Supporting Information

Boosting Natural Killer Cell-Based Cancer Immunotherapy with Selenocystine/Transforming Growth Factor-Beta Inhibitor-Encapsulated Nanoemulsion

Chang Liu‡, Haoqiang Lai‡, Tianfeng Chen*

The First Affiliated Hospital, and Department of Chemistry, Jinan University, Guangzhou 510632, China

‡ These authors contributed equally to this work.

E-mail: tchentf@jnu.edu.cn.

MATERIALS AND EXPERIMENT

LDH Assay. LDH cytotoxicity assay kit was used following the operating instructions. Briefly, MDA-MB-231 cells were seeded in 96-well plate at the density of 4×10^4 cells/mL and incubated for 24 h, SeC + SB and SSB NMs (16 μM Se) were added and incubated for 12 h, then NK cells (effector-to-target (E:T) = 5:1) were added for 8 h. Lysis buffer was added before 30 min before the end of the experiment (high control). The plate was centrifuged at 250 g for 2 min and 100 μL of the clarified media from each sample was transferred to another 96-well plate and 100 μL of working solution was added and incubated for 20 min at room temperature. The absorbance was measured at 490 nm using Cytation™ 5 (BioTek). LDH activity released by cells without lysate and drugs was the low control. The percentage cytotoxicity was calculated as LDH activity (%) = [(experimental value – low control) / (high control – low control) × 100%].

Flow Cytometric Analysis. MDA-MB-231 cells at the density of 1×10^5 cells/mL were seeded in 10 cm dish overnight. Then SSB NMs (8 μM Se) was added and treated for 12 h. Cells were collected by centrifugation at 500 g for 5 min after trypsinization. After that, 1×10^6/mL cells were stained with 10 μg/mL of the primary antibody for 30 min

at room temperature. After washed with PBS twice, fluorochrome-labeled secondary antibody (1:2000) in 100 μL PBS was added and incubated for another 30 min. The labeled cells were collected (1500 rpm, 5 min), rinsed with PBS twice and resuspended in 400 μL PBS, and then analyzed by flow cytometry (Beckman Coulter). Effects of the nanosystem on MDA-MB-231 cells cycle distribution and cells apoptosis analysis were carried out according to previously published articles[54]. Briefly, MDA-MB-231 cells at the density of 4×10^3 cells/mL were seeded in 6 cm dish for 24 h. After treated with SeC solution, Sec NMs and SSB NMs (8 μM of Se) for indicated times, cells were collected and fixed with 70% cold ethanol for 4 h. After stained with PI, flow cytometry (Beckman Coulter) was employed to analyze the cell cycle distribution. The results were analyzed by ModFit LT 5.0 software.

***In Vitro* Migration Assay.** 4×10^4 cells/mL of MDA-MB-231 cells were seeded in 6-well plate for 24 h. After that cells were starved with medium containing with 0.5% of FBS for 12 h. Cells were wounded by pipette tips and washed with PBS for 3 times and then fresh medium without FBS were replaced with or without 2 ng/mL of TGF-β. Two hours later, SeC NMs and SSB NMs (8 μM of Se) were added. Migrated cells were stained with Hoechst 33342 and photographed under fluorescence microscope and then quantified by manual counting after incubation predetermined times.

Western Blot Analysis. 1×10^5 cells/mL MDA-MB-231 cells were inoculated in 10 cm dishes for 24 h, then indicated concentration of SSB NMs was added and continue incubated for determined times. Total cell lysates were harvested by 12000 rpm for 10 min after cells lysed by lysate buffer (Beyotime, #P0013B) at 4 ℃, and the protein concentrations were determined by BCA kit. 40 μg proteins were adjusted for 12% SDS-PAGE gel electrophoresis under the condition of 70 V and 120 min. Then proteins were transferred to nitrocellurose membrane at 110 V and 90 min. After that, the membrane was subjected to the blocking buffer (5% of skim milk, Sigma) for 2 h and then incubated with primary antibodies (1:1000) overnight at 4 ℃. After 3 washes with TBST, the second antibody (1:3000) was added and incubated for 2 h at 4 ℃. The protein bands were conducted for chemiluminescence analysis by using HRP Substrate (Sigma) and visualized under Tanon 5200 chemiluminescence imaging system.

Comet Assay. 4×10^3cells/mL of MDA-MB-231 cells were seeded in 2 cm dish overnight. Then cells were treated with indicated concentration of SeC + SB and SSB

NMs for 12 h. After that, cells were collected by centrifugation (1500 rpm, 5 min) and resuspended in 500 μL PBS. Then cells were mixed with melted agarose (1%) at 1:9 (v/v). Then 100 μL of the mixture was pipetted on the surface of the slide and refrigeration at 4 ℃ for 30 min followed by immersing in the cold lysis solution for 90 min. After that, the slide was transfered to the prepared alkaline solution for 20 min and then subjected to electrophoresis at 19 V for 30 min. Finally, DNA was stained with AO and visualized under the fluorescence microscope (Life technologies EVOS®FL Auto, 200 ×).

Immunofluorescence. A total of MDA-MB-231 cells at the density of 4×10^3 cells/mL were plated in 2 cm glass bottom dishes overnight and treated with indicated concentration of SSB NMs and SeC for 12 h. After rinsed with PBS twice, cells were fixed with 4% formalin in PBS for 20 min followed by twice washes in PBS. Then 5% BSA was used to block the cells for 1 h and incubated with primary antibody of phosphor-H2A.X (Ser139) (1:500) overnight at 4 °C. After 3 washes with PBS, second antibody (Alexa Fluor® 488, 1:500) was added and treated for 1 h at room temperature. Finally, the expression of phospho-H2A.X (Ser139) was analyzed by using fluorescence microscope.

RESULTS

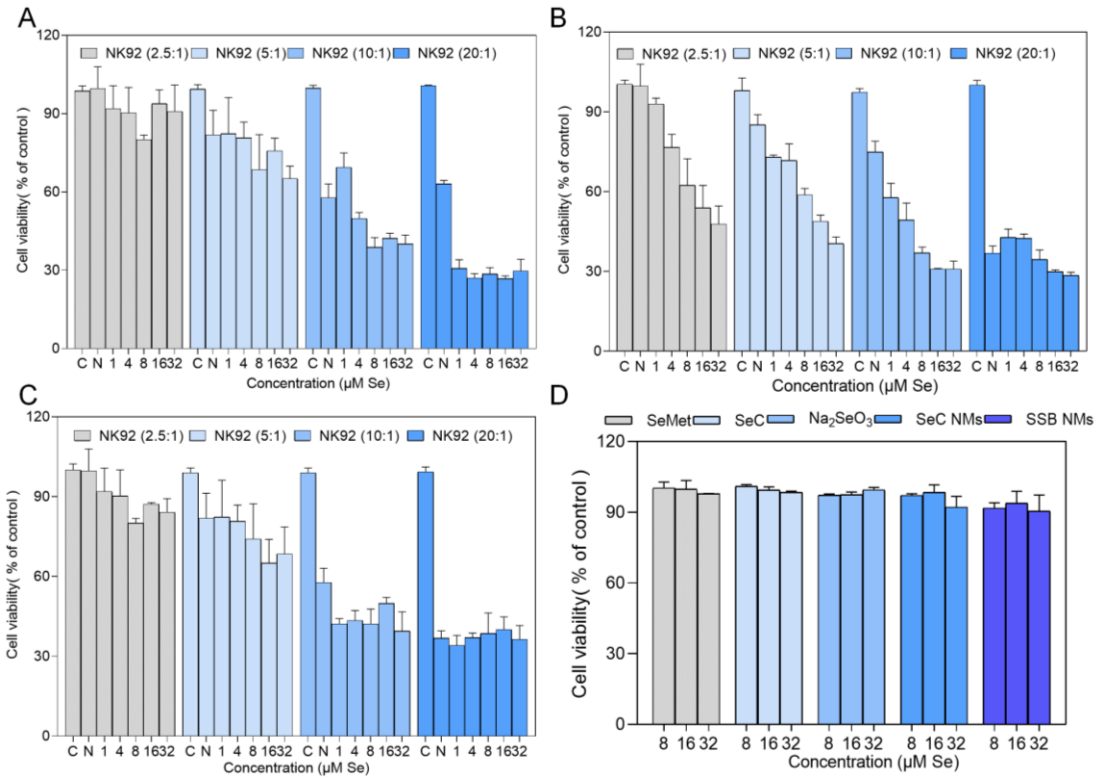

Figure S1. Immunosensitizing effects of NK92 cells induced by SSB NMs against MDA-MB-231 cancer cells. Cytolytic activities of NK92 cells toward cancer cells under the simultaneous treatment of NK92 cells and SSB NMs (A), SSB NMs pretreatment followed by NK92 cells (B) and SSB NMs preincubated with NK92 cells for 12 h and then treated cancer cells (C). (D) Cytotoxicity of SeMet, SeC, Na_2SeO_3, SeC NMs and SSB NMs on T cells derived from humans. Cell viability was examined by MTT assay as described in the Experimental section. C: Control, N: NK92 cells.

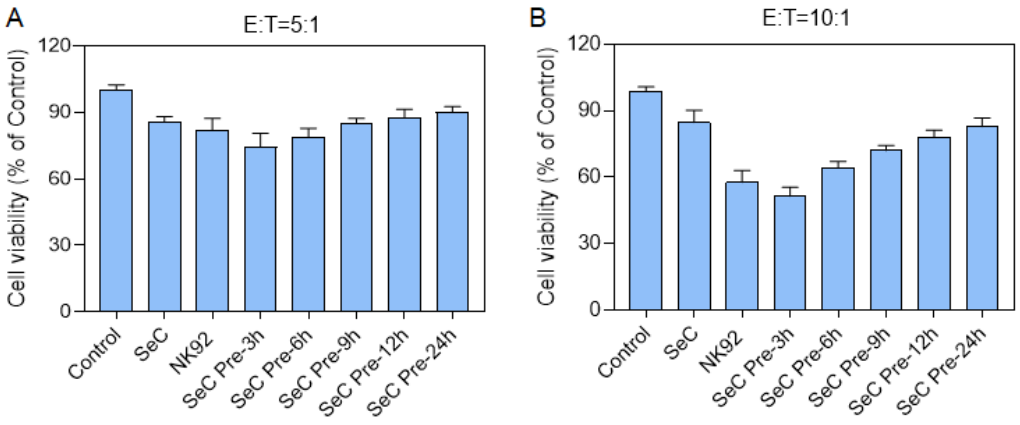

Figure S2. Cytolytic activities of NK92 cells against MDA-MB-231 cells. The killing effects of NK92 cells that pretreated with SeC (8 μM Se) for different times against MDA-MB-231 cells at E:T = 5:1 (A) and E:T = 10:1 (B). NK92 cells were preincubated with SeC for 0, 3, 6, 9, 12 and 24 h then added to cancer cells and coincubated for 8 h. After that, NK92 cells were removed gently and rinsed with PBS 3 times. The viability of cancer cells was examined by MTT assay as described in the experimental section.

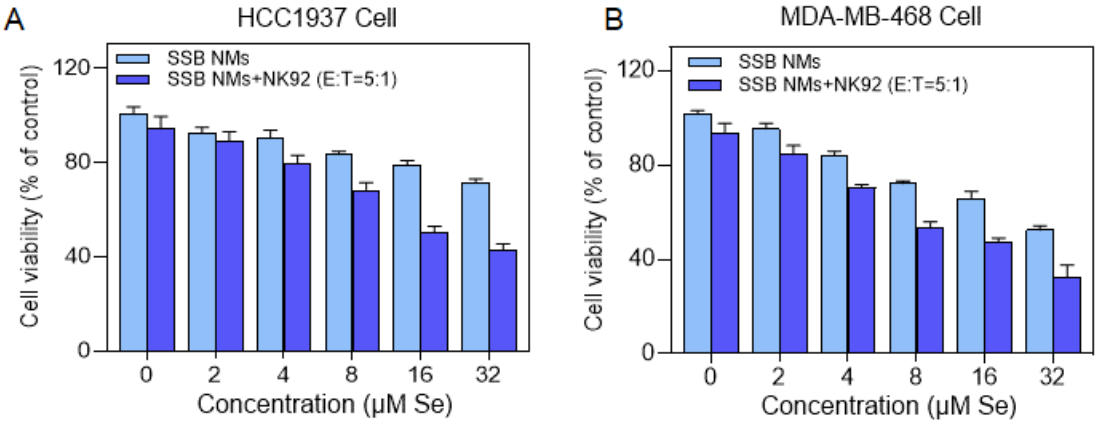

Figure S3. SSB NMs augments the cytolytic activities of NK92 cells on HCC1937 cells (A) and MDA-MB-468 cells (B). HCC1937 and MDA-MB-468 cells (4000 cells of per well) were seeded in 96-well plates and incubated overnight, and then different concentration of SSB NMs was added and incubated for 12 h. After that, NK92 cells E:T = 5:1) were added and cotreated for 8 h, and the lytic potency of NK92 cells was determined by MTT assay.

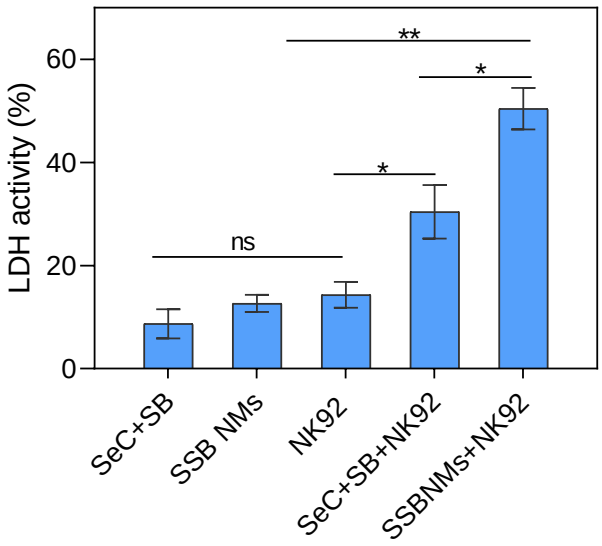

Figure S4. SSB NMs augments the cytolytic activities of NK92 cells by determining the LDH release. E:T = 5:1. n = 3, **P* < 0.05, ***P* < 0.01.

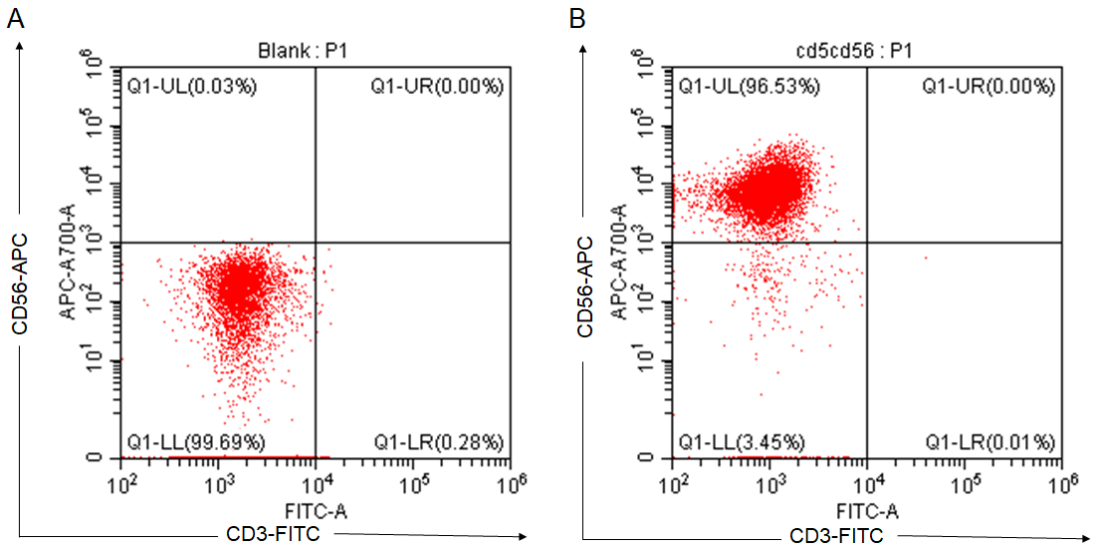

Figure S5. Characterization of patient-derived NK cells by flow cytometry assay. (A) NK cells unstained or stained (B) with CD3-FITC and CD56-APC antibody.

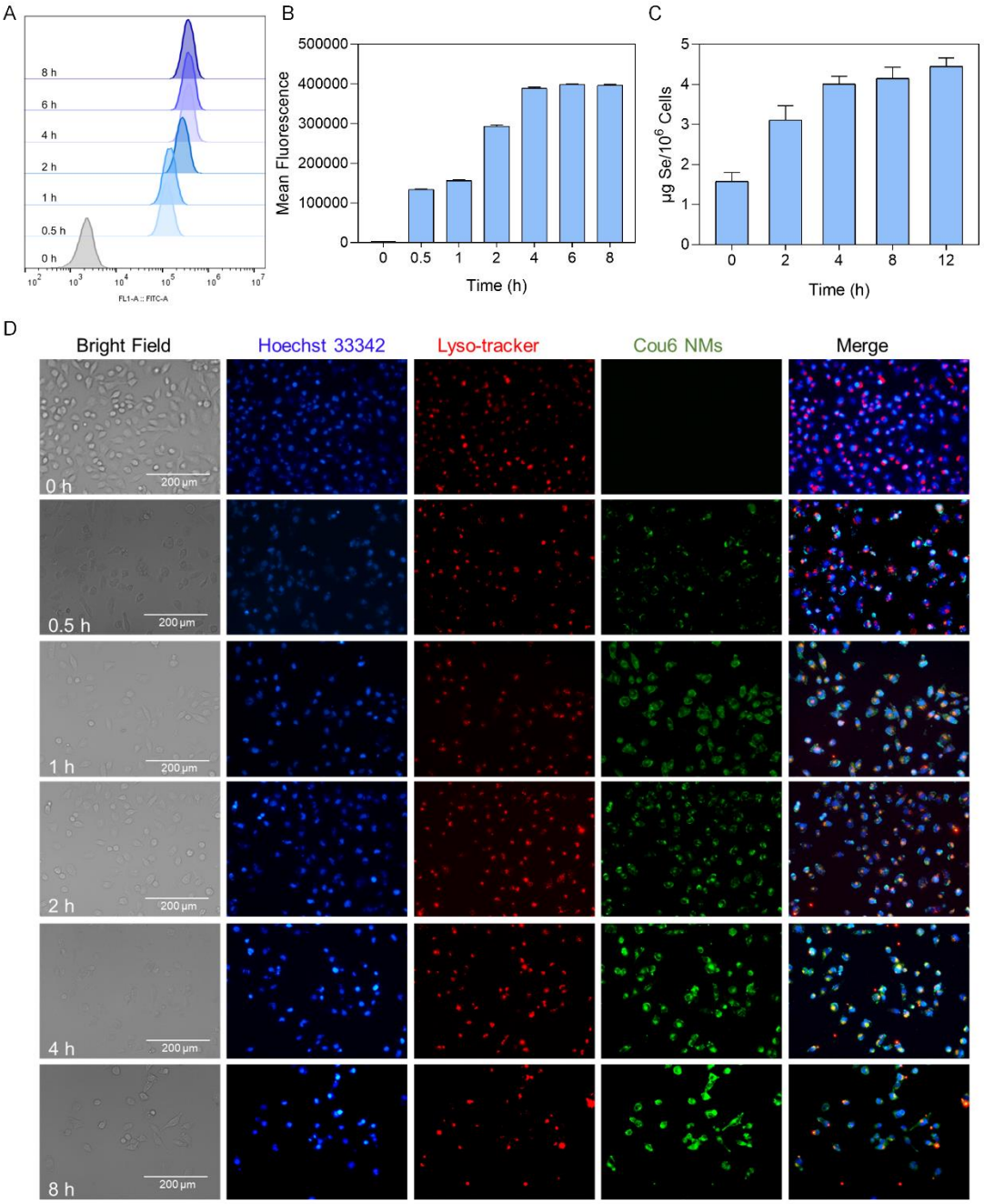

Figure S6. Cellular uptake and intracellular localization evaluation. (A) Cellular uptake of Cou6-labeled NMs by MDA-MB-231 cells was examined by flow cytometry assay. (B) Representative of Cou6 fluorescence intensity in MDA-MB-231 cells. MDA-MB-231cells were seeded in 6-cm dishes (4×10^5 cells/dish) and incubated overnight, then Cou6-labeled NMs was added and incubated for predetermined times, and then the uptake of NMs in MDA-MB-231cells was analyzed by flow cytometry assay. (C) Cellular uptake of SSB NMs in MDA-MB-231 cells was examined by detecting the content of Selenium. Briefly, MDA-MB-231 cells (10×10^4 cells/mL) in 10 cm dishes were treated with SSB NMs (8 μM Se) for indicated times. After that, 1×10^6 cells were harvested and analyzed by determining the content of Se by AFS assay. The results are expressed as means ± standard deviation of triplicates. (D) Intracellular localization of

Cou6-labeled NMs (green) and lysosomes (red) in MDA-MB-231 cells. MDA-MB-231 cells (4×10^3 cells/mL) were seeded in 2-cm dishes and incubated overnight. After the lysosomes and nucleus were labeled with lysosomes tracker and Hoechst 33342, Cou6-labeled NMs was added and incubated for indicated times. The cellular distribution of NMs was visualized by fluorescence microscope.

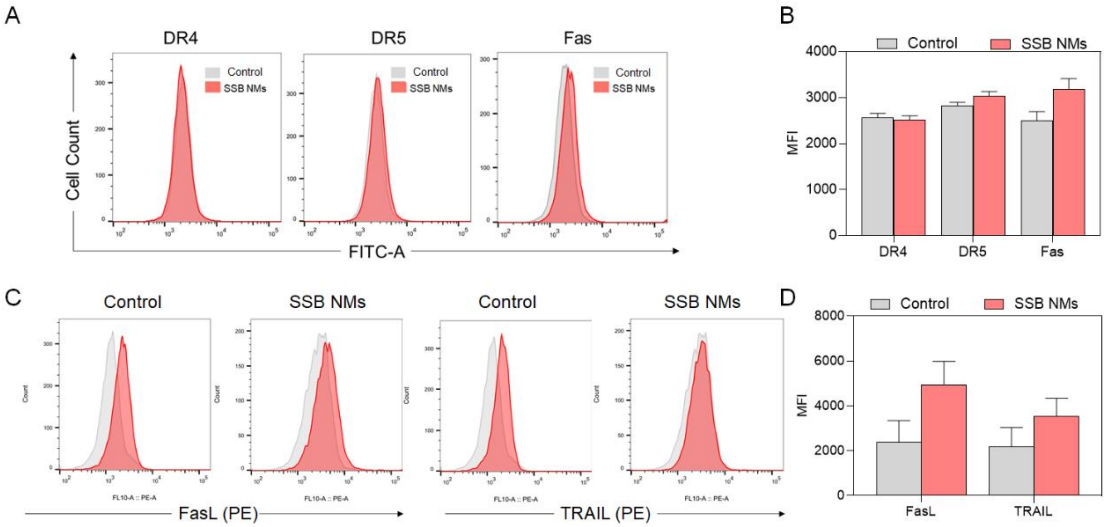

Figure S7. Evaluation of the surface expression of DR4/5, TRAIL and FasL. (A) Expression of DR4, DR5 and Fas on the surface of MDA-MB-231 cells, which were incubated with SSB NMs (8 μM of Se) for 12 h, were analyzed by flow cytometry assay. (B) Mean fluorescence intensity of the expression of DR4, DR5 and Fas. (C) FasL and TRAIL expression on the surface of NK92 cells after incubation with SSB NMs (8 μM Se) for 12 h was examined by flow cytometry assay. (D) Representative mean fluorescence intensity of the expression of TRAIL and FasL.

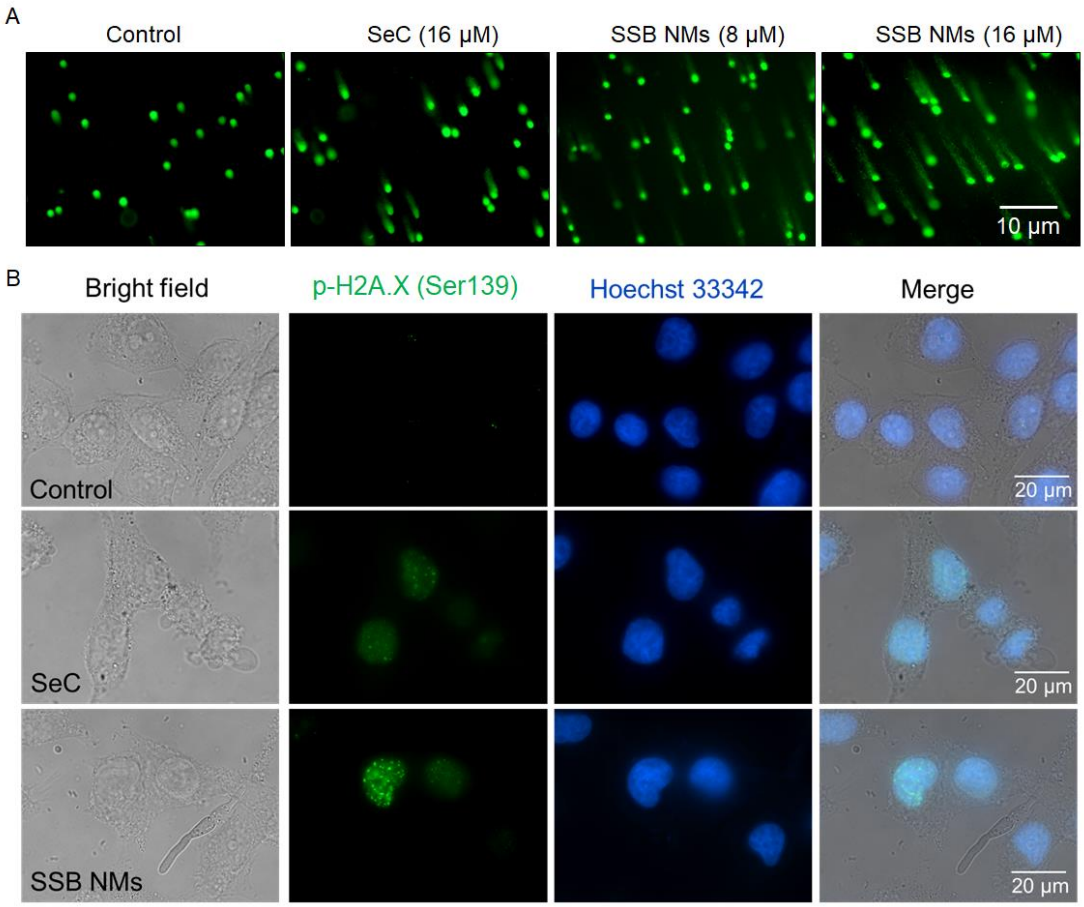

Figure S8. Evaluation of the DNA damage inducing capacity of SeC and SSB NMs. (A) Effects of DNA damage induced by SSB NMs and SeC as examined by comet assay. (B) Examination of the expression of phospho-H2A.X (Ser139) in MDA-MB-231 cells. 4×10^3 cells/mL of MDA-MB-231 cells were seeded in 2 cm dish overnight. Then cells were treated with indicated concentration of SeC (16 μM Se) and SSB NMs (8 and 16 μM Se) for 12 h. After that, cells were collected for agarose gel electrophoresis and DNA damage was stained with AO (17.5 μg/mL) and visualized under the fluorescence microscope. As for the immunofluorescence assay, cells were fixed with 4% formalin in PBS, blocked with 5% BSA and incubated with phospho-H2A.X (Ser139) primary antibody (1:500) overnight. After incubated with Alexa Fluor® 488 second antibody (1:500), the expression of phospho-H2A.X (Ser139) was analyzed by using fluorescence microscope.

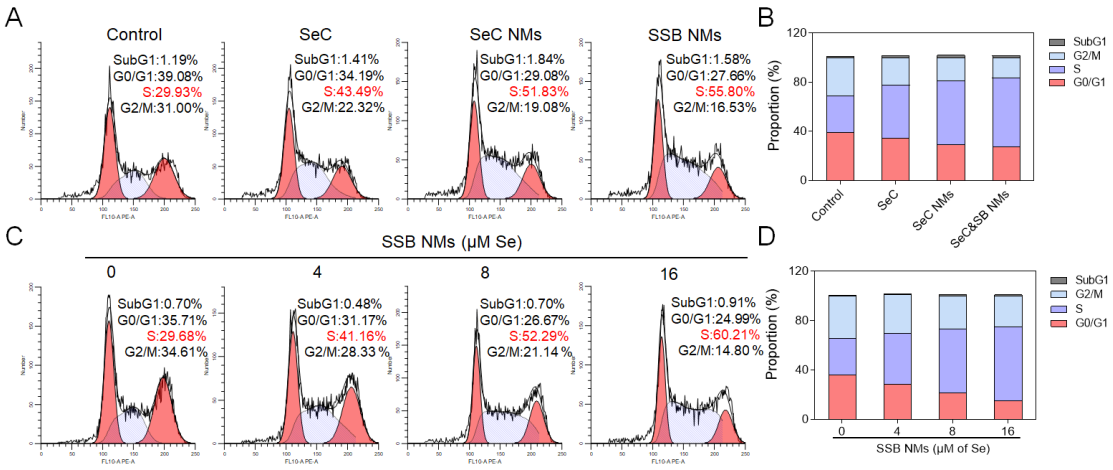

Figure S9. Cell cycle distribution analysis by flow cytometry. (A-B) Cell cycle analysis of MDA-MB-231 cells after treated with PBS, SeC solution, Sec NMs and SSB NMs (8 μM of Se). (C-D). Cell cycle analysis of MDA-MB-231 cells treated with different concentration of SSB NMs and the proportion analysis.

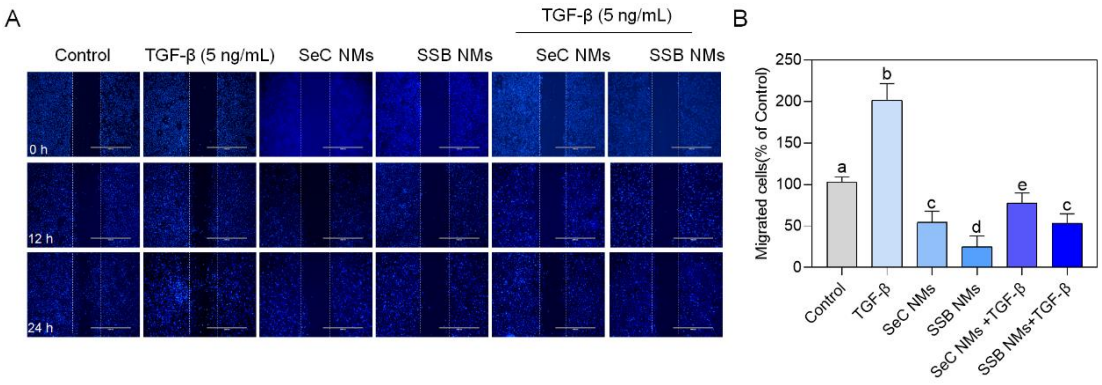

Figure S10. Anti-migration effects of SeC NMs and SSB NMs against MDA-MB-231 cells. (A). Low dosage of Sec NMs and SSB NMs (4 μM of Se) inhibited TGF-β-induced MDA-MB-231 migration. (B) Quantitative analysis of the migrated cells by manual counting. Bars with different characters a, b, c, d and e are statistically different at $P < 0.05$ level.

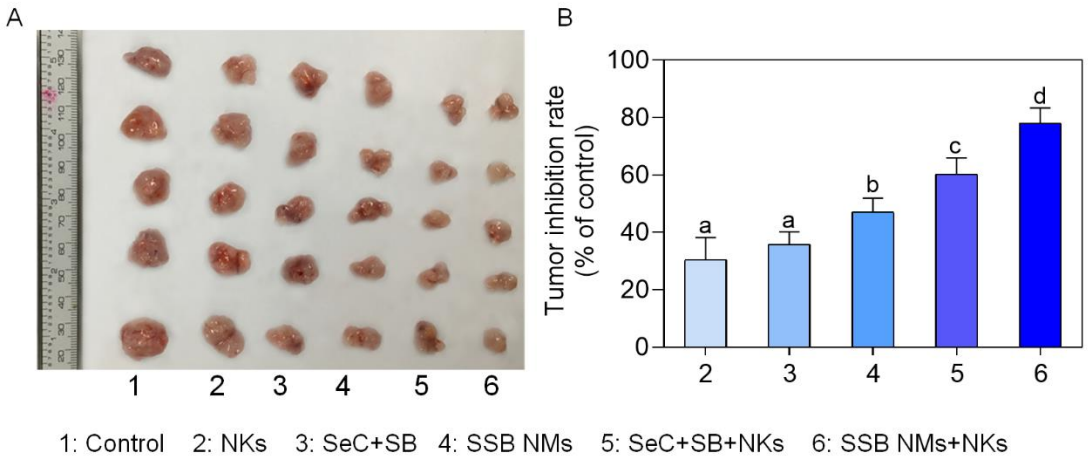

Figure S11. Tumor images (A) and tumor inhibition ratio (B) after treatment of NK92 cells, SeC + SB, SSB NMs, SeC + SB + NK92 and SSB NMs + NK92 in the subcutaneous tumor model assay. Bars with different characters a, b, c and d were statistically different at $P < 0.05$ level.

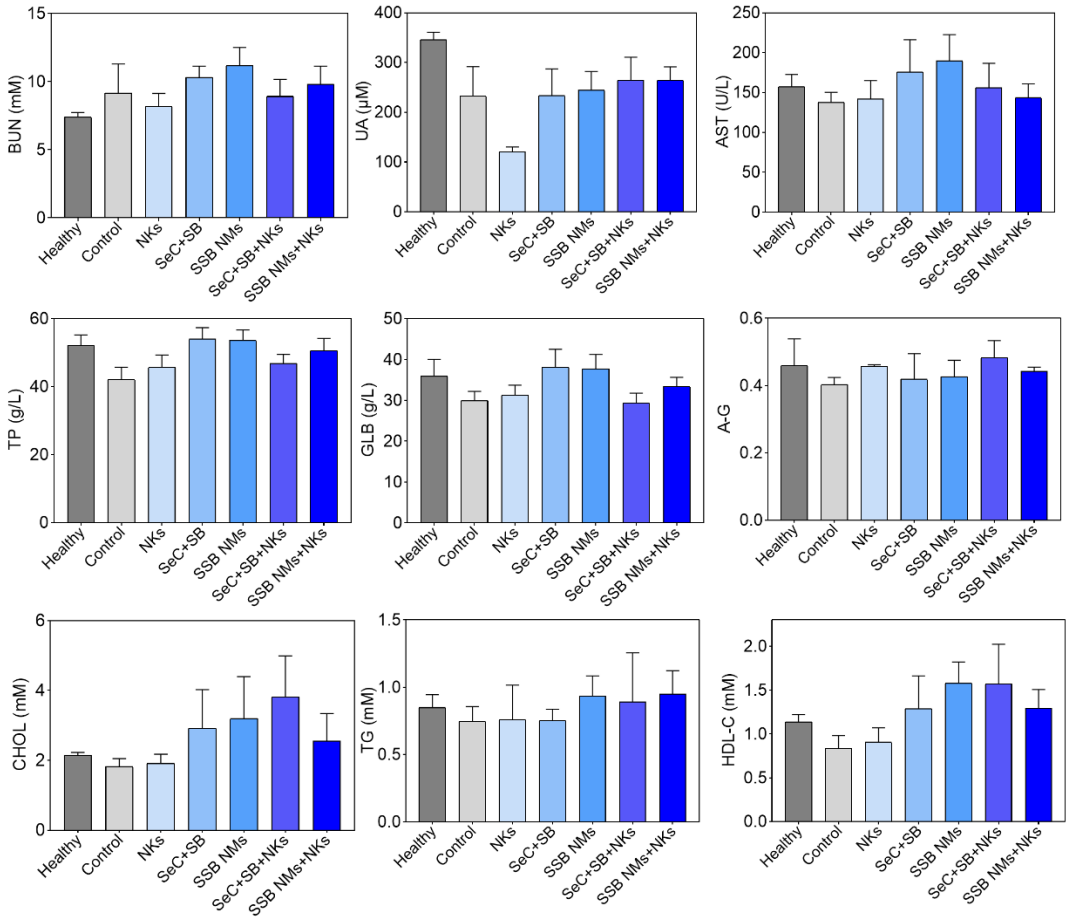

Figure S12. Hematological analysis of liver-function markers, kidney-function markers, heart-function markers, blood glucose and blood lipid index. Serum from mice that treated with NKs, SeC + SB, SSB NMs, SeC + SB + NKs and SSB NMs + NKs in the orthotopic tumor model was subjected to hematological analysis. Data were expressed as mean ± SD, n = 3.

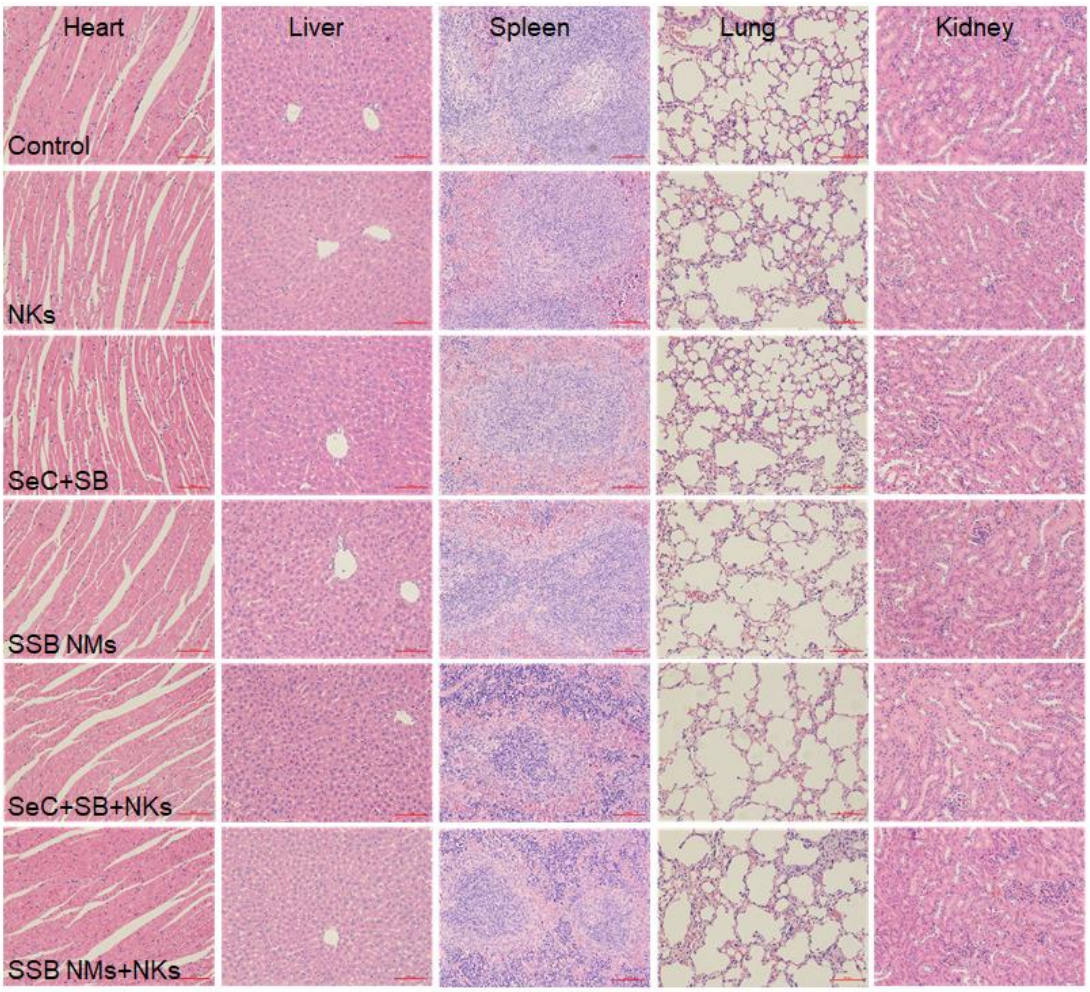

Figure S13. H&E-stained slice images of major organs in xenograft mouse model.

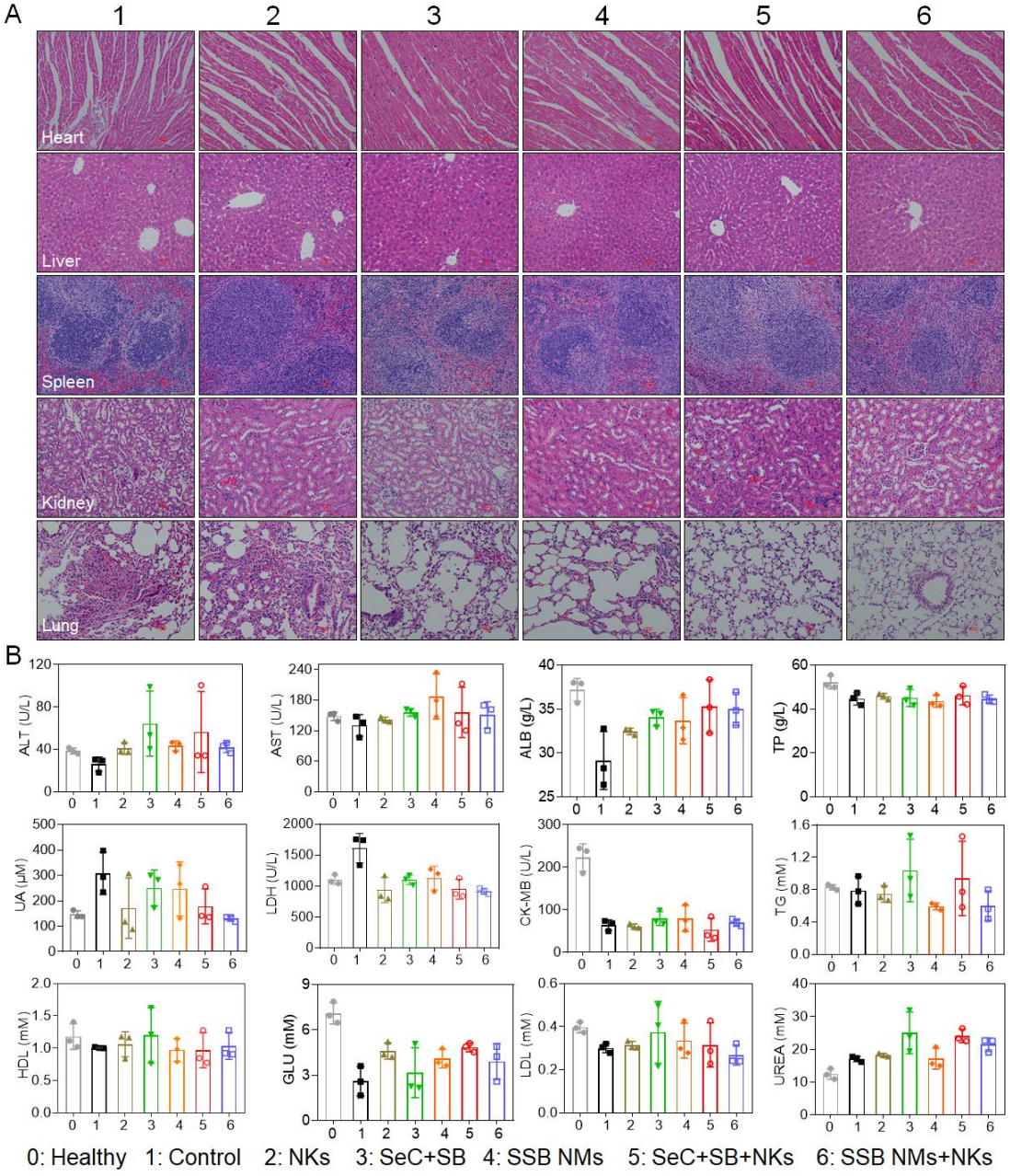

Figure S14. H&E-stained slice images and clinical chemistry evaluation in orthotopic tumor model. (A) Representative H&E-stained slice images of heart, liver, spleen, kidney and lung for each treatment groups. (B) Hematological and clinical chemistry evaluation of the liver, kidney, and heart function for each treatment groups. Data were expressed as mean ± SD, n = 3.