Supporting Information

Treating Immunologically Cold Tumors by Precise Cancer Photoimmunotherapy with an Extendable Nanoplatform

Wenqian Yu, Junlin Sun, Feng Liu, Shuyi Yu, Jialing Hu, Yun Zhao, Xiuyuan Wang, Xiaoqing Liu*

College of Chemistry and Molecular Sciences, Wuhan University, Wuhan 430072, P. R. China.

* To whom correspondence should be addressed.

E-mail: xiaoqingliu@whu.edu.cn
# Table of Contents

**Experimental Section** .................................................................S3-S4

**Figure S1.** TEM image of HA/ZIF-8@ICG@IMQ........................................S5

**Figure S2.** Time-dependent hydrodynamic diameter of HA/ZIF-8@ICG@IMQ.....S6

**Figure S3.** Standard linear calibration curve of ICG......................................S7

**Figure S4.** Standard linear calibration curve of IMQ....................................S8

**Figure S5.** Standard linear calibration curve of FITC-labeled HA....................S9

**Figure S6.** Cell viability of NCM460 cells treated with HA/ZIF-8@ICG@IMQ......S10

**Figure S7.** Cell viability of CT26 cells treated with HA/ZIF-8@ICG@IMQ........S11

**Figure S8.** Apoptosis analysis of different nanoparticles...............................S12

**Figure S9.** Hemolytic analysis........................................................................S13

**Figure S10 In vivo fluorescence imaging........................................................S14

**Figure S11.** Temperature change induced by different nanoparticles...............S15

**Figure S12.** Ex vivo fluorescence images of the major organs.........................S16

**Figure S13.** Tumor weight of primary tumors................................................S17

**Figure S14.** Tumor weight of distant tumors..................................................S18

**Figure S15.** H&E analysis of the major organs..............................................S19

**Figure S16.** ICP-MS analysis.........................................................................S20

**Figure S17.** Blood circulation curve...............................................................S21

**Figure S18.** MDSCs in the primary tumors......................................................S22

**Figure S19.** Cytotoxic CD8\(^+\) T cells in the distant tumors.........................S23

**Figure S20.** Immumohistochemical staining of distant tumors.......................S24

**Figure S21.** TNF-\(\alpha\) levels in mice serum.....................................................S25

**Figure S22.** IL-6 levels in mice serum.............................................................S26

**Figure S23.** IFN-\(\gamma\) levels in mice serum.....................................................S27
Experimental Section

Materials. Zinc nitrate hexahydrate (Zn(NO)₃·6H₂O, 98%), 2-methylimidazole (99%), indocyanine green (ICG), imiquimod (IMQ) and hyaluronic acid (HA) were purchased from Sigma-Aldrich. Apoptosis Detection Kit and Live/dead Cell Staining Kit were purchased from Best Bio Science Co., Ltd. Dimethyl sulfoxide (DMSO) were purchased from Sinopharm Chemical Reagent Co. Trypsin–EDTA (0.25%) was purchased from Gibco. DMEM high glucose (L-glutamine 4.00 mM, glucose 4500 mg/L) and phosphate buffer saline (0.0067 M, none of calcium and magnesium) were purchased from HyClone™. Antibodies against cell surface markers for flow cytometry assay were obtained from Biolegend.

Cellular experiments. CT26 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 ℃ and 5% CO₂ in a humidified incubator.

In vivo experiment. All animal experiments were carried out following the criteria of The National Regulation of China for Care and Use of Laboratory Animals. The experiments were used under protocols approved by the Institutional Animal Care and Use Committee of Wuhan University. For humane reasons, BALB/C mice were sacrificed when the solid tumor volume exceeded 2000 mm³.

Hemolysis Assay. The fresh mouse blood was centrifuged, washed with PBS and diluted with PBS to obtain red blood cells (RBCs). Then, 0.2 mL of RBC suspension was added into 0.8 mL of HA/ZIF-8@ICG@IMQ (0-200 μg/mL) in PBS. Meanwhile, the same volume of distilled water was added into RBC suspension as the positive control. After standing for 4 h, the supernatants were collected by centrifugation at 4000 rpm for 5 min, and the absorbance at 541 nm was measured.

Flow cytometry analysis for anti-tumor immune responses. To analyze the immune cells of tumors in different models, tumors were harvested from mice, cut into pieces and digested using collagenase (1 mg/mL, Sigma), hyaluronidase (0.1 mg/mL, Sigma), and DNase (0.1 mg/mL, Sigma) at 37 ℃ for about 30 min. Afterwards, cells were filtered, washed with PBS containing 1% FBS and resuspended in the buffer solution. After washed with PBS, the single-cell suspension
was stained incubated with anti-CD3-FITC (Biolegend), anti-CD4-APC (Biolegend), anti-CD8a-PE (Biolegend) antibodies. After 30 min later, those cells were washed with PBS and determined using flow cytometry.

To analyze the DCs in lymph nodes, lymph nodes were harvested from mice and ground into a single-cell suspension, then washed three times and stained with anti-CD11c-PE (Biolegend), anti-CD86-APC (Biolegend) and anti-CD80-FITC (Biolegend) and determined by flow cytometry.

To analyze the effector memory T cells, spleens were harvested from mice and ground into a single-cell suspension, then incubated with anti-CD8-PE (Biolegend) anti-CD44-FITC (Biolegend) and anti-CD122-APC (Biolegend). 30 min later, those cells were washed with PBS and determined using flow cytometry.

**Detection of cytokines.** The pro-inflammatory cytokines including TNF-α, IFN-γ and IL-6 from sera were measured by using ELISA kits according the manufacturer's instruction book.

**Statistical analysis.** The statistical significance of different treatments was conducted using a one-way ANOVA with Tukey's post hoc test. All statistical analyses were performed using the SPSS 22.0 software. Significance was indicated as follows: *p < 0.05, **p < 0.01 or ***p < 0.001. Error bar represents standard deviation (SD).
Figure S1. TEM image of HA/ZIF-8@ICG@IMQ.
**Figure S2.** Time-dependent hydrodynamic diameter of HA/ZIF-8@ICG@IMQ in water, PBS and culture medium.
Figure S3. Standard curve of ICG by calculating the absorbance of ICG standard solutions at 790 nm.
Figure S4. Standard curve of IMQ by calculating the absorbance of IMQ standard solutions at 330 nm.
**Figure S5.** Standard linear calibration curve of the FITC-labeled HA.
**Figure S6.** Cell inhibition rates of HA/ZIF-8@ICG@IMQ on NCM460 cells under laser irradiation (1 W/cm², 5 min) after incubation for 24 h. Statistical significance was calculated via one-way ANOVA with Tukey post-hoc test (**: p < 0.01, ***: p < 0.001); Data are represented as mean ± SD; n = 3 each group.
Figure S7. Cell inhibition rates of HA/ZIF-8@ICG@IMQ on CT26 cells under laser irradiation (1 W/cm$^2$, 5 min) after incubation for 24 h. Statistical significance was calculated via one-way ANOVA with Tukey post-hoc test (**: $p < 0.01$, ***: $p < 0.001$); Data are represented as mean ± SD; $n = 3$ each group.
Figure S8. Analysis of apoptosis of CT26 cells treated with different nanoparticles by Annexin V/PI staining and flow cytometry.
Figure S9. Hemolysis percentage of different concentrations of HA/ZIF-8@ICG@IMQ nanoparticles. Inset: hemolysis of red blood cells after different treatments. Data are represented as mean ± SD; n = 3 each group.
**Figure S10** Time-dependent fluorescence images of tumor-bearing mice after the intravenous injection of HA/ZIF-8@IMQ@ICG.
Figure S11. Temperature change at the tumor site of tumor-bearing mice upon laser irradiation after intravenous injected with PBS, HA/ZIF-8, HA/ZIF-8@ICG, ZIF-8@ICG@IMQ and HA/ZIF-8@ICG@IMQ separately for 8 h.
Figure S12  (A) *Ex vivo* fluorescence images of heart, liver, spleen, lung, kidney and tumor isolated from CT26 tumor-bearing mice treated separately with ZIF-8@ICG@IMQ and HA/ZIF-8@ICG@IMQ. The tumor-bearing mice were sacrificed after intravenously injected with ZIF-8@ICG@IMQ or HA/ZIF-8@ICG@IMQ for 24 h, and the major organs were collected for imaging. (B) Total fluorescence intensity of isolated organs and tumors at 24 after injection as shown in (A).
Figure S13. (A) Weights of the primary tumors measured at the end of separate treatments with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5). (B) The final morphology of isolated primary tumors as shown in (A). Statistical significance was calculated via one-way ANOVA with Tukey post-hoc test (**: $p < 0.01$, ***: $p < 0.001$); Data are represented as mean ± SD; $n = 5$ each group.
Figure S14. Weights of the distant tumors measured at the end of separate treatments with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5). (B) The final morphology of isolated distant tumors as shown in (A). Statistical significance was calculated via one-way ANOVA with Tukey post-hoc test (**: $p < 0.01$, ***: $p < 0.001$); Data are represented as mean ± SD; $n = 5$ each group.
**Figure S15.** Images of H&E staining for heart, liver, lung, kidney and spleen of mice after various treatments. Scale bar, 50 μm.
Figure S16. ICP-MS analysis of Zn content in different organs. The tumor-bearing mice were sacrificed after intravenously injected with HA/ZIF-8@ICG@IMQ for 24 h, and the major organs were collected and digested to detect the amount of Zn. Data are represented as mean ± SD; n = 3 each group.
Figure S17. Blood circulation curve of HA/ZIF-8@ICG@IMQ in CT26 tumor bearing mice by measuring the blood concentration of Zn at different time points. Data are represented as mean ± SD; n = 3 each group.
Figure S18. Flow cytometry plots showing MDSCs in the primary tumors after various treatments. The mice were treated with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5), separately.
**Figure S19.** Flow cytometry plots showing cytotoxic CD8⁺ T cells in the distant tumors after various treatments. The mice were treated with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5), separately.
Figure S20. CD4+ T cells and CD8+ T cells in the distant tumor after treatments through immunohistochemical staining. Scale bar, 50 μm.
Figure S21. TNF-α levels in serum of mice re-challenged with secondary tumors. The mice were treated with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5), separately. Statistical significance was calculated via one-way ANOVA with the Tukey post-hoc test (**: p < 0.01); Data are represented as mean ± SD; n = 3 each group.
Figure S22. IL-6 levels in serum of mice re-challenged with secondary tumors. The mice were treated with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5), separately. Statistical significance was calculated via one-way ANOVA with the Tukey post-hoc test (**: $p < 0.01$); Data are represented as mean ± SD; $n = 3$ each group.
Figure S23. IFN-γ levels in serum of mice re-challenged with secondary tumors. The mice were treated with PBS (1), HA/ZIF-8@ICG@IMQ (2), ZIF-8@ICG@IMQ and NIR (3), HA/ZIF-8@ICG and NIR (4), and HA/ZIF-8@ICG@IMQ and NIR (5), separately. Statistical significance was calculated via one-way ANOVA with the Tukey post-hoc test (*: $p < 0.05$, ***: $p < 0.001$); Data are represented as mean ± SD; $n = 3$ each group.