Supporting Information

Bacteria engineered with intracellular and extracellular nanomaterials for hierarchical modulation of antitumor immune responses

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Materials and methods

Materials and Strains

Copper chloride ($CuCl_2$) was purchased from Sigma-Aldrich (Shanghai, China). Sodium sulfide ($Na_2S·9H2O$) was purchased from Xilong Chemical Co., Ltd (Shantou, China). NLG919 was purchased from Macklin (Shanghai, China), and bovine serum albumin (BSA), N, N′-Bis (acryloyl) cystamine and p-dimethylamino benzaldehyde were purchased from Aladdin (Shanghai, China).

Growth of VNP20009

Attenuated Salmonella strain VNP20009 (ATCC 202165) was provided by the American Type Culture Collection. VNP20009 was inoculated into 50 mL of liquid LB medium and placed in a rotary shaker with 37 °C overnight. Then, the culture was diluted by fresh liquid LB medium in a ratio of 1:50 and further cultured at 37 °C for 3 h. Bacteria were collected by centrifugation at 4,000 g for 10 min and resuspended in ice-cold sterile saline to achieve an optical density (OD 600) of 0.8, corresponding to a bacterial concentration of 1×10^8 colony forming units (CFU mL^{-1}).

Preparation of $_{CuS}$VNP20009, NB NPs and $_{CuS}$VNP20009$_{NB}$

$_{CuS}$VNP20009: VNP20009 was cultured in LB medium at 37 °C with a rotary shaker (200 rpm) for 12 h until the late stationary phase. The cells were harvested by centrifugation (5,000 g, 4 °C, 5 min) and washed two times with PBS. The initial OD at 600 nm was adjusted at about 0.8 using fresh LB medium containing 25 mmol L^{-1} $CuCl_2$. After 12 h of co-culture (37 °C, 200 rpm), the mixture was centrifuged (5,000 g, 8 min) to remove the supernatant, followed by incubation with LB medium containing 25 mmol

L^{-1} Na_2S for another 12 h. Then, the medium was centrifuged (5,000 g, 8 min) and $_{CuS}$VNP20009 was collected and washed thrice by PBS buffer for use.

NB NPs: 80 mg BSA was dissolved in 2 mL of deionized water and the pH was then adjusted to 10 using 0.1 mol L^{-1} sodium hydroxide (NaOH). Then, 8 mL of 1.5 mg mL^{-1} NLG919 ethanol solution was added drop-wise in a rate of 1.0 mL min^{-1}. Finally, 0.8 mL of 10 mg mL^{-1} N, N′-Bis (acryloyl) cystamine (BAC) ethanol solution was added to induce intra-particle cross-linking. The solution was stirred continuously for 48 h at 500 rpm and 50 °C. The resulting NB NPs were obtained by centrifugation at 12,000 rpm for 15 min to remove unload NLG 919 and washed three times with deionized water. The amounts of unloaded NLG 919 were determined by UV–vis absorption spectra at 280 nm, and the loading capacity (LC) of NLG919 in NB NPs was determined as following:

$$LC(\%) = \frac{NLG919[total\ weight] - NLG919[unload\ weight]}{NB[weight]} \times 100\%$$

$_{CuS}$VNP20009$_{NB}$: 100 μL of 1 mg mL^{-1} NB NPs and 100 μL of 10 mg mL^{-1} tannic acid aqueous solutions were added to 4 mL of PBS solution. After 5 min of stirring, the mixture was further mixed with 1 mL of 7.8×10^7 CFU mL^{-1} $_{CuS}$VNP20009 (equivalent to bacteria concentration). After 1 h, the suspension was centrifuged (5,000 g, 8 min), washed three times with PBS and resuspended in PBS for further use. 1×10^6 CFU mL^{-1} $_{CuS}$VNP20009$_{NB}$ (equivalent to VNP20009 content) contained 2 mg mL^{-1} NB NPs and 2.2 mg mL^{-1} CuS NMs.

Characterization

The morphology of NB NPs, thinly sectioned VNP20009 and $_{CuS}$VNP20009 were taken by a transmission electron microscope (JEM-2010, Japan). Energy-dispersive X-

ray spectroscopy (EDX) was used to further identify Cu and S elements. The crystal structure of CuS was detected by XRD. Chemical composition of the samples was analyzed by the X-ray photoelectron spectra (XPS, Thermo SCIENTIFIC ESCALAB 250Xi). The size distribution and zeta potential of NB NPs were determined using DLS measurements (Malvern Instruments Ltd.). The morphology of VNP20009, $_{CuS}$VNP20009 and $_{CuS}$VNP20009$_{NB}$ was characterized by scanning electron microscope (JSM-6700F, Japan).

Imaging of bacterial cells by Nano-CT

Synchrotron soft X-ray transmission microscope (TXM, Nano-CT) at the beamline BL07W of the National Synchrotron Radiation Laboratory (NSRL, Hefei, China) was used to observe a single bacterial imaging. VNP20009 and $_{CuS}$VNP20009 were fixed in 2.5% glutaraldehyde overnight at 4 ℃, then, the above bacterial solutions were centrifuged (2000rpm, 10 min) and dehydrated through treating with 30, 55, 75, 85 and 95% gradient ethanol for 15 min and 100% ethanol for 20 min. We dispersed the bacterial cells with PBS buffer and dropped 5 μL onto a 100-mesh nickel grid. After bacterial cells deposition, the nickel grid was immersed in liquid ethane and then inserted into a sample holder that was placed in liquid nitrogen. The sample holder with the nickel grid was transferred to the chamber of TXM. The soft X-ray beam was focused onto the bacterial cells and the bacterial cells were magnified on a CCD camera with a 9 μm field of view and 30 nm spatial resolution using a zone plate as an objective at an energy of 520 eV. Then, 116 consecutive projection images of bacterial cells rotated from −60° to +55° were taken by TXM at 1° intervals with an exposure time of 1 s. Next, we aligned the tilt series

by XMController, performed 3D tomographic reconstruction by XMReconstruction, and visualized bacterial cell structures and captured 3D morphological characteristics by Amira v.5.3.1 software (FEI Visualization Science Group, France).

Photothermal measurement

1 mL of 0.2 mg mL^{-1} $_{CuS}VNP20009_{NB}$ (equivalent to CuS content) was irradiated with 808 nm laser at the density of 0.75 W cm^{-2} for 10 min, and the temperature of the solution was detected by a thermocouple probe with accuracy of ± 0.1 ºC and recorded every 30s.

GSH-responsive NLG919 release

5 mL of 2 mg mL^{-1} $_{CuS}VNP20009_{NB}$ suspension (equivalent to NB NPs content) was transferred to a dialysis bag (molecular weight cut-off: 10 kDa) and soaked in 20 mL of PBS buffer solution in the presence or absence of 10 mmol L^{-1} GSH at 37 °C. 500 μL of the dialysis solution was taken at the predetermined time points to determine the concentration of NLG919 based on its absorbance at 280 nm.

Bacterial viability assay

100 μL of 1×10^6 CFU mL^{-1} VNP20009, $_{CuS}$VNP20009 or $_{CuS}VNP20009_{NB}$ (equivalent to VNP20009 colony) was stained with 5 μmol L^{-1} SYTO 9 and 5 μmol L^{-1} PI (Thermo Fisher Scientific) for 20 min. Live/Dead bacteria was analyzed by fluorescence microscope (Olympus BX-51 optical system microscope, Tokyo, Japan) and BD Accuri™ C6 flow cytometer.

Anaerobic migration

Anaerobic migration of $_{CuS}VNP20009_{NB}$ was investigated using a Transwell system

(polycarbonate membrane: 3 μm pore size, 6.5 mm diameter and 0.33 cm^2 membrane surface area) (Corning, USA). 200 μL of 1.5×10^7 CFU mL^{-1} $_{CuS}VNP20009_{NB}$ (equivalent to VNP20009 colony) was added to the upper chamber, while 0.4 mL of glucose solution (0.4 mg mL^{-1}), glucose oxidase (0.5 KU) and catalase (0.5 KU) were added to the lower chamber to simulate hypoxic condition. In comparison, the lower chamber was filled with 0.4 mL of glucose solution (0.4 mg mL^{-1}) to remain normoxic condition. The bacteria in the lower chamber were stained with SYTO9 (5 μmol L^{-1}) and their quantity was detected by flow cytometry.

Cell lines and cell culture

B16F1 murine melanoma cell line and RAW264.7 cell line were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) medium with 10% FBS and 1% antibiotics at 37 ° C in a humidified atmosphere containing 5% CO_2. BMDCs were freshly separated from mice and cultured in DMEM medium containing 20 ng mL^{-1} GM-CSF, 20 ng mL^{-1} IL-4, 10% FBS and 1% antibiotics at 37 °C in a humidified atmosphere containing 5% CO_2.

Cell uptake

SYTO 9-labeled VNP20009, $_{CuS}VNP20009$ and $_{CuS}VNP20009_{NB}$ were used to investigate the cellular uptake by fluorescence microscopy and flow cytometry. B16F1 or RAW 264.7 cells were seeded into a 24 well plate at a density of 4×10^4 cells with a volume of 0.4 mL and incubated for 24 h. Then, the medium was changed with fresh medium containing SYTO9-labeled VNP20009, $_{CuS}VNP20009$ and $_{CuS}VNP20009_{NB}$ (equivalent to 5×10^4 CFU mL^{-1} VNP20009). For fluorescence microscopy analysis, after

12 h of treatment, the cells were washed with PBS three times and observed by fluorescence microscope (Olympus BX-51 optical system microscope). For flow cytometry analysis, after12 h of treatment, the cells were washed with PBS three times, detached by trypsin and resuspended in PBS for analysis by BD Accuri™ C6 flow cytometer.

Cell viability assessment

First, B16F1 cells (1×10^4 cell/well) were seeded in 96-wells plates and cultured for 24 h. Then, the cells were incubated with various concentrations of VNP20009, NB NPs, $_{CuS}$VNP20009 and $_{CuS}$VNP20009$_{NB}$. For NIR laser irradiation group, after 6 h of incubation, the cells were irradiated by 808 nm laser (0.75 W cm^{-2}) for 5 min, followed by additional 18 h of incubation. For no irradiation group, cells were incubated for 24 h. For both groups, CCK-8 assay (Sigma-Aldrich) was employed to assess the cell viability according to manufacturer's protocol.

Live/dead cell staining and cell apoptosis detection *in vitro*

B16F1 cells were seeded in 24-well plates at 4×10^4 cells/well and cultured for 24 h. Then, the cells were treated with VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 5×10^4 CFU mL^{-1} VNP20009 or 100 μg mL^{-1} NB NPs). For NIR laser irradiation group, after 6 h of incubation, the cells were irradiated by 808 nm laser (0.75W cm^{-2}) for 5 min, followed by additional 18 h of incubation. For no irradiation group, cells were incubated for 24 h. For live/dead cell staining assay, the cells were stained with calcein-AM (1 μmol L^{-1})/PI (1 μmol L^{-1}) for 15 min at room temperature in the dark, and the cellular fluorescence image was observed by fluorescence microscopy. For cell apoptosis detection, Annexin V-FITC/PI apoptosis detection kit

(Dalian Meilunbio Biotechnology Co., Ltd., Dalian, China) was used to analyze apoptotic cells by BD Accuri™ C6 flow cytometer.

Evaluation of IDO-1 enzymatic activity.

B16 F1 cells were seeded into a 96 well plate at a density of 1×10^4 cells per well and allowed to grow overnight. Then cell culture medium was changed with fresh medium at pH 7.4 or 5.0. Recombinant mouse IFN-γ (50 ng mL^{-1}) was added to each well for stimulation of IDO-1 expression. Then, various concentrations of VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ were used to treat the cells. After 48 h of incubation, 150 μL of the supernatant extracted from each well was incubated with 75 μL of trichloroacetic acid (30%) at 50°C for 30 min to hydrolyse N-formylkynurenine to kynurenine. Then, the mixture was incubated with an equal volume of Ehrlich reagent (2% p-dimethylamino benzaldehyde w/v in glacial acetic acid) for 10 min at room temperature. The absorbance of the reaction product was measured at 490 nm using a microplate reader.

ICD effect and DC maturation

B16F1 cells were seeded in the 12-well plate at a density of 8×10^4 cells/well. After 24 h pre-incubation, the cells were treated with VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 5×10^4 CFU mL^{-1} VNP20009 or 100 μg mL^{-1} NB NPs). For NIR laser irradiation group, cells were treated for 6 h and irradiated by 808 nm laser (0.75W cm^{-2}) for 5 min, followed by additional 18 h of incubation. For no irradiation group, cells were incubated for 24 h. For CRT detection, the cells were collected to incubate with CRT primary antibody (Beyotime Biotechnology, Shanghai, China) for 1 h and further incubated with Alexa Fluor 488-conjugated secondary antibody (Beyotime

Biotechnology, Shanghai, China) for 2 h. Finally, the cells were stained with PI and detected by flow cytometry to identify cell surface CRT. The fluorescence intensity of stained cells was gated on PI-negative cells. For HMGB1 detection, the cell culture supernatant was collected for Elisa measurement using a HMGB1 assay kit (Shanghai Enzyme-linked Biotechnology Co, Ltd, Shanghai, China) according to manufacturer's instructions.

B16F1 cells were seeded in the lower chamber (4×10^4 cells /well) of a 24-well transwell plate for overnight growth, then 1×10^5 cells/well of BMDCs were cultured in the upper chamber and VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 5×10^4 CFU mL^{-1} VNP20009 or 100 μg mL^{-1} NB NPs) was added to the lower chamber. For NIR laser irradiation group, after 6 h of incubation, B16F1 cells were irradiated by 808 nm laser (0.75W cm^{-2}) for 5 min, followed by additional 18 h of incubation. For no irradiation group, cells were incubated for 24 h. After 24 h, BMDCs in the upper chamber of the transwell system were collected, and stained with anti-CD11c-APC, anti-CD86-PE, and anti-CD80-FITC antibodies for 30 min in the dark. The stained cells were acquired in BD Accuri™ C6 flow cytometer, and analyzed with Flow Jo software.

***In vitro* assessments of macrophage repolarization**

RAW264.7 were seeded into a 12-well plate with a density of 8×10^4 cells/well. After 24 h of growth, cells were treated with 10 ng mL^{-1} of IL-4 for 24 h, and further treated with VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 5×10^4

CFU mL^{-1} VNP20009 or 100 μg mL^{-1} NB NPs) for 24 h. The supernatant of each group was collected to measure the content of TNF-α and IL-10 using ELISA kits (BioLegend, San Diego, CA, USA) according to manufacturer's instructions.

Animals and tumor-bearing mice model establishment

Female C57BL/6 mice were purchased from Beijing Vital River Experiment Animal Technology Co. Ltd, and fed distilled water and disinfected food at approximately 20 °C and normal humidity in stainless steel cages. All animal procedures were carried out in accordance with the Guidelines for Care and Use of Laboratory Animals of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences, and approved by the Animal Ethics Committee of Changchun Institute of Applied Chemistry, Chinese Academy of Sciences. 100 μl of PBS containing 1×10^6 B16F1 cells/mL was implanted subcutaneously into the backs of female C57BL/6 mice to establish B16F1 tumor-bearing mice.

***In vivo* distribution**

When the tumor reached around 100 mm^3, B16F1 tumor-bearing mice were intravenously injected with VNP20009, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 1×10^5 CFU VNP20009/mice). At 24 h post-injection, the mice were sacrificed. The heart, liver, spleen, lung, kidney and tumor tissues with the same mass were harvested and homogenized. The homogenates were diluted in a 10-fold gradient and plated in plates, and solid LB agar medium was added and the amounts of colony were counted after 24 h of incubation. For determination of Cu contents, tumor, heart, liver, spleen, lung and kidney were collected and weighed, and then dissolved in fresh aqua reggae. The

concentration of copper element was determined by inductively coupled plasma mass spectrometry (ICP-MS).

***In vivo* therapeutic efficiency assessment**

When the tumor reached around 100 mm^3, the mice were randomly divided into 10 groups (three mice per group): a) PBS; b) VNP20009; c) NB NPs; d) $_{CuS}$VNP20009; e) $_{CuS}$VNP20009$_{NB}$; f) PBS+NIR laser; g) VNP20009+NIR laser; h) NB NPs+NIR laser; i) $_{CuS}$VNP20009+NIR laser; j) $_{CuS}$VNP20009$_{NB}$+NIR laser. Mice were intravenously injected with various formulations (equivalent to 10^5 CFU VNP20009/mice or 10 mg NB NPs/kg mice). For NIR laser irradiation, at 24 h post-injection, the tumor region of mice was irradiated with 808 nm laser for 5 min at 0.75 W cm^{-2}. The tumor volume and body weight of mice were monitored throughout the whole experiment period. The B16F1 tumor volume was defined as follows: $V = L \times W^2 / 2$, where W was the minor axis of the tumor and L represented the major axis of the tumor. After 14 days of treatments, the mice were sacrificed, and the plasma, major organs and tumors were harvested for ELISA assay, H&E analysis and flow cytometry analysis. The tumors and the major organs were dipped in 4 % paraformaldehyde to prepare tissue sections, and the slices were stained with hematoxylin and eosin for optical observation by light microscopy. Meanwhile, the plasma in each group was also collected for measurement of INF-γ and TNF-α using ELISA kits (BioLegend, San Diego, CA, USA).

ICD induction, DC maturation and macrophage repolarization *in vivo*

The tumor tissues of each group were harvested after the tumor-bearing mice were sacrificed at 5 days post-injection. Tumor tissues were minced and filtered twice through

a 40 μm cell strainer, and the cells were centrifuged at 1,500 rpm for 5 min at 4 °C to be processed into single-cell suspension. For CRT expression and HMGB1 release *in vivo*, the tumor cells were detected by flow cytometer using anti-CRT mouse monoclonal antibody (1:1000) (Beyotime Biotechnology, Shanghai, China) and FITC-labeled goat anti-mouse lgG (H+L) (Beyotime Biotechnology, Shanghai, China) according to standard protocol. Moreover, the supernatant was also collected for measurement the intracellular HMGB1 release using ELISA kit (Shanghai Enzyme-linked Biotechnology Co, Ltd, Shanghai, China).

For TAM polarization, anti-CD80-PE, anti-CD206-APC and anti-F4/80-FITC were added to single-cell suspension and incubated 30 min in the dark, then the cells were detected by flow cytometer.

The inguinal lymph nodes (LNs) were harvested at 5 days post-injection. LNs were minced and filtered twice through a 40 μm cell strainer, and the cells were centrifuged at 2100 rpm for 5 min at 4 °C to prepare a single-cell suspension. To investigate the DC maturation, anti-CD11c-APC, anti-CD86-PE, and anti-CD80-FITC antibodies were added and incubated for 30 minutes in the dark for flow cytometry analysis.

Quantification of *in vivo* Trp/Kyn ratio and tumor-infiltrating immune cells

To determine the ratio of Trp/Kyn, the blood samples were extracted at 5 days post-injection and centrifuged at 3,000 rpm for 5 min. The obtained supernatant (200 μL) was mixed with 40 μL of 30% trichloroacetic acid at 50 °C for 30 min. After that, the mixed solution was centrifuged again at 12,000 rpm for 30 min. The supernatant was collected and diluted with acetonitrile for HPLC analysis to determine Trp and Kyn.

To quantify the tumor-infiltrating immune cells, tumors were cut into small pieces and then minced completely by using sterile blades. Subsequently, single cell suspensions were obtained by filtration through a 200-mesh sieve. The cells were collected by centrifugation at 1200 rpm for 5 min. Regulatory T cell ($CD3^+$ $CD4^+$ $Foxp3^+$ T cells), helper T cell of $CD4^+$ T cell ($CD3^+CD4^+$ T cells) and cytotoxic T cell of $CD8^+$ T cell ($CD3^+CD8^+$ T cells) were stained by using anti-CD3-FITC, anti-CD4-PE, anti-CD8-APC and anti-Foxp3-APC-labeled antibodies for flow cytometry analysis.

***In Vivo* biosafety analysis**

At the end of 14 days of treatments, the blood was collected and the levels of hematological parameters, including white blood cells, red blood cells, platelets, lymphocytes, eosinophils and basophils, were tested by automated hematology analyzer (Sysmex KX-21, Cobe, Japan).

After 20 days of tumor ablation in $_{CuS}$VNP20009$_{NB}$ with laser irradiation, the mice were sacrificed and major organs (heart, liver, spleen, lung, kidneys) and tumor were collected and homogenized with sterile H_2O containing 0.1% Triton X-100, and then plating a series of diluted homogenates onto solid LB agar plates. The colonies on the plates were counted after being placed at 37 ℃ for 24 h to assess whether the VNP20009 can be cleared from the body.

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis of data was performed with a two-tailed unpaired Student's t test with GraphPad Prism6. The level of significance was defined at *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

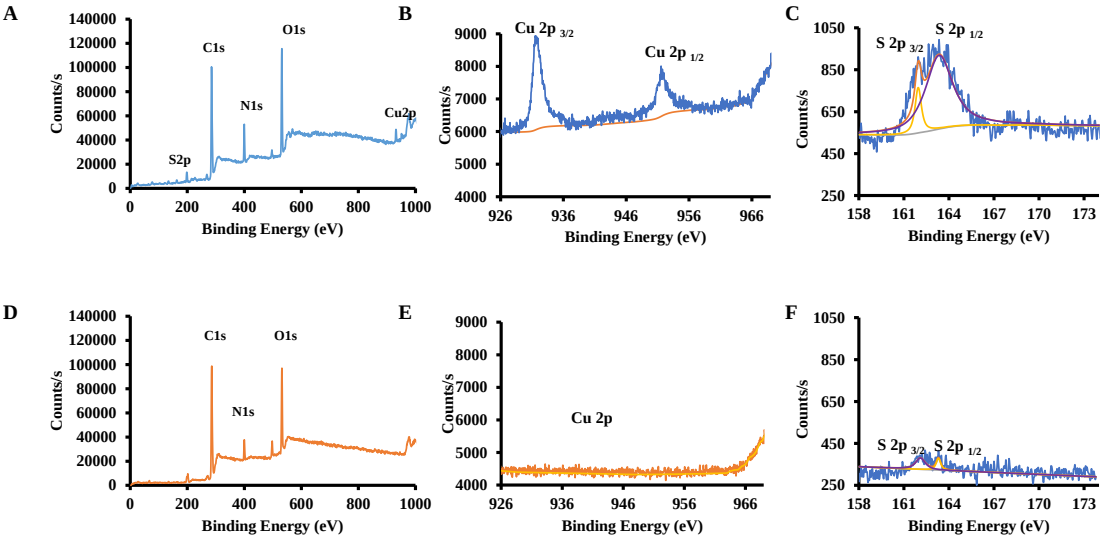

Figure S1. XPS survey spectra (A), high-resolution XPS spectra of Cu 2p (B) and S 2p (C) of $_{CuS}$VNP20009$_{NB}$; XPS survey spectra (D), high-resolution XPS spectra of Cu 2p (E) and S 2p (F) of VNP20009.

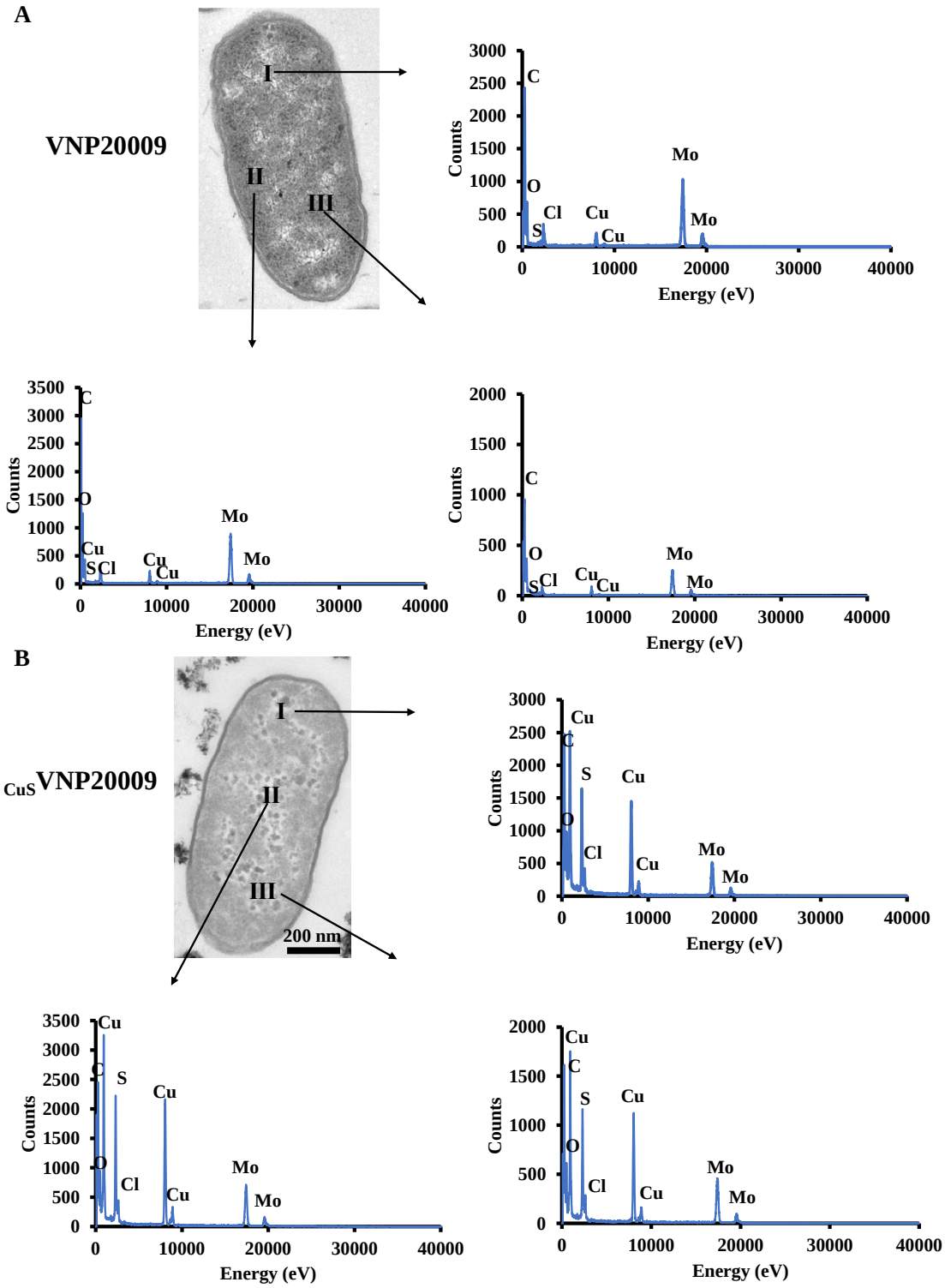

Figure S2. EDX spectra of the microtome-sliced VNP20009 (A) and $_{CuS}$VNP20009 (B).

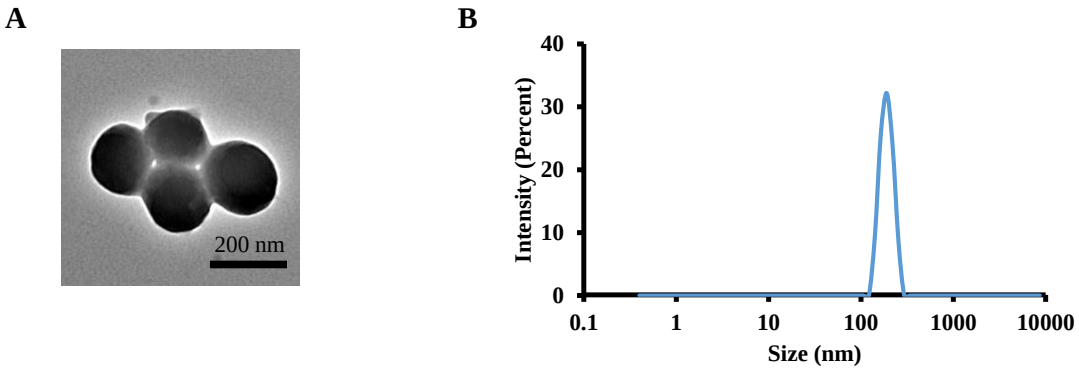

Figure S3. (A) TEM image and (B) Hydrodynamic size distribution of NB NPs.

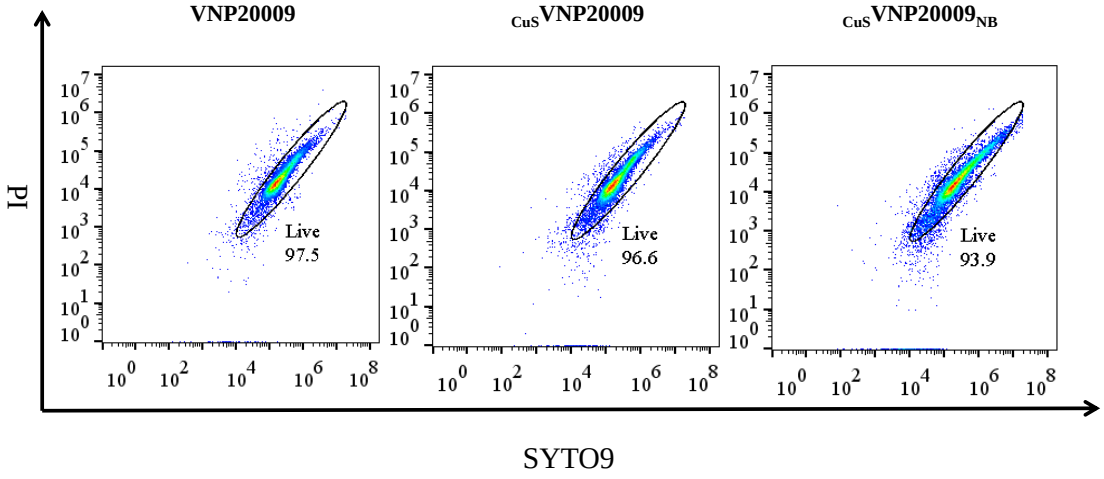

Figure S4. Bacterial viability assessments by flow cytometry using SYTO 9/PI staining assay.

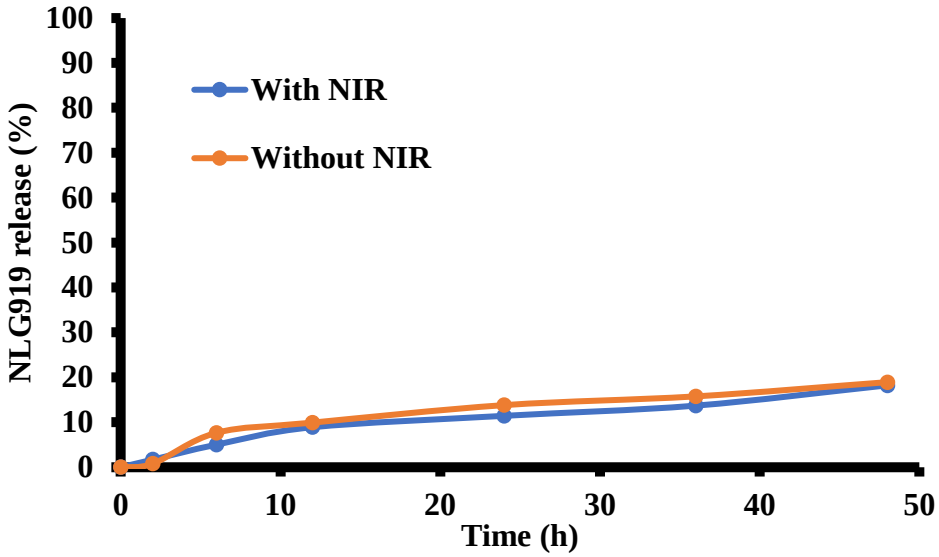

Figure S5. NLG919 release profiles from $_{CuS}$VNP20009$_{NB}$ without or with NIR (808 nm, 0.75 W cm^{-2}, 5 min).

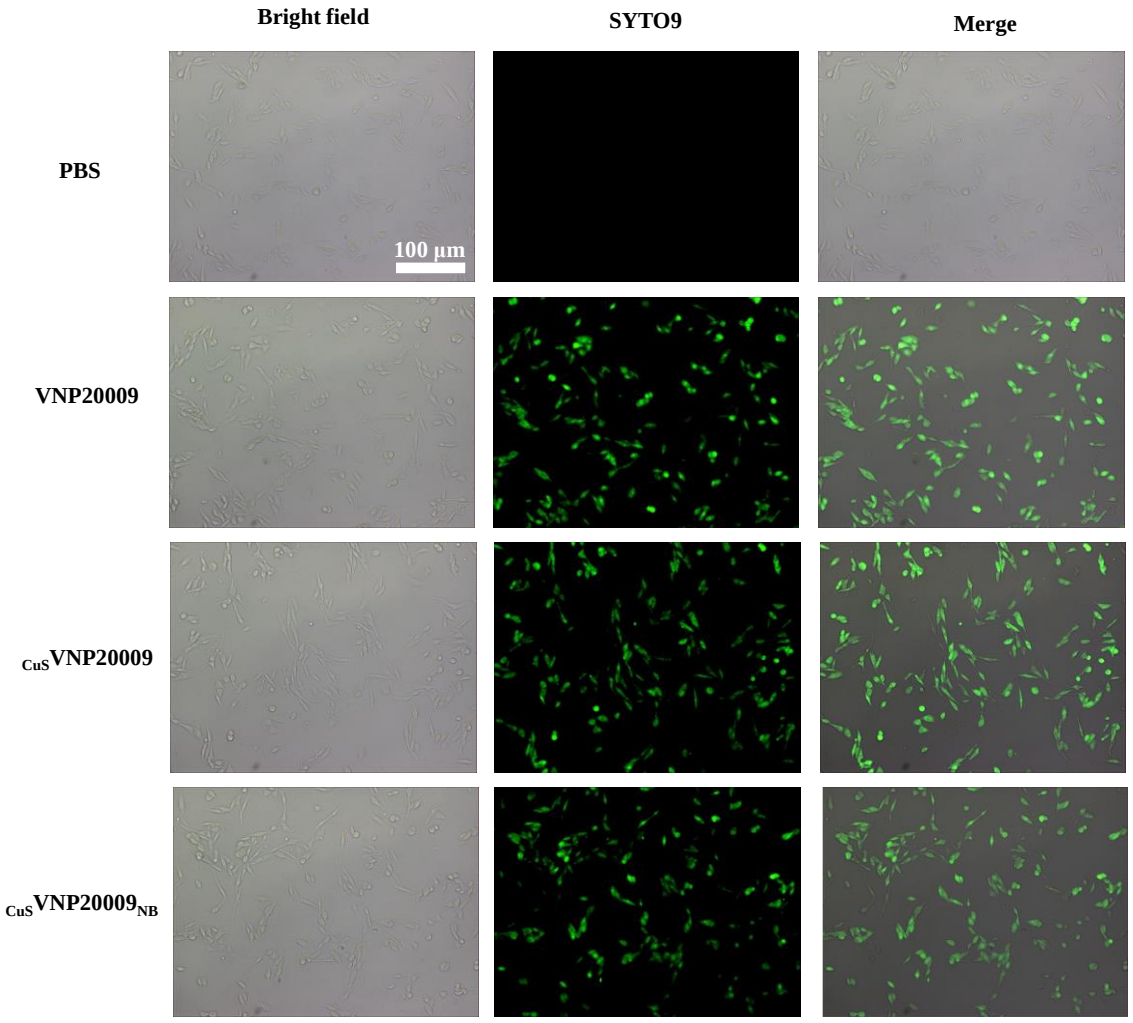

Figure S6. Fluorescence images for cellular uptake of B16F1 cells exposed to VNP20009, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ for 12 h.

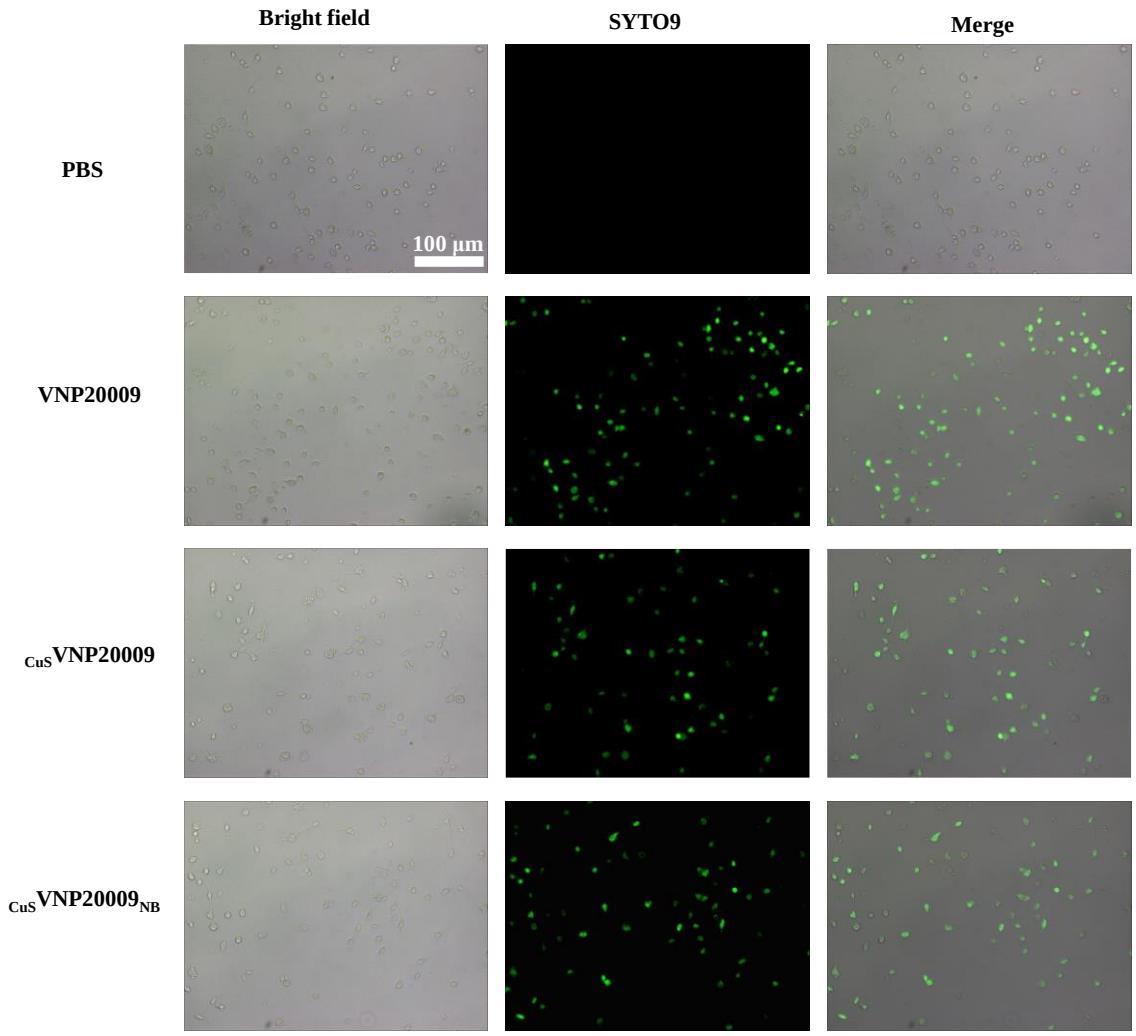

Figure S7. Fluorescence images for cellular uptake of RAW264.7 cells exposed to VNP20009, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ for 12 h.

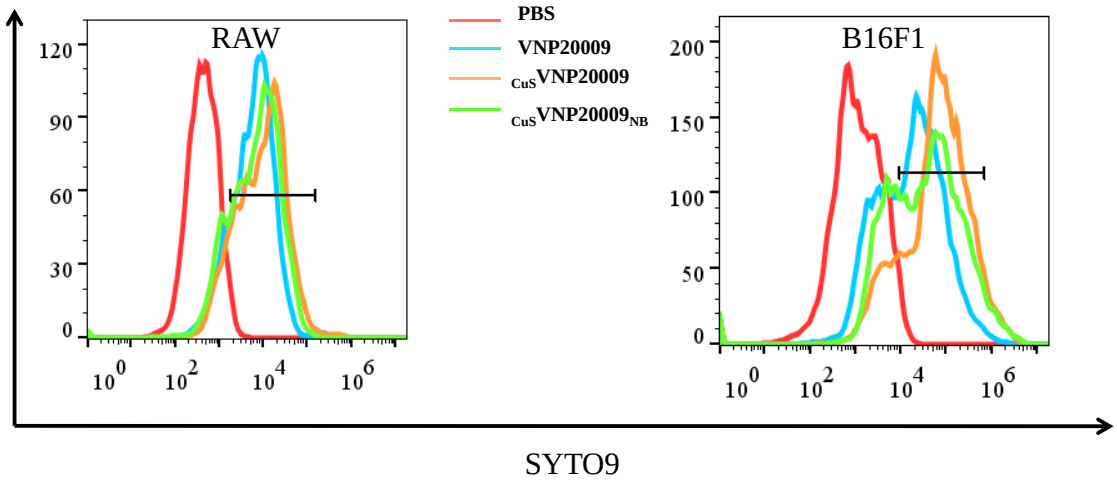

Figure S8. Flow cytometric analysis for cellular uptake of RAW264.7 and B16F1 cells exposed to VNP20009, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ for 12 h.

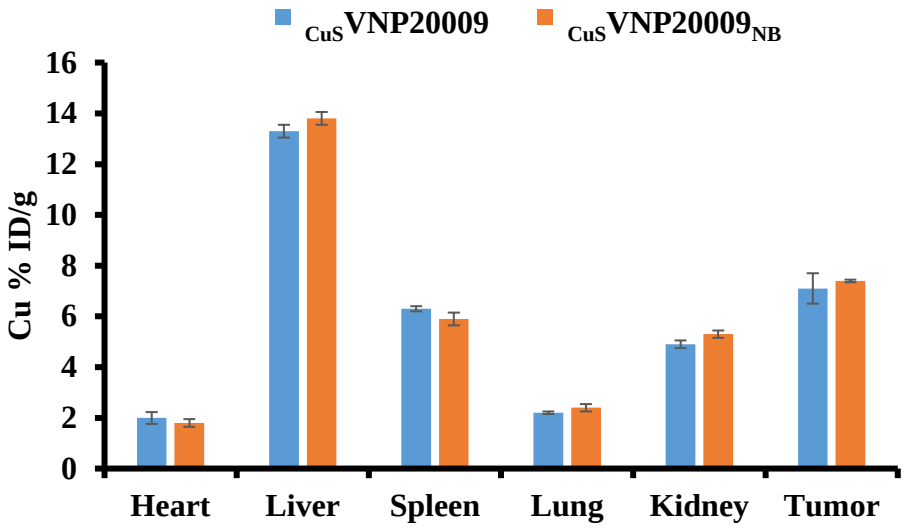

Figure S9. Biodistribution of $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ in B16F1 tumor-bearing C57 mice at 24 h post-injection based ICP-MS analysis of Cu element.

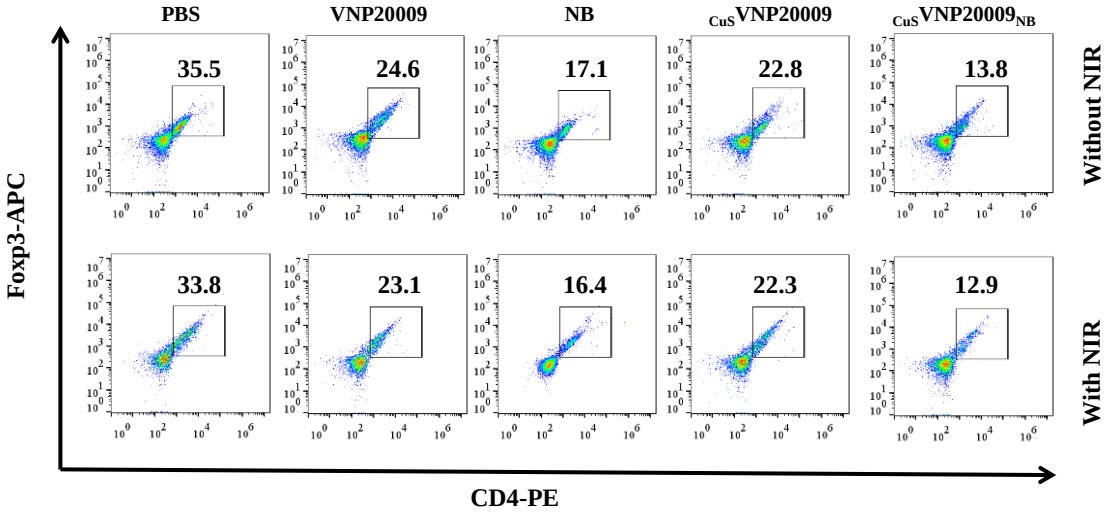

Figure S10. Frequency of intratumoral Treg cells ($CD3^+$ $CD4^+$ $Foxp3^+$) with or without NIR laser irradiation as analyzed by flow cytometry (gated on $CD3^+$ T cells).

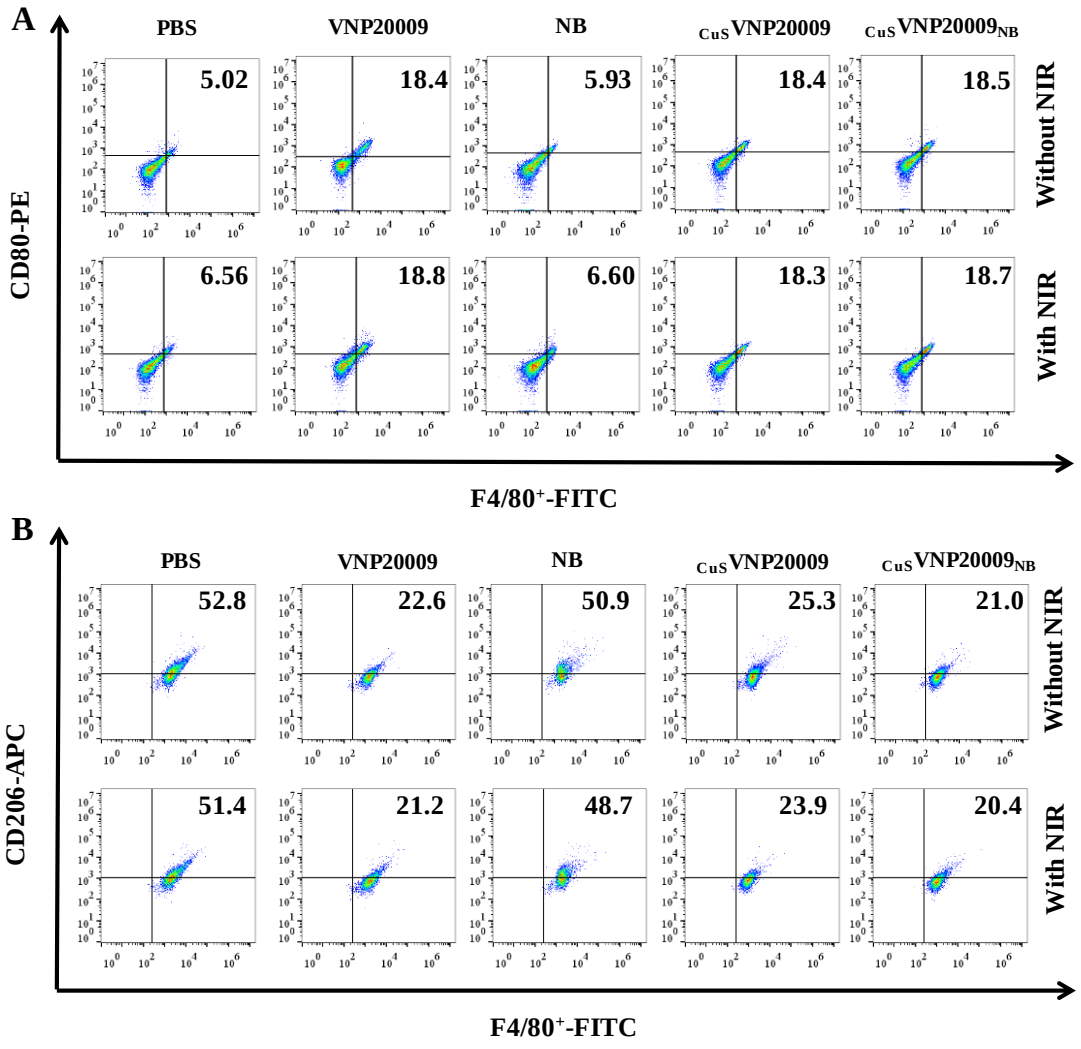

Figure S11. Frequency of the M1 macrophage ($F4/80^+$ $CD80^+$) (A) and M2 macrophage ($F4/80^+$ $CD206^+$) (B) in the B16F1 tumors. B16F1 tumor-bearing mice were intravenously injected with PBS, VNP20009, NB, V_{CuS}VNP20009 and $_{CuS}$VNP20009$_{NB}$ (equivalent to 10^5 CFU VNP20009/mice or 10 mg NB/kg mice), and tumor regions were irradiated with or without 808 nm laser (0.75W cm^{-2}, 5 min) at 24 h post-injection, and tumor tissues were harvested at 5 days post-injection to detect M1 and M2 macrophages by flow cytometry.

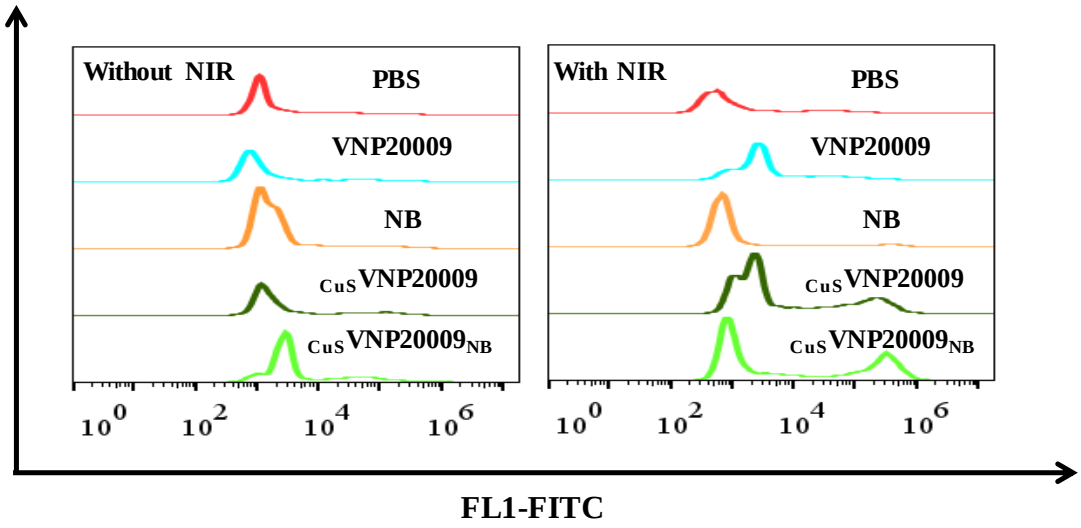

Figure S12. Flow cytometric examination of CRT expression in B16F1 tumor. B16F1 tumor-bearing mice were intravenously injected with PBS, VNP20009, NB NPs, $_{CuS}$VNP20009 or $_{CuS}$VNP20009$_{NB}$ (equivalent to 10^5 CFU VNP20009/mice or 10 mg NB/kg mice), and tumor regions were irradiated with or without 808 nm laser (0.75W cm^{-2}, 5 min) at 24 h post-injection, and tumor tissues were harvested at 5 days post-injection to detect CRT expression.

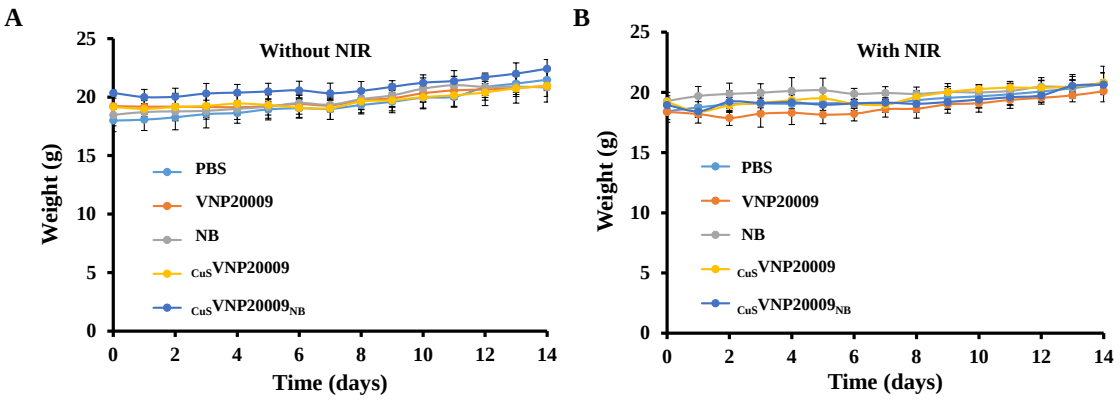

Figure S13. Body weight of B16F1 tumor-bearing mice during the treatment period without (A) or with NIR laser irradiation (B).

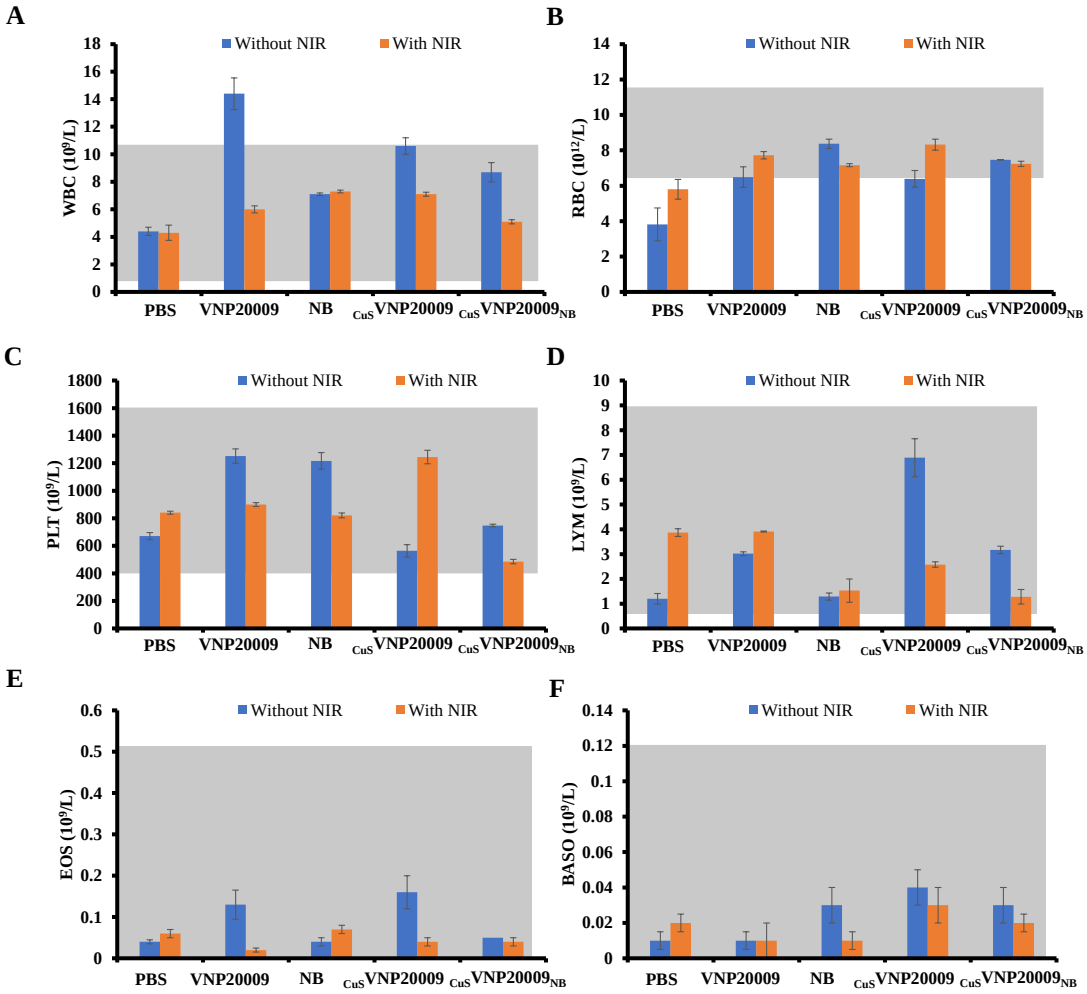

Figure S14. Analysis of hematological parameters, including white blood cells (A), red blood cells (B), platelets (C), lymphocytes (D), eosinophils (E) and basophils (F) after 14 days of treatment with PBS, VNP20009, NB, $_{CuS}$VNP20009 and $_{CuS}$VNP20009$_{NB}$. Upper and lower limits of the normal range were depicted as shades of gray; all parameters were from Beijing Vital River Experiment Animal Technology Co. Ltd. (http://www.vitalriver.com).

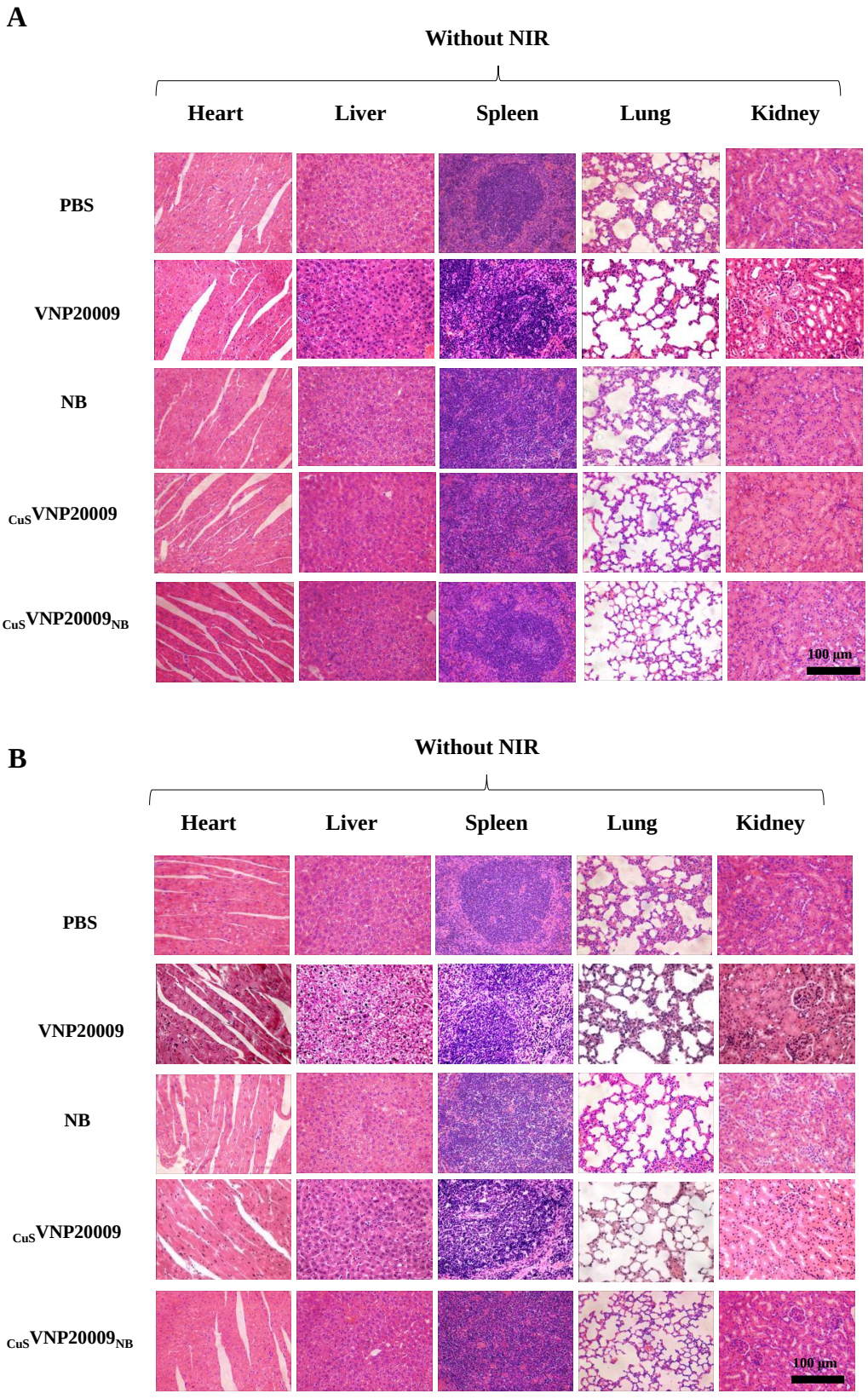

Figure S15. H&E staining of major organs at the end of treatment without (A) or with (B) NIR laser irradiation.

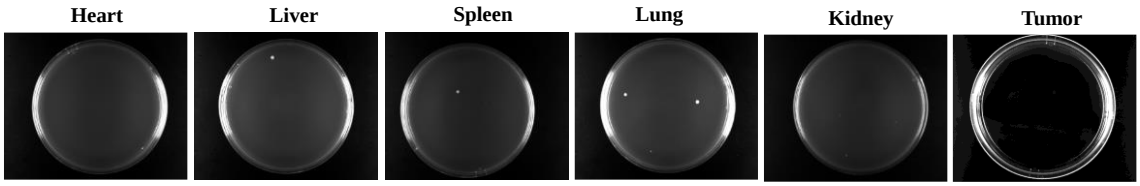

Figure S16. Colony distribution in major organs (heart, liver, spleen, lung, kidney) and tumor of mice treated with $_{CuS}$VNP20009$_{NB}$ under laser irradiation after 20 days of tumor ablation.