Supporting Information

Near infrared light activation of injectable whole-cell cancer vaccine for cancer immunoprophylaxis and immunotherapy

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Materials

Pluronic F127 was purchased from Beijing Solibao Technology Co., Ltd. p-Toluenesulfonic acid monohydrate, 3-mercaptopropionic acid, DL-dithiothreitol (DTT), ascorbic acid, chloroauric acid ($HAuCl_4$), sodium borohydride, cetrimonium bromide (CTAB), hydrochloric acid, dimethyl sulfoxide (DMSO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Sigma-Aldrich (St. Louis). Benzyl alcohol and silver nitrate ($AgNO_3$) was bought from Innochem. ATP detection assay kit, BCA protein concentration assay kit, Coomassie blue staining kit, Hoechst 33342, trypan blue and DiR were purchased from Shanghai Beyotime Biotechnology Co., Ltd. Annexin V-FITC/Propidium Iodide (PI) cell apoptosis kit was bought from Hangzhou Lianke Biotechnology Co., Ltd. Mouse calreticulin (CRT) ELISA kit was purchased from Beijing Huabo Deyi Biotechnology Co., Ltd. Mouse high mobility group protein (HMG) ELISA kit was purchased from Jiangsu Boshen Biotechnology Co., Ltd.

Instruments

The formed structures were analyzed with a JEM 1400 TEM instrument with an acceleration voltage of 120 kV (JEOL, Japan). Zeta potential and size analysis was performed on a Nano-ZS ZEM3600 (Malvern, UK). UV−vis absorbance spectra were measured with an UV-2600

spectrophotometer (SHIMADZU, Japan). Cytotoxicity assay was measured with a microplate reader at 570 nm (Bio-Rad, model 550, USA). The small animal *in vivo* imaging was performed by In-Vivo FX PRO (BRUKER, Germany). Flow cytometry (BD FACSAria TM III) analysis was conducted for the quantitative detection of cellular fluorescence. Trypan blue, H&E, and live/dead cell staining were acquired by an L55 inverted fluorescence microscope (PerkinElmer). All the cell morphologies were captured on Ti2-A inversion fluorescence microscope (Nikon, Japan). The mean fluorescence intensity of cells was analyzed with cytoFLEX (BECKMEN, USA). The thermographic images were acquired by an infrared thermal camera (FLIR, USA). Phototherapy on cells or mice was performed with a 793 nm laser (Stone, China). All the parameters of blood biochemistry were analyzed by Pointcare M3 (MNCHIP, China).

Cells and Animals

4T1 murine breast tumor cells and RAW 264.7 macrophage cells were obtained from ATCC. 4T1 cells were cultured in roswell park memorial institute (RPMI) 1640 supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin-streptomycin. RAW 264.7 cells were cultured in dulbecco's modified eagle medium (DMEM) supplemented with 10 % FBS and 1 % penicillin-streptomycin. All the reagents for cell culture were bought from Gibco. BALB/c female mice (6 weeks of age) were obtained from Laboratory Animal Center of Southern Medical University. All animal procedures were performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Southern Medical University and approved by the Animal Ethics Committee of Southern Medical University (permit number: SYXK 2016-0041).

Experimental Section

Synthesis of HS-F127-SH: HS-F127-SH was synthesized according to the previous report.[1] Briefly, F127 (1 mmol), 3-mercaptopropionic acid (20 mmol), p-toluenesulfonic acid monohydrate (2 mmol) and DL-dithiothreitol (10 mmol) in toluene (200 ml) were charged into a flask equipped with a Dean-Stark trap. The solution was then refluxed until no water was evolved. After isolation and purification, HS-F127-SH was obtained.

Synthesis of GNRs: GNRs were synthesized via a seed-mediated method with slight modifications.[2, 3] $HAuCl_4$ solution (0.25ml, 10 mmol) was added into CTAB solution (10 ml, 100 mmol), followed by the addition of ice-cold NaBH4 solution (0.60 ml, 10 mmol). The

resulting seed solution was stirred vigorously for 5 min and kept at 30 °C. After 2 h, seeds solution (24 μl) was added into a growth solution (10 ml) containing CTAB (100 mmol), $HAuCl_4$ (0.5 mmol), ascorbic acid (0.8 mmol), $AgNO_3$ (0.12 mmol), and HCl (19 mmol). The mixture was standing at 30 °C for 2 h. The solution was then centrifuged (8000 rpm, 30 min) and washed three times with H_2O.

Synthesis of GNR-HS-F127-SH: Aqueous solutions of HS-F127-SH (15 wt %) was first prepared as stock solution. In a typical synthesis, GNRs (1.5 wt %) were dispersed in HS-F127-SH stock solution. The resulting solution was stirred at room temperature for 12 h. The resultant mixture was centrifugally separated, and the products were collected and washed with H_2O for three times.

Fabrication of InLCCV: InLCCV was prepared through a facile mixing method. Briefly, HS-F127-SH (19 wt %) was added into PBS (0.01 M) solution containing GNRs-HS-F127-SH (10 μg ml^{-1}) and LPS (100 pg ml^{-1}). The mixture was kept at 4 °C for 12 h to form GNRs/LPS-F127 sol. The log-phase 4T1 cells were trypsinized, counted, and re-suspended in the GNRs/LPS-F127 sol (4T1 cell concentration was 1×10^7 ml^{-1}), InLCCV was obtained.

Photothermal effect of InLCCV and its laser-triggered antigen release: InLCCV with different GNRs concentrations were irradiated with a 793 nm laser at different laser power density for 10 min. Laser-induced temperature increasement was recorded using an infrared thermal camera.

To investigate the laser-triggered release of tumor antigen from InLCCV, 100 μl of InLCCV was added into a 1.5 ml EP tube and kept at 37 °C. 200 μl of PBS solution (pH 7.4) containing 0.5 wt % Tween-20 was then added on top of the hydrogel subsequently. The hydrogel was then irradiated with a 793 nm laser at different photo density for 10 min respectively. The supernatant (200 μl) was replaced at predetermined time intervals. All experiments were performed at 37 °C. The collected solutions were centrifuged at 12,000 rpm for 10 min and the supernatant was removed. Proteins from tumor antigen were analyzed using BCA protein concentration assay kit. The SDS-PAGE analysis was performed to estimate the released proteins of the 4T1 cells. Briefly, equal amounts of proteins from different samples (quantified with BCA protein concentration assay kit) were used for SDS-PAGE analysis. The gel was treated with coommassie brilliant blue for stained and imaged.

Safety evaluation of InLCCV *in vitro*: To evaluate the effect of InLCCV sol on cell viability, 4T1 cells were seeded onto the 96-well plates with a density of 6×10^3 cells per well and cultured for 12 h. The cells were treated with InLCCV sol containing different GNRs concentrations. After incubating for 12 h, the cells were then irradiated with a 793 nm laser for 10 min (0.5 W cm^{-2}) and the resulting cells were further cultured for 2 h. The cell viability was measured by MTT assay. The dark control experiment was conducted under the identical conditions with the experimental group except for NIR laser irradiation.

Apoptosis analysis was performed using Annexin V-FITC/PI apoptosis detection kit. 4T1 cells were seeded onto the 24-well plates (2.5×10^4 cells per well) and cultured for 12 h. The cells were treated with PBS and InLCCV sol (GNRs concentration 10 μg ml^{-1}), respectively. After 2 h of incubation, the cells were irradiated with a 793 nm laser for 10 min (0.5 W cm^{-2}), followed by another 10 h incubation. The cell apoptosis was detected by Annexin V-FITC/PI cell apoptosis kit using flow cytometry. The dark control experiment was conducted under the identical conditions with the experimental group except for NIR laser irradiation.

Dead cells were visualized using the propidium iodide (PI). Briefly, 4T1 cells were seeded onto the 6-well plates (1×10^6 cells per well) and cultured for 12 h. The cells were treated with PBS and InLCCV sol (GNRs concentration 10 μg ml^{-1}), respectively. After 2 h of incubation, the cells were irradiated with a 793 nm laser for 10 min (0.5 W cm^{-2}), followed by another 10 h incubation. Then calcein-AM and propidium iodide (PI) co-staining was performed for 10 min and the fluorescence was immediately measured using a fluorescence microscope. The dark control experiment was conducted under the identical conditions with the experimental group except for NIR laser irradiation.

Detection of ICD biomarkers: For immunofluorescence analysis, 4T1 cells were seeded onto the 6-well chamber slides (1×10^5 cells per well) and cultured for 12 h. InLCCV sol (10 μg ml^{-1} of GNRs) was added and incubated with the cells for 1 h. The cells were then irradiated with a 793 nm laser (0.5 W cm^{-2}) for 10 min. After 12 h incubation, the cells were washed with PBS three times and then incubated with anticalreticulin antibody at 4 °C for 12 h. The resulting cells were washed and incubated with Fluorescein (FITC)-conjugated secondary antibody (ProteinTech, USA) for 1 h. After staining with DAPI, the cells were observed under inverted fluorescent microscope.

Briefly, 4T1 cells were seeded onto the 12-well plates (8×10^4 cells per well) and cultured for 12 h. The cells were treated with InLCCV sol (10 μg ml^{-1} of GNRs) for 1 h and the cells were irradiated with a 793 nm laser for 10 min (0.5 W cm^{-2}). After incubating for another 8 h, the

cell supernatant was collected. The release of CRT, HMG, and ATP was detected by the CRT Elisa kit, HMG ELISA kit, and chemiluminescence ATP determination kit, respectively.

***In vitro* immune response induced by InLCCV:** To analyze the effect of InLCCV on the migration capacity of murine macrophages (RAW 264.7), Transwell migration assay was performed with Transwell chambers (24-well, 8 μm pore size; Corning, USA). 100 μl of PBS, LPS (1 μg ml^{-1}), and InLCCV were added into the lower chamber and incubation was at 37 °C for 10 min. 200 μl cell suspension (about 1×10^5 cells) was added into the upper chamber, while the lower chamber was filled with 500 μl DMEM medium containing 10 % fetal bovine serum. Transwell chambers were incubated at 37 °C in 5 % CO_2 for 30 min. The lower chamber was exposed to 793 nm laser (0.5 W cm^{-2}) irradiation for 10 min. After cultured at 37 °C in 5 % CO_2 for 24 h, the upper chamber was fixed by 4 % paraformaldehyde for 10 min at 37 °C. After washing with PBS, the cells on the inside of each upper chamber were swabbed gently and the underside of each insert was stained by 0.1% crystal violet. The migrating cells were imaged using a light microscopy in 3 randomly chosen fields and quantified by counting cell numbers. To investigate the immunological effects of InLCCV, bone marrow-derived dendritic cells (BMDCs) were separated from BALB/c mice and cultured for 8 d. Then the cells were seeded (1×10^5 cells) into the lower chambers and cultured (37 °C, 5 % CO_2) for 12 h. 100 μl of PBS, LPS (1 μg ml^{-1}), and InLCCV were added into the upper chambers, respectively. The upper chambers were then exposed to a 793 nm laser (0.5 W cm^{-2}) irradiation for 10 min. After culturing for another 24 h, the cells were harvested and co-stained with anti-CD11c-FITC (eBioscience, Clone: N418, Catalog: 11-0114-85), anti-CD80-PE (eBioscience, Clone: 16-10A1, Catalog: 12-0801-82) and anti-CD86-APC antibodies (eBioscience, Clone: GL1, Catalog: 17-0862-81), and the resulting cells were then examined with flow cytometry.

***In vivo* immune response and biosafety of InLCCV:** DiR was incorporated to investigate the *in vivo* retention time of InLCCV. Briefly, mice were subcutaneously injected with 100 μl of PBS/DiR or InLCCV/DiR at same DiR concentration of 5 mmol. The retention of the hydrogel vaccine was then examined by an animal imaging system at predetermined time points post-injection (Ex= 748 nm, Em = 780 nm for DiR).

36 healthy female BALB/c mice were randomly divided into 2 groups and subcutaneously injected with 100 μl InLCCV or PBS. After 30 min, 9 mice were randomly selected from each group and then exposed to laser irradiation (793 nm; 0.5 W cm^{-2}) for 10 min. The mice without irradiation were used as a control group. On day 1, 7 and 14, animals were sacrificed and vital

organs (heart, liver, spleen, lungs, and kidneys) and blood were collected. The collected organs were examined by hematoxylin and eosin (H&E) staining to evaluate the biosafety of the InLCCV. In order to further demonstrate the biosafety of our vaccine and immune response induce by vaccination, collected blood was used directly for hematological and biochemical analysis. The rest blood was used for the preparation of serum. The concentrations of TNF-α, IFN-γ, and IL-6 were further analyzed with ELISA kits.

Prevention effect of InLCCV: In order to study preventive effects of the vaccine, 20 healthy female BALB/c mice were randomly divided into 4 groups and subcutaneously injected with 100 μl InLCCV (2 groups) and PBS (2 groups) into the left side of fat pad. After 30 min, the mice were randomly selected from each group and the injection site of was exposed to laser irradiation (793 nm; 0.5 W cm^{-2}) for 10 min. After vaccinating for 7 days, 1×10^5 4T1 tumor cells were then transplanted into the right flank of each mouse. Body weight and tumor growth were monitored every 2 days. When the tumor size reached to 20 mm in any direction or the mice displayed restriction, inability to access food and water, then the mice were euthanized.

Anti-tumor and anti-metastatic activity of InLCCV: To evaluate the antitumor effect of InLCCV, the BALB/c mice with subcutaneous 4T1 xenografts on right fat pad were divided into four groups (n = 5). The mice were subcutaneously injected with PBS or InLCCV into the left side of fat pad, respectively. After 30 min, the injection site was irradiated with laser for 10 min (793 nm; 0.5 W cm^{-2}). During the NIR laser irradiation, the real-time temperature and thermal images were recorded by an infrared thermal-imaging camera. The treatment was repeated for three times at a time interval of 5 days. The tumor size and body weight were monitored every 2 days. The tumor volume was calculated according to the following formula: Tumor volume=length×$width^2$×0.5, where length represents the largest diameter and width the smallest diameter of the tumor. All the major organs and tumors were collected and examined by H&E staining.

To study the anti-metastatic activity of InLCCV, lung metastasis tumor model was established by injecting 4T1 cells (1×10^5) through the tail vein after vaccinating for 7 days. The mice were sacrificed 14 days post tumor cell injection. The lungs were observed for the metastasis nodules, then sectioned and subjected to H&E staining.

All animal experiments were performed in compliance with the guidelines for the care and use of laboratory animals.

Statistical Analysis: Unless otherwise stated, the mean ± SD were determined for all the treatment groups. Statistical analysis was performed by Student's *t*-test (two-tailed) between two groups. $P < 0.05$ was considered representative of a statistically significant difference between two groups.

Supplementary figures:

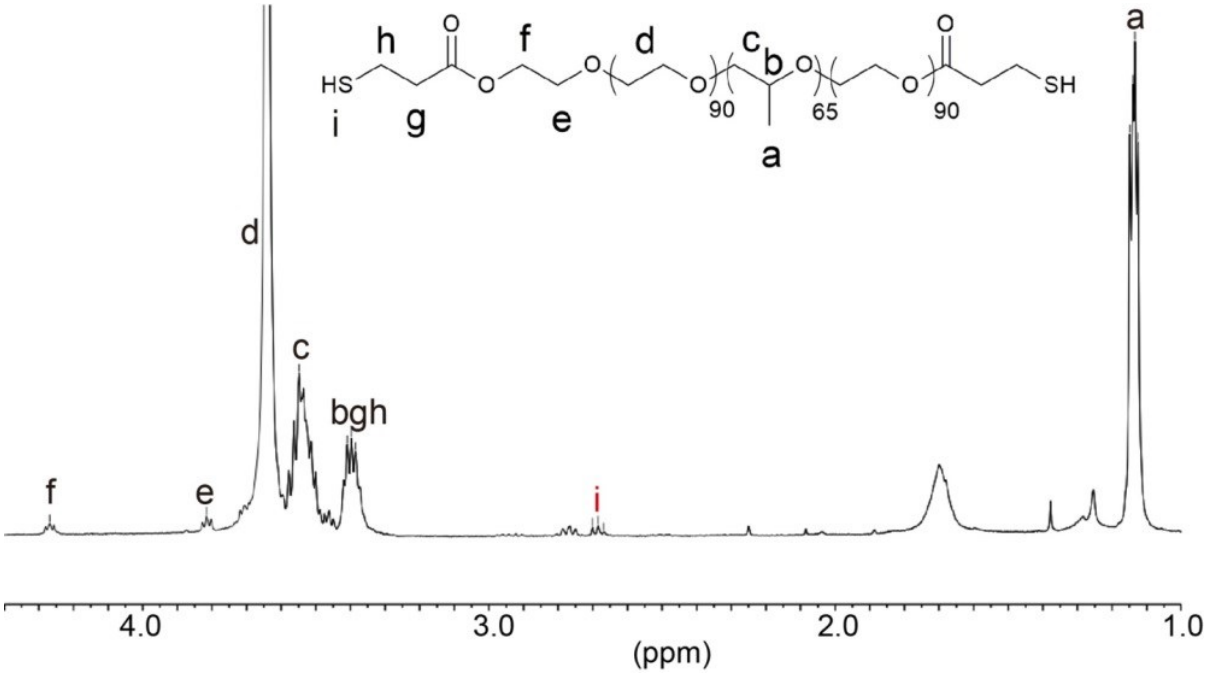

Figure S1. ^{1}H-NMR spectrum of thiol functionalized F127 (HS-F127-SH).

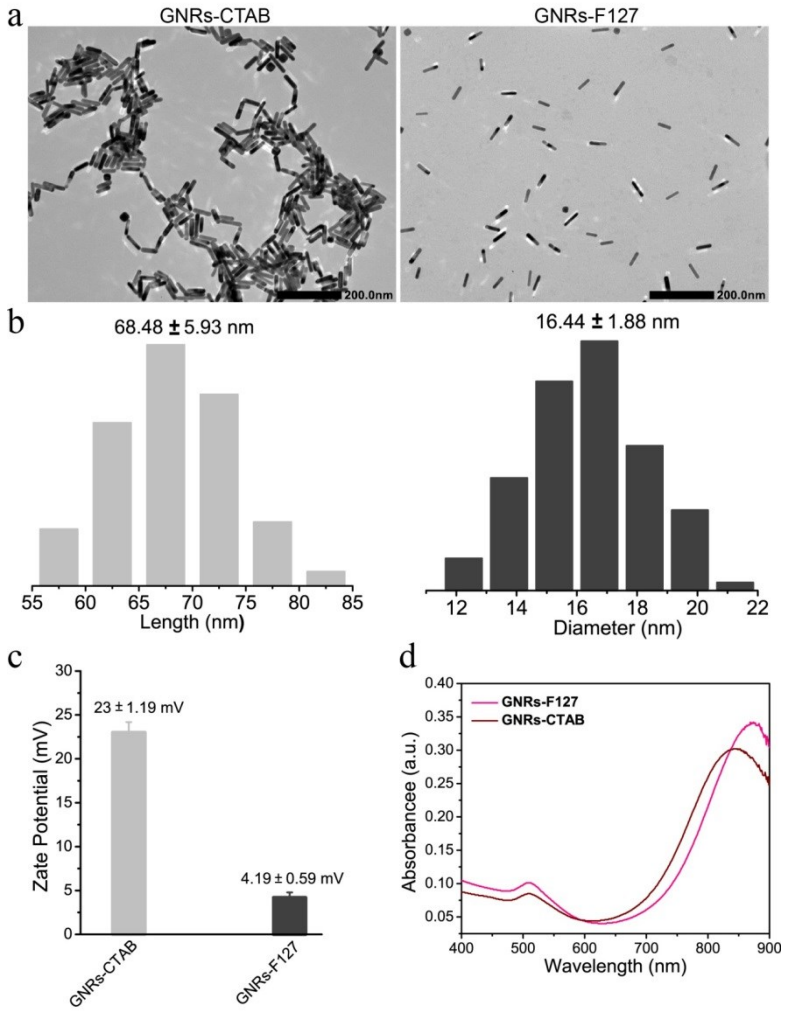

Figure S2. (a) TEM images of GNRs-CTAB and GNRs-F127. (b) Statistical analysis of the length and the diameter of GNRs based on the TEM images (100 particles). (c) Surface Zate potential of GNRs-CTAB and GNRs-F127. (d) UV-vis-NIR absorption spectra of GNRs-CTAB and GNRs-F127 in water.

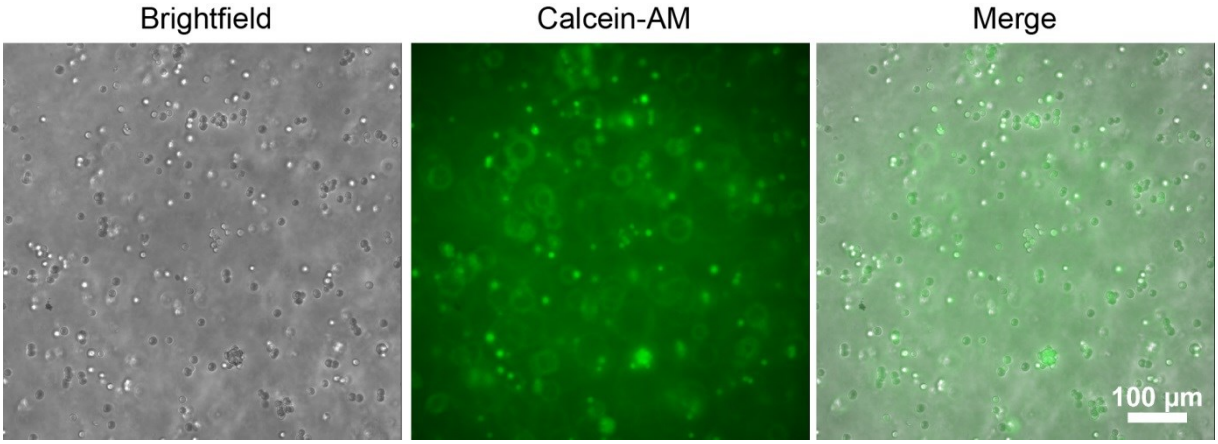

Figure S3. Micrograph of 4T1 cells in InLCCV. Green fluorescence indicates live 4T1 cells stained with calcein-*AM*.

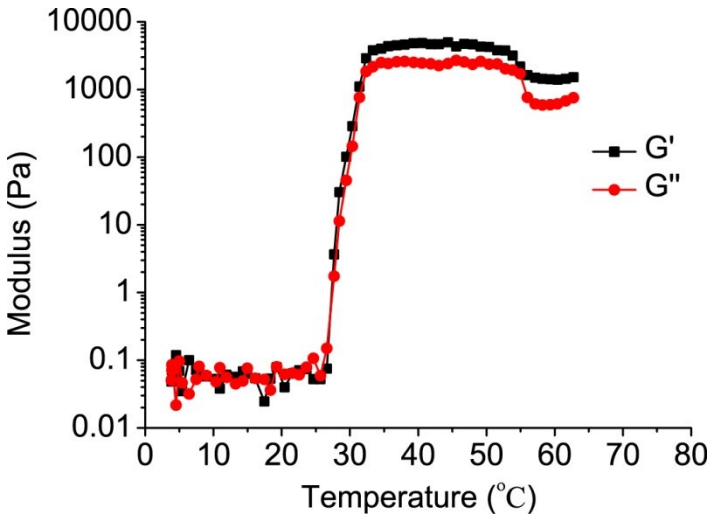

Figure S4. Variation of the storage modulus (G') and loss modulus (G") value of the InLCCV hydrogels function of a temperature during the sol-gel transition.

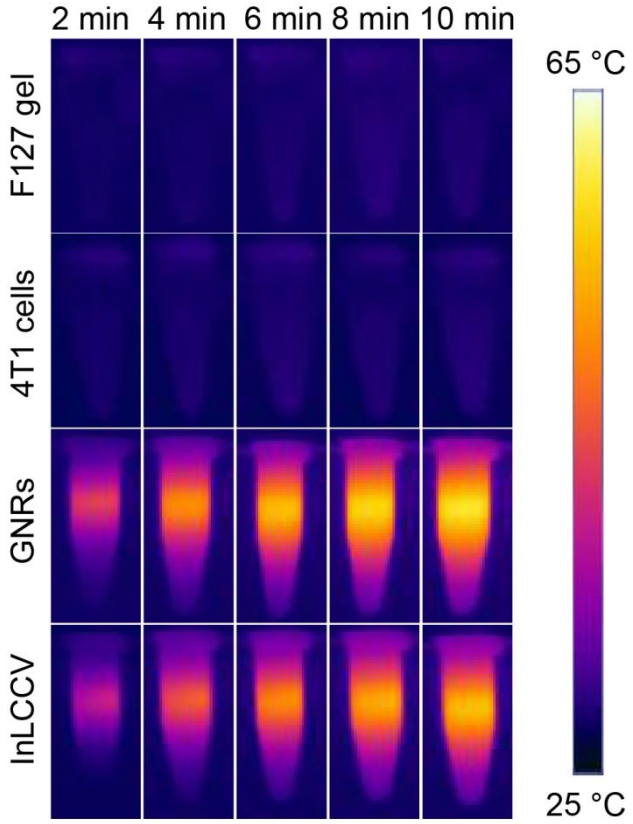

Figure S5. Infrared thermal images of InLCCV, GNRs, 4T1 cells and F127 gel under near-infrared laser (793 nm; 1 W cm^{-2}) irradiation for 10 min.

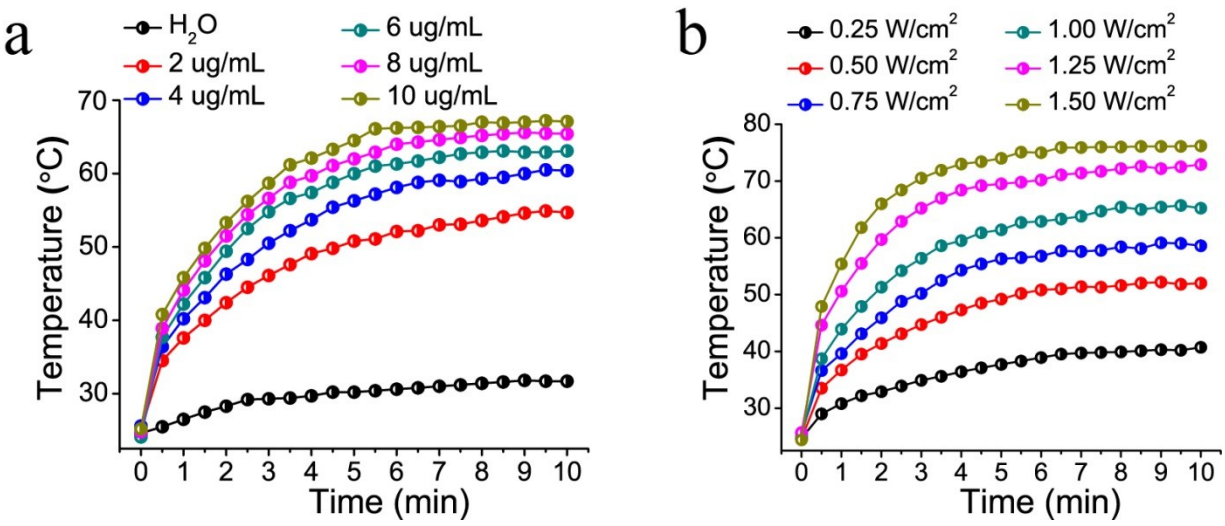

Figure S6. (a) Temperature increase curve of InLCCV with different GNRs concentrations under near-infrared laser irradiation. (b) Temperature increase profile of InLCCV under irradiation with different laser power.

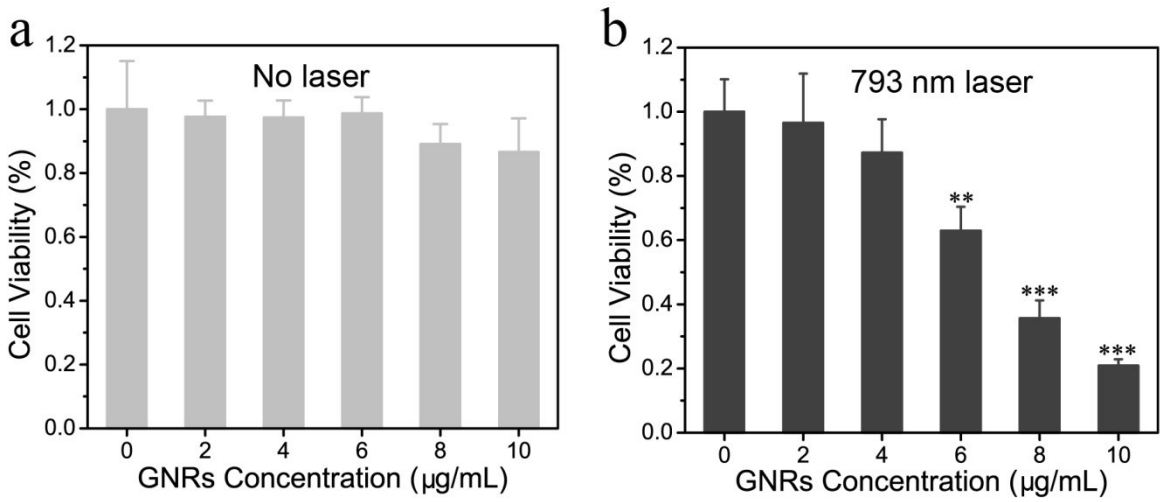

Figure S7. (a) Relative viability of the 4T1 tumor cells after incubation with GNRs-127 (GNRs concentrations of 0, 2, 4, 6, 8 and 10 μg ml^{-1}) for 12 h. (b) Relative viability of the 4T1 tumor cells after incubation with GNRs-F127 (GNRs concentrations of 0, 2, 4, 6, 8 and 10 μg ml^{-1}) for 4 h after irradiation with the 808 nm laser (0.5 W cm^{-2}) for 10 min. Data are means ± S.D. (n = 5). **P < 0.05; ***P < 0.01, compared with the GNRs-F127 concentration of 0 group.

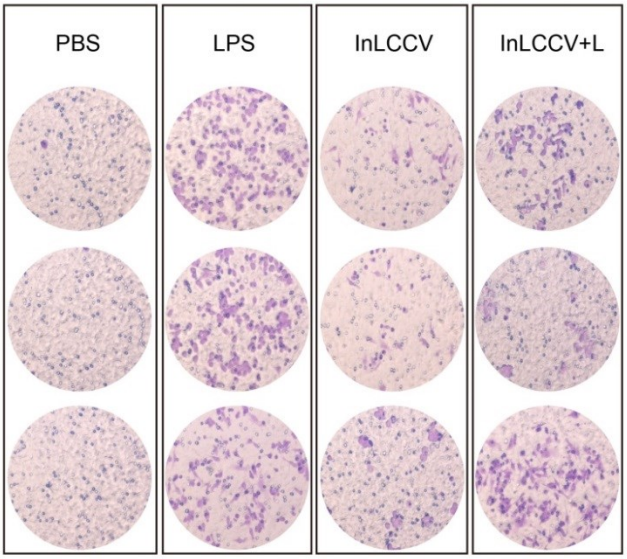

Figure S8. Representative images of crystal violet-stained macrophages that migrating from the insert chamber of the transwell (pore size, 8 μm) to the lower chamber after incubation with InLCCV.

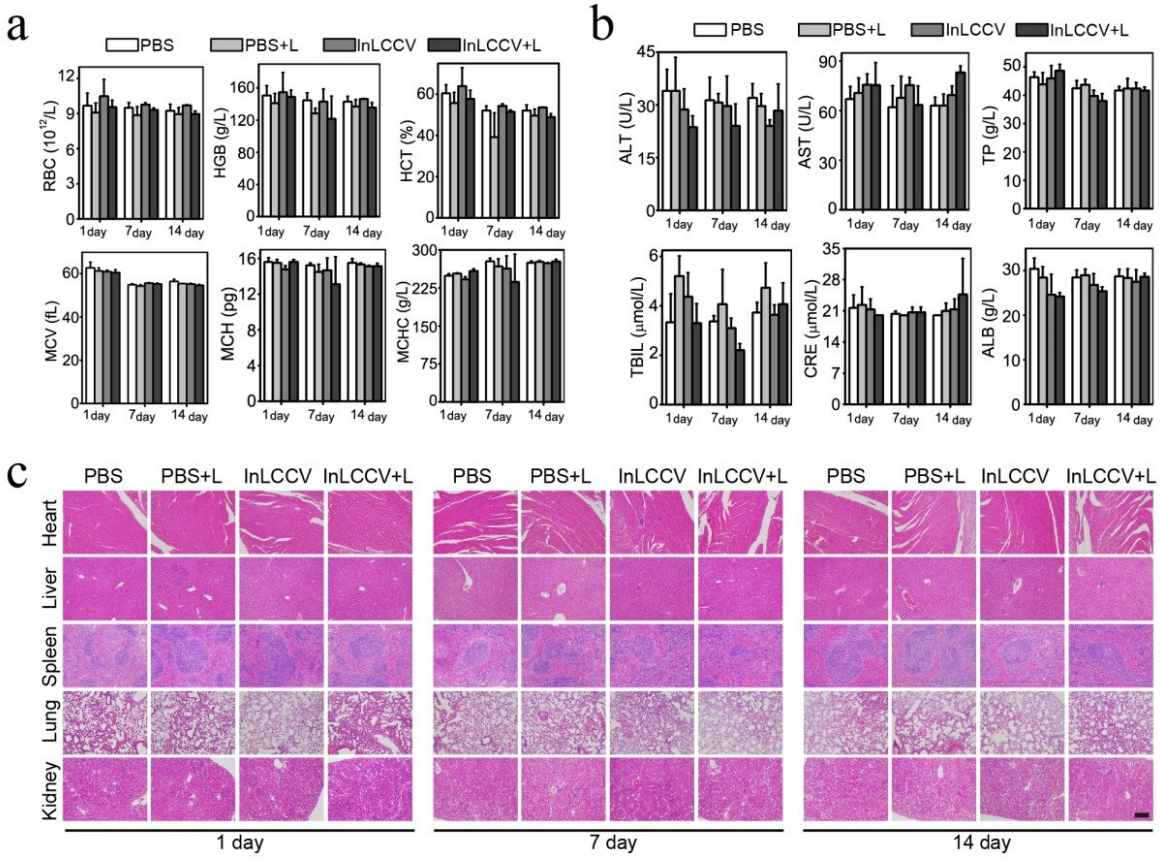

Figure S9. (a) Haematological data of the mice at 1, 7 and 14 day post-injection of InLCCV. (b) Blood biochemical analysis of the vaccinated mice at 1, 7 and 14 day post-injection. (c) H&E staining of the heart, liver, spleen, kidney, and lung of mice after vaccinating for 1, 7, and 14 day (Scale bars = 200 μm).

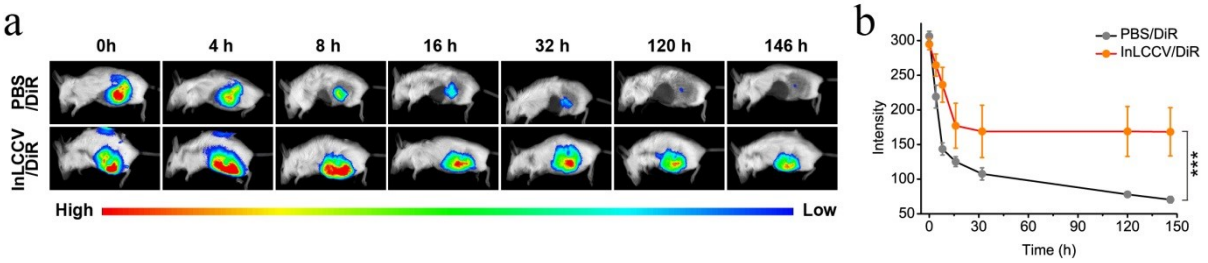

Figure S10. (a) *In vivo* fluorescence images of mice at different time points after subcutaneous injection of free PBS/DiR or InLCCV/DiR. (b) Normalized *in vivo* DiR fluorescence intensity of subcutaneous injected PBS/DiR and InLCCV/DiR with time. (Ex = 748nm, Em = 780 nm). The significance of differences was analyzed by Unpaired student's *t*-test (two-tailed) ****p* < 0.01. Data represent mean ± S.D. (n = 3).

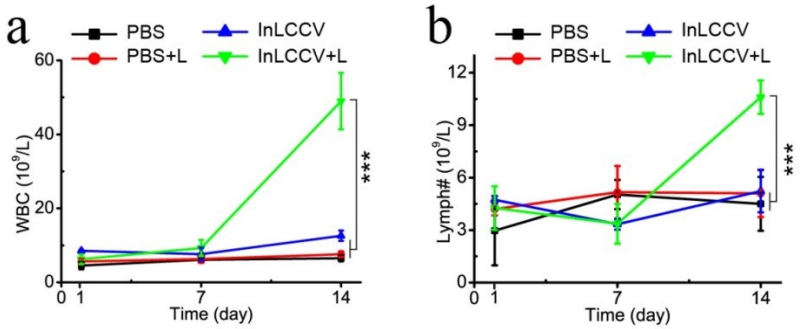

Figure S11. Vaccinated mice had blood collected on Days 1, 7, and 14. (a) white blood cell count. (b) absolute numbers of lymphocytes. The significance of differences was analyzed by Unpaired student's *t*-test (two-tailed) *** p < 0.01. Data are expressed as mean ± S.D. (n = 3).

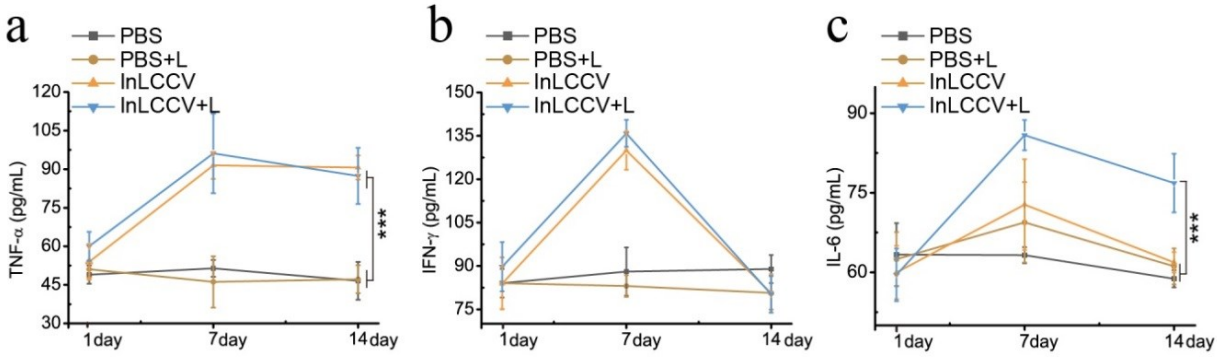

Figure S12. Serum concentrations of TNF-α (a), IFN-γ (b), and IL-6 (c). The significance of differences was analyzed by Unpaired student's *t*-test (two-tailed) *** p < 0.01. Data are expressed as mean ± S.D. (n = 3).

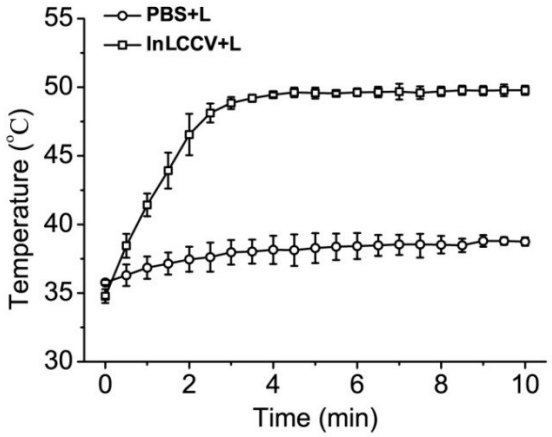

Figure S13. Temperature curve of the mice injected with InLCCV followed by 793 nm laser irradiation (10 min, 0.5 W cm^{-2}).

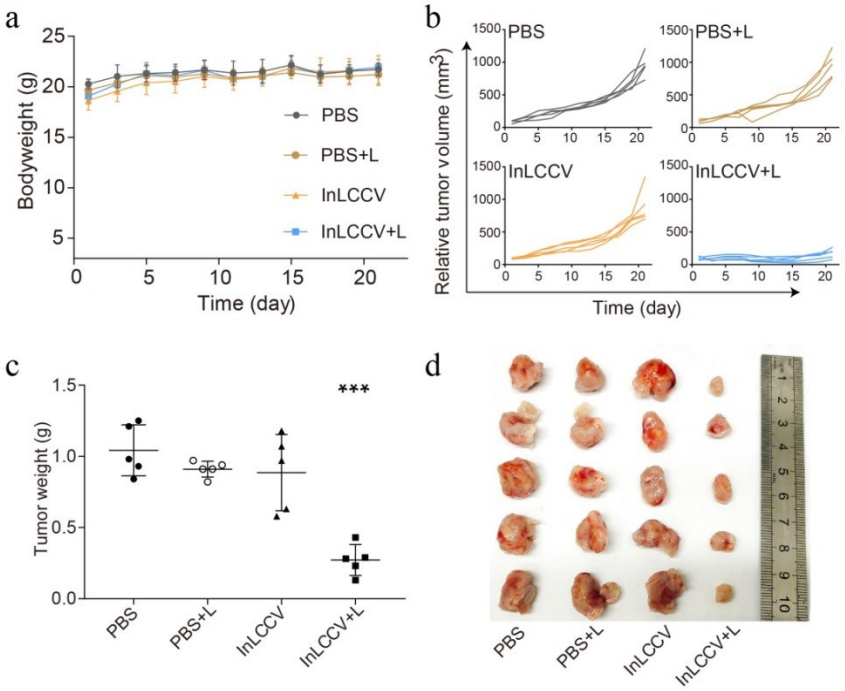

Figure S14. (a) Body weight of the mice during the vaccine treatment. (b) Individual growth curves of abscopal tumor after treating with InLCCV. (c) Tumor and tumor weight of the mice from each group. (d) Collected tumors of the mice from different groups. The significance of differences was analyzed by Unpaired student's *t*-test (two-tailed) ***$p < 0.01$. Data represent mean ± S.D. (n = 5).

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