Science Advances
AAAS

advances.sciencemag.org/cgi/content/full/6/50/eabc3013/DC1

Supplementary Materials for

Cryo-shocked cancer cells for targeted drug delivery and vaccination

Tianyuan Ci, Hongjun Li, Guojun Chen, Zejun Wang, Jinqiang Wang, Peter Abdou, Yiming Tu, Gianpietro Dotti, Zhen Gu*

*Corresponding author. Email: guzhen@zju.edu.cn

Published 9 December 2020, *Sci. Adv.* **6**, eabc3013 (2020)
DOI: 10.1126/sciadv.abc3013

This PDF file includes:

Materials and Methods

Characterization of LNT cells

The LNT cell structure was analyzed *via* fluorescence staining with Hoechst (Invitrogen) and AF488 conjugated phalloidin (Invitrogen). Briefly, 1×10^6 LNT cells were suspended in 1 mL PBS. 25 μL phalloidin stock solution (6.6 μM) was added and the cells were stained at room temperature for 20 min. After that, the cells were centrifuged at 500 g for 3 min and washed with PBS. After the cells re-suspending in 1 mL PBS, 10 μL Hoechst stock solution (10 mg/mL) was added and stained the cells for 10 min. After washing with PBS, the cells were suspended in 500 μL PBS and analyzed by confocal microscopy (Zeiss LSM 880). The live C1498 cells were first fixed with 4 % paraformaldehyde (Thermo Scientific) for 15 min and treated with 0.1 % Triton X-100 (Thermo Fisher Scientific) for 15 min. The following staining process was similar with LNT cells.

For cell viability analysis, the cells were stained with Live/Dead viability kit (ThermoFisher Scientific #L3224) according to the manufacturer's protocol. After staining, the cells were analyzed by confocal microscopy. In addition, about 200 cells were captured and the cellular size was measured with the Nano Measurer software.

For cell death analysis, LNT cells were stained with Annexin V/PI apoptosis kit (Beyotime #C1062S) according to the manufacturer's protocol and analyzed by Flow cytometry.

For scanning electron microscopy (SEM) characterization, the cells were fixed in 3.5 % glutaraldehyde for 4 hours. After washing with 0.1 M sodium cacodylate buffer (Electron Microscopy Sciences) three times, the cells were fixed for 1 h with 1 % osmium tetroxide (Electron Microscopy Sciences). After washing with 0.1 M sodium cacodylate buffer, the cells were dehydrated with graded ethanol (30 %, 50 %, 70 %, 85 %, 90 % once for 15 min, and 100 % twice for 30 min). The cells suspended in 100 % ethanol were dropped on silicon. After drying, the silicon was coated by a thin layer of gold and analyzed by SEM (Zeiss Supra 40VP).

Cell proliferation of LNT cells

For *in vitro* cell proliferation, both live cells and LNT cells were suspended in the cell culture medium (DMEM, no phenol red, 10 % FBS) and added to 96-well plates with a cell density of 8×10^3 per well. After culturing for 0.5 h, 24 h, 48 h and 72 h, 10 μL cell counting kit-8 solution (CCK-8, Sigma-Aldrich) was added to each well. After incubation for 3 h, the absorbance was measured at 450 nm using a microplate reader (Tecan).

For *in vivo* cell proliferation, 2×10^6 live or LNT luciferase and DsRed tagged C1498 cells were injected into the mice intravenously. The proliferation of cells was monitored by detecting the bioluminescence signal at day 7, day 14 and day 21. After 10 min of the intraperitoneal

injection of the substrate D-Luciferin (150 mg/kg), the mice were imaged with the IVIS Spectrum Imaging System (PerkinElmer). At day 20, 200 μL blood was collected through the orbital vein. After treatment with ACK buffer (Gibco), the remaining cells were centrifuged at 800 g for 10 min. After suspension in PBS, the cells were analyzed by flow cytometry (BD LSRII). The fluorescence signal of DsRed was recorded.

Protein expression of LNT cells

Whole-cell protein expression was analyzed by SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The proteins were extracted from live and LNT C1498 cells by using RIPA lysis and extraction buffer (ThermoFisher) with protease inhibitor cocktail added (ThermoFisher). Protein concentration was determined by BCA assay (ThermoFisher) according to the manufacturer's instructions. Loading samples were prepared using Laemmli sample buffer (Bio-Rad) with the protein amount of 20 μg per well. After the proteins in the loading samples were denatured for 10 minutes at 95 °C, the loading samples were analyzed by SDS-PAGE in a Stain-Free™ Precast Gel (Bio-Rad #4568094). The gel was imaged with the Bio-Rad ChemiDoc MP Imaging System using the stain-free gel imaging mode with 5 min UV activation.

The expressions of CD44 and CXCR4 of the cells were analyzed by confocal microscopy and flow cytometry. For CD44, both live and LNT cells were suspended in cell staining buffer (Biolegend) and stained with APC-CD44 for 1 h. After centrifugation and re-suspension in PBS, the cells were imaged by confocal microscopy and analyzed by flow cytometry. For CXCR4, the live cells were first fixed with 4 % paraformaldehyde for 15 min and treated with 0.1 % Triton X-100 in PBS for 15 min, then suspended in the cell staining buffer and stained with APC-CXCR4 for 1 h. The LNT cells were stained with same process but without treatment with paraformaldehyde and Triton X-100.

For quantitive determination of the expression of CD44 and CXCR4 in LNT cells, western blotting analysis was adopted. Briefly, after SDS-PAGE, the separated protein on the gel were transferred to PVDF membrane (Merck Millipore). Samples aiming to different target proteins (CD44 or CXCR4) were run in separate gel. After blocking in 5 % milk for 2 h, the membranes were incubated overnight at 4 °C with diluted primary antibodies against CXCR4 (Novus Biologicals, NB100-56437) and CD44 (eBioscience, 14-0441-82). After washing with PBST, the membranes were further incubated with HRP-conjugate secondary antibody for 2 h. After thoroughly washing with PBST, the membranes were immersed in SuperSignal Sensitivity Substrate (Thermo Fisher Scientific). After imaging with Bio-Rad ChemiDoc MP Imaging System,

the relative protein expressions were reflected as the grayscale of the bands of targeted protein in comparison to reference protein, which were analyzed with ImageJ software.

In vivo biodistribution of LNT cells

Live cells and LNT cells were first incubated in cy5.5-NHS (Lumiprobe) containing PBS for 0.5 h to obtain cy5.5 labeled cells. In addition, some of the cy5.5-labeled live C1498 cells were treated with 4 % paraformaldehyde for 1 h to denature proteins as the control group. The cy5.5-labeled live and LNT C1498 cells as well as cy5.5-labeled paraformaldehyde-fixed C1498 cells were intravenously injected into the mice with cy5.5 dose of 30 nmol/kg. Six hours later, the mice were euthanized and the organs of heart, liver, spleen, lung, kidneys and the hind limb bones were isolated for fluorescence imaging by IVIS imaging system (Perkin Elmer).

The bone slices were also prepared to observe the bone marrow accumulation of LNT cells. In particular, cy5-labeled live and LNT C1498 cells were intravenously injected into the mice with cy5 dose of 60 nmol/kg. 4 h later, the bones were taken out and fixed in 4 % paraformaldehyde for 2 days. After decalcification in EDTA solution for 10 days, the bones were dehydrated in 30 % sucrose solution. Then the bone tissues were frozen in O.C.T. medium for slicing. After nucleus staining, the bone slides were observed with fluorescence microscopy.

In vivo clearance of LNT cells

The mice were intravenously injected with cy5.5-labeled LNT C1498 cells with cy5.5 dose of 60 nmol/kg. At time intervals of 5 min, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h, 50 μL blood was taken out from the orbital vein. The fluorescence images of the blood as well as the whole body of the mice were captured and analyzed with IVIS imaging system (Perkin Elmer).

Characterization of DOX-loaded LNT cells

The drug release profile of DOX from LNT cell/DOX was determined. Briefly, 1 mL releasing medium of PBS was added in the well of 12-well plate equipped with 3 μm Transwell, and 200 μL LNT cell/DOX was added in the chamber of Transwell. The plate was kept in 37 °C incubator (Corning LSE Shaking Incubator) with a shaking rate of 120 rpm. At specified time points, 1 mL of the releasing medium in the well was withdrawn and refreshed with same volume PBS. The DOX concentration was determined by a microplate reader with excitation and emission wavelengths of 480 nm and 598 nm, respectively.

The *in vitro* cytotoxicity of LNT cell/DOX was determined *via* MTT assay. Briefly, C1498 cells were cultured in 24-well plate equipped with 1 μm Transwell with a cell density of 2×10^6 per

well. And LNT cell/DOX solution with different DOX concentrations was added in the chamber of Transwell. 24 h later, the Transwell was discarded and 80 μL MTT solution (5 mg/mL) was added to each well. The cells were incubated for further 4 h at 37 °C. The upper medium was gently aspirated and 600 μL DMSO was added to dissolve the formed formazan. OD value was detected at 490 nm. IC_{50} values were analyzed by Graphpad Prism 7.0.

The pharmacokinetics of DOX was monitored after intravenous injection of free DOX and LNT cell/DOX (DOX 2.5 mg/kg). At time points, 150 μL blood was collected *via* the orbital vein and centrifuged at 5000 rpm for 10 min to get the plasma. 100 μL cold acetonitrile was added to 50 μL plasma and the mixture was centrifuged at 10000 rpm for 10 min to eliminate proteins. The supernatant was withdrawn and detected with fluorescence detector (Tecan Inifinite M Plex).

For DOX accumulation in the bone marrow, the femur and tibia bones of the mice were carefully isolated 3 h post-administration of free DOX and LNT cell/DOX (DOX 2.5 mg/kg), and the bone marrow was flushed with 300 μL DMSO. After centrifugation and filtration with 0.22 μm filter, the sample was analyzed by high performance liquid chromatography (HPLC) equipped with a reverse-phase column of 5 μm C_{18} (150 mm×4.6 mm, Inertsil ODS-3). The mobile phase was composed of 20.5 % acetonitrile, 20 % methanol and 59.5% 0.2 M NaH_2PO_4 (v/v/v, pH 4.0). The detection wavelength was set as 480 nm.

Activation of dendritic cells

Bone marrow dendritic cells (BMDC) were collected from the femur and tibia of the mice. Briefly, after the mice were euthanized, the femur and tibia were harvested. Both ends of each bone were cut open, and the bone marrow was flushed with cell culture medium. The cells were first pelleted at 600 g for 5 min and suspended in 3 mL ACK buffer for 3 min. After centrifugation, the cells were washed with PBS twice. Then the cells were cultured in RPMI-1640 medium (10 % FBS) with granulocyte/macrophage-colony stimulating factor (GM-CSF, 20 ng/mL, R&D Systems) and IL4 (5 ng/mL, Biolegend) for 7 days. The medium was changed every three days. At day 6, the cells were collected with the cell scraper and cultured in 6-well plates at a cell density of 1×10^6. At day 7, 1×10^6 LNT C1498 cells were added to the well without changing the medium. The group of blank medium without LNT cells was set as control. 48 h later, the cells were collected. After suspending in cell staining buffer (Biolegend), the cells were stained with BV421-CD11c, PE-CD80, APC-CD86, APC-CD40 and PE-MHC-II.

In vivo treatment of AML

AML model was established by intravenous injection of 5×10^6 C1498 cells on day 0. On day 7, day 11 and day 17, saline, LNT cell, free DOX and LNT cell/DOX were administrated intravenously, with DOX dose of 5 mg/kg. The bioluminescence images of mice were captured every 3 days with IVIS imaging system (Perkin Elmer) after 10 min of the intraperitoneal injection of D-Luciferin (150 mg/kg). The exposure time was 2 min.

For analysis of immunological effects of LNT C1498 cells, AML-bearing mice were established by intravenous injection of 5×10^6 C1498 cells on day 0. On day 7, saline and LNT cell were administrated intravenously. On day 11, 400 μL blood was collected *via* the orbital vein. 200 μL blood was treated with ACK buffer and centrifuged at 800 g for 8 min to get the pellets of white blood cells. After washing with PBS and suspended in cell staining buffer (Biolegend), the cells were stained with BV421-CD3, PE-CD4, and APC-CD8. 200 μL blood was collected in blood serum collection tubes (BD Microtainer 365967) and centrifuged at 3000 rpm for 10 min. The serum was detected with the IFN-γ ELISA kit (BioLegend 430804).

In vivo prophylactic efficiency against AML

Different groups of saline, Adjuvant, LNT cell+Adjuvant were intravenously injected at day 0, day 7 and day 14 (LNT cell 5×10^6 per mouse, MPLA 20 μg per mouse). On day 21, 1×10^6 live C1498 cells were intravenously injected into the mice. The tumor growth was monitored *via* bioluminescence intensity by IVIS after 10 min of the injection of D-Luciferin (150 mg/kg). The exposure time was 2 min. At day 5 and day 24, 400 μL blood was collected *via* the orbital vein. 200 μL blood was treated with ACK buffer and centrifuged at 800 g for 8 min to get the pellets of white blood cells. After washing with PBS and suspended in cell staining buffer (Biolegend), the cells were stained with BV421-CD3, PE-CD4, and APC-CD8. 200 μL blood was collected in blood serum collection tubes (BD Microtainer 365967) and centrifuged at 3000 rpm for 10 min. The serum was detected with the following ELISA kits: IFN-γ (BioLegend 430804), TNF-α (BioLegend 430904), IL-12 (BioLegend 433604) and IL-6 (BioLegend 431304).

Preparation and characterization of LNT 4T1 cells

4T1 cells were centrifuged at 250 g for 3 min and suspended in the medium, which was composed of 80 % DMEM and 20 % FBS. Above medium was immediately immersed in liquid nitrogen for 12 h. Before use, the cell-containing medium was thawed at 37 °C to obtain LNT 4T1 cells.

The cellular structure of live and LNT 4T1 cells were first captured with microscopy under bright field. After staining with DAPI and TRITC-phalloidin, both live and LNT 4T1 cells were imaged with confocal microscopy.

In vitro proliferation of LNT 4T1 cells were further analyzed *via* CCK8 assay. Briefly, LNT 4T1 cells were seeded in the 96-well plate in the cell density of 3000 cells per well. Same density of live 4T1 cells was set as control. After 24 h, 48 h and 72 h, the upper cell culture medium was withdrawn and the wells were washed with PBS twice. Then 110 μL medium (100 μL cell culture medium + 10 μL CCK8 stock solution) was added in each well. After incubation for another 3 hours, the UV absorbance at 450 nm was measured with the microplate reader.

CD44 expressions of live and LNT 4T1 cells were analyzed with flow cytometry after staining with APC-CD44 antibody. The cells without staining was set as control.

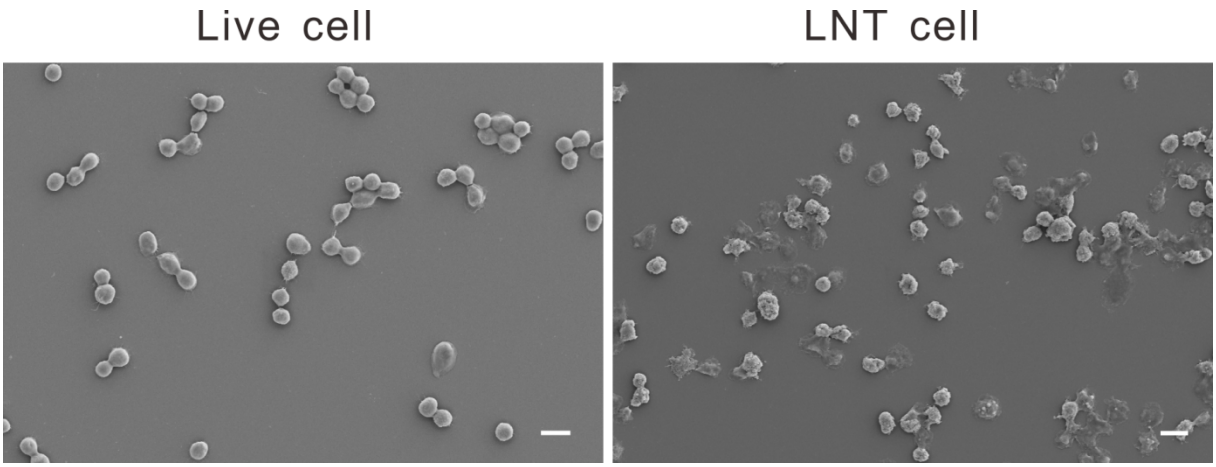

Fig. S1. SEM images of live and cryo-treated cells. Typical images of live and LNT C1498 cells. Scale bars, 10 μm.

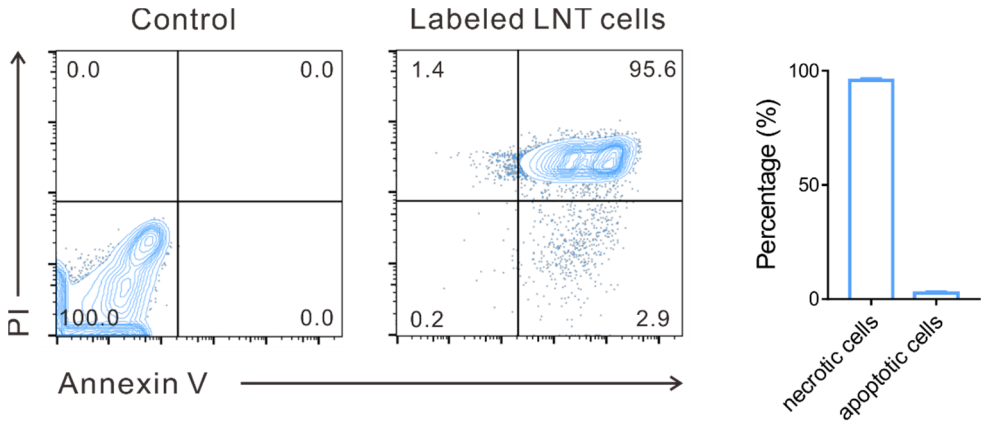

Fig. S2. Analysis of cell death of LNT cells by Annexin V-FITC/PI staining. Representative flow cytometry plots of control and LNT C1498 cells stained with Annexin-V/PI. Necrotic cells are referred as Annexin V^+PI^+ cells, while apoptotic cells are referred as Annexin V^+PI^- cells. Error bars represent the s.d. (n = 3).

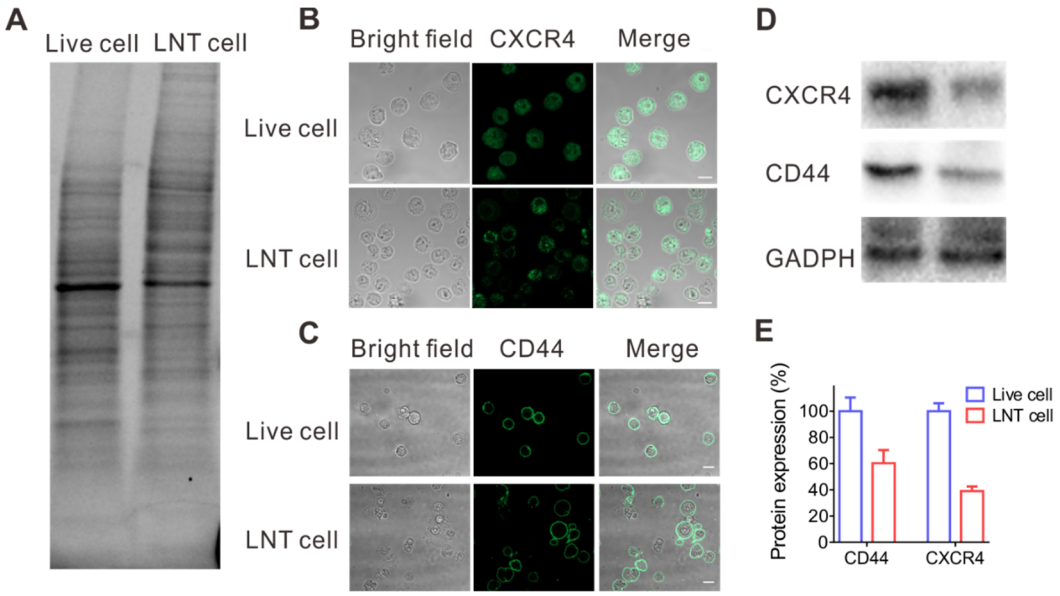

Fig. S3. Protein expression in LNT cells. (A) SDS-PAGE of whole cell lysate proteins obtained from live and LNT C1498 cells. (**B**) CXCR4 expression in live and LNT C1498 cells. Live C1498 cells were treated with paraformaldehyde and Triton X-100 before antibody staining. Scale bars, 10 μm. (**C**) CD44 expression in live and LNT C1498 cells. Scale bars, 10 μm. Representative western blotting (**D**) and quantification (**E**) of CD44 and CXCR4 in LNT C1498 cells. Error bars represent the s.d. (n = 3).

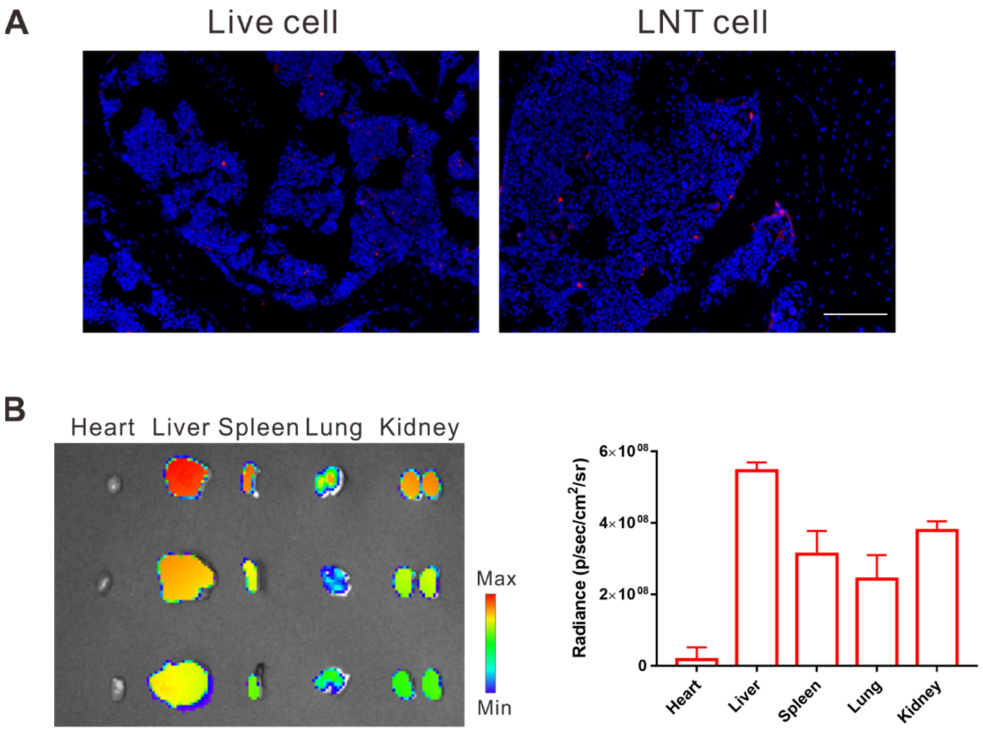

Fig. S4. *In vivo* biodistribution of LNT cells. (A) Fluorescence microscopy images of the bone tissue after injection of cy5-labeled live and LNT C1498 cells. Red fluorescence: cy5; blue fluorescence: nucleus. Scale bar, 100 μm. (B) IVIS image of typical organs and relative fluorescence intensities of the mice 6 h-post injection of cy5.5-labeled LNT cells. Error bars represent the s.d. (n = 3).

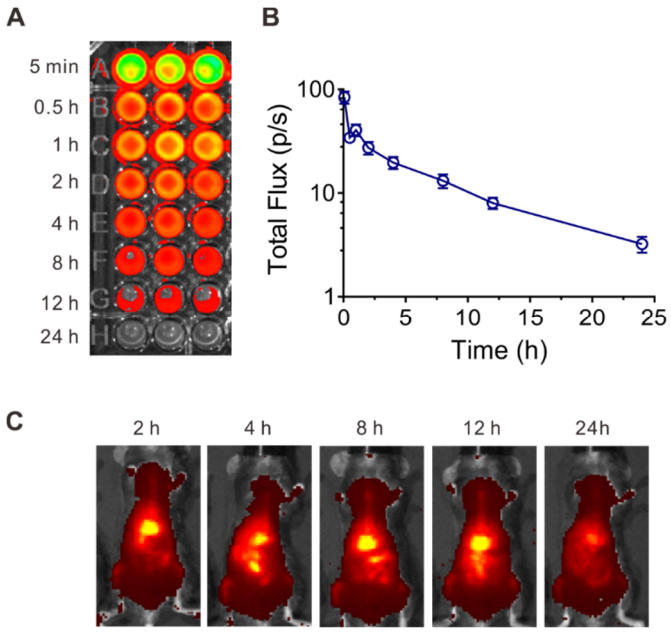

Fig. S5. ***In vivo*** **clearance of LNT C1498 cells.** IVIS images (**A**) and fluorescence intensities (**B**) of the blood collected from the mice at indicated time points after intravenous injection of cy5.5-labeled LNT C1498 cells. Error bars represent the s.d. (n = 3). (**C**) IVIS images of the whole body of mice after challenge with cy5.5-labeled LNT C1498 cells.

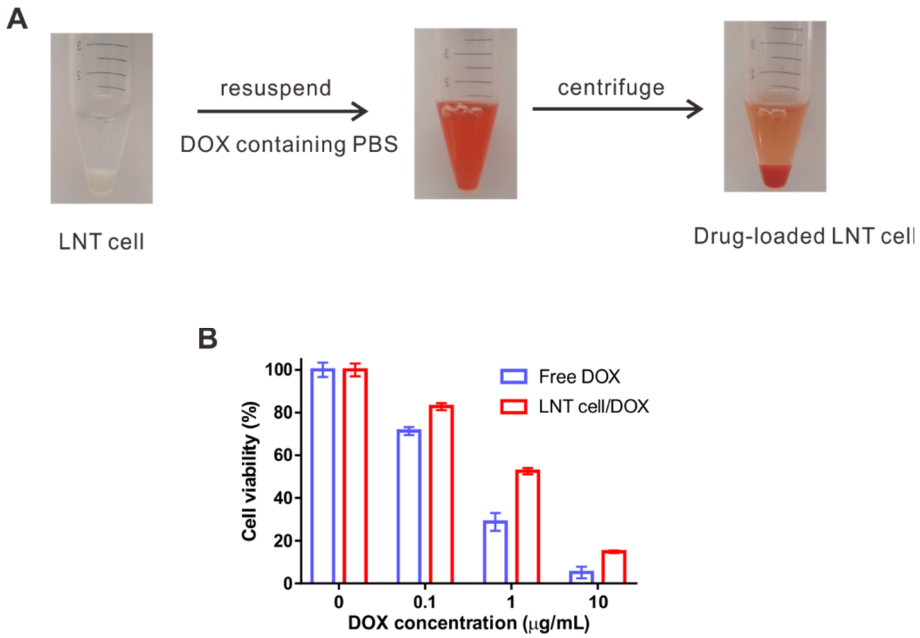

Fig. S6. Preparation and *in vitro* cytotoxicity of DOX-loaded LNT cells. (**A**) Schematic of the procedure to prepare DOX-loaded LNT cells. (**B**) The *in vitro* cytotoxicity of indicated formulations. Data are presented as means ± s.d. (n = 3).

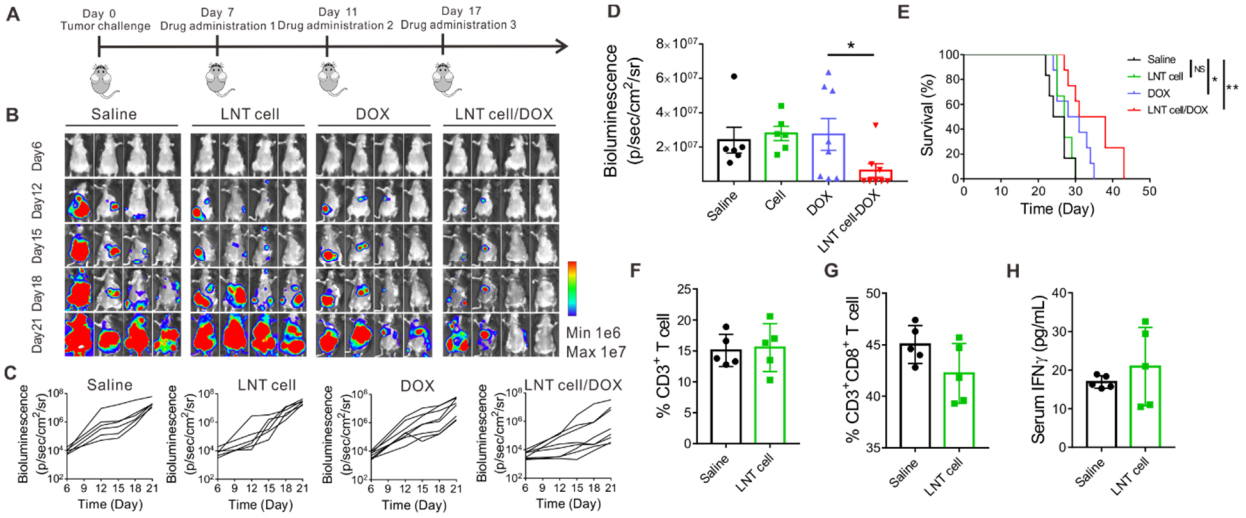

Fig. S7. Therapeutic efficacy in AML-bearing mice. (**A**) Schematic of the murine treatment model. (**B**) Representative tumor bioluminescence images in response to intravenous injection of saline, LNT cell, free DOX and LNT cell/DOX (DOX: 5 mg/kg; LNT cells: 1-2 $\times 10^7$). (**C**) Quantification of tumor bioluminescence of different treatment groups. (**D**) Tumor bioluminescence intensity at day 21. Data are presented as means ± s.e.m. ($n = 6$ for saline and LNT cell groups, $n = 8$ for DOX and LNT cell/DOX groups). Statistical significance was calculated *via* one-way ANOVA (nonparametric), $^*P < 0.05$. (**E**) Survival of the mice of different groups ($n = 6$ for saline and LNT cell groups, $n = 8$ for DOX and LNT cell/DOX groups). Statistical significance was calculated *via* the log-rank (Mantel-Cox) test, $^*P < 0.05$, $^{**}P < 0.01$. Quantification of $CD3^+$ T cells (**F**), $CD3^+CD8^+$ T cells (**G**) and serum IFN-γ level (**H**) in AML-bearing mice 4 days post-challenge of LNT C1498 cells. Saline injection was used in the control group. Data are presented as means ± s.d ($n = 5$). Statistical significance was calculated *via* Student's *t*-test, $^*P < 0.05$.

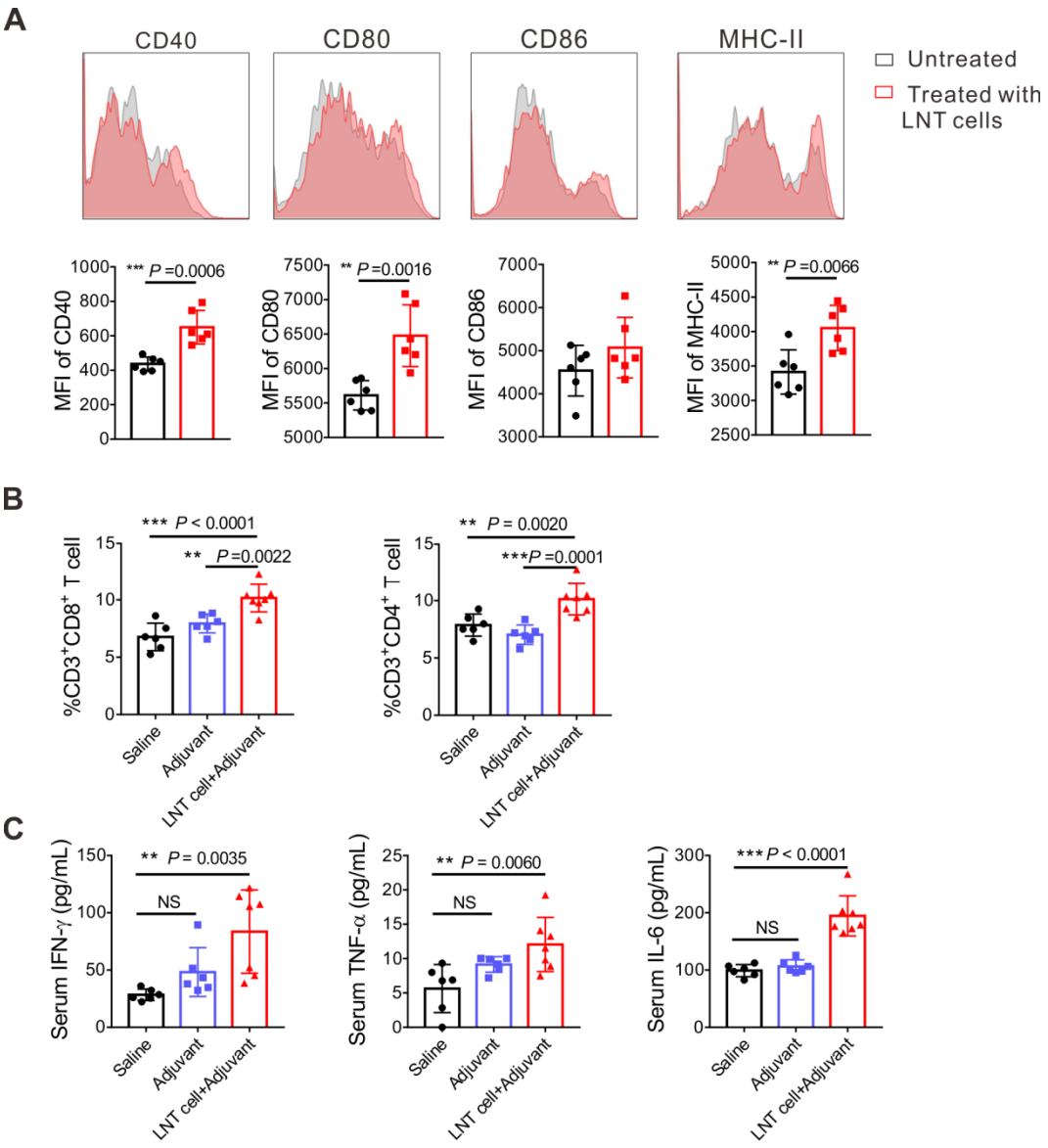

Fig. S8. Activation of immune responses of LNT tumor cells. (**A**) *In vitro* activation of DCs by LNT C1498 cells. Typical flow cytometry images and surface marker fluorescence intensities of untreated DCs and DCs treated with LNT C1498 cells. Data are presented as means ± s.d. ($n = 6$). Statistical significance was calculated *via* Student's *t*-test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**B**) Proportions of $CD3^{+}CD8^{+}$ T cells and $CD3^{+}CD4^{+}$ T cells in peripheral leukocytes 5 days post-injection of indicated formulations. Data are presented as means ± s.d. ($n = 7$ for LNT cell+Adjuvant, $n = 6$ for other groups). Statistical significance was calculated *via* ordinary one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (**C**) Serum cytokine levels of IFN-γ, TNF-α and IL-6 5 days post-injection of indicated formulations. Data are presented as means ± s.d. ($n = 7$ for LNT cell+Adjuvant, $n = 6$ for other groups). Statistical significance was calculated *via* ordinary one-way ANOVA, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

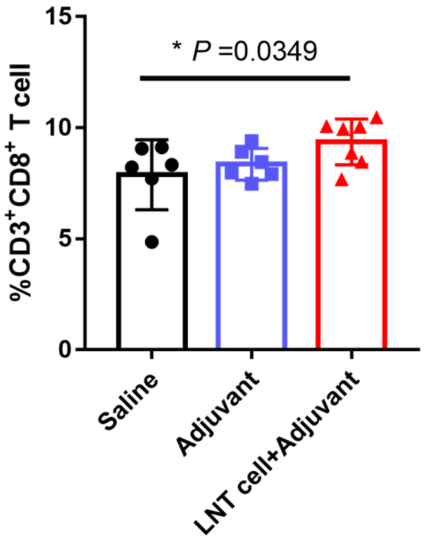

Fig. S9. Peripheral proportion of CD3$^+$ CD8$^+$ T cells. Proportion of CD3$^+$ CD8$^+$ T cells on the gate of peripheral leukocytes after challenge of live C1498 cells. The mice were pre-immunized with indicated formulations. Data are presented as means ± s.d. ($n = 6$ for Saline and Adjuvant, $n = 7$ for LNT cell+Adjuvant). Statistical significance was calculated *via* ordinary one-way ANOVA, $^*P < 0.05$.

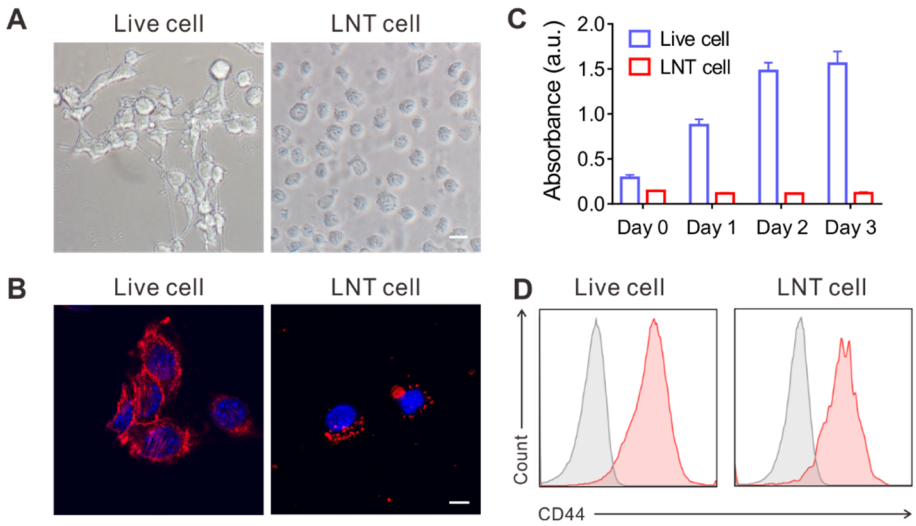

Fig. S10. Characterization of LNT 4T1 cells. (**A**) Microscopy images of live and LNT 4T1 cells. Scale bar, 20 μm. (**B**) Cellular structure of live and LNT 4T1 cells analyzed by confocal microscopy. Cell nucleus was stained by DAPI and cytoplasm F-actin was stained by TRITC-phalloidin. Scale bar, 10 μm. (**C**) *In vitro* proliferation of live and LNT 4T1 cells assessed by CCK8 assay. Error bars represent the s.d. (n = 6). (**D**) CD44 expression in live and LNT 4T1 cells analyzed by flow cytometry. The cell suspension medium before liquid nitrogen treatment was 80% DMEM and 20% FBS.