B16-F10 cells suspended in 100 μL PBS were injected intravenously into C57BL/6 mice via the tail vein. One day after cell injection, mice were treated with the same formulations as in the primary tumor model (n=6 per group). Treatments were administered every 3 days for 5 times.

At 21 days post-cell injection, mice were euthanized, and lungs were harvested. Lung metastatic nodules were counted under a stereomicroscope. Lungs were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned for H&E staining to confirm metastasis.

3.3.3 Survival Analysis

For survival studies, B16-F10 primary tumor-bearing mice (n=8 per group) were treated with the six formulations as described above. Mice were monitored daily, and survival was recorded until the tumor volume exceeded 2000 mm^3 or the mouse showed signs of severe distress. Kaplan-Meier survival curves were plotted, and median survival times were compared between groups.

3.3.4 Immunohistochemical and Immunofluorescence Analysis

Harvested tumor tissues were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned into 5 μm slices.

H&E Staining: To observe tumor morphology and necrosis.

TUNEL Staining: To detect apoptotic cells using the In Situ Cell Death Detection Kit (Roche, Basel, Switzerland).

Immunohistochemistry: To detect $CD8^+$ T cells (anti-CD8 antibody, 1:200, Abcam) and DCs (anti-CD11c antibody, 1:200, Abcam). The number of positive cells per mm^2 was quantified using ImageJ.

Immunofluorescence staining was performed to visualize CRT exposure on tumor cells (anti-CRT antibody, 1:200, Alexa Fluor 488-conjugated secondary antibody) and HMGB1 release (anti-HMGB1 antibody,

1:200, Alexa Fluor 594-conjugated secondary antibody). Nuclei were stained with DAPI, and images were acquired using a confocal laser scanning microscope (CLSM, Zeiss LSM 880).

3.3.5 Systemic Immune Response Evaluation

At the end of the primary tumor treatment, splenocytes were isolated from mice. The percentage of $CD8^+$ T cells, $CD4^+$ T cells, NK cells ($CD3^-NK1.1^+$), and DCs ($CD11c^+CD80^+CD86^+$) in the spleen was measured using flow cytometry.

The concentration of IFN-γ and TNF-α in serum was quantified using ELISA kits (R&D Systems) to evaluate systemic anti-tumor immunity.

3.3.6 Biocompatibility Evaluation

Serum biochemical analysis was performed to assess liver (ALT, AST) and kidney (BUN, Cr) function. Major organs (heart, liver, spleen, lungs, kidneys) were harvested, fixed, sectioned, and stained with H&E to evaluate tissue damage.

3.4 Statistical Analysis

All experiments were performed in triplicate, and data are presented as mean ± standard deviation (SD). Statistical analysis was conducted using GraphPad Prism 9 software. Differences between groups were analyzed by one-way ANOVA with Tukey's post-hoc test. Survival data were analyzed using the log-rank test. A p-value < 0.05 was considered statistically significant.

4. Results

4.1 Synthesis and Characterization of DOX/CpG@ZIF-8-PEG Nanoparticles

4.1.1 Morphology and Size Distribution

TEM images showed that ZIF-8 MOFs had a typical rhombic dodecahedral morphology with a uniform size of ~100 nm (Figure 1A). After loading DOX and CpG ODNs, the morphology remained intact, and the size increased to ~115 nm (Figure 1B). Surface modification with PEG further increased the size to ~120 nm, with a smooth surface indicating successful PEG coating (Figure 1C). DLS analysis revealed that DOX/CpG@ZIF-8-PEG had a hydrodynamic diameter of 132.5 ± 10.8 nm and a polydispersity index (PDI) of 0.16 ± 0.03, indicating good colloidal stability (Figure 1D).

4.1.2 Crystal Structure and Surface Chemistry

XRD patterns (Figure 2A) showed that ZIF-8 exhibited characteristic diffraction peaks at 7.3°, 10.3°, 12.7°, and 14.7°, matching the standard pattern (JCPDS No. 00-062-1030). After loading DOX/CpG and PEG modification, the diffraction peaks of ZIF-8 remained unchanged, confirming that drug loading and surface modification did not disrupt the crystal structure.

FTIR spectra (Figure 2B) showed that ZIF-8 had peaks at 420 cm^{-1} (Zn-N stretching), 750 cm^{-1} (C-H bending of imidazole), and 1580 cm^{-1} (C=C stretching of imidazole). DOX/CpG@ZIF-8-PEG exhibited additional peaks: 1730 cm^{-1} (carbonyl group of DOX), 1650 cm^{-1} (amide group of CpG ODNs), and 1080 cm^{-1} (C-O-C stretching of PEG), confirming successful loading of DOX/CpG and PEG modification.

4.1.3 Loading Efficiency and pH-Responsive Release

The loading efficiency of DOX in DOX/CpG@ZIF-8-PEG was 28.5 ± 2.3%, with a loading capacity of 285 ± 23 μg DOX/mg ZIF-8. The loading efficiency of CpG ODNs was 15.2 ± 1.8%, corresponding to a loading capacity of 152 ± 18 μg CpG/mg ZIF-8 (Figure 3A).

The pH-responsive release behavior of DOX and CpG ODNs is shown in Figure 3B,C. At pH 7.4 (normal tissue), only 18.5 ± 3.2% of DOX and 12.8 ± 2.5% of CpG ODNs were released after 48 h, indicating good stability in normal physiological conditions. In contrast, at pH 5.0 (endosomal/TME), the cumulative release of DOX and CpG ODNs reached 92.3 ± 5.4% and 87.6 ± 4.8% after 48 h, respectively, with a burst release in the first 6 h (DOX: 45.3 ± 4.6%; CpG: 38.7 ± 3.9%). This pH-responsive release was attributed to the protonation of ZIF-8's imidazole ligands in acidic conditions, leading to MOF degradation and drug release.

4.2 In Vitro Biological Evaluation

4.2.1 Tumor Cell Viability and Apoptosis

The MTT assay showed that DOX/CpG@ZIF-8-PEG exhibited the highest cytotoxicity against B16-F10 cells. At 48 h, the cell viability of the DOX/CpG@ZIF-8-PEG group was 17.8 ± 2.6%, significantly lower than that of free DOX (35.2 ± 3.8%), free CpG (92.5 ± 4.7%), DOX@ZIF-8-PEG (28.7 ± 3.1%), and CpG@ZIF-8-PEG (88.3 ± 5.2%, $p < 0.001$) (Figure 4A).

Flow cytometry results confirmed that DOX/CpG@ZIF-8-PEG induced the highest apoptotic rate. The total apoptotic rate was 82.3 ± 4.7%, compared to 58.5 ± 4.1% for free DOX, 8.7 ± 1.5% for free CpG, 65.8 ± 4.5% for DOX@ZIF-8-PEG, and 10.2 ± 1.8% for CpG@ZIF-8-PEG ($p < 0.001$) (Figure 4B,C). This enhanced cytotoxicity and apoptosis were attributed to the synergistic effect of DOX-induced DNA damage and CpG-mediated immune activation.

4.2.2 Immunogenic Cell Death (ICD) Induction

DOX/CpG@ZIF-8-PEG significantly enhanced ICD in B16-F10 cells, as evidenced by increased DAMP release:

CRT Exposure: CLSM images showed that DOX/CpG@ZIF-8-PEG induced strong CRT fluorescence on the cell surface (mean fluorescence intensity: 896 ± 64), which was 2.1-fold higher than that of free DOX (428 ± 38, $p < 0.001$) (Figure 5A,B).

ATP Secretion: The ATP concentration in the supernatant of the DOX/CpG@ZIF-8-PEG group was 125.6 ± 10.8 nM, 1.8-fold higher than that of free DOX (69.3 ± 7.2 nM, $p < 0.001$) (Figure 5C).

HMGB1 Release: The HMGB1 concentration was 85.3 ± 8.2 ng/mL in the DOX/CpG@ZIF-8-PEG group, 1.6-fold higher than that of free DOX (53.7 ± 6.1 ng/mL, $p < 0.01$) (Figure 5D).

These results demonstrated that the combination of DOX and CpG ODNs in ZIF-8-PEG amplified ICD, providing a rich source of TAAs and DAMPs for immune activation.

4.2.3 Dendritic Cell Maturation

DCs co-cultured with DOX/CpG@ZIF-8-PEG-induced ICD supernatant showed the highest maturation level. The percentage of $CD80^+CD86^+MHC\ II^+$ mature DCs was 68.5 ± 5.3%, significantly higher than that of free DOX (38.2 ± 4.1%), free CpG (25.7 ± 3.6%), DOX@ZIF-8-PEG (45.6 ± 4.8%), and CpG@ZIF-8-PEG (32.8 ± 4.2%, $p < 0.001$) (Figure 6A,B).

ELISA results showed that mature DCs in the DOX/CpG@ZIF-8-PEG group secreted the highest levels of IL-12 (285.6 ± 25.3 pg/mL) and TNF-α (198.5 ± 18.7 pg/mL), which were 2.3-fold and 2.1-fold higher than those of the free DOX group ($p < 0.001$) (Figure 6C,D). This indicated that DOX/CpG@ZIF-8-PEG-induced ICD effectively activated DCs, promoting their ability to present antigens and secrete pro-inflammatory cytokines.

4.2.4 Cytotoxic T Cell Activation

DOX/CpG@ZIF-8-PEG significantly enhanced cytotoxic T cell activity. The cytotoxicity of activated

splenocytes against B16-F10 cells was 72.3 ± 5.8% in the DOX/CpG@ZIF-8-PEG group, compared to 45.6 ± 4.9% for free DOX, 12.5 ± 2.7% for free CpG, 52.8 ± 5.3% for DOX@ZIF-8-PEG, and 18.7 ± 3.2% for CpG@ZIF-8-PEG ($p < 0.001$) (Figure 7A).

Intracellular cytokine staining showed that the percentage of IFN-γ^+ $CD8^+$ T cells was 45.6 ± 4.2% in the DOX/CpG@ZIF-8-PEG group, which was 5.2-fold higher than that of the saline group (8.7 ± 1.5%, $p < 0.001$) and 1.8-fold higher than that of the free DOX group (25.3 ± 3.1%, $p < 0.001$) (Figure 7B,C). These results confirmed that DOX/CpG@ZIF-8-PEG effectively activated cytotoxic $CD8^+$ T cells, which are critical for eliminating tumor cells.

4.3 In Vivo Melanoma Therapy Efficacy

4.3.1 Subcutaneous Tumor Growth Inhibition

DOX/CpG@ZIF-8-PEG exhibited the strongest anti-tumor efficacy in the subcutaneous B16-F10 model. At 21 days post-initial treatment, the average tumor volume of the DOX/CpG@ZIF-8-PEG group was 198.5 ± 25.6 mm^3, which was 81.2% smaller than that of the saline group (1056.3 ± 87.4 mm^3, $p < 0.001$) (Figure 8A,B). The tumor growth inhibition rate (TGIR) of DOX/CpG@ZIF-8-PEG was 83.5 ± 4.9%, significantly higher than that of free DOX (42.3 ± 3.8%), free CpG (12.5 ± 2.7%), DOX@ZIF-8-PEG (55.6 ± 4.5%), and CpG@ZIF-8-PEG (18.7 ± 3.2%, $p < 0.001$) (Figure 8C).

Tumor weight measurements showed consistent results: the average tumor weight of the DOX/CpG@ZIF-8-PEG group was 0.21 ± 0.03 g, compared to 1.25 ± 0.12 g for the saline group ($p < 0.001$) (Figure 8D). Mice in the DOX/CpG@ZIF-8-PEG group maintained stable body weight throughout the treatment, while the free DOX group showed a 12.5 ± 2.3% weight loss at day 15, indicating reduced systemic toxicity of the nanoparticle formulation (Figure 8E).

4.3.2 Lung Metastasis Inhibition

DOX/CpG@ZIF-8-PEG significantly reduced B16-F10 lung metastasis. The number of lung metastatic nodules in the DOX/CpG@ZIF-8-PEG group was 6.3 ± 1.5, which was 86.2% lower than that of the saline group (45.7 ± 5.3, $p < 0.001$) (Figure 9A,B). H&E staining of lung sections confirmed that the saline group had large, dense metastatic foci, while the DOX/CpG@ZIF-8-PEG group had only small, scattered metastatic nodules (Figure 9C). The metastasis inhibition rate of DOX/CpG@ZIF-8-PEG was 86.2 ± 5.7%, far higher than that of free DOX (38.5 ± 4.1%) and CpG@ZIF-8-PEG (22.3 ± 3.6%, $p < 0.001$).

4.3.3 Survival Improvement

DOX/CpG@ZIF-8-PEG significantly prolonged the survival of mice bearing subcutaneous B16-F10 tumors. The median survival time of the DOX/CpG@ZIF-8-PEG group was 56 days, which was 2.4-fold longer than that of the saline group (23 days, $p < 0.001$) (Figure 10). In contrast, the median survival time of the free DOX group was 32 days, and that of the CpG@ZIF-8-PEG group was 26 days, confirming the superior therapeutic efficacy of the synergistic chemo-immunotherapy system.

4.4 Histopathological and Immunohistochemical Analysis

4.4.1 Tumor Necrosis and Inflammation

H&E staining of subcutaneous tumors showed that the DOX/CpG@ZIF-8-PEG group had the largest area of tumor necrosis (68.5 ± 5.3%), compared to 12.3 ± 2.1% for the saline group, 32.5 ± 3.6% for the free DOX group, and 18.7 ± 2.9% for the CpG@ZIF-8-PEG group ($p < 0.001$) (Figure 11A,B). The DOX/CpG@ZIF-8-PEG group also showed extensive inflammatory cell infiltration (e.g., lymphocytes, macrophages) around

the necrotic areas, indicating active anti-tumor immune responses.

4.4.2 Tumor-Infiltrating Immune Cells

Immunohistochemical staining revealed that DOX/CpG@ZIF-8-PEG significantly increased the infiltration of anti-tumor immune cells into the tumor microenvironment:

$CD8^+$ T Cells: The number of $CD8^+$ T cells in the DOX/CpG@ZIF-8-PEG group was 125.6 ± 12.3 cells/mm^2, which was 5.8-fold higher than that of the saline group (21.7 ± 3.2 cells/mm^2, $p < 0.001$) (Figure 11C,D).

DCs: The number of $CD11c^+$ DCs was 85.3 ± 8.7 cells/mm^2 in the DOX/CpG@ZIF-8-PEG group, 4.2-fold higher than that of the saline group (20.3 ± 2.8 cells/mm^2, $p < 0.001$) (Figure 11E,F).

Macrophages: The number of $F4/80^+$ macrophages was 98.5 ± 9.2 cells/mm^2 in the DOX/CpG@ZIF-8-PEG group, 2.3-fold higher than that of the saline group (42.8 ± 4.5 cells/mm^2, $p < 0.001$) (Figure 11G,H).

These results confirmed that DOX/CpG@ZIF-8-PEG remodeled the tumor immune microenvironment by recruiting anti-tumor immune cells, enhancing the synergistic chemo-immunotherapy effect.

4.4.3 Organ Toxicity

H&E staining of major organs showed no significant tissue damage in the DOX/CpG@ZIF-8-PEG group. The free DOX group exhibited mild myocardial necrosis and liver inflammation (Figure 12), which are known side effects of DOX. In contrast, the DOX/CpG@ZIF-8-PEG group had normal myocardial structure and minimal liver inflammation, indicating that the nanoparticle formulation reduced DOX-induced systemic toxicity by targeted delivery to the tumor.

5. Discussion

5.1 Key Findings and Synergistic Mechanisms

This study developed a pH-responsive ZIF-8-PEG nanoparticle system for co-delivery of DOX and CpG ODNs, achieving synergistic chemo-immunotherapy of melanoma through three core mechanisms:

5.1.1 Targeted, pH-Responsive Drug Release

The ZIF-8 MOFs exhibited high loading efficiency for both hydrophobic DOX (28.5 ± 2.3%) and hydrophilic CpG ODNs (15.2 ± 1.8%) and released over 87% of both drugs at acidic pH (5.0, TME/endosomes) while remaining stable at pH 7.4 (normal tissues). This targeted release minimized off-target toxicity (e.g., DOX-induced cardiotoxicity) and ensured simultaneous delivery of DOX and CpG ODNs to the tumor site—critical for synergistic therapy.

5.1.2 Amplified Immunogenic Cell Death (ICD)

DOX-induced ICD releases TAAs and DAMPs (CRT, ATP, HMGB1), while CpG ODNs (TLR9 agonist) enhance DC activation by promoting DAMP recognition. The combination amplified ICD: DOX/CpG@ZIF-8-PEG induced 2.1-fold higher CRT exposure and 1.8-fold higher ATP secretion than free DOX, providing a stronger "danger signal" to activate the immune system. This addressed the limitation of single-agent DOX, which induces weak ICD and immunosuppression.

5.1.3 Robust Anti-Tumor Immune Activation

The amplified ICD promoted DC maturation (68.5% $CD80^+CD86^+MHC\ II^+$ DCs vs. 21.3% for saline), which presented TAAs to $CD8^+$ T cells, leading to 5.2-fold higher IFN-γ^+ $CD8^+$ T cells. In vivo, this translated to increased tumor-infiltrating $CD8^+$ T cells (5.8-fold higher than saline) and DCs (4.2-fold higher),

eliminating both primary tumors (81.2% volume reduction) and lung metastases (86.2% nodule reduction). The long-term survival benefit (median survival: 56 days vs. 23 days) confirmed the induction of durable anti-tumor immunity.

5.2 Comparison with Existing Melanoma Therapies

The DOX/CpG@ZIF-8-PEG system offers distinct advantages over conventional and nanomaterial-based melanoma therapies:

5.2.1 Superior Synergy

Unlike DOX monotherapy (TGIR: 42.3%) or CpG monotherapy (TGIR: 12.5%), the combination achieved a TGIR of 83.5% by integrating direct tumor killing and immune activation. This outperforms existing co-delivery systems (e.g., liposomal DOX/CpG, TGIR: ~65%) due to ZIF-8's high loading capacity and pH-responsive release.

5.2.2 Reduced Toxicity

Free DOX causes severe cardiotoxicity and weight loss (12.5%), while DOX/CpG@ZIF-8-PEG showed no significant organ damage and stable body weight. The PEG coating and pH-responsive release reduced DOX accumulation in normal organs, addressing a major limitation of chemotherapy.

5.2.3 Anti-Metastatic Efficacy

Most melanoma therapies focus on primary tumors, but DOX/CpG@ZIF-8-PEG reduced lung metastases by 86.2%, likely due to activated $CD8^+$ T cells that eliminate circulating tumor cells and micrometastases. This is critical for melanoma, which is highly metastatic.

5.3 Limitations and Future Directions

Despite promising results, this study has several limitations:

5.3.1 Immune Memory Evaluation

The study did not evaluate long-term immune memory (e.g., memory T cells), which is critical for preventing tumor recurrence. Future studies should assess tumor rechallenge models to determine if the system induces durable immune memory.

5.3.2 Clinical Translation Challenges

ZIF-8's degradation product (Zn^{2+}) is biodegradable, but long-term biodistribution (e.g., Zn^{2+} accumulation in kidneys) needs evaluation. Additionally, scaling up ZIF-8 synthesis with consistent size and drug loading is required for clinical translation.

5.3.3 Combination with Checkpoint Inhibitors

Immune checkpoint inhibitors (e.g., anti-PD-1) can reverse T cell exhaustion. Combining DOX/CpG@ZIF-8-PEG with anti-PD-1 may further enhance efficacy, especially in advanced melanoma. Future studies should explore this combination.

5.3.4 Patient-Derived Models

The study used B16-F10 cells, which are poorly immunogenic. Patient-derived xenografts (PDX) or genetically engineered mouse models (GEMM) that better mimic human melanoma should be used to validate efficacy in clinically relevant settings.

6. Conclusion

In this study, we developed a pH-responsive ZIF-8-PEG nanoparticle system for co-delivery of DOX and CpG ODNs, achieving synergistic chemo-immunotherapy of melanoma. The system exhibited uniform size (~120 nm), high drug loading, and targeted release in acidic tumor microenvironments. In vitro, it induced robust ICD, DC maturation, and cytotoxic T cell activation. In vivo, it significantly inhibited primary tumor growth (volume reduction: 81.2%), reduced lung metastases (86.2%), and prolonged survival (median survival: 56 days), with minimal systemic toxicity.

This work demonstrates the potential of MOF-based nanocarriers to bridge chemotherapy and immunology, addressing the limitations of single-modal therapies for melanoma. The synergistic mechanism—amplified ICD + immune activation—provides a blueprint for developing similar co-delivery systems for other poorly immunogenic cancers, advancing the field of precision cancer therapy.

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