# Electronic Supplementary Information

## $^{68}$Ga-labeled dendrimer-entrapped gold nanoparticles for PET/CT dual-modality imaging and immunotherapy of tumors

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**Part of experimental section:**

**Materials.** G5 PAMAM dendrimers were purchased from Dendritech, Inc. (Midland, United States). 2,2',2''-(10-(2-(2,5-Dioxopyrrolidin-1-yloxy)-2-oxoethyl)-1,4,7,10-tetraazacyclododecane-1,4,7-tril) triacetic acid (DOTA-NHS) was from CheMatech (Dijon, France). D-(+)-Glucosamine (DG) were acquired from Aladdin (Shanghai, China). PEG with one end of the maleimide group and the other end of carboxyl group (COOH-PEG-MAL, Mw=2000) and PEG monomethyl ether with one end of carboxyl group (*m*PEG-COOH, Mw=2000) were purchased from Shanghai Yanyi Biotechnology Corporation (Shanghai, China). Ethidium bromide (95%),1-ethyl-3-(3-dimethylaminopropyl) carbo- diimide hydrochloride (EDC), N-hydroxy-succinimide (NHS), HAuCl4·4H2O, sodium borohydride ($NaBH_4$) and all the other chemicals and solvents were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The Primary Amino Nitrogen (PANOPA) Assay Kit was obtained from Megazyme (Wicklow, Ireland). Agarose was from Gene Tech (Shanghai, China). Single-stranded CpG-ODN-1826 (5'-TCCATGACGTTCCTGACGTT-3') was purchased from Sangon Biotech (Shanghai, China) Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, and trypsin (0.25%) were from Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Bone marrow-derived dendritic (BMDCs) were from Shanghai Cancer Center, Fudan University and melanoma (B16) cells were obtained from the Shanghai Institutes for Biological Sciences, the Chinese Academy of Sciences. Cell Counting Kit-8 (CCK-8) was acquired from Services Co., Ltd (Shanghai, China) and 4',6-diamidino-2-Phenylindole (DAPI) was from Bestbio Biotechnology Corporation (Shanghai, China). Nylon wool was purchased from Kisker, Germany. Anti-CD4-PE (phycoerythrin), anti-CD8-FITC (fluorescein isothiocyanate), anti-CD86 PE, and anti-CD80 FITC, were from Thermo Fisher Scientific (Waltham, MA). Water used in all experiments was purified using a Milli-Q Plus 185 water purification system (Millipore, Bedford,

MA) with a resistivity higher than 18.2 MΩ·cm. Regenerated cellulose dialysis membranes with a molecular weight cut-off (MWCO) of 500 or 10000 were acquired from Fisher (Pittsburgh, PA).

**Characterization Techniques.** $^1$H NMR measurements were performed on a Bruker DRX 500 NMR spectrometer (400 MHz). Samples were dissolved in $D_2O$ before measurements. UV-vis spectra were recorded using a Lambda 25 UV-vis spectrophotometer (PerkinElmer, Boston, MA). Samples were dissolved in water with a concentration of 1 mg/mL before analysis. The concentration of Au for the Au DENPs was determined by Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH). The samples were digested by aqua regia and diluted with water before measurements. The number of the primary amines of Au DENPs was determined using a Megazyme PANOPA Assay Kit (Wicklow, Ireland) according to the manufacturer's instruction. Before measurements, the Au DENPs were dispersed in water (2 mg/mL). Transmission electron microscopy (TEM) was used to observe the size and morphology of the formed Au core particles of the Au DENPs. The Au DENPs were dispersed in water (2mg/mL), deposited on a carbon-coated copper grid, and air dried before TEM measurements using a JEOL 2100F analytical electron microscope (Tokyo, Japan) at an accelerating voltage of 200 kV.

**X-ray Attenuation Property.** To analyze the X-ray attenuation property, the DG-Au DENPs, NT-Au DENPs and Omnipaque (the clinical CT contrast agent) with different Au or iodine (I) concentrations (0.005, 0.01,0.02, 0.03 and 0.04 M, respectively) were performed using a dual-source SOMATOM Definition Flash CT system (Siemens, Erlangen, Germany) with 140 kV and a slice thickness of 0.6 mm. In addition, the evaluation of the X-ray attenuation intensity was carried out by a standard display program. Contrast enhancement was determined in Hounsfield units (HU) for each sample with different Au or I concentrations.

**Cytotoxicity Assay.** BMDCs and B16 cells were regularly cultured in DMEM and RPMI 1640, respectively. The used DMEM and RPMI 1640 were supplemented with 10% FBS, 1% penicillin-

streptomycin. The cell culture was maintained at 37 ℃ in a 5% $CO_2$ incubator.

In brief, $1\times10^4$ BMDCs and B16 cells were respectively seeded into each well of a 96-well plate with 0.1 mL DMEM or RPMI 1640 overnight based on our previous work.[1] After that, the medium was replaced with fresh medium containing the Au DENPs or Au DENPs/CpG polyplexes at different Au concentrations (0, 100, 500, 1000, 2000, 3000 μM, respectively) for 24 h. After that, the medium in each well was discarded and CCK-8 solution (100 μL medium containing 10 μL CCK-8) was added and the cells were incubated for 4 h. Then, the absorbance was measured at a wavelength of 450 nm by Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). The assay was carried out according to the manufacturer's instructions. For each concentration of Au DENPs, mean and standard deviation of the quadruplicate wells were reported.

**Cellular Uptake Assay**. B16 cells were seeded in 12-well plates at a density of $1\times10^5$ cells per well in 1 mL of RPMI 1640 and incubated at 37℃ and 5 % $CO_2$ overnight. After that, the medium was substituted by 1mL of fresh RPMI 1640 containing DG-Au DENPs/CpG, NT-Au DENPs/CpG, at different dendrimer concentrations (0, 1000, 2000, and 4000 μM, respectively, 1 μg of CpG for each polyplexes). After 4 h incubation, the cell number of each well was counted and then the cells were digested by aqua regia solution for 4 h, followed by ICP-OES analysis of Au content.

To observe the intracellular uptake of the DG-Au DENPs/CpG or NT-Au DENPs/CpG polyplexes, B16 cells were seeded in confocal culture dishes at a density of $1\times10^5$ cells per dish with 1 mL RPMI 1640 medium, and cultured overnight till the adherence of cells reached 80%. Then, the medium of each dish was replaced with fresh complete RPMI 1640 medium (1 mL) containing DG-Au DENPs/CpG, NT-Au DENPs/CpG, at different dendrimer concentrations (0, 1000, 2000, and 4000 μM, respectively, 1 μg of CpG-Cy3 for each polyplexes) and the cells were incubated for 3 h. Cells treated with PBS under the same conditions were used as controls. After that, the cells were washed three times with PBS, fixed with 4% glutaraldehyde at 4 °C for 15 min, stained with DAPI

for 15 min, washed with PBS for 3 times, and observed to check their red fluorescence signals with a ZEISS LSM-700 laser scanning confocal microscope (Jena, Germany).

***In Vitro* Cytokines Secreted by Dendritic Cells.** To further verify the function of the induced BMDCs, some specific inflammatory cytokines secreted in the supernatant medium of BMDCs were examined using an ELISA kit. The BMDCs culture medium on the 7th day after induction by cytokines was collected. After centrifugation, about 1 mL of the supernatant was used to detect IL-12, IFN-α and IL-6 cytokines secreted by BMDCs by ELISA kit.

***In Vivo* Biodistribution.** The B16 tumor-bearing ICR mice (20-25 g) were subjected to in vivo biodistribution analysis to evaluate the metabolic pathway of the DG-Au DENPs/CpG or NT-Au DENPs/CpG. After intravenous injection of the DG-Au DENPs/CpG or NT-Au DENPs/CpG (Au = 0.1 M, in 100 μL PBS for each mouse). Then, the mice were sacrificed at 1,6,12,24, and 48 h post-injection, respectively. The major organs (heart, liver, spleen, lung, and kidney) and tumors were harvested, cut into small pieces, and digested with *aqua regia* for a week. The amount of Au in these organs was analyzed by ICP-OES.

***In Vivo* Antitumor Therapeutic Efficacy**. When the tumors grew to approximately 50 $mm^3$ in volume, the mice were divided into seven groups randomly with 6 mice in each group as follows: Group 1, PBS (0.1 mL); Group 2, CpG (4 μg, in 0.1 mL PBS); Group 3, DG-Au DENPs/CpG, NT-Au DENPs/CpG (1.12 mg/mL, in 0.1 mL PBS ); Group 4, DG-Au DENPs (5 mg/kg, in 0.1 mL PBS); Group 5, NT-Au DENPs/CpG (5 mg/kg, CpG = 4 μg, in 0.1 mL PBS) + laser; Group 6, DG-Au DENPs/CpG (5 mg/kg, CpG = 4 μg, in 0.1 mL PBS). All groups were treated through tail-vein intravenous injection, The injection dose was applicable for each mouse. The injection is given every five days for a total of four doses. The tumor size and body weight were monitored every two days. The tumor volume (V, $mm^3$) was calculated using the formula of $V = 3\pi ab^2/4$, where a and b refer to the length and width of the tumor, respectively. Relative tumor volume was calculated as $V/V_0$ ($V_0$,

tumor volume before treatment; V, tumor volume after treatment at a given time point). Tumors and major organs (heart, liver, spleen, lungs, and kidneys) were surgically harvested at the end of the treatment. The tissues were collected, fixed, sectioned, and hematoxylin & eosin (H&E) or terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) stained. The tumor tissues were also sectioned for CD4 and CD8 immunostaining, while the spleens were sectioned for CD4 and CD8 immunostaining.

**Cytokines Detection.** Serum samples were isolated from mice on day 18 and diluted for analysis. Tumor necrosis factor α (TNF-α), interferon-γ (IFN-γ), and interleukin 6 (IL-6) were analyzed with ELISA kits.

***In Vivo* Tumoral Distribution of Immune Cells.** The mice were killed by cervical dislocation and immersed in 75% alcohol for 2-5 min. The tumors were obtained under sterile conditions, extracted, and stored in PBS solution. The tumor tissues were grinded in a 200-mesh sieve to obtain a single cell suspension of tumor-infiltrating tissue. Then the lymphocytes suspension in the tumor tissues was obtained according to the instructions of the Tumor Infiltrating Lymphocyte Cell Separation Medium Kit, Mice. The lymphocytes separation solution and an equal volume of prepared single cell suspension were mixed and then centrifuged (500 g, 30 min) at room temperature. The second lymphocyte layer was separated, washed with 10-mL cell-washing solution, and collected. Then, the cells were resuspended in 5 mL of PBS and centrifuged (250 g, 10 min) to collect the tumor-infiltrating lymphocytes. The obtained lymphocytes were filtered by the nylon wool column for 6-8 times to obtain T cells. Further, the collected cells were incubated with IgG isotype control, anti-CD3-FITC, or anti-CD4-PE/anti-CD8-FITC antibodies according to the standard protocols to determine the contents of $CD4^+$ and $CD8^+$ T cells in the tumors through flow cytometry[2].

**Statistical Analysis.** One-way ANOVA statistical analysis was performed to evaluate the

significance of the experimental data between groups. A p value of 0.05 was selected as the significance level, and the data were indicated with * for $p < 0.5$, ** for $p < 0.01$, and *** for $p < 0.001$, respectively.

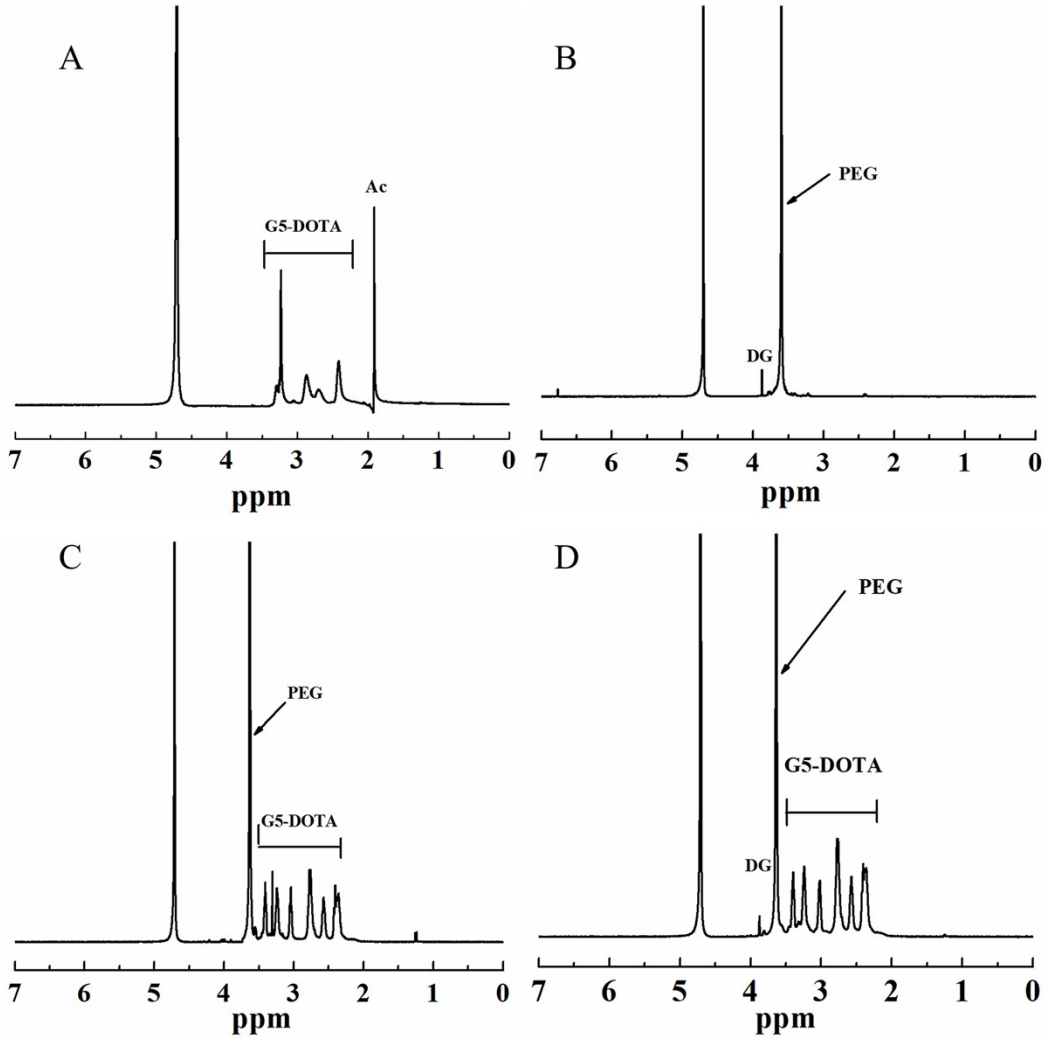


**Figure S1.** $^{1}$H NMR spectra of (A) G5-DOTA, (B) DG-PEG, (C) G5-DOTA-PEG and (D) G5-DOTA-(DG-PEG).

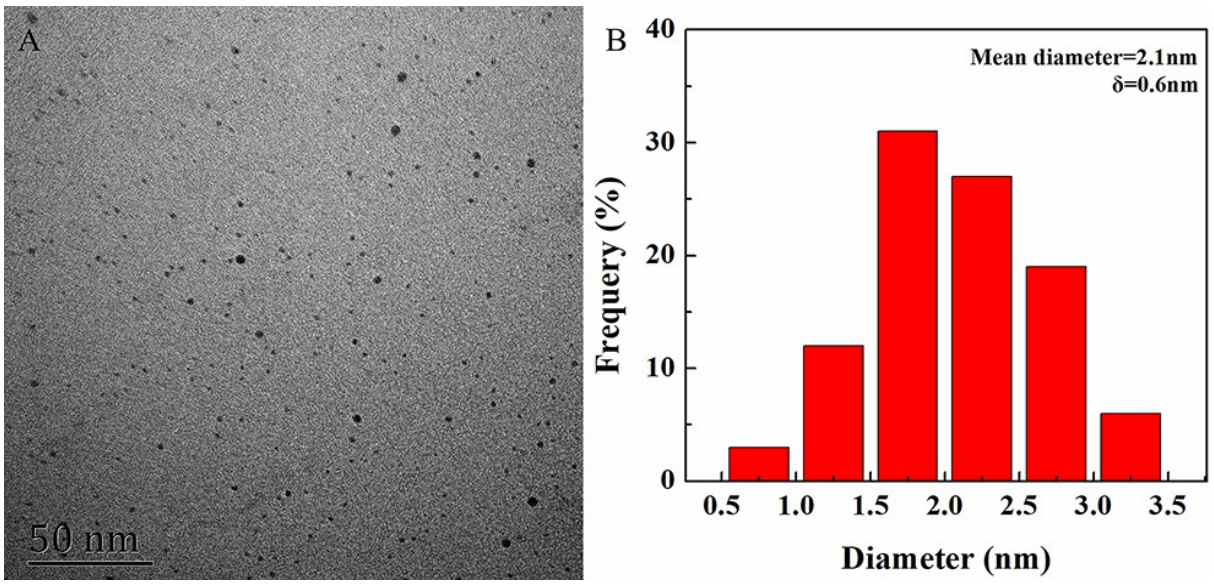


**Figure S2.** TEM images (A) and size distribution histogram (B) of NT-Au DENPs

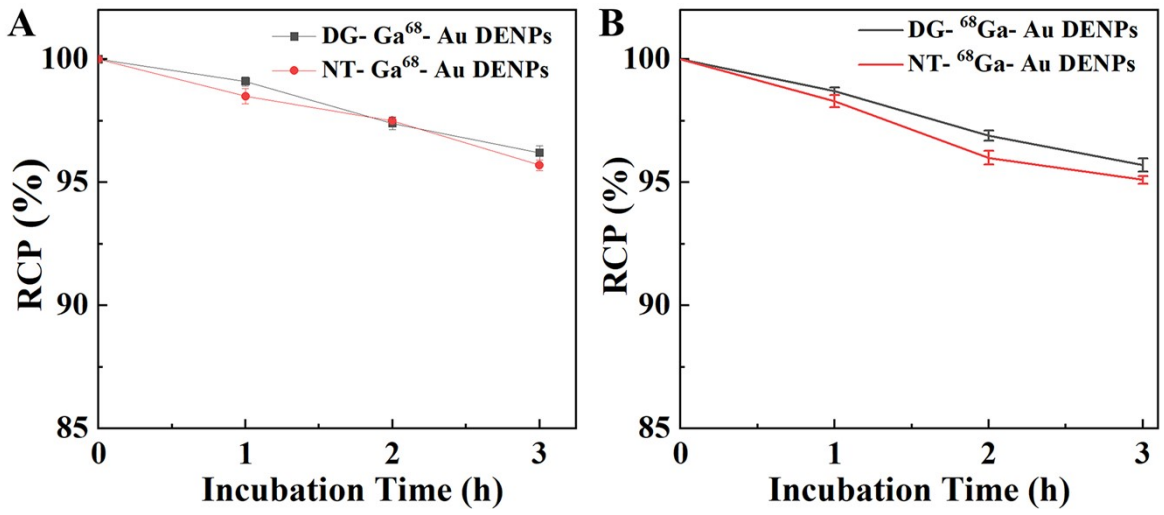


**Figure S3.** The radiochemical purity of the DG-$^{68}$Ga-Au DENPs and NT-$^{68}$Ga-Au DENPs in normal saline (A) and DMEM (B) at 0 h, 1 h, 2 h and 3 h.

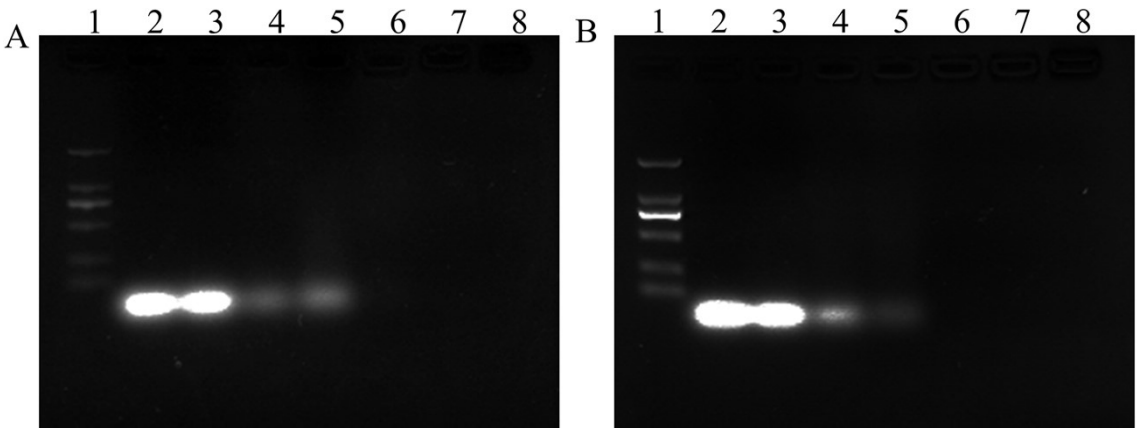


**Figure S4.** Gel retardation assay of CpG complexed with the (A) DG-Au DENPs and (B) NT-Au DENPs at different N/P ratios (Lane1: Marker; Lane 2: free CpG; Lanes 2-8 represent N/Ps of 0.25, 0.5, 1, 2, 3, and 4, respectively).

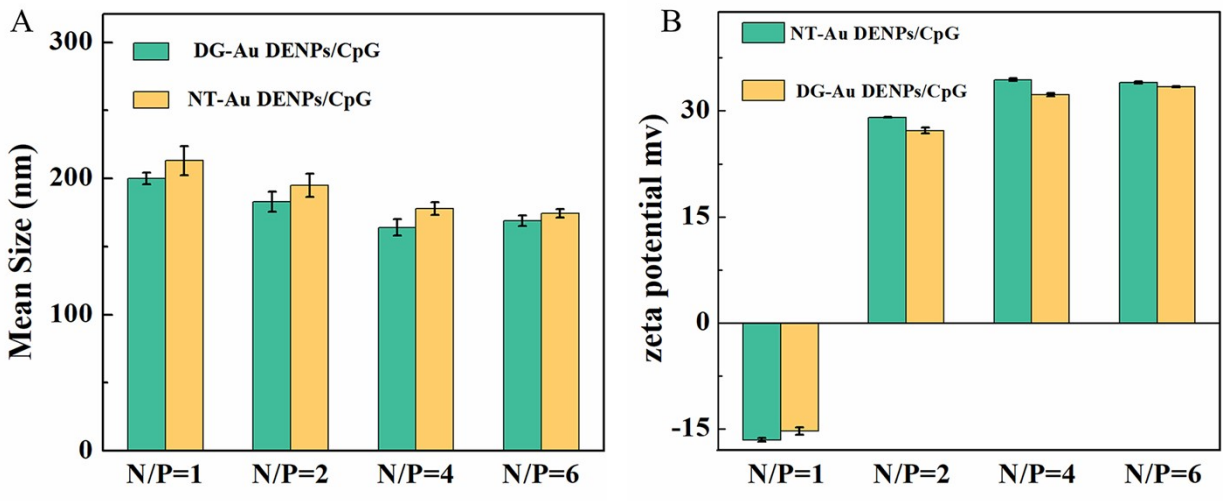


**Figure S5.** (A) Mean hydrodynamic size and (B) Zeta potential of DG-Au DENPs/CpG or NT-Au DENPs/CpG polyplexes at various N/P ratios (n = 3).

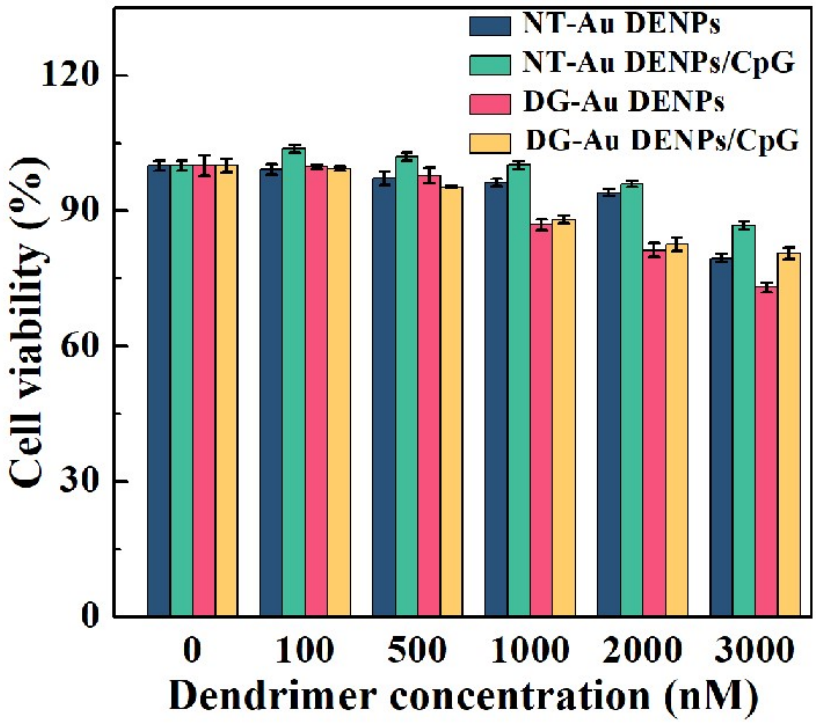


**Figure S6.** Viability of B16 cells treated with the NT-Au DENPs, NT-Au DENPs/CpG, DG-Au DENPs and DG-Au DENPs/CpG Polyplexes at dendrimer concentration for 24 h.

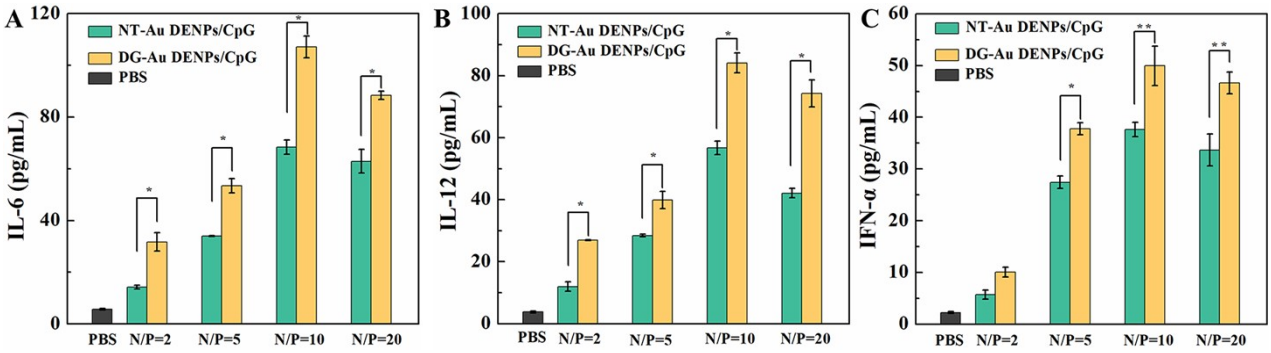


**Figure S7.** Detection of (A) IFN-α, (B)IL-6 and (C) IL-12 cytokines in culture supernatant after different treatments by ELISA.

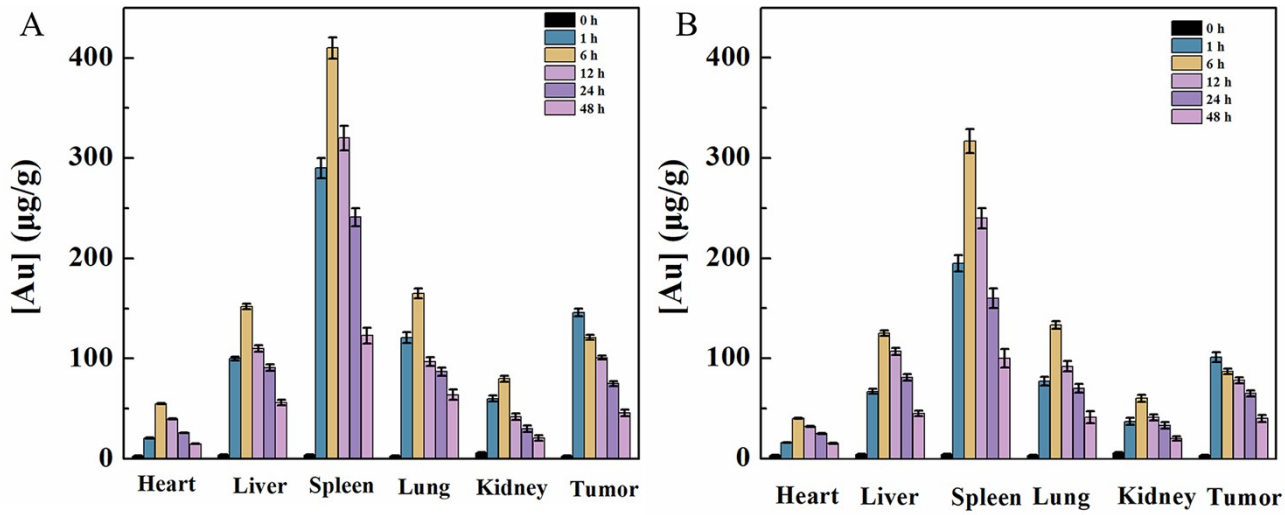


**Figure S8.** Biodistribution of Au in B16 tumor-bearing mice at different time points post intravenous injection of (A) DG-Au DENPs/CpG or (B) NT-Au DENPs/CpG polyplexes. The Au contents in heart, liver, spleen, lung, kidney and tumor was determined by ICP-OES (n = 3 for each sample).

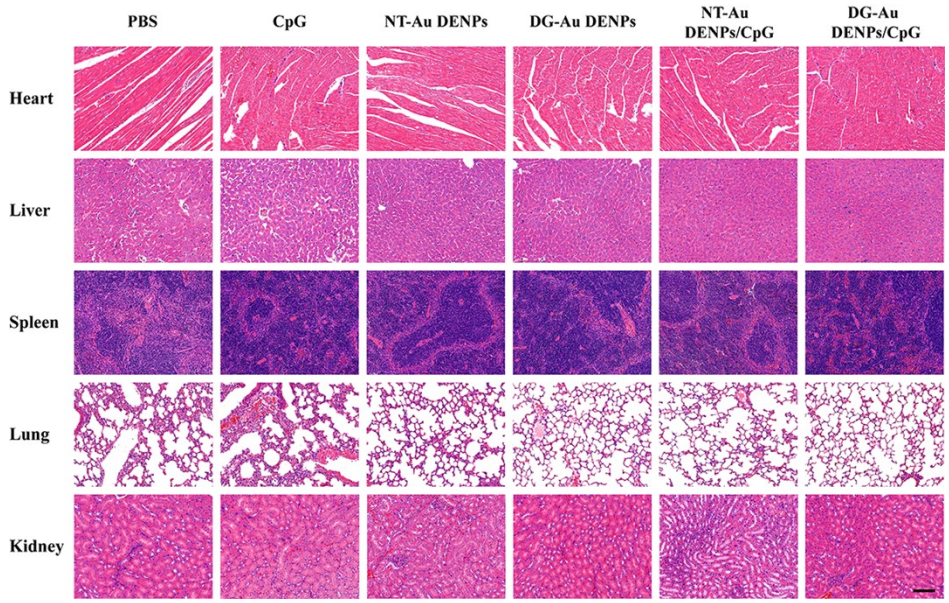


**Figure S9.** H&E-stained slices of major organs of B16 tumor-bearing mice after different treatments. Scale bar = 100 μm.

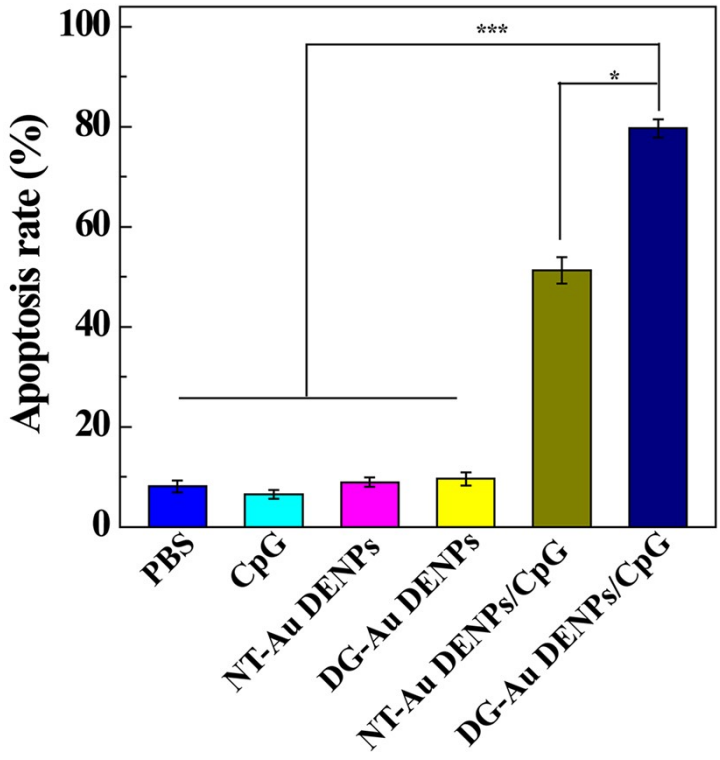


**Figure S10.** Tumor cell apoptosis rate from TUNEL staining.

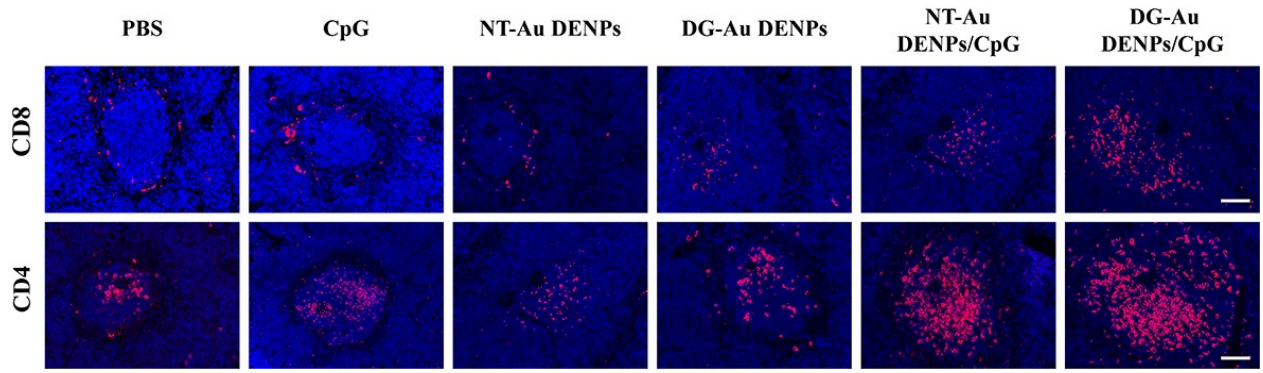


**Figure S11.** Representative immunofluorescence staining of $CD4_+$ and $CD8_+$ T cells in spleen. Scale =100 μm.

## References

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2. W. Hu, T. T. Xiao, D. Li, Y. Fan, L. X. Xing, X. P. Wang, Y. L. Li, X. Y. Shi and M. W. Shen, *Advanced Science*, 2021, **8**.