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Polarization of macrophages to an anti-cancer phenotype through *in situ* uncaging of a TLR 7/8 agonist using bioorthogonal nanozymes

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General information

All chemicals and materials were purchased from Fisher Scientific or Sigma-Aldrich without further purification. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker ADVANCE 400 instrument. Electrospray ionization Mass Spectrometry (ESI-MS) was performed using a Bruker MicroTOF-II. Nanoparticle and nanozyme concentrations were measured *via* a previously reported method, using absorbance at 506 nm.[1] Absorbance and fluorescence were measured with a Molecular Devices SpectraMax M2 microplate reader. Transmission electron microscopy (TEM) images of samples were prepared by placing 10 μL of the desired nanoparticle solution (~5 μM) onto a 300-mesh Cu grid coated with carbon film and photographed using JEOL CX-100 electron microscopy. Dynamic light scattering of nanoparticles (~1 μM) was measured using a Malvern Zetasizer Nano ZS instrument at a measuring angle of 173° (backscatter).

RAW 264.7 cells and GFP-U2OS cells were purchased from American Type Culture Collection (ATCC), and cultured in Dulbecco's Modified Eagle's Medium (DMEM, high glucose) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Primary bone marrow-derived macrophages (BMDMs) were isolated from freshly euthanized C57/B6J mice, donated generously by Dr. Jessie Mager from the Department of Veterinary and Animal Science, UMass Amherst and Dr. Hang Xiao from the Department of Food Science, UMass Amherst. The BMDMs were isolated, differentiated, and cultured according to previously reported methods.[2] Macrophages were polarized to M2-like states by incubating with interleukin 4 (IL-4) at 50 ng/mL concentration for 24 h. The expression of cytokines was determined by following the user's guide of the enzyme-linked immunosorbent assay (ELISA) kit purchased from BD Biosciences. The

expression of nitrite was measured using the Invitrogen Griess assay kit. Cells were seeded in glass bottom MatTek confocal dishes (35mm) for imaging experiments.

Synthesis of TTMA-NP

TTMA-NP was synthesized by performing a ligand exchange reaction using TTMA ligand and pentanethiol-coated gold core following a previously reported method.[3]

Fabrication of TTMA-NZ

TTMA-NZ was synthesized by encapsulating the Pd catalyst into **TTMA-NP** using nanoprecipitation. Pd catalyst (1 mg) was dissolved in 1 mL of acetone and tetrahydrofuran (v/v=1:1). The Pd solution was added dropwise to 10 mL **TTMA-NP** solution (~100 nM) in water. The resulting solution was filtered by a 0.22 μm PES filter and applied to a 10 kDa molecular cut-off filter eight times to remove excess catalysts and obtain **TTMA-NZ**.

Catalyst loading

Catalyst loading was quantified by using inductively coupled plasma mass spectrometry (ICP-MS) using a Perkin-Elmer NexION 300X ICP mass spectrometer. ^{106}Pd and ^{197}Au were measured under the standard mode: nebulizer flow rate: 0.95 L/min; rf power: 1600 W; plasma Ar flow rate: 18 L/min; dwell time: 50 ms. Standard solutions of Multi-Element Standard Solution 4 (ME4) (0, 0.2, 0.5, 1, 2, 5, 10, 20 ppb) were prepared via serial dilutions for the calibration curve. Nanozyme samples were diluted. 20uL of diluted sample was transferred to 15 mL clean centrifugal tubes, followed by adding 0.5 mL of fresh *aqua regia* and then diluting to 10 mL with MilliQ water.

Table S1. Pd amount per nanozyme

TTMA-NZ	Au [ppb]	Pd [ppb]	Au [nM]	Pd [nM]	NP [nM]	Pd per NP	Ave	SD
NZ-1	675675	59357	3430402	554790	19058	29.1	29.3	0.82
NZ-2	697354	60283	3540466	563445	19669	28.6		
NZ-3	723461	65934	3673012	616261	20406	30.2		

Kinetic study of TTMA-NZ

In a 96-well black plate, **TTMA-NZ** and **pro-Rho** were mixed in PBS to obtain a 100 μL solution containing 200 nM **TTMA-NZ** and 10 μM **pro-Rho**. **Pro-Rho**-only and **TTMA-NZ**-only

samples were used as negative controls. The kinetic results were obtained by tracking the fluorescence (λ_{ex}: 488 nm, λ_{em}: 521 nm, cutoff: 515 nm) using a Molecular Devices SpectraMax M2 plate reader at 37 °C for 60 min continuously.

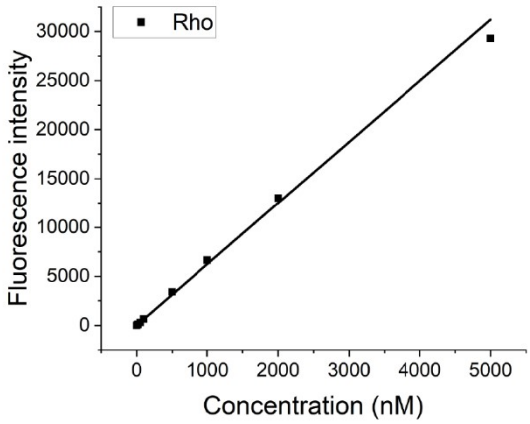

Figure S1. Calibration curve of **Rho** in PBS.

Cytotoxicity of TTMA-NZ on RAW 264.7 cells

RAW 264.7 cells were seeded in a 96-well plate at a density of 20,000 cells (for 24 h treatment), 7,500 cells (for 48 h treatment) and 4,000 cells (for 72 h treatment) per well. The following day, fresh media containing various concentrations of nanozymes was added to cells. After incubation for 24 h, 48 h or 72 h, cells were washed with phosphate-buffered saline (PBS) four times and treated with cell culture media containing 10% Alamar Blue. Three hours later, the supernatant was transferred to a 96-well black plate and cell viability was determined by determining the fluorescence using a SpectraMax M2 plate reader (λ_{ex}: 560 nm, λ_{em}: 590 nm).

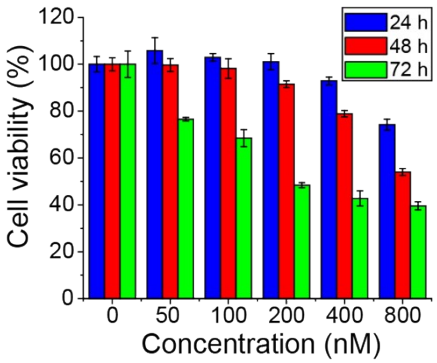

Figure S2. Cytotoxicity of **TTMA-NZ** on RAW 264.7 cells. Data shown is the average of three biological replicates and error bars represent the standard deviation.

Immunogenicity of TTMA-NZ and free catalysts

RAW 264.7 cells were seeded in a 24-well plate at a density of 80,000 cells per well. Cell culture media containing 400 nM **TTMA-NZ** or 11.6 μM Pd catalyst was added to the cells, which were then incubated for 24 h. After incubation, cells were washed with PBS three times, and RNA was extracted from macrophages using TRIzol reagent (Ambion Inc.). Approximately 2 μg RNA was used to generate cDNA using the SuperScript IV First-Strand Synthesis System (ThermoFisher Scientific), according to the manufacturer's protocols. RT-PCR was then performed on prepared cDNA using a CFX Connect real-time system (Biorad) with iTaq Universal SYBR Green Supermix (Biorad). All primers were purchased from Integrated DNA Technologies. The primer sequences used were as follows:

β-Actin (Forward): 5'-GATCAGCAAGCAGGAGTACGA-3';

β-Actin (Reverse): 5'-AAAACGCAGCGCAGTAACAGT-3';

TNF-α (Forward): 5'-CCTGTAGCCCACGTCGTAG-3';

TNF-α (Reverse): 5'-GGGAGTCAAGGTACAACCC-3'.

Confocal imaging

RAW 264.7 cells (80,000) were seeded in a glass-bottom confocal microscopy dish. Nanozymes (400 nM) were incubated with cells for 24 h, followed by washing four times with PBS. **Pro-Rho** (100 μM) was then added to the cells and incubated for another 24 h. The next day, cells were washed with PBS four times and stained with Lysotracker® deep red and Hoechst 23342 for 30 min. Cells were washed with PBS once more and imaged using a Nikon A1 spectral detector confocal microscope (A1SP) using a 40x objective at the Light Microscopy Core Facility at UMass Amherst.

Synthesis of pro-Imq

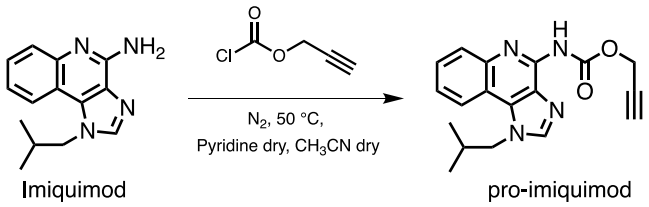

Figure S3. Synthesis of pro-Imq.

Pro-Imq was prepared by modifying a reported protocol.[4] In a two-neck 50 mL round flask, imiquimod (161 mg, 0.67 mmol, 1 eq) was dissolved in dry acetonitrile (12 mL, freshly distilled over CaH_2). Dry pyridine (220 μL) was added, and the mixture was heated to 50 °C under nitrogen

atmosphere. To the heated mixture, propargyl chloroformate (115 µL, 140 mg, 1.18 mmol, 1.7 eq) dissolved in dry acetonitrile (3 mL) was added dropwise over 90 min. The formation of the product was monitored by thin layer chromatography (TLC; AcOEt, R_f = 0.56; a weak spot of the di-propargylate was also visible at R_f = 0.72). After 150 min no further changes were visible via TLC and the reaction was cooled to room temperature. The resulting milky-white dispersion was filtered and the solvent was evaporated yielding a sticky oil. The crude product was purified twice with a flash column on silica (CH_2Cl_2:AcOEt 2:5) yielding pro-imiquimod as a waxy white solid (30 mg, 0.093 mmol, yield 14%). Note: to minimize the formation of the di-propagate by-product, chloroformate should not be used in excess. ^{1}H-NMR ($CDCl_3$, 400MHz, δ ppm): 8.254 (d, J = 8.2 Hz, 1H), 8.030 (s, 1H), 8.023 (d, J = 8.2 Hz, 1H), 7.683 (t, J = 7.6 Hz, 1H), 7.581 (t, J = 7.6 Hz, 1H), 4.925 (d, J = 2.5 Hz, 2H), 4.380 (d, J = 7.5 Hz, 2H), 2.515 (t, J = 2.5 Hz, 1H), 2.339 (m, J = 7.0 Hz, 1H), 1.052 (d, J = 7.0 Hz, 6H). ^{13}C-NMR ($CDCl_3$, 125 MHz, δ ppm): 151.583, 144.412, 144.235, 140.189, 134.960, 128.976, 128.787, 126.936, 126.019, 120.164, 115.733, 77.462, 75.424, 55.341, 53.631, 28.865, 19.795. ESI Mass calculated for MH^+ [$C_{18}H_{18}N_4O_2$ + H^+] 323.15, found 323.21.

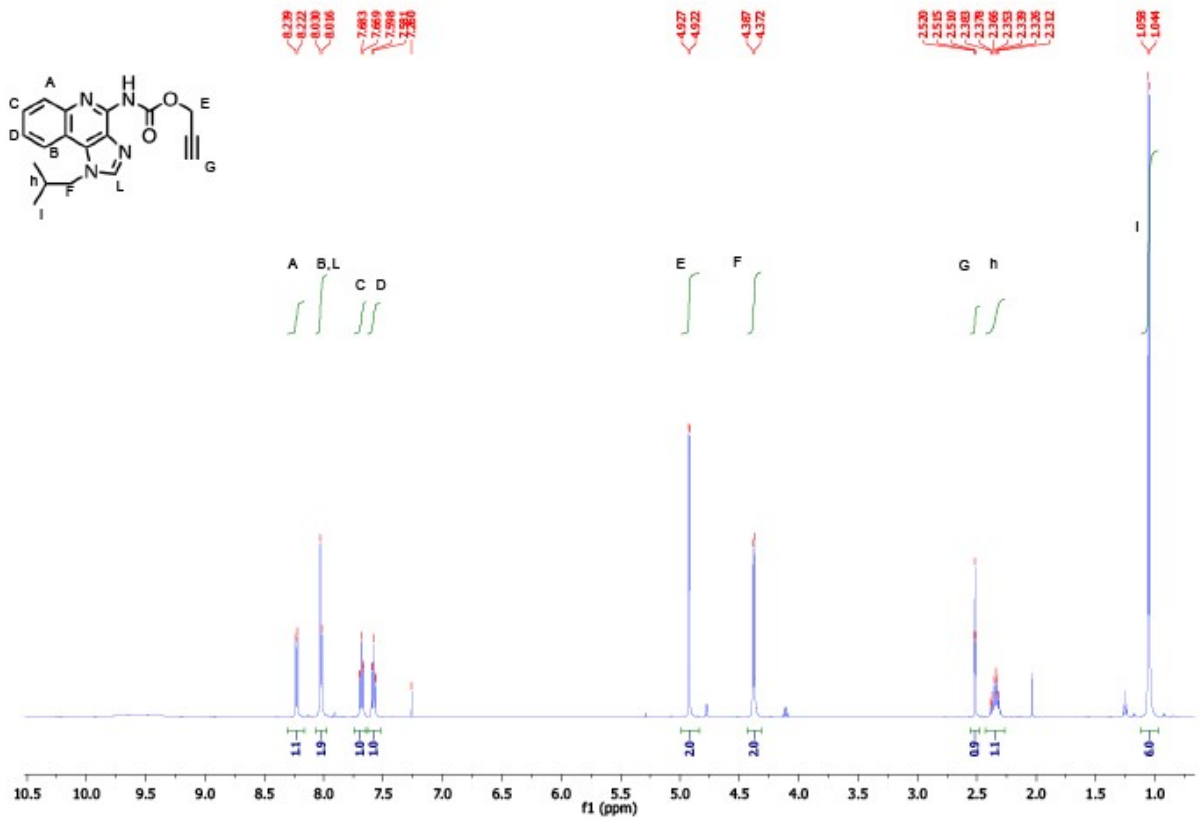

Figure S4. ^{1}H-NMR of **pro-Imq**.

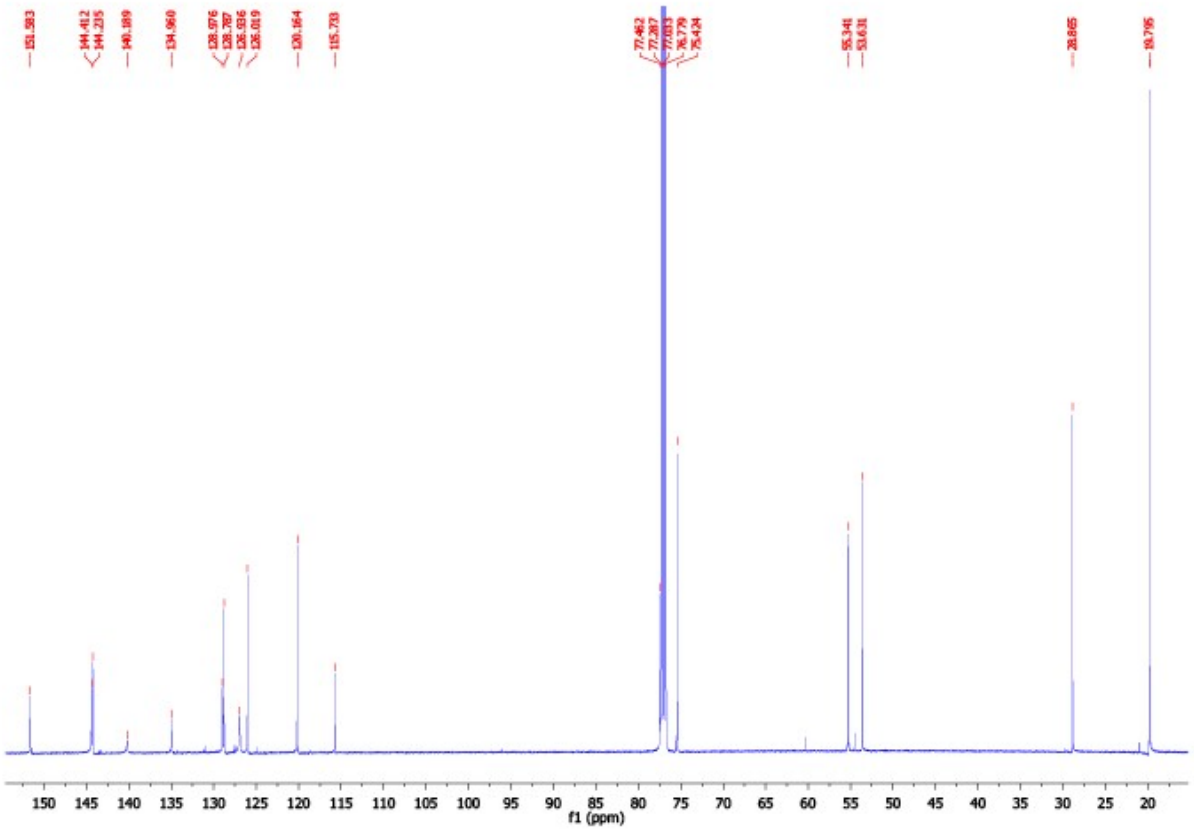

Figure S5. ^{13}C-NMR of **pro-Imq**.

<u>Cytotoxicity and immunogenicity of Imq and pro-Imq toward RAW 264.7 cells</u>

RAW 264.7 cells were seeded in a 96-well plate at the density of 20,000 cells (for 24 h treatment), 7,500 cells (for 48 h treatment) and 4,000 cells (for 72 h treatment) per well. On the following day, fresh media containing various concentrations of **Imq** and **pro-Imq** were added to the cells and incubated for 24 h, 48 h or 72 h. Afterward, the cell viability was measured by Alamar Blue assay, and the expression of TNF-α was measured using ELISA sets (BD Bioscience) by following the manufacturer's protocol.

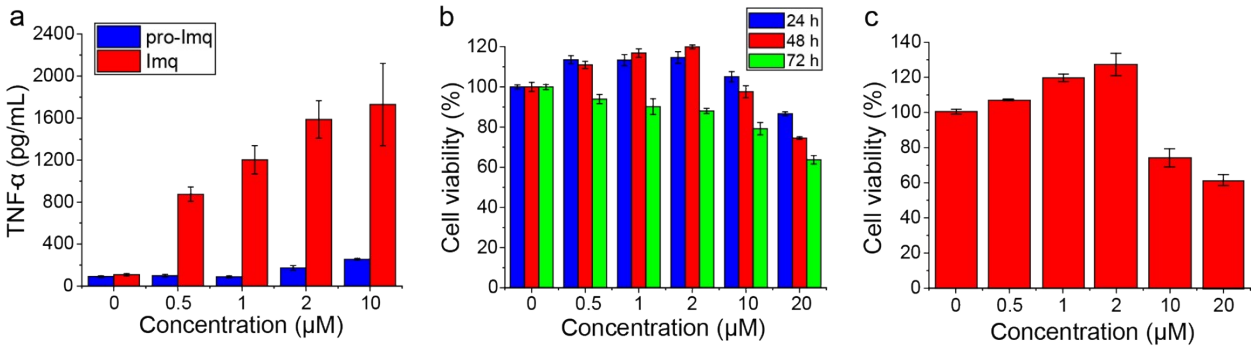

Figure S6. (a) TNF-α expression of RAW 264.7 cells after 24 h treatment with **Imq** or **pro-Imq**. **Pro-Imq** resulted in substantially lower expression of TNF-α versus **Imq**. **(b)** Cytotoxicity of **pro-Imq** toward RAW 264.7 cells. **(c)** Cytotoxicity of **Imq** toward RAW 264.7 cells after 24 h treatment.

Cytotoxicity of TTMA-NZ and pro-Imq toward Primary bone marrow-derived macrophages (BMDMs)

BMDMs were seeded in a 96-well plate at the density of 30,000 cells per well. The following day, fresh media containing various concentrations of **TTMA-NZ** and **pro-Imq** were added to the cells, which were then incubated for 24 h, 48 h, or 72 h. Afterward, cell viability was measured by Alamar Blue assay.

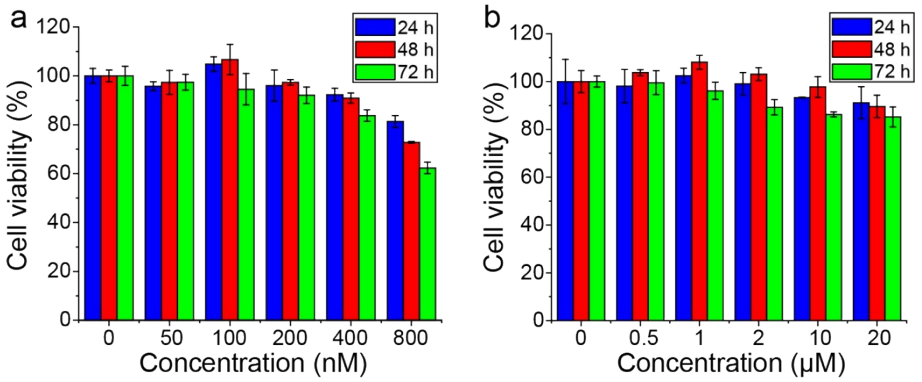

Figure S7. (a) Cytotoxicity of **TTMA-NZ** toward BMDMs. **(b)** Cytotoxicity of **pro-Imq** toward BMDMs.

Assessment of pro-inflammatory marker expression

RAW 264.7 cells or BMDMs were seeded with IL-4 in a 24-well plate to achieve an M2-like phenotype (60,000/well for RAW 264.7 cells and 200,000/well for BMDMs). The following day, cells were treated with 400 nM nanozyme for 24 h followed by washing four times with PBS and incubating designated samples with **pro-Imq** (10 μM) for another 24 h. Nanozymes alone and pro-Imq alone were used as negative controls, while **Imq** (2 μM) was used as the positive control.

The supernatant was collected for ELISA (TNF-α and IL-6) and Griess assay by following the manufacturers' protocols. Cells were harvested and incubated with allophycocyanin (APC)-labeled CD80 antibody (from Sigma-Aldrich) at a concentration of 60 ng per one million cells in FACS buffer (1% FBS in PBS) for one hour. The expression of CD80 was acquired by a Becton Dickinson (BD) LSR Fortessa 5 Laser Cell Analyzer and analyzed using FlowJo software at the University of Massachusetts Institute of Applied Life Sciences Flow Cytometry Core Facility.

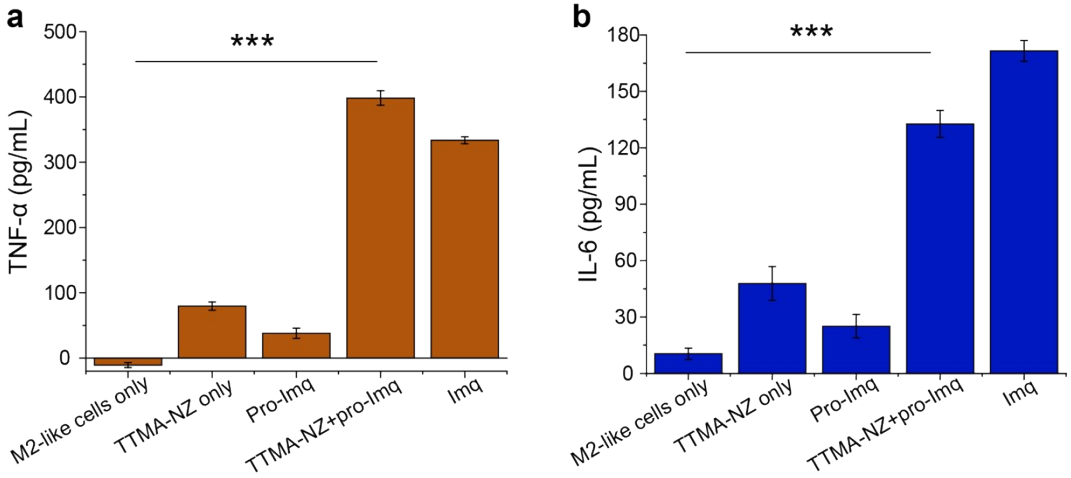

Figure S8. Quantification of (a) TNF-α and (b) IL-6 expression of BMDMs using enzyme-linked immunosorbent assay (ELISA).

Cytotoxicity of Imq toward GFP-U2SO cells

GFP-U2SO cells were seeded in a 96-well plate at the density of 20,000 cells per well. The following day, fresh media containing various concentrations of **Imq** were added to the cells and incubated for 24 h, 48 h, or 72 h. Afterward, cell viability was measured by Alamar Blue assay.

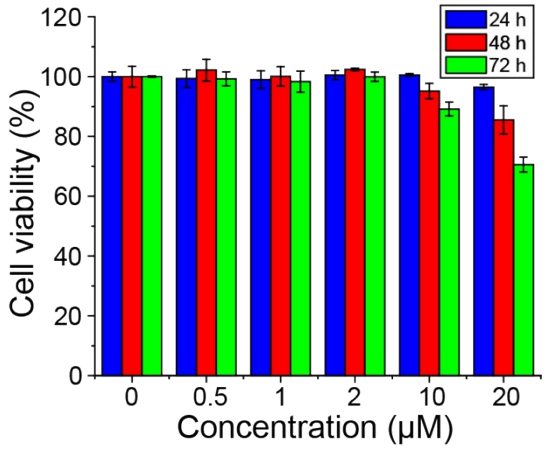

Figure S9. Cytotoxicity of **Imq** toward GFP-U2OS cells.

Phagocytosis assay

The phagocytosis assay was performed similarly to a previous report.[5] Briefly, M2-like macrophage cells were treated with 400 nM nanozymes for 24 h followed by washing four times with PBS and incubating with **pro-Imq** (10 μM) for another 24 h. Nanozymes alone and **pro-Imq** alone were used as negative controls, while **Imq** (2 μM) was used as the positive control. After treatment, macrophages were harvested and stained by PE anti-mouse F4/80 antibody (eBioscience™) at 4°C for 30 min. Afterward, the cells were washed with PBS three times to remove excess/non-associated antibodies and incubated with GFP-U2OS cells at a ratio of 300,000 (macrophages) to 100,000 (GFP-U2OS) for 4 h. After incubation, cells were washed with PBS three more times, resuspended (10, 000 cells) in FACS buffer (1% FBS in PBS), and analyzed using a BD LSR Fortessa 5 Laser Cell Analyzer. The phagocytosis percentages were calculated as the percentages of GFP-positive cells within the PE-positive macrophage gates using FlowJo.

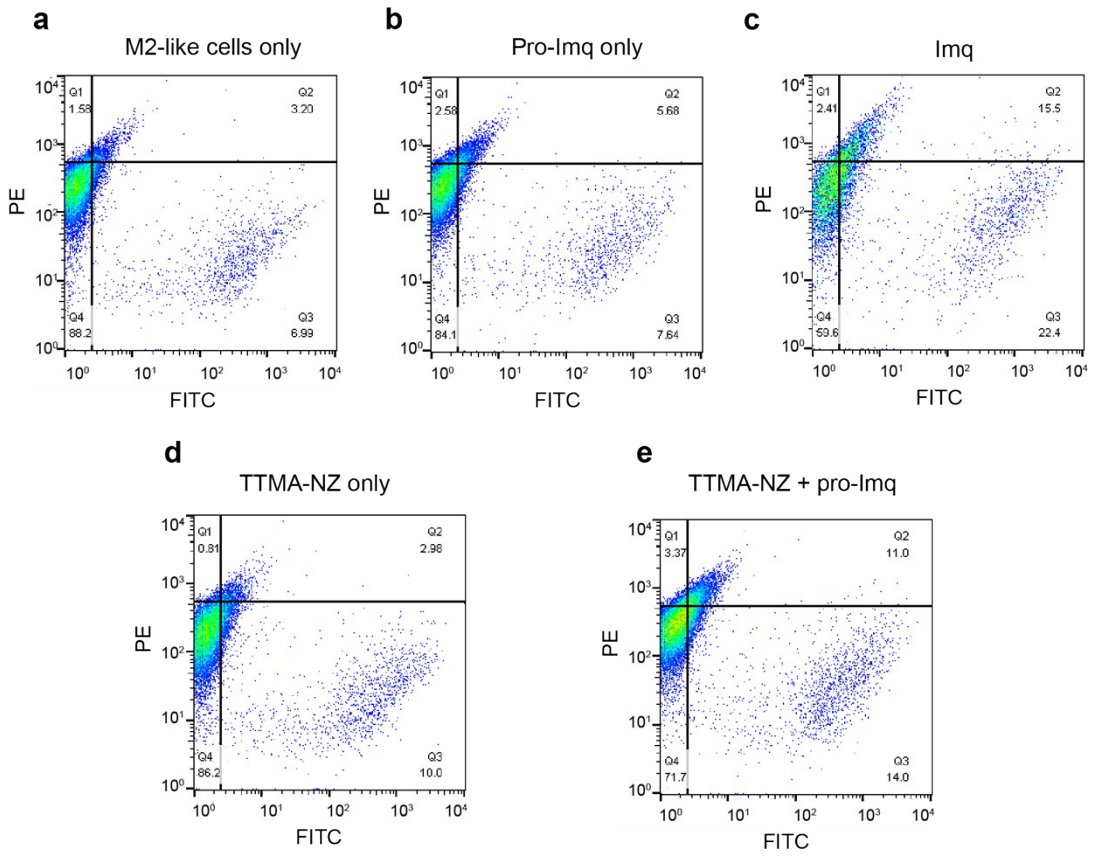

Figure S10. Flow cytometry experiments to determine effects on macrophage phagocytosis. Representative scatterplots are shown for **(a)** M2-like macrophages treated with **(b) pro-Imq**, **(c) Imq**, **(d) TTMA-NZ**, and **(e) TTMA-NZ** + **pro-Imq** sequentially. Macrophages were labeled with PE-F4/80 antibody, and U2OS cancer cells expressed GFP (FITC channel).

Co-culture cancer killing

M2-like macrophage cells were co-cultured with GFP-U2OS cells in a confocal microscopy dish at densities of 300,000 and 100,000 cells, respectively. Then, cells were treated with **TTMA-NZ** (400 nM) for 24 h followed by washing four times with PBS and incubating with **pro-Imq** (10 μM) for another 24 h. **Pro-Imq** and **TTMA-NZ** alone were used as negative controls, and **Imq** was used as the positive control. After incubation, cells were stained with Hoechst 23342 for 30 min and imaged using by Nikon A1 spectral detector confocal microscope (A1SP) using a 10x objective from Light Microscopy Core Facility at UMass Amherst, and the fluorescence intensity was quantified by Image J. The experiment was performed three times.

References

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