Supporting Information

A biomimetic therapeutic nanovaccine based on dendrimer-drug conjugates coated with metal-phenolic networks for combination therapy of nasopharyngeal carcinoma: An *in vitro* investigation

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Experimental

Materials

Tannic acid (TA) and 4-carboxyphenylboronic acid (PBA) were from J&K Scientific (Beijing, China). Generation 5 (G5) poly(amidoamine) (PAMAM) dendrimers (G5.NH_2) with ethylenediamine core were purchased from Dendritech (Midland, MI). Toyocamycin (Toy) was from Shanghai Wei Huan Biological Technology Co., Ltd. (Shanghai, China). CpG (5'-TCCATGACGTTCCTGACGTT-3')-cholesterol with or without Cy3 fluorescent probe labeled was from Sangon Biotech (Shanghai) Co., Ltd. (Shanghai, China). Acetic anhydride (Ac_2O), triethylamine (TEA), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), N-hydroxysuccinimide (NHS), indocyanine green carboxylic acid (ICG-COOH), manganese (II) chloride ($MnCl_2$), hydrogen peroxide (H_2O_2), methylene blue (MB), and all the other chemicals and solvents were from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Sodium dodecyl sulfate (SDS) sample loading buffer and SDS-polyacrylamide gel were from Tanon Science & Technology Co., Ltd. (Shanghai, China). The 3-dimensional (3D) Petri Dish was from Microtissues (Providence, RI). Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 3500 and phosphate buffered saline (PBS) were from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm. FAT 7 cells (established from a nasal squamous cell carcinoma induced by formaldehyde inhalation in an adult male rat) was from ATCC (catalog number CRL2109, Manassas, VA). L929 cells (a mouse fibroblast cell line) and RAW 264.7 cells (a mouse macrophage cell line) were acquired from Institute of Biochemistry and Cell Biology (the Chinese Academy of Sciences, Shanghai, China). Dendritic cells (DCs) were from Shanghai Cancer Center, Fudan University. Dulbecco's Modified Eagle Medium (DMEM) was from HyClone Lab., Inc. (Logan, UT). Penicillin-streptomycin and trypsin (0.25%) were from Gino Biomedical Technology Co., Ltd. (Hangzhou, China). Cell counting kit-8 (CCK-8) was from 7sea Biotech. Co., Ltd. (Shanghai, China). 4',6-Diamidino-2-phenylindole (DAPI) was acquired from

BestBio Biotechnology Co., Ltd. (Shanghai, China). BeyoRT™ III first strand cDNA synthesis kit, RNAeasy plus animal RNA isolation kit, BeyoFast™ SYBR green qPCR mix (2×, Low ROX), bicinchoninic acid (BCA) assay kit, annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, glutathione (GSH)/oxidized GSH disulfide (GSSG) assay kit, adenosine triphosphate (ATP) detection kit, 2, 7-dichlorofluorescin diacetate (DCFH-DA), anti-calreticulin (CRT) rabbit polyclonal antibody, and mitochondrial membrane potential assay kit with a JC-1 probe were from Beyotime Biotech Co., Ltd. (Shanghai, China). The lipid peroxidation (LPO) sensor of C11-BODIPY581/591 was from Shanghai Hongye Biotechnology Co., Ltd. (Shanghai, China). CD80-PE monoclonal antibody, CD86-FITC monoclonal antibody and CD206-FITC monoclonal antibody were from Proteintech Group, Inc. (Wuhan, China). Stimulator of interferon genes (STING)/p-STING rabbit pAb, TANK-binding kinase 1 (TBK1)/p-TBK1 rabbit pAb, interferon regulatory factor 3 (IRF3)/p-IRF3 rabbit pAb and β-Actin rabbit pAb were from Wuhan Servicebio Technology Co., Ltd. (Wuhan, China). Enzyme linked immunosorbent assay (ELISA) kits of mouse interleukin-6 (IL-6), interferon-β (IFN-β), tumor necrosis factor-α (TNF-α) and high mobility group protein 1 (HMGB-1) were obtained from Multisciences (Lianke) Biotech (Hangzhou, China).

Synthesis of G5.NHAc-ICG-Toy (GIT)

$G5.NH_2$ dendrimers were reacted with 20 molar equiv. of PBA through an EDC/NHS-mediated covalent conjugation according to the literature protocols.[1] Briefly, NHS (45.0 mg, in 5 mL dimethyl sulfoxide, DMSO) and EDC (65.0 mg, in 5 mL DMSO) were added to the PBA solution (6.5 mg, in 5 mL DMSO) under stirring for 3 h at room temperature. After that, the above mixture was slowly dropped into the $G5.NH_2$ dendrimer solution (50.0 mg, in 5 mL DMSO) under stirring for 24 h at room temperature. Subsequently, the reaction mixture was dialyzed against water (9 times, 2 L) using a dialysis membrane with an MWCO of 3500 Da for 3 days, and then lyophilized to get the $G5.NH_2$-PBA product.

Next, NHS (45.0 mg, in 5 mL dimethyl sulfoxide, DMSO) and EDC (65.0 mg, in 5 mL DMSO) were added to the ICG-COOH solution (13.7 mg, in 5 mL DMSO) under stirring for 3 h at room temperature.

The activated ICG-COOH was reacted with the G5.NH_2-PBA dendrimers (55.4 mg, in 10 mL water) under stirring for 24 h at room temperature. The obtained reaction mixture was then dialyzed and lyophilized under the same conditions described above to obtain the G5.NH_2-PBA-ICG product.

Afterwards, the G5.NH_2-PBA-ICG dendrimers (50 mg, in 10 mL water) were mixed with 128 × 5 molar equiv. of TEA (210 μL) under stirring for 30 min, and then added with Ac_2O (120 μL) under stirring for 24 h to neutralize the remaining dendrimer terminal amines. Then the mixture solution was dialyzed against water for 3 days (MWCO = 3500 Da; 9 times, 2 L) and lyophilized to get the G5.NHAc-PBA-ICG product.

For Toy conjugation, the G5.NHAc-PBA-ICG dendrimers (65.0 mg, in 10 mL water) were reacted with 16.5 molar equiv. of Toy (8.0 mg, in 15 mL water) under stirring for 2 days. Then, the mixture solution was dialyzed against water for 3 days (MWCO = 3500 Da; 9 times, 2 L) and lyophilized to get the G5.NHAc- ICG-Toy product.

Synthesis of GIT@TM/C-aCM nanoparticles (NPs)

The G5.NHAc-ICG-Toy dendrimers (10 mg) were dispersed in 5 mL of water, then added with 100 μL of Tris-HCl buffer (pH = 7.5-8). Subsequently, 100 μL of $MnCl_2$ solution (4 mg/mL) was added, and the mixture solution was vortexed for 10 s, followed by addition of 100 μL of TA solution (20 mg/mL) and vortexed for 10 s. After that, the mixture solution was dialyzed against water for 3 days (MWCO = 3500 Da; 9 times, 2 L) to get the G5.NHAc- ICG-Toy@TM product (GIT@TM).

Extraction and characterization of apoptotic cancer cell membranes (aCM)

FAT 7 cells were maintained in Ham's F12K medium, supplemented with insulin (0.01 mg/mL), hydrocortisone (250 ng/mL), transferrin (90%, 0.0025 mg/mL), fetal bovine serum (FBS, 10%), and antibiotics (penicillin, streptomycin, and amphotericin B, GIBCO, Carlsbad, CA), and incubated at 37°C in a 95%/5% air/CO_2 incubator. When the culture was near confluence, the cells were subcultured using a 0.05% trypsin-ethylenediamine tetraacetic acid (EDTA) solution (GIBCO).

For aCM extraction, FAT 7 cells cultured to 80% confluence were treated with free ICG (50 μg/mL), and the intracellular fluorescence intensity of the cells at different culture time points was measured by flow cytometry. It was observed that the highest intracellular fluorescence intensity was achieved after 6 h of incubation. Hence, the optimal cellular uptake was selected at 6 h. Further, after ICG uptake, we induced apoptosis of FAT 7 cells through laser irradiation at different power densities (0.5, 0.75, 1, 1.25 or 1.5W/cm^2) and found that the apoptosis rate was close to 50% at the power density of 1.5 W/cm^2. In order to avoid excessive necrosis, the cell membranes were harvested in the apoptotic state. Under the optical cell culture conditions, FAT 7 cells were cultured to 80% confluence in flasks, treated with ICG at a concentration of 50 μg/mL, irradiated using an 808-nm laser with a power density of 1.5 W/cm^2 after 6 h of incubation, and then collected using a cell spatula after overnight incubation. Then, the apoptotic cell membranes were extracted using the Cell Membrane Protein Extraction Kit, and coated onto the surface of the GIT@TM to get the product of GIT@TM/aCM. For the synthesis of GIT@TM/aCM NPs, the prepared GIT@TM NPs (200 μg) were mixed with 0.5 mL of aCM suspension (4 mg/mL) and the mixture was extruded 11 times using an Avanti mini extruder (Avanti Polar Lipids, Inc., Alabaster, AL). Subsequently, the mixture was centrifuged at 12000 rpm for 8 min at 4 ℃ to obtain the GIT@TM/aCM NPs in the precipitate. Lastly, CpG-cholesterol (labeled with Cyanine 3 (Cy3) dye) was inserted into the surface of the GIT@TM/aCM NPs by incubation at 37 ℃ for 4 h according to a GIT@TM/aCM/CpG mass ratio 10: 1. The mixture was stirred at 37 ℃ for 4 h, centrifuged at 8000 rpm for 5 min, and washed with PBS for 3 times to obtain the final product of GIT@TM/C-aCM.

Characterization techniques

^{1}H NMR was performed using a Bruker NMR (400 MHz) spectrometer. The G5.NH_2-PBA dendrimers were dissolved in D_2O (7 mg/mL) before measurements. Zeta potentials and hydrodynamic sizes of all samples were measured using a Malvern Zetasizer Nano ZS system model ZEN3600 (Worcestershire, UK) equipped with a standard 633-nm laser. Each sample was dispersed in water at a concentration of 1 mg/mL before measurements. Fourier transform infrared (FTIR) spectra were recorded using a Nicolet

6700 spectrometer (Thermo Electrion Corporation, Madison, WI). The size and morphology of GIT@TM/C-aCM were observed by transmission electron microscopy (TEM) using an FEI Tecnai F20 analytical electron microscopy (FEI, Hillsboro, OR) under an operating voltage of 200 kV. The GIT@TM/C-aCM NPs were dispersed in water (0.1 mg/mL), then dropped onto the carbon-coated copper grid and air dried before measurements. The size of GIT@TM/C-aCM NPs was measured by ImageJ software (https://imagej.nih.gov/ij/download.html), and the average size was obtained by measuring the size of more than 100 particles in the TEM images. UV-vis spectra were obtained using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Waltham, MA) and all samples were dispersed in water before measurements. The content of Mn in the GIT@TM/C-aCM NPs was directly analyzed by inductively coupled plasma-optical emission spectroscopy (ICP-OES, Leeman Prodigy, Hudson, NH).

Detection of ·OH generation by an Mn^{2+}-mediated Fenton-like reaction

Methylene blue (MB) was introduced to detect the hydroxyl radical (•OH) generated by the prepared GIT@TM/C-aCM NPs. Briefly, the GIT@TM/C-aCM NPs ([Mn] = 0.1 mM) were dispersed in water, well mixed with an H_2O_2 solution (10 mM) and an MB solution (10 μg/mL), and incubated at room temperature for 3 h. The OH•-induced MB degradation was quantified by UV-vis spectroscopy at 665 nm. For comparison, the blank MB solution and H_2O_2/MB mixture solution were also tested under the same conditions.

***In vitro* Toy and Mn release**

To investigate the reactive oxygen species (ROS)- and pH-responsive Toy release, the GIT@TM/C-aCM NPs (4 mg) were dispersed in 1.0 mL of phosphate buffer with two different pHs (6.5 and 7.4) in the presence or absence of 10 mM H_2O_2. Each dispersion was placed in a dialysis bag with an MWCO of 3500, and the bag was submerged into 9 mL of the corresponding buffer medium. The entire system was kept in a constant temperature vibrator at 37 °C. At each predetermined time interval, 1.0 mL of the outer phase buffer medium was taken out and the released Toy was measured by UV-vis spectrometry at 279 nm. The volume of outer phase was maintained constant by adding 1.0 mL of the corresponding buffer

solution. Similarly, to investigate the pH-responsive Mn release kinetics, the GIT@TM/C-aCM NPs (4 mg) were dispersed in 1.0 mL of phosphate buffer with two different pHs (6.5 and 7.4). The release experiment was similar to the above, and the released Mn element was measured by ICP-OES.

Photothermal property of the GIT@TM/C-aCM NPs

To evaluate the photothermal conversion property of the GIT@TM/C-aCM NPs, the temperature change of GIT@TM/C-aCM in aqueous suspension at varying concentrations ([ICG] = 0, 2.5, 5, 10, or 20 μg/mL) under an 808-nm laser (Shanghai Xilong Optoelectronics Technology Co. Ltd., Shanghai, China) irradiation (1.0 W/cm^2) for 10 min. Typically, the GIT@TM/C-aCM solution ([ICG] = 10 μg/mL) was laser irradiated for 10 min under an output power density of 1.0 W/cm^2, and real-time temperature changes were recorded every 5 s by an online DT-8891E thermocouple thermometer (Shenzhen Everbest Machinery Industry Co., Ltd., Shenzhen, China). To quantify the photothermal conversion efficiency (η) of the GIT@TM/C-aCM NPs, a heating and cooling cycle of the sample was carried out, and η was calculated according to the literature protocols.[2, 3] Furthermore, to evaluate the photothermal stability of the GIT@TM/C-aCM NPs, 5 heating and cooling (laser on-off) cycles were performed to record the temperature changes.

Cellular uptake assay

The uptake of the GIT@TM/aCM NPs by FAT 7 cells was assessed by BD FACS Calibur Flow cytometry (Franklin Lakes, NJ). In brief, cells were plated in a 12-well plate at a density of 1 × 10^5 cells per well with 1.0 mL Ham's F12K medium and incubated overnight at 37 °C and 5% CO_2. The next day, the medium of each well was replaced with fresh medium containing GIT@TM/aCM NPs at different concentrations, and the cells were incubated for 6 h. After that, the cells were washed three times with PBS, trypsinated, centrifuged and resuspended in 300 μL PBS to measure the mean red fluorescence intensity by flow cytometry. Each measurement was repeated for 3 times.

The cellular uptake was also studied by confocal laser scanning microscopy (CLSM). Briefly, FAT 7 cells were seeded in confocal dishes at a density of 1.5 × 10^5 cells per dish with 1.5 mL complete Ham's

F12K medium and cultured overnight. After that, the medium of each dish was replaced with fresh medium containing GIT@TM/aCM or GIT@TM NPs at different concentrations, and the cells were cultured for 6 h. The cells were washed three times with PBS, fixed with 4% glutaraldehyde at 4 °C for 15 min, stained with DAPI for 10 min, washed with PBS 3 times, and observed by a ZEISS LSM-700 CLSM (Jena, Germany) to check their red fluorescence signals.

Cell viability assay

Firstly, FAT 7 cells were regularly cultured and passaged in Ham's F12K medium containing 10% FBS and 1% penicillin-streptomycin, and then a cell viability assay was carried out using a CCK-8 kit according to the manufacturer's instructions. In brief, FAT 7 cells were seeded in 96-well plates at a density of 1×10^4 cells per well in 0.1 mL of medium overnight, then the medium of each well was replaced with fresh medium containing GIT, GIT@TM or GIT@TM/C-aCM at different Toy concentrations (0, 0.5, 2.5, 5, 10, 20 and 30 μg/mL, respectively), and the cells were cultured for 24 h. After that, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). Subsequently, the medium of each well was replaced with the CCK-8-containing medium (10 μL CCK-8 in 0.1 mL FBS-free medium), and cells were incubated for 2 h. The absorbance of each well was then recorded using a Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) at a wavelength of 450 nm. Each sample was tested in three parallel wells. Cells treated with PBS were used as control, and the corresponding cell viability was recorded as 100%. Similarly, the viability of FAT 7 cells incubated with GIT@TM/NM (GIT@TM coated by L-929 cell membranes, as a control for normal cell membranes, for short, NM) or GIT@TM/aCM at different Toy concentrations for 24 h was also evaluated.

Live/dead cell staining assay

To further qualitatively confirm the photothermal effects of different materials *in vitro*, a live/dead cell staining assay was performed. Briefly, FAT 7 cells were seeded into a 12-well plate at a density of 1×10^5 cells per well in 1 mL of medium. After overnight incubation at 37 ℃ and 5% CO_2, the medium of each

well was replaced with fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM, and the cells were incubated for 24 h. Next, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). Subsequently, the cells were washed three times with PBS and stained by PI (staining dead cells, in red) and calcein-AM (staining live cells, in green) for 30 min. The cells were then observed using an inverted Carl Zeiss fluorescence microscope (Axio V ert. Al, Jena, Germany).

Cancer cell apoptosis *in vitro*

To check the cell apoptosis after different treatments, an annexin V-FITC/PI kit was used to stain cells before flow cytometry measurements. Cells were seeded in 12-well plates at a density of 1 ×10^5 cells per well in 1 mL of medium overnight, then treated with fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 12 h. After 12 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). Subsequently, the cells were regularly incubated for 4 h, then trypsinized to detach them from the plate, and annexin V-FITC/PI stained according to the manufacturer's instruction before flow cytometry assay. Each measurement was repeated for 3 times.

Detection of ROS and LPO levels

The ROS and LPO levels in FAT 7 cells after different treatments were detected using DCFH-DA and C11-BODIPY probe, respectively, according to the manufacturer's instructions. For ROS detection, FAT 7 cells were seeded in confocal dishes at a density of 1.5×10^5 cells per dish in 1 mL of medium overnight, and then incubated with fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 4 h. After 4 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). The cells were then stained with DCFH-DA (10 μM, in 1 mL FBS-free medium) for 30 min, fixed with 4% glutaraldehyde for 15 min, stained with DAPI for 10 min, and washed with PBS to observe the green fluorescence signals using a ZEISS LSM-700 laser scanning confocal microscope under a 63× oil immersion objective lens.

Meanwhile, FAT 7 cells seeded in 12-well plates at the above density were treated under the same incubation and DCFH-DA staining protocols. Then, the cells were washed, trypsinized, centrifuged, harvested in PBS and analyzed using a BD FACS Calibur Flow Cytometer to quantitatively determine the green fluorescence intensity. Each measurement was sampled with 1×10^4 cells and repeated for 3 times.

Similarly, for LPO detection, FAT 7 cells were seeded in confocal dishes at a density of 1×10^5 cells per well in 1 mL of medium overnight, and then incubated with fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 4 h. After 4 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). The cells were then stained with C11-BODIPY (5 μM, in 1 mL PBS) for 20 min, fixed with 4% glutaraldehyde for 15 min, stained with DAPI for 10 min, and washed with PBS to be observed by a ZEISS LSM-700 laser scanning confocal microscope under a 63× oil immersion objective lens.

Intracellular GSH consumption assay

The intracellular GSH levels in FAT 7 cells after different treatments were determined using a GSH/GSSG assay kit to investigate the GSH depletion efficiency. In brief, FAT 7 cells were seeded in 6-well plates at a density of 2×10^5 cells per well in 2 mL of medium and incubated overnight. Then, the medium was discarded, and the cells were cultured with fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 4 h. After 4 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). Subsequently, the cells were trypsinized, harvested, ultrasonically disrupted and centrifuged to collect the supernatant for GSH measurements using the GSH/GSSG assay kit according to the manufacturer's instruction. The intracellular GSH content in PBS-treated FAT 7 cells was set as 100%.

Singlet oxygen generation by GIT@TM/C-aCM NPs

The intracellular singlet oxygen level was determined using Singlet Oxygen Sensor Green (SOSG). In the presence of singlet oxygen, SOSG could generate an endoperoxide product, which emitted bright green

fluorescence to indicate the intracellular singlet oxygen levels. To test the generation of singlet oxygen, water, H_2O_2, GT@TM/C-aCM or GIT@TM/C-aCM with or without H_2O_2 and mixed with SOSG, laser irradiated (808 nm, 1.0 W/cm^2 for 1 min). Then, the fluorescence intensity of cells in different groups was measured and recorded using a fluorescence spectrophotometer (RF-6000 SHIMADZU, Kyoto, Japan) at Ex/Em of 488/525 nm. The fluorescence intensities of the mixed solutions at 525 nm (the characteristic peak of SOSG) before laser irradiation were defined as F_0. Then, the mixed solutions were irradiated by an 808-nm laser for different time periods, and their fluorescence intensities at 525 nm was measured and defined as F. The $^1O_2$ generation was evaluated by calculating the fluorescence intensity enhancement (F/F_0).

Mitochondrial membrane potential measurements

The mitochondrial membrane potential detection kit with a JC-1 probe was used to analyze the changes of mitochondrial membrane potential within FAT 7 cells after different treatments. Briefly, FAT 7 cells were seeded in 6-well plates at a density of 2.0 × 10^5 cells per well in 2 mL of medium overnight. Then, cells were treated with PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 12 h. After 6 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min) and then incubation for an additional 6 h. Subsequently, the cells were washed with PBS for 3 times, and incubated with JC-1 dye at 37 °C in the dark for 30 min according to the manufacturer's instructions. Then, the cells were trypsinized, centrifuged, and resuspended in JC-1 buffer (0.4 mL) before flow cytometry analysis. For each sample, the measurement was repeated for 3 times. Cells treated with PBS were used as control.

Tumor-associated macrophage (TAM) polarization *in vitro*

After different treatments, the TAM polarization behavior was analyzed by flow cytometry. In brief, RAW264.7 cells were seeded into 6-well plates at a density of 2 × 10^5 cells/well in 2 mL of DMEM for 12 h. Next, cells were treated with PBS, GI, Toy, GIT, GIT@TM, GIT@TM/C-aCM, GIT@TM/C-aCM or LPS (positive control, 2 μg/mL) for 24 h at 37 °C and 5% CO_2. After 6 h incubation, the GIT@TM/C-

aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). The cells were washed 2 times with PBS, collected and resuspended in 500 μL PBS. Then, 5 μL of anti-CD206 (FITC)/anti-CD86 (PE) was added into each cell suspension and the cells were incubated for 30 min in the dark, followed by centrifugation to remove the excessive antibody. The cells were washed and resuspended in PBS for flow cytometry analysis. Each measurement was repeated for 3 times. Additionally, the macrophage culture supernatant in each group was also collected for ELISA assay to determine the contents of TNF-α and IL-6.

Endoplasmic reticulum stress (ERS) assay

Quantitative real time-polymerase chain reaction (RT-PCR) was used to analyze the expression of ERS-related genes of glucose-regulated protein 78 (*GRP78*), unspliced X-box binding protein 1 (*XBP1u*), splicing X-box binding protein 1 (*XBP1s*) and C/eBP homologous protein (*CHOP*). In brief, FAT 7 cells were seeded and treated with PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 6 h. After 6 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). After that, the cells were washed with PBS for three times and collected for RNA extraction using an RNAeasy Plus Animal RNA Isolation Kit. Then, the first strand cDNA was synthesized by BeyoRT^{TM} III First Strand cDNA Synthesis Kit according to the manufacturer's instructions. Subsequently, the obtained cDNA was mixed with a specific primer (Table S2) and BeyoFast^{TM} SYBR-Green qPCR Mix to analyze the expression of *GRP78*, *XBP1u*, *XBP1s* and *CHOP* by RT-PCR (7500, Applied Biosystems, Waltham, MA, n = 3). The amplification was performed for 40 cycles by a fluorescence detection system with SYBR green fluorescence. Each cycle consisted of heat denaturation at 95 °C for 15 s, annealed at 62 °C for 15 s, and extended at 72 °C for 30 s, and each sample was quantified by a comparative cycle threshold method to calculate the relative gene expression using the reference gene *Actin*.

Induction of immunogenic cell death (ICD) *in vitro*

To verify the ICD of cancer cells induced by different treatments, the release of HMGB-1 and ATP was detected. In brief, FAT 7 cells were seeded in 12-well plates at a density of 1×10^5 cells per well with 1 mL medium overnight. Then, the culture medium in each well was replaced by fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM, and cells were cultured for 24 h. After 6 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm2 for 5 min). After that, the culture medium from each well was collected and used for the detection of HMGB-1 using an HMGB-1 ELISA assay kit or ATP using an ATP detection kit, respectively according to the manufacturer's instructions.

The expression level of CRT on the surface of cancer cells was next checked through immunofluorescence staining. To be brief, FAT 7 cells were seeded in confocal dishes at a density of 1.5×10^5 cells per dish with 1.5 mL medium and incubated overnight. Then, the culture medium in each dish was replaced by fresh medium containing PBS, GI, Toy, GIT, GIT@TM, GIT@TM/C-aCM, respectively for 12 h. After 6 h incubation, the GIT@TM/C-aCM treatment was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min). Subsequently, the cells were regularly incubated for 6 h. Cells treated with PBS were used as control. Then, the cells were washed with PBS for three times and fixed with glutaraldehyde (2.5%) for 15 min. After that, the cells were treated with immunol staining blocking buffer for 60 min, incubated with anti-CRT (primary antibody) for another 60 min, washed with PBS and incubated with FITC-labeled secondary antibody for 60 min. Finally, the cells were stained with DAPI for 15 min at 37 ℃ before CLSM observation.

ICD-induced maturation of DCs *in vitro*

To investigate the ICD-induced maturation of DCs *in vitro*, a 6-well transwell system with 0.4-μm polycarbonate porous membranes was used. Briefly, FAT 7 cells were seeded in the upper wells at a density of 1×10^5 cells per well with 1 mL medium and incubated overnight. Then, the culture medium in each upper chamber was replaced by fresh medium containing PBS, GI, Toy, GIT, GIT@TM or GIT@TM/C-aCM for 24 h. Meanwhile, DCs were seeded in the lower chamber at a density of 2×10^5 cells

per well with 2 mL medium and incubated for 24 h. Subsequently, the FAT 7 cells in the upper chamber were merged with the lower wells for the mixed culture of both FAT 7 cells and DCs, and GIT@TM/C-aCM was divided into two groups of GIT@TM/C-aCM and GIT@TM/C-aCM + Laser (808 nm, 1.0 W/cm^2 for 5 min), and the laser group received laser irradiation at 4 h incubation of the material. After incubation for 24 h, DCs were digested, collected, and stained with anti-CD86 FITC and anti-CD80 PE for 30 min. After centrifugation, DCs were resuspended in 0.4 mL of PBS for flow cytometry assays. Each measurement was repeated for 3 times. Meanwhile, the cell medium in the lower chambers was also collected to determine IFN-β secretion using an IFN-β ELISA assay kit according to the manufacturer's instructions.

Penetration and antitumor therapy of 3D tumor spheroids

We constructed 3D tumor spheroids composed of FAT 7 cells by a hanging drop technique. Briefly, 500 μL of agarose solution (2%, w/v, sterile saline) was dropped into the mold in the 3D petri dish. Air bubbles were removed *via* pipet suction. After that, the solidified gels were separated from the mold, placed in each well of a 12-well plate, and equilibrated for more than 15 min with medium. Then, a cell suspension (190 μL) containing 5 × 10^5 FAT 7 cells was slowly added to each well of the 12-well plate, and after standing for 10 min, 2.5 mL of fresh cell culture medium was slowly added to each well to let the cells to aggregate and grow. When the tumor spheroids reached an appropriate size, the penetration capacity and the therapeutic efficacy of the GIT@TM/C-aCM to the 3D tumor spheroids were evaluated. To study the penetration of free ICG, GIT@TM or GIT@TM/C-aCM NPs, the FAT 7 tumor spheroids were incubated with free ICG, GIT@TM or GIT@TM/C-aCM NPs at an ICG concentration of 10 μg/mL for 24 h. Then, the tumor spheroids were washed three times carefully with PBS and observed using CLSM with Z stack scanning.

The antitumor therapy effect *in vitro* was investigated by incubating the FAT 7 tumor spheroids with NS, GIT@TM or GIT@TM/C-aCM (with or without laser irradiation for 5 min after 6 h incubation) for

48 h. The tumor spheroids were monitored by a phase contrast microscope for 48 h to check the size changes.

Statistical analysis

One-way analysis of variance statistical analysis was performed using GraphPad Prism 8.0.2 software (San Diego, CA) to evaluate the significance of the experimental data. A p value of 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Hydrodynamic sizes and zeta potentials of different NPs (n = 3).

Sample	Hydrodynamic size (nm)	Zeta potential (mV)	Polydispersity index (PDI)
GIT	110.6 ± 1.9	13.51 ± 0.6	0.28 ± 0.042
GIT@TM	78.1 ± 2.3	-15.4 ± 1.2	0.32 ± 0.015
GIT@TM/C-aCM	93.3 ± 0.7	-12.1 ± 0.9	0.21 ± 0.023

Table S2. Primers used to detect the mRNA expression of the ER stress-related genes.

Gene	Oligo name	Sequence 5‘-3’
GRP78	GRP78-F	TCATCGGACGCACTTGGAA
	GRP78-R	CAACCACCTTGAATGGCAAGA
XBP1u	XBP1u-F	GACAGAGAGTCAAACTAACGTG G
	XBP1u-R	GTCCAGCAGGCAAGAAGGT
XBP1s	XBP1s-F	AAGAACACGCTTGGGAA TGG
	XBP1s-R	CTGCACCTGCTGCGGAC
CHOP	CHOP-F	GTCCCTAGCTTGGCTGACAGA
	CHOP-R	TGGAGAGCGAGGGCTTTG
β-actin	β-actin-F	AGAGGGAAA TCGTGCGTGAC
	β-actin-R	GCGTCCACGTAGTAGTAGCC

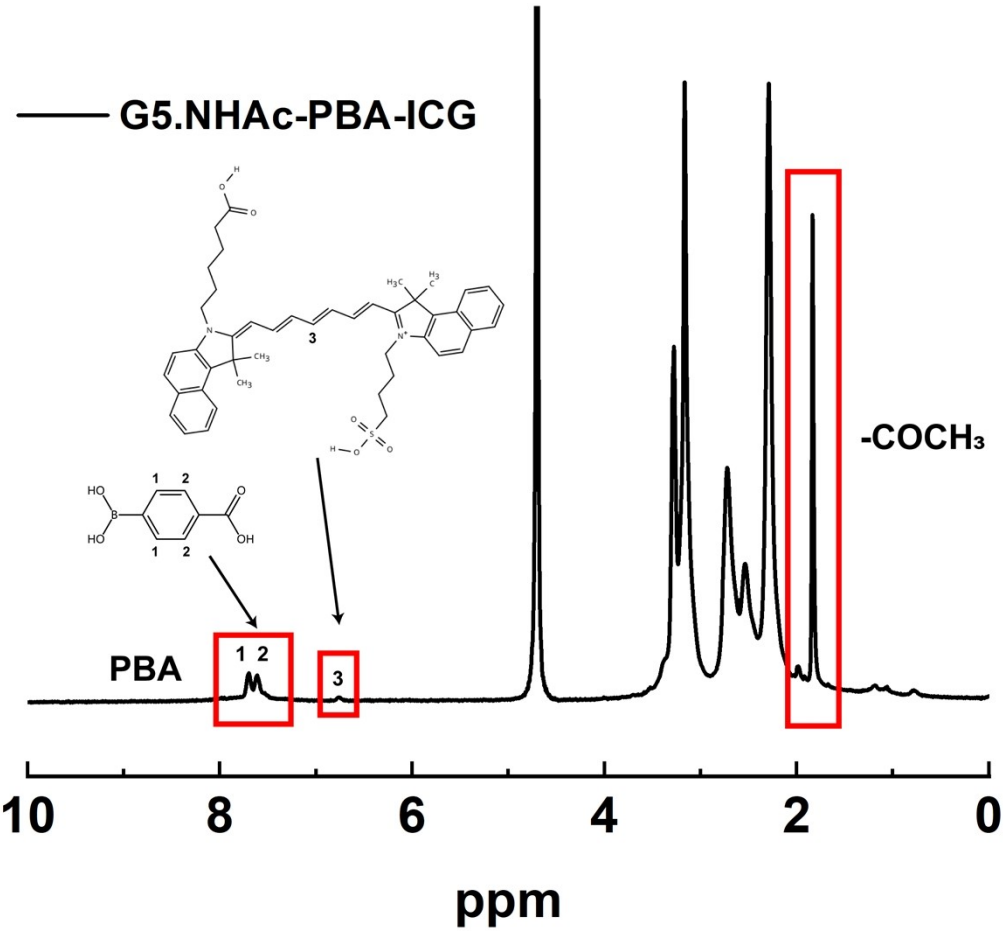

Figure S1. ^{1}H NMR spectrum of G5.NHAc-PBA-ICG dendrimer.

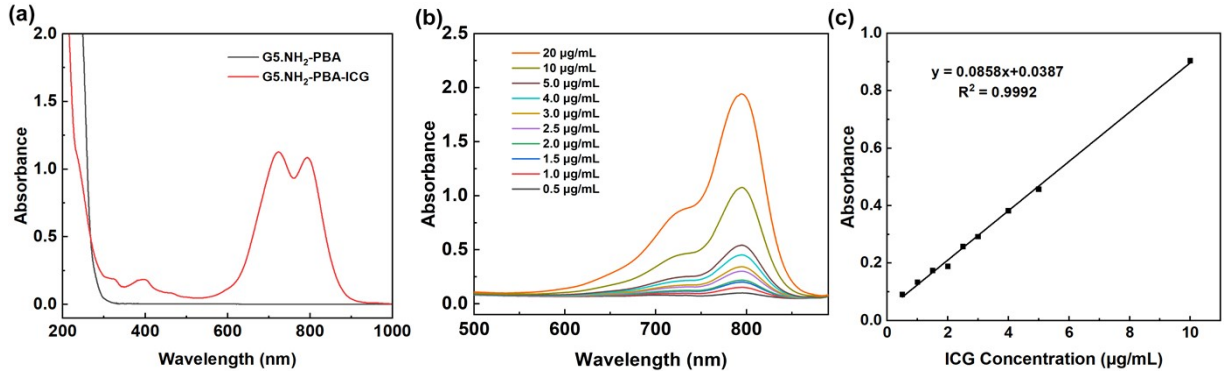

Figure S2. (a) UV–vis spectra of G5.NH_2-PBA and G5.NH_2-PBA-ICG. (b) UV-vis spectra of free ICG dissolved in water at different concentrations. (c) Calibration curve ICG absorbance at 808 nm versus concentration.

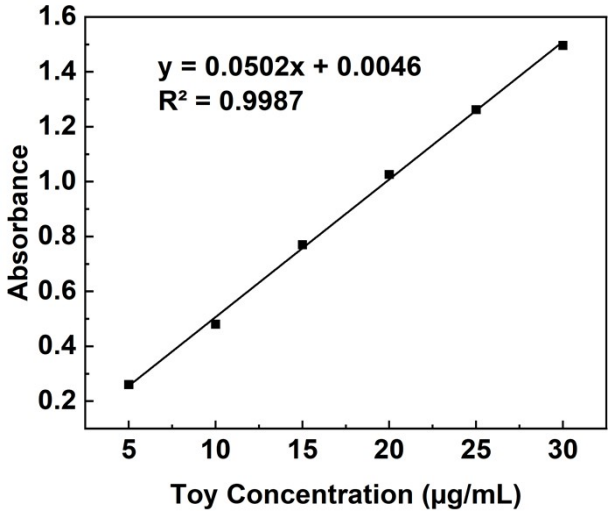

Figure S3. Calibration curve Toy absorbance at 280 nm versus concentration.

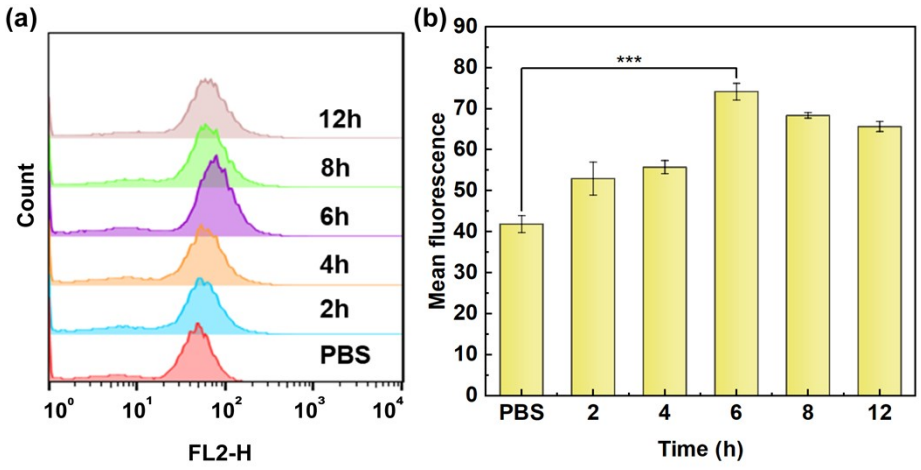

Figure S4. (a) Flow cytometric histograms and (b) mean fluorescence intensity of FAT 7 cells treated with PBS and free ICG at different time periods (n =3, *** represents $p < 0.001$).

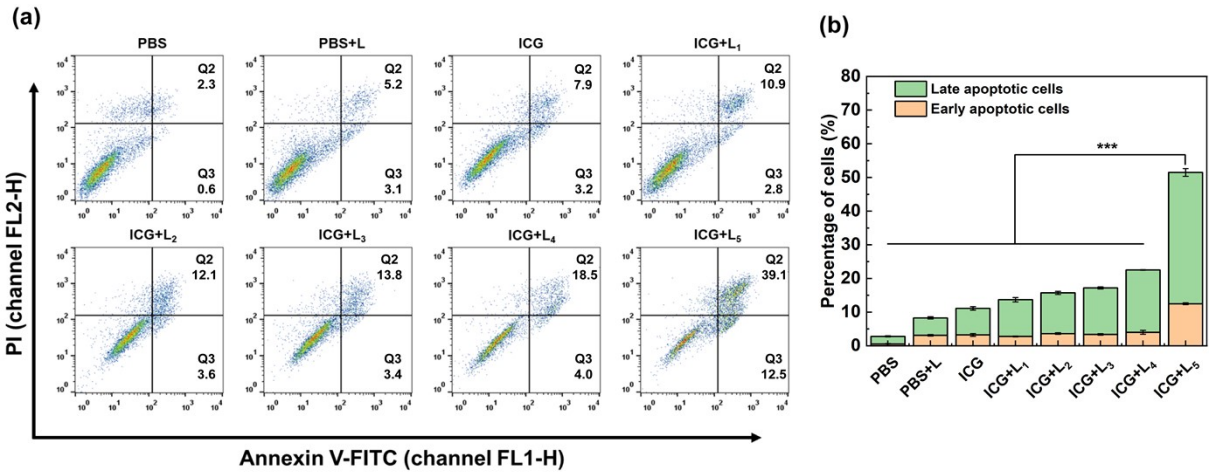

Figure S5. (a) Flow cytometry analysis and (b) quantification of early apoptotic and late apoptotic FAT 7 cells after treatment with PBS, PBS+L (L denotes laser, power density of 1.5 W/cm^2 for 5 min), ICG and ICG+L (at power densities of 0.5 W/cm^2 (L_1), 0.75 W/cm^2 (L_2), 1 W/cm^2 (L_3), 1.25 W/cm^2 (L_4), and 1.5 W/cm^2 (L_5), respectively, and the ICG concentration was 50 μg/mL). For (b), n =3, and *** represents $p < 0.001$.

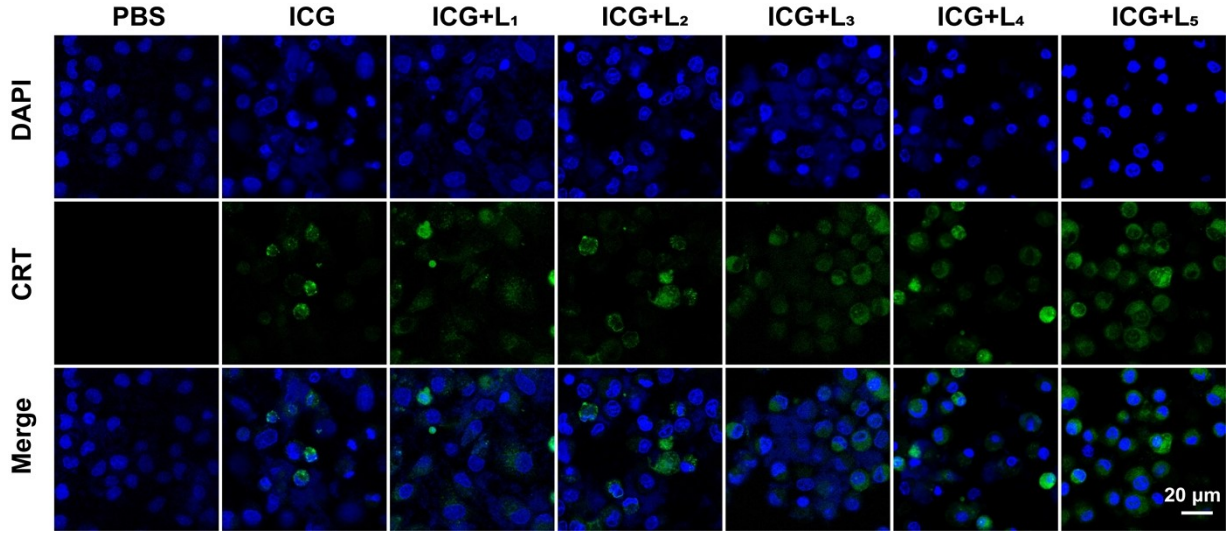

Figure S6. CLSM images of CRT expression on FAT 7 cells after treatment with PBS, ICG and ICG+L at powers densities of 0.5 W/cm^2 (L_1), 0.75 W/cm^2 (L_2), 1 W/cm^2 (L_3), 1.25 W/cm^2 (L_4), and 1.5 W/cm^2 (L_5), respectively (the ICG concentration was 50 μg/mL, scale bar = 20 μm for each panel).

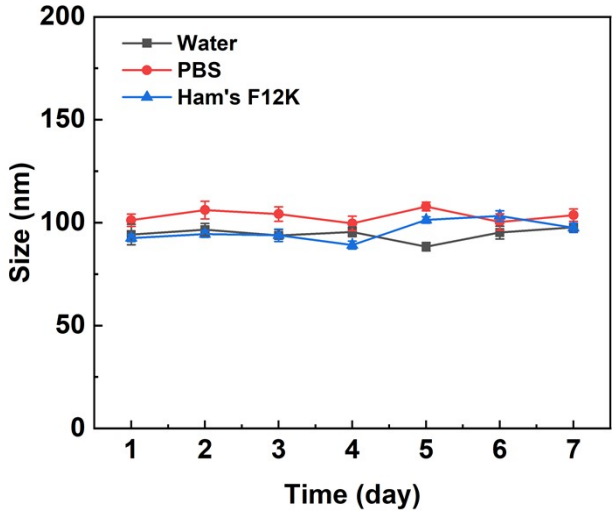

Figure S7. The hydrodynamic size of GIT@TM/C-aCM dispersed in water, PBS, or Ham's F12K medium (containing 10% FBS) for one week at room temperature (n =3).

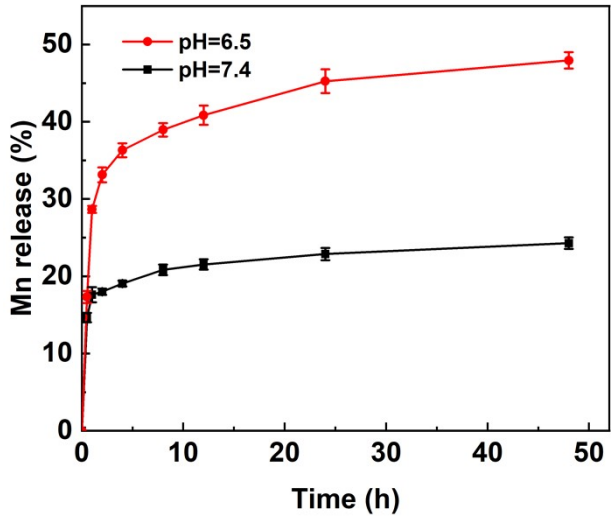

Figure S8. (a) The accumulative Mn^{2+} release from GIT@TM/C-aCM at pH 7.4 or pH 6.5 (n = 3).

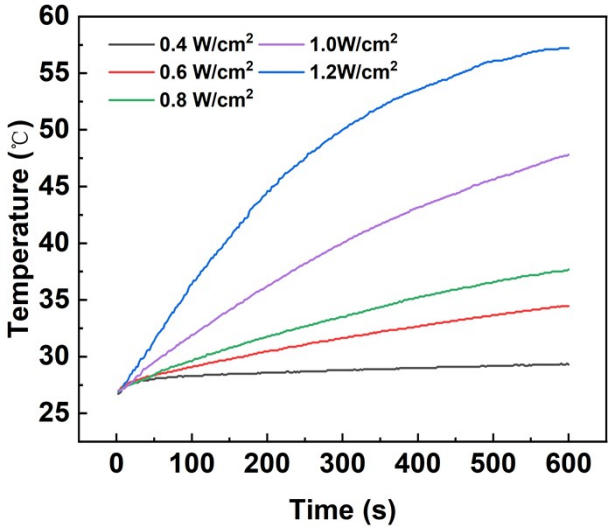

Figure S9. The temperature change of GIT@TM/C-aCM aqueous suspension at an ICG concentration of 10 μg/mL under different laser power densities (0.4, 0.6, 0.8, 1.0, or 1.2 W/cm^2) under a 808-nm laser irradiation for 10 min.

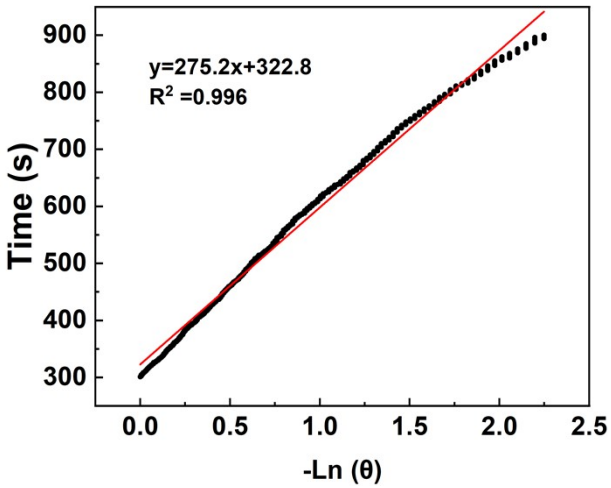

Figure S10. Plot of the cooling time vs –ln θ.

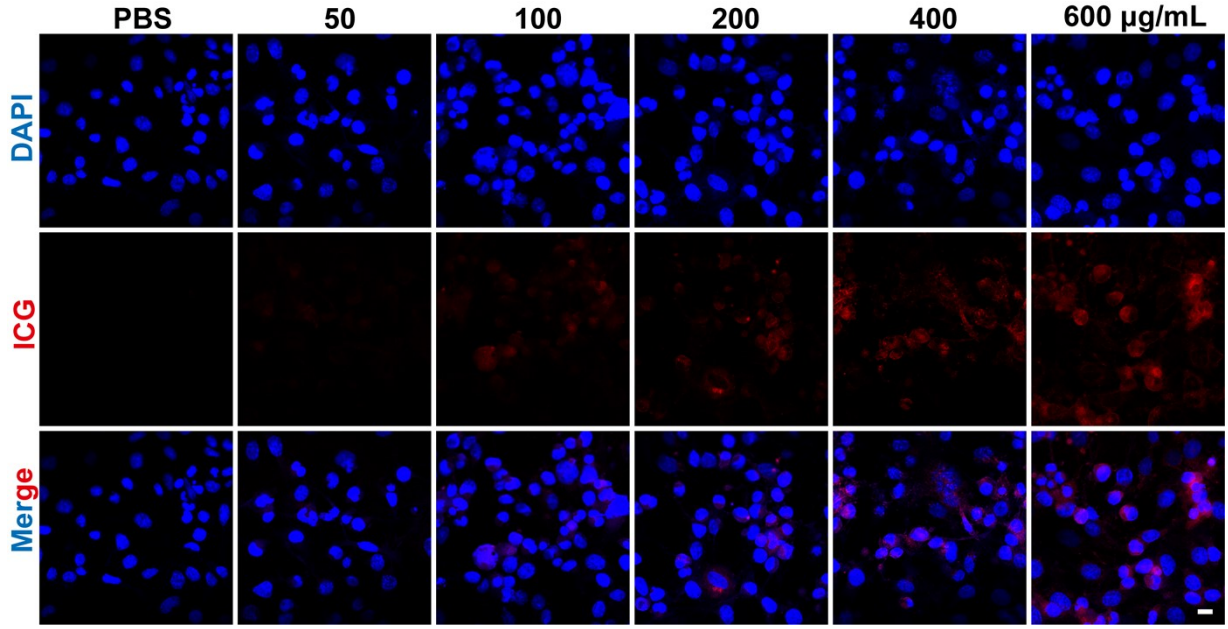

Figure S11. CLSM images of FAT 7 cells treated with PBS and GIT@TM/C-aCM at various concentrations for 6 h (scale bar represents 20 μm for each panel).

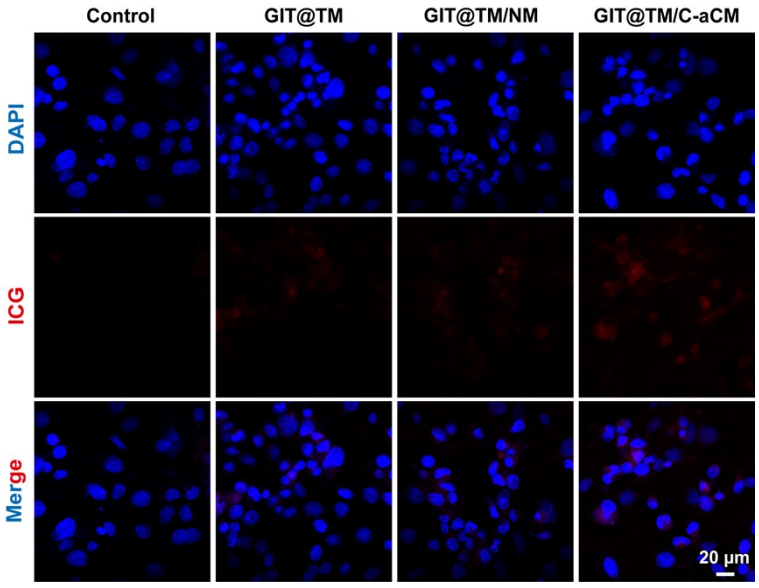

Figure S12. CLSM images of FAT 7 cells treated with PBS, GIT@TM, GIT@TM/NM and GIT@TM/C-aCM for 6 h (scale bar represents 20 μm for each panel).

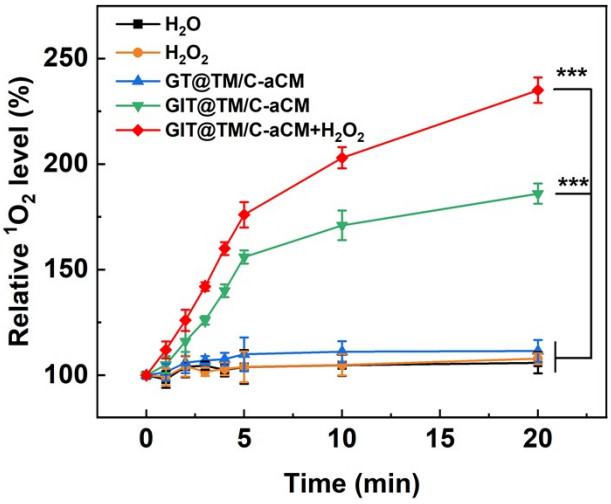

Figure S13. Elevated generation of singlet oxygen upon laser irradiation (808 nm, 1.0 W/cm^2 for 1 min) for different time periods in water, H_2O_2, GT@TM/C-aCM, or GIT@TM/C-aCM solution with or without H_2O_2. Singlet oxygen was detected by the fluorescence probe of SOSG (n = 3).

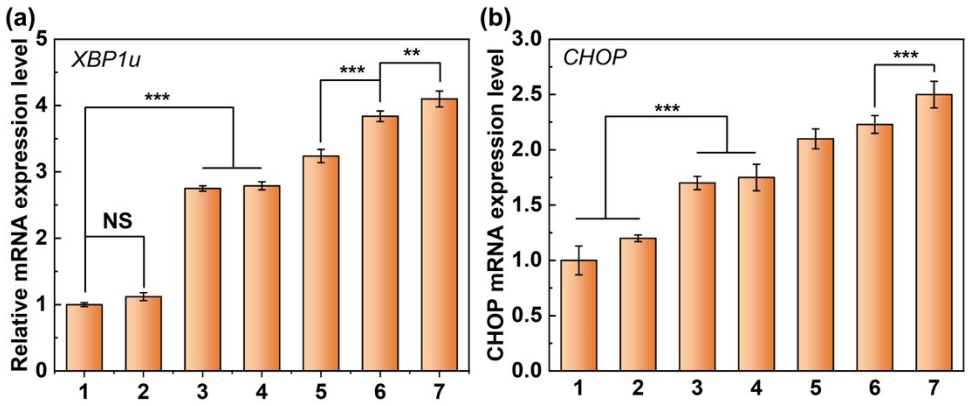

Figure S14. RT-PCR analyses of ERS-related representative factors of (a) XBP1u and (b) CHOP in FAT 7 cells after incubation with PBS, GI, Toy, GIT, GIT@TM, GIT@TM/C-aCM, or GIT@TM/C-aCM + L for 6 h (n = 3, ** and *** represent $p < 0.01$ and $p < 0.001$, respectively). Legend: 1, PBS; 2, GI; 3, Toy; 4, GIT; 5, GIT@TM; 6, GIT@TM/C-aCM; and 7, GIT@TM/C-aCM + L, and laser power density was 1 W/cm^2 (5 min).

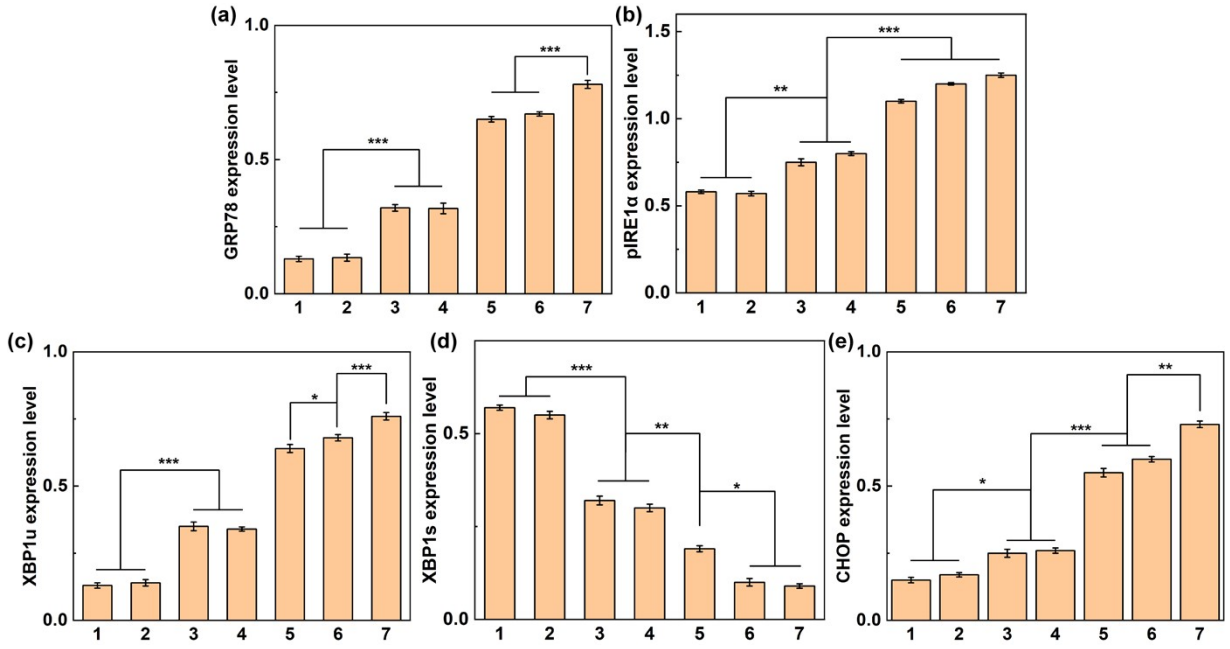

Figure S15. The quantification of the expression levels of (a) GRP78, (b) pIRE1α, (c) XBP1u, (d) XBP1s and (e) CHOP relative to β-actin after respective incubation with PBS, GI, Toy, GIT, GIT@TM, GIT@TM/C-aCM, or GIT@TM/C-aCM + L for 24 h (n = 3, and * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$, respectively). Legend: 1, PBS; 2, GI; 3, Toy; 4, GIT; 5, GIT@TM; 6, GIT@TM/C-aCM; 7, GIT@TM/C-aCM + L, and laser power density was 1 W/cm^2 (5 min).

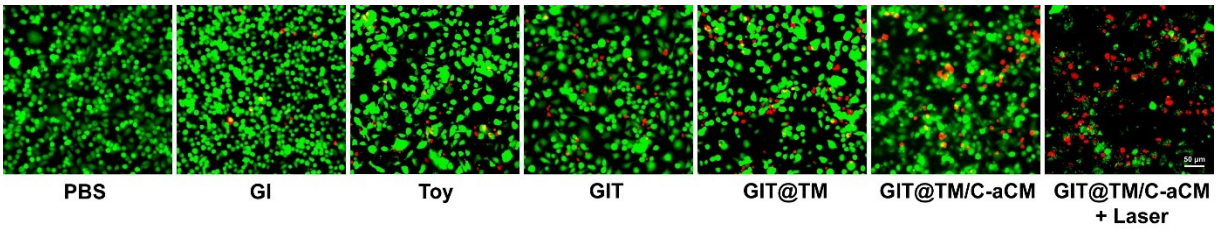

Figure S16. Fluorescence microscopic images of calcein AM- and PI-co-stained FAT 7 cells with live cells (green) or dead cells (red) after different treatments. PBS was used as control. Scale bar = 50 μm for each panel.

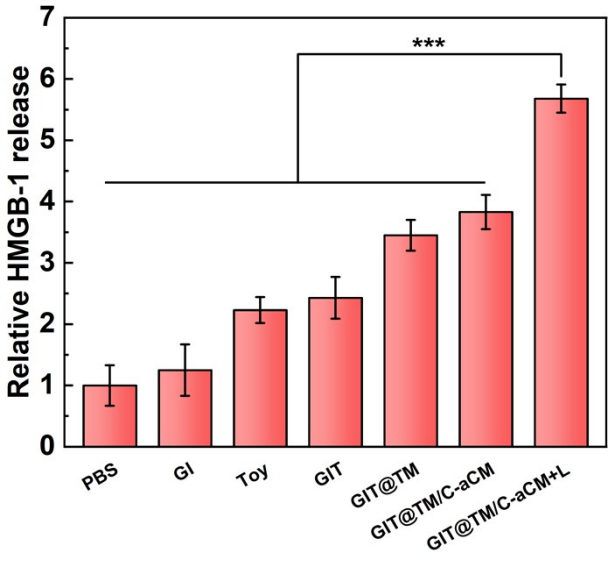

Figure S17. Detection of HMGB-1 release from FAT 7 cells after different treatments for 24 h (n = 3, and *** for $p < 0.001$).

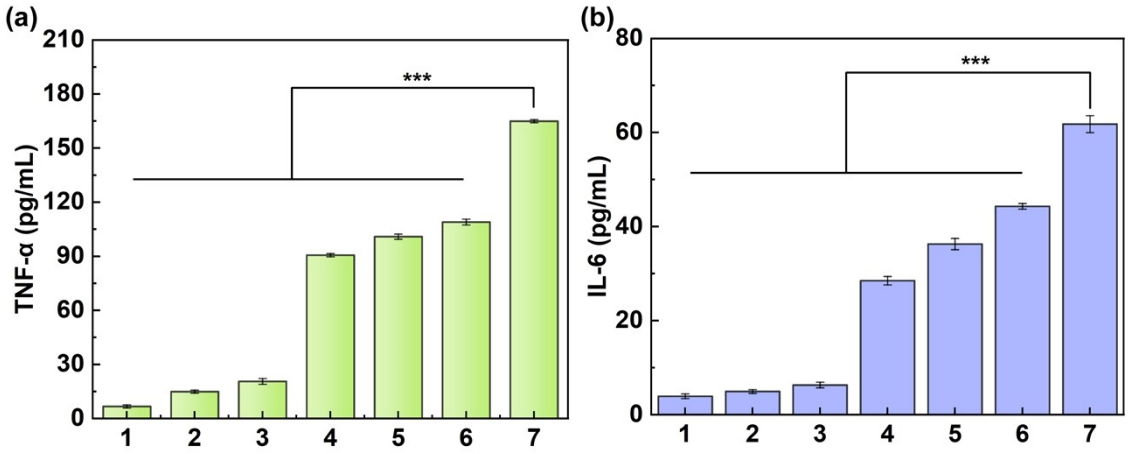

Figure S18. ELISA of (a) TNF-α and (b) interleukin-6 (IL-6) in RAW264.7 cell culture supernatant after different treatments for 24 h (n = 3, and *** represents $p < 0.001$). Legend: 1, PBS; 2, GI; 3, Toy; 4, GIT; 5, GIT@TM; 6, GIT@TM/C-aCM; and 7, GIT@TM/C-aCM + L, and Laser = 1 W/cm^2 (5 min).

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