Supplementary Information

The Influence of Tumor-induced Immune Dysfunction on Immune Cell Distribution of Gold Nanoparticles in Vivo

Chenxi Wu,[a] Hongmei Chen,[a] Xuan Wu,[a,b] Xiuxiu Cong,[a,b] Li Wang,[d] Yucai Wang,[d] Yongguang Yang,[a,b,c] Wei Li*[a] and Tianmeng Sun*[a,b]

a. The First Hospital of Jilin University, Changchun, China.

b. Institute of Immunology, Jilin University, Changchun, China.

c. Columbia Center for Translational Immunology, Columbia University College of Physicians and Surgeons, New York, NY, USA.

d. School of Life Science, University of Science & Technology of China, Hefei, Anhui, China.

***Email: tsun41@jlu.edu.cn (T. Sun); Jdyylw@163.com (W. Li)**

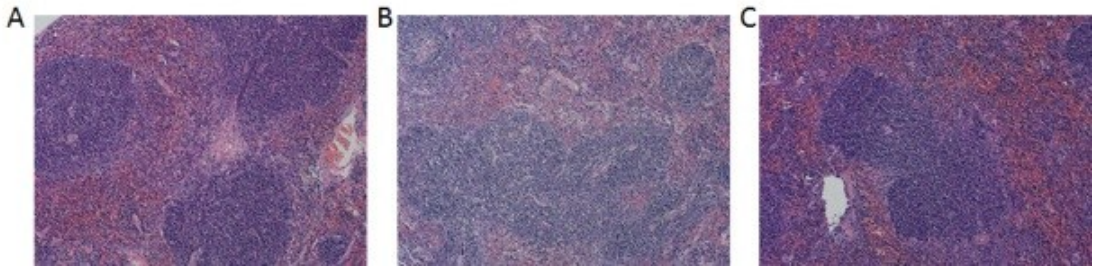

Figure S1. The H&E staining of the spleen slices from the (A) tumor free mouse, (B) small tumor bearing mouse and (C) big tumor bearing mouse.

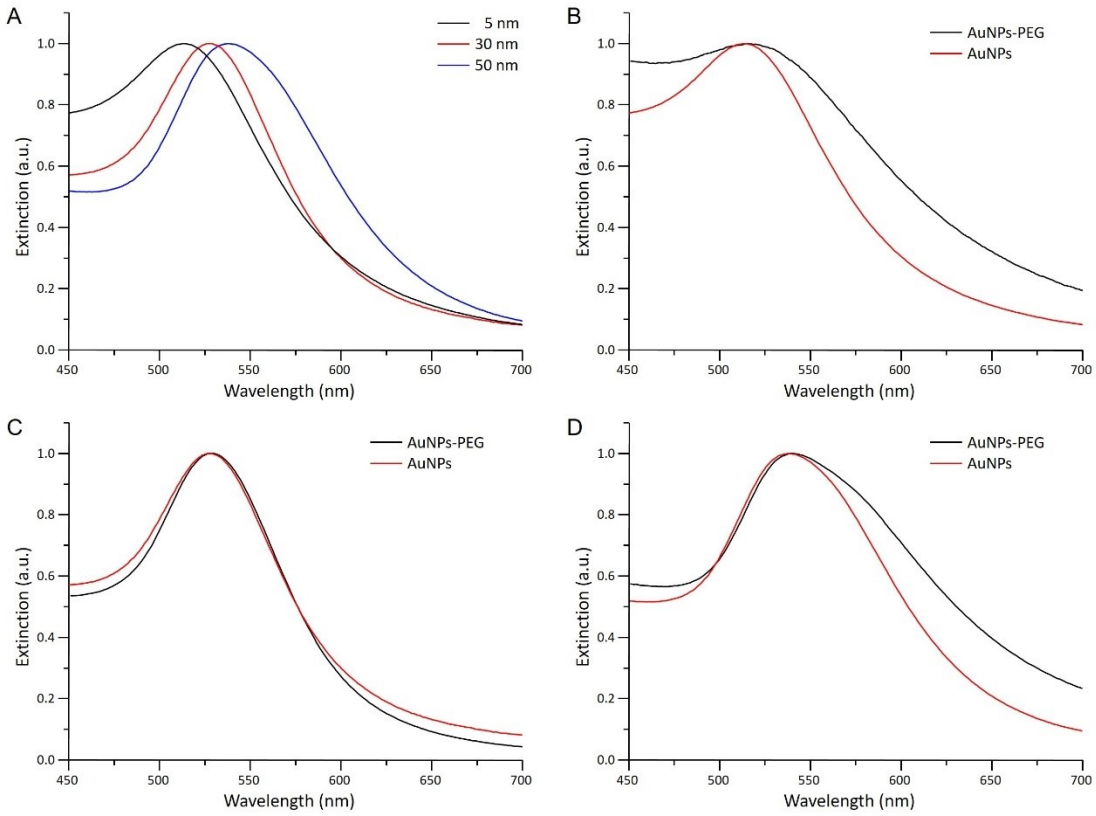

Figure S2. (A) UV-vis-NIR spectra of the Au nanoparticles of various sizes: 5, 30 and 50 nm in aqueous suspension. (B-D) UV-vis-NIR spectra of the citric acid stabilized AuNPs and PEGylated Au nanoparticles (AuNPs-PEG) in aqueous suspension. The diameters of the Au nanoparticles are 5 nm (B), 30 nm (C) and 50 nm (D).

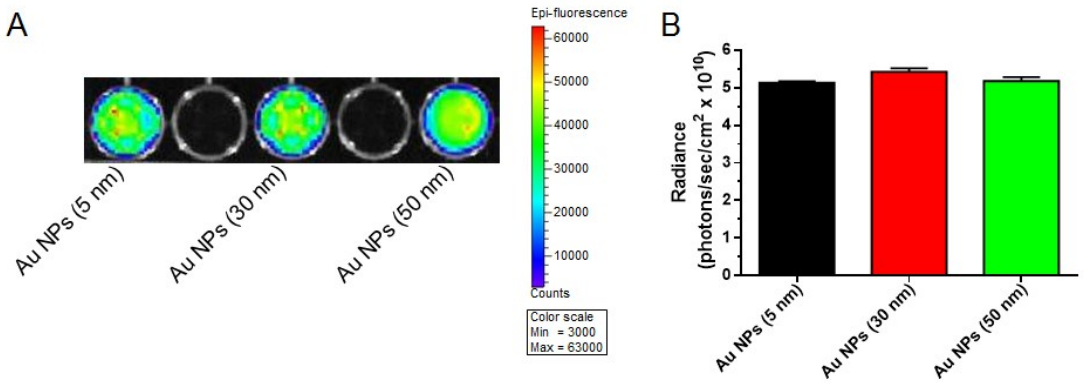

Figure S3. (A) Fluorescence image of Cy5 conjugated AuNPs with different sizes in aqueous suspension acquired by Xenogen IVIS 200 Imaging System. (B) Fluorescence intensities of the Cy5 dye conjugated to the AuNPs with different diameters (n = 3).

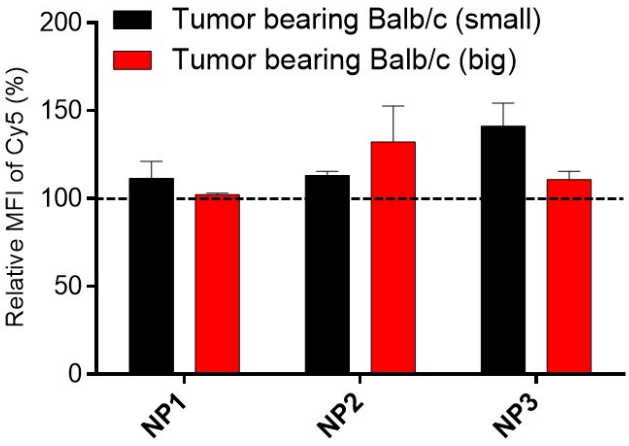

Figure S4. Relative mean fluorescence intensity of AuNPs associated Cy5 signal within tumor cells in tumor bearing Balb/c mice 24 h after AuNPs intravenous injection (n=3).

Materials and methods

1 Chemicals and materials

Histopaque®-1083 was purchased from sigma-aldrich (St. Louis, MO, USA) mPEG-SH was purchased from Laysan Bio (Arab, AL, USA). SH-PEG-NH_2 was purchased from ToYong Bio (Shanghai, China). The dialysis bag was purchased from Spectrum (Middleton, WI ,USA). Dulbecco's modified eagle medium (DMEM), phosphate buffer saline (PBS), Penicillin/streptomycin, and L-glutamine were all obtained from Invitrogen (Carlsbad, CA, USA) and 0.25% trypsin was obtained from Hyclone (Pittsburgh, PA, USA). Fetal bovine serum (FBS), AQUA, and Collagenase (Type IV, powder) were all purchased from Thermo Fisher Scientific (Waltham, MA, USA). Red blood cell lysis buffer was purchased from Solarbio (Beijing, China). Fluorescence-labeled rat anti-mouse F4/80, CD49b, Gr-1, CD45, CD11b, CD11c, CD19, CD3, CD16/32 and CD4 mAbs were obtained from Biolegend (San Diego, CA, USA). Fluorescence-labeled rat anti-mouse CD8, and LY-6G were purchased from BD (Franklin Lakes, New Jersey, USA) In all of these experiments, we used deionized water with a resistivity of 18.2 MΩ/cm, which was prepared by using an ultrapure water system (Millipore, Billerica, MA, USA).

2 Animals

Female BALB/c mice (4-6 weeks), obtained from Charles River (Beijing, China), were raised in a specific pathogen-free environment and were given free access to food and water. All animals received care in accordance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals. All procedures were ratified by the Jilin University Animal Care and Use Committee.

3 Synthesis of the AuNPs

Briefly, gold nanoparticles (AuNPs) with sizes 5, 30 and 50 nm were prepared by the citrate reduction of chloroauric acid. In this method, 300 μL of 1% chloroauric acid was added to 30 mL of doubled distilled water and brought to boil. Next, 900,

300 and 240 mL of 1% citric acid was added to the solution to produce nanoparticles with the sizes 5, 30 and 50 nm, respectively. Refluxing of the solution continued until the color of the boiling solution changes from dark purple to red vine color. The final product was collected by centrifugation (55,000 rpm, 40 min for 5 nm; 14,500 rpm 15 min for 30 and 50 nm) and resuspended in deionized water at a particle concentration of 0.5 nM.

The AuNPs were first PEGylated. Amine-PEG-thiol (MW 3,400) was mixed with AuNPs (5, 30 and 50 nm) at a molar ratio of 40:1 and the mixture was stirred overnight at 4 ^{o}C to allow complete conjugation via an Au-S bond. Then PEG-thiol (MW 3,400) was added into the AuNPs solution at a molar ratio of 10,000:1 and the mixture was stirred for another 2 h at 24 ^{o}C to allow complete conjugation via an Au-S bond. The mixture was then purified by centrifugation as described above. The supernatant was removed, and the pellet was resuspended in 1 mL PB solution (pH 8.4).

To label the PEGylated AuNPs, 10 μL Cy-5-NHS dye (1 mg/mL) was added into 3 mL PEGylated AuNPs and was reversed gently at room temperature for 2 hours. Then the Cy5 labeled PEGylated AuNPs was transferred into a dialysis bag and dialyzed in 500 mL deionized water for 2 h to remove the free dye. The water was changed every 30 min. Then the dialysis bag was placed on dry PEG solid to be condensed for 20 min. Finally, the NPs solution was divided into three parts averagely to be injected into 3 mice respectively. All the procedures above were kept away from light.

4 Characterization of Au nanoparticles

Sizes and morphologies of Au nanoparticles were observed by a transmission electron microscope (TEM) operated at 120 kV (HT7700, Hitachi, Tokyo, Japan).

5 Cell culture

The mouse breast cancer cell line 4T1 was purchased from American Type Culture Collection (ATCC) and were cultured in DMEM supplemented with 10%

FBS at 37 ^{o}C incubator with 5% CO_2.

6 Eastablishment of tumor model

First, the 4T1 cells got a starvation treatment for 4 h with DMEM without FBS and then digested by 0.25% trypsin for 5 min at 37 ^{o}C and terminated by PBS. Then, the cells were suspended in PBS (3 x 10^6/mL). To eastablish the tumor model, 150 μL cell suspension was injected into the breast pad of the anesthetized mice orthotopicly.

7 Flow cytometer Analysis

Mice (tumor-bearing or tumor free) were divided into 4 groups (3 mice/group) randomly and were given intravenous injection of AuNPs (5, 30 and 50 nm) or PBS respectively. After 24 h, the mice were sacrificed for the collection of tumors and organs including blood, spleen and bone marrow.

The tumor was cut into small pieces and then put into a 15-mL centrifuge tube containing 5 mL PBS with 0.07% (W/V) collagenase IV. Then the tube was incubated in a 37 ^{o}C water bath shaker. After 80 min, the digested tumor tissue was washed with 5 mL PBS by centrifugation (4 ^{o}C, 1,650 rpm, 5 min) and the cells were resuspended into single-cell suspension using 1 mL PBS. Then the cell suspension was gentlely layered onto the top of 1 mL histopaque and centrifuged for 30 min at 2,000 rpm at 4 ^{o}C in a swing-out bucket. Later, the whitish coat (immunocytes) formed in the interphase between histopaque and PBS was aspirated and washed with PBS by centrifugation.

Spleen and bone marrow were gently grinded into single-cell suspension with PBS. All the organs got the removal of red blood cells by RBC lysis buffer.

Before antibody staining, all the cells were blocked by CD16/32 antibody for 15 min at room temperature. Then the cells from different organs were all stained with the antibodies of CD11b, CD11c, F4/80, Gr-1, CD45, CD3, CD4, CD19, CD49b and AQUA. The results were analyzed by FlowJo Vx.0.7.

8 Histopathological analysis.

Spleens were collected form the naïve mice or tumor bearing mice and fixed in 10% formalin solution. After 24 h, these tissues were sliced in to 5 μm thickness. After hematoxylin-eosin (H&E) staining, the slides were observed and photograghed by Zeiss LSM 880 confocal laser scanning microscope using a 4× objective.

9 Statistical analysis.

Statistical analyses were performed using ANOVA test to measure statistical differences among groups. Data with $p<0.05$ were considered to be statistically significant.