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Supplemental Information

Ginseng-derived nanoparticles potentiate immune checkpoint antibody efficacy by reprogramming the cold tumor microenvironment

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Supplementary Materials and Methods

Mice，cell lines and ethics statement

Male and female 6-week-old BALB/c mice and C57BL6 mice were purchased from the Comparative Medicine Centre, Yangzhou University (Yangzhou, Jiangsu, China). All mice received access to food and water *ad libitum* and were housed in a temperature-controlled colony room with a 12/12-hour dark/light cycle. All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Nanjing University of Chinese Medicine.

The murine melanoma cell line B16-F10, murine colon cancer cell line CT26, murine luciferase expressed breast cancer cell line 4T1-Luc, and murine colon cancer cell line MC38 were purchased from the Institute of Biochemistry and Cell Biology, Academy of Science (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) or Roswell Park Memorial Institute (RPMI) 1640, supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin (all from Thermo Fisher Scientific, USA). All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Patient samples

Our study was conducted and archived colorectal carcinoma specimens (n = 52, 2020LWKYZ052) were collected according to the protocol of a human research ethics committee at Affiliated Hospital of Integrated Traditional Chinese and Western

Medicine (Nanjing, China) with patient's written formal consent. These patients have been followed over time.

Study design

This study was designed to characterize the efficiency of GDNPs combined with PD-1 mAb by analyzing samples from mice with tumors. Three murine tumor models (CT26, 4T1-luc, MC38) were selected to evaluate the combinatorial treatment efficiency. Six-week-old male mice were inoculated subcutaneously with 3×10^5 cells in the right upper flank ($n = 8$ per group, day 0). The first treatment was scheduled until the tumor was around 50 - 100 mm^3 on day 8. The control group received IgG (200 μg/100 μL/mouse/*i.p.*, clone 2AE, Bio X Cell, Lebanon, USA). PD-1 mAb (200 μg/100 μL/mouse/*i.p.*, clone BE0146, Bio X Cell, Lebanon, USA), GDNPs (200 μg/100 μL/mouse/*i.p.*) were injected intraperitoneally on day 8, 11, 14, 17 and 20 as Figure 2A demonstrated. The tumors were measured every other day with a caliper and the volume was calculated (length × $width^2/2$). Mice with no visible and touchable tumors on consecutive days were considered to have complementally regressed tumors. Mice were sacrificed when tumor volume was over 2000 mm^3. Tumor weight was calculated using an electronic weighing machine.

The rechallenge study: To evaluate whether Combo treatment exerted specific and long-term therapeutic effects, we performed a rechallenge assay. Mice were re-challenged with 3×10^5 CT26 murine colon cancer cells in the right lower flank or 2×10^5 4T1 murine breast cancer cells inoculated in the left breast fat pad in the left lower

flank without any subsequent treatment (on day 85 since the original tumor implanted in the Combo group or healthy mice of the same age) (Figure 5B). Thereafter, tumor volume was recorded continuously for 17 days.

The 4T1 murine breast cancer lung metastasis study: Six-to-eight-week-old female BALB/c mice were inoculated with 2×10^5 4T1 murine breast cancer cells in the right lower breast pad on day 0. On day 5, 1×10^5 4T1-luc murine breast cancer cells were intravenously injected to mimic the breast cancer lung metastasis. The treatment was started when the tumor volume in breast pad was around 50-100 mm^3. The luciferase images were taken on days 13, 16, and 19. The intensity of luciferin signal in mouse chest were measured using IVIS Series *In Vivo* Imaging Systems (PerkinElmer, USA). Mice were sacrificed on day 20 and their lungs were harvested and stored in Bouin's buffer (RS 4140, G-CLONE).

$CD4^+/CD8^+$ T lymphocyte or macrophage depletion assay: Six-week-old male BALB/c mice were inoculated with 3×10^5 CT26 murine colon cancer cells in the right upper flank on day 0. $CD8^+$ T, $CD4^+$ T, or macrophage depletion was performed by intraperitoneally injecting with anti-mouse CD8A (200 μg/100 μL/mouse/*i.p.*; clone 53-6.7; rat IgG2a; Bio X Cell)/ Isotype control (200 μg/100 μL/mouse/*i.p.*; clone 2A3; rat IgG2a; Bio X Cell) or CD4 (200 μg/100 μL/mouse/*i.p.*; clone YTS191; rat IgG2b; Bio X Cell)/ Isotype control (200 μg/100 μL/mouse/*i.p.*; clone LTF-2; Rat IgG 2b; Bio X Cell) or clodronate liposome (1 mg/200 μL/mouse/*i.p.*; Yeasen)/PBS liposome (1 mg/200 μL /mouse/*i.p.*; Yeasen) twice a week before Combo treatment began. PD-1

mAb (200 μg/100 μL/mouse/*i.p.*, clone BE0146, Bio X Cell) and GDNPs (200 μg/100 μL/mouse/*i.p.*) were administered every two days from day 8 for 5 times in total. Mice were sacrificed on day 21 and the tumor weight was calculated by an electronic weighing machine.

CCL5 and CXCL9 ***in vivo*** neutralization assay: Six-week-old male BALB/c mice were inoculated with 3×10^5 CT26 murine colon cancer cells in the right upper flank on day 0. $CD8^+$ T, $CD4^+$ T, or macrophage depletion was performed by intraperitoneally injecting with anti-mouse CCL5 (50 μg/100 μL/mouse/*i.p.*; clone 53405; rat IgG2a; R&D)/Isotype control (50 μg/100 μL/mouse/*i.p.*; Catalog: MAB006; rat IgG2a; R&D) or anti-mouse CXCL9 (100 μg/100 μL/mouse/*i.p.*; clone MIG-2F5.5; Armenian Hamster IgG κ; Bio X Cell)/ Isotype control (100 μg/100 μL/mouse/*i.p.*; Catalog: BE0091; Armenian Hamster IgG; Bio X Cell) twice a week after Combo treatment began. PD-1 mAb (200 μg/100 μL/mouse/*i.p.*, clone BE0146, Bio X Cell) and GDNPs (200 μg/100 μL/mouse/*i.p.*) were administered every two days from day 8 for 5 times in total. Mice were sacrificed on day 21 and the tumor weight was calculated by an electronic weighing machine.

GDNPs preparation

GDNPs were isolated from fresh *Panax ginseng C. A. Mey* root and prepared as previously published protocol.[21] The Bicinchoninic Acid Kit (Beyotime, China) was used to better quantify the GDNPs concentration. GDNPs measurement were performed using Nanoparticle Tracking Analyses NS3000 with NanoSight software.

For Transmission electron microscope image, 10 μL purified GDNPs was deposited onto the surface of formvar-coated copper grids, followed by incubation with 1% uranyl acetate for 15s. The samples were left to dry at room temperature and observed using a HITACHI H-7650 electron microscope operated at 80 kV at a magnification of 20, 000×. A sensitive LC-MS method has been developed for determination of Ginsenoside Re in GDNPs. For quantitative analysis, the separation of the multi-components was carried out by using the Waters Quattro Micro (series 2695; Waters, USA) liquid chromatography equipped with a quaternary pump, an online vacuum degasser, an autosampler, a thermostatic column compartment. All data collected were analyzed and processed using the Masslynx (Waters, USA). Chromatographic separation was performed on an Agilent HC-C_{18} column (4.6 mm × 250 mm, 5 μm, Agilent, USA) using gradient elution of acetonitrile-0.1% formic acid in water with a flow rate of 0.4 ml/min. The mass spectrometer was run in electrospray ionization (ESI +) mode by using multiple reaction monitoring (MRM).

Flow cytometry analysis of immune cells in the TME, splenocytes and peripheral blood

Immune cells were isolated by using Percoll (17-0891-09. GE Healthcare) from tumor cell suspension. These cells were incubated with CD16/32 (clone 93, BioLegend) for 15 min on ice and then were stained with various combinations of following fluorochrome-conjugated antibody at the appropriate dilutions for 30 min on ice, namely, CD3-APC/Cy7 (clone 145-2C11, BioLegend), CD8a-phycoerythrin (PE; clone

53-6.7, BioLegend), CD45-fluorescein isothiocyanate (FITC; clone 30-F11, BioLegend), CD11b-APC/Cy7 (clone M1/70, BioLegend), F4/80-PE/Cy7 (clone BM8, BioLegend), F4/80-BV421(clone BM8, BioLegend), CD8a-APC (clone 53-6.7, Biolegend), CD4-APC (clone GK1.5, BioLegend), CD4-PE/Cy7 (clone GK1.5 BioLegend), FVD506 (eflour 506, Invitrogen), CD45-BV510 (clone 30-F11, BioLegend), TIM3-PE (clone 5D12, BD Pharmingen), ICOS-PE/Cy7 (clone 7E.17G9, Invitrogen), PD-1-APC (clone 29F-1A12, BioLegend).

For T cell derived anti-tumor cytokines, 2×10^6 splenocytes or TILs were incubated in RPIM 1640 with Cell Stimulation Cocktail Plus Protein Transport Inhibitors (500×) for 6 h. Then cells were stained with surface markers and fixed and permeabilized using Fixation/Permeabilization kit (00-5123-43, 00-5223-56, Invitrogen), IFN-γ-PE/Cy7 (clone XMG1.2 BioLegend), granzyme B-FITC (clone NGZB, BioLegend), TNF-α-BB700 (clone MP6-XT22, BD Horizon) were diluent in Permeabilization buffer 10× (1:20) for 45 min.

Stained cells were analysed on a FACS Aria II Flow Cytometer, BD Biosciences) using BD FACSDiva software (BD Bioscience, USA) and data were processed using Flowjo Version 10 (BD Bioscience, USA).

Milliplex Luminex assay

Milliplex Luminex assay (Merk, Germany) was performed to examine the concentration of IL-2, IL-12 p40, and IL-12 p70 in mice plasma according to

manufacturer's instructions.

Reverse transcription PCR assay

Total RNA was isolated from 50,000 cells of M2-BMDM + GDNPs/PBS cultured with TRIzol reagent (Invitrogen) for 3min. The total RNA was reverse-transcribed using HiScript III RT SuperMix for qPCR (+gDNA wiper; category number R323-01; Vazyme Biotech), ChamQ Universal SYBR qPCR Master Mix (category number Q711-02; Vazyme Biotech). Primers were listed in the Supplemental Materials. Real-time PCR was performed using a 7500 Real-Time PCR System (Applied Biosystems, USA).

RNA-seq analyses

Total RNA of M2-BMDM + GDNPs/PBS was extracted using a TRIzol reagent kit (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The RNA quality was examined using RNAse free agarose gel electrophoresis and processed using a 2100 Bioanalyzer Instrument (Agilent Technologies, USA). After total RNA was extracted, eukaryotic mRNA was enriched using Oligo(dT)beads, while prokaryotic mRNA was enriched by removing rRNA using Ribo-Zero TM Magnetic Kit (Epicenter, USA). Then, the enriched mRNA was fragmented using fragmentation buffer and reverse transcribed into cDNA with random primers. The second-strand cDNA was synthesized using DNA polymerase I, RNase H, dNTP and buffer. Then the cDNA fragments were purified with a QiaQuick PCR extraction kit (Qiagen, Netherlands), end repaired, poly(A) added, and ligated to Illumina sequencing adapters.

The ligation products were selected by size using agarose gel electrophoresis, PCR amplified, and sequenced using Illumina HiSeq2500 by Gene Denovo Biotechnology (Guangzhou, China).

Immunohistochemistry

Fresh organs such as heart, liver, spleen, lung, and kidney were fixed. Hematoxylin-eosin staining was performed to analyze microscopic pathological changes in murine main organs under Combo treatment by an optical microscope (Olympus, Japan). Fresh tumor samples were fixed with 4% paraformaldehyde for several days and embedded in paraffin, sectioned into 5-μm-thick sections and observed using a Leica RM 2235 (Leica, Germany) after being mounted on adhesive glass slides. BSA (Service Bio, G50001), and CD8 (GB13429, Service, 1:200) were used for staining to evaluate the infiltration of CTLs in tumors.

Immunofluorescent

For immunofluorescence, BMDMs on the microscope cover glass were fixed in ice-cold 4% paraformaldehyde. Sections were washed thrice with PBS for 5 min and mounted using ProLong Gold Antifade Mountant with DAPI (4′,6-diamidino-2-phenylindole; Thermo Fisher Scientific, USA) and imaged using 60× magnification with an Olympus FV10i confocal microscope (Olympus, Japan). The resultant digital images were analysed using the Olympus FluoView software version 4.0b. Images of three nonoverlapping optical fields covering the tumor sections surface were captured.

Image analysis was performed in ImageJ with the area measurement application or manual counting.

For OCT mounted CT26 murine colon tumor tissue sections, sliced samples were fixed in ice-cold 4% paraformaldehyde, after been incubated with 5% BSA, they were incubated with CD3-PE, CD8-APC overnight in 4 ℃. Following steps were same to BMDMs on the microscope cover glass.

Bioinformatic analysis

Correlation analysis between gene transcriptomes, such as CXCR3 and CCL9, CCR5 and CCL5 in COAD and BRCA patients in TCGA by using TIMER2.0 (http://timer.cistrome.org/).

Statistical analysis

The results are expressed as the mean ± standard error of mean. All data were analyzed using GraphPad Prism 7.0 (GraphPad Software, USA) by unpaired Student's t test, one-way or two-way analysis of variance (ANOVA), and log-rank (Mantel-Cox) test. $p < 0.05$ was considered statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$)

Supplementary Figures

Supplementary Figure 1

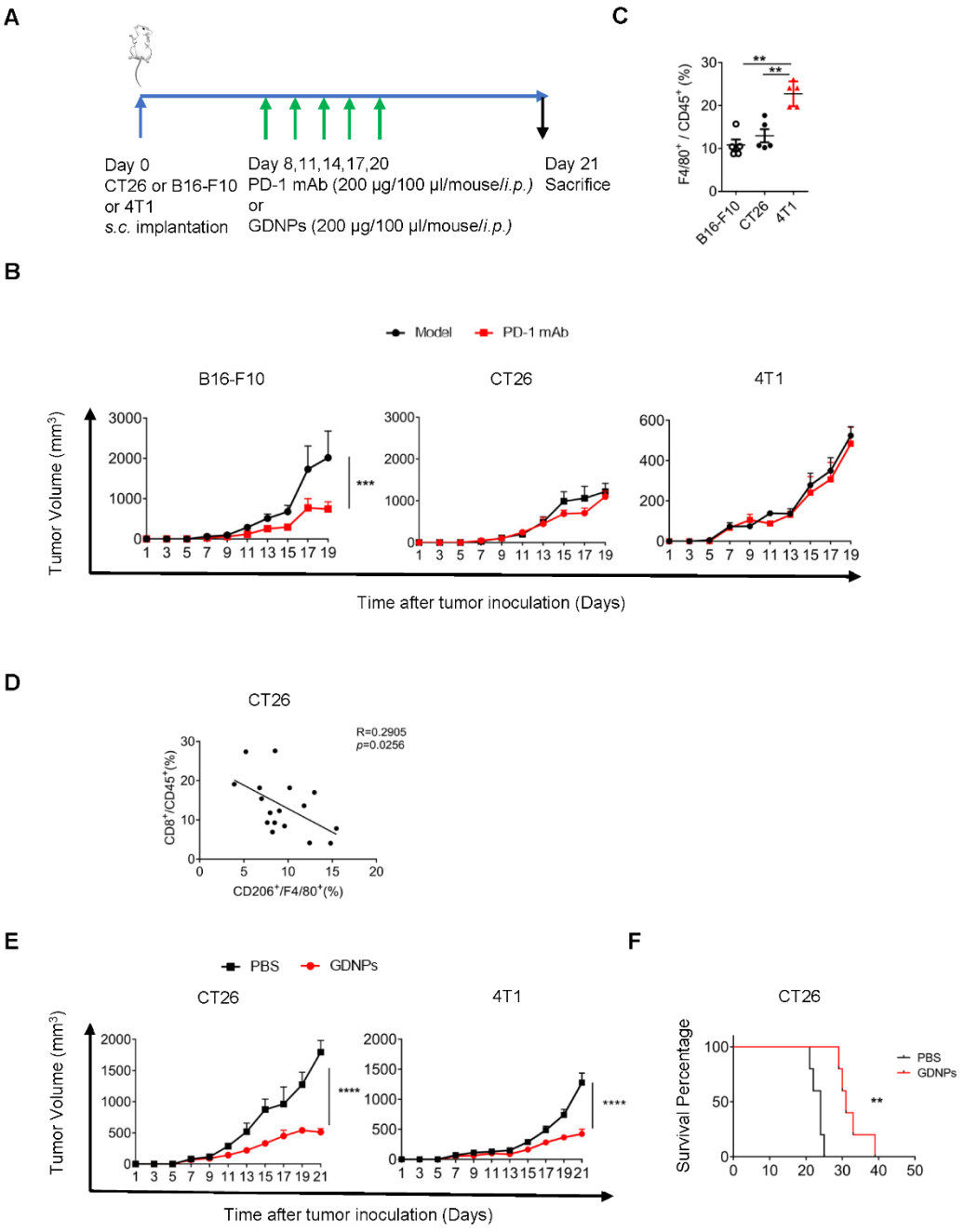

Figure S1. PD-1 mAb and GDNPs independent therapy in three tumor bearing mouse models. (A) Schematic of B16-F10 murine melanoma, CT26 murine colon

tumor, 4T1 murine breast tumor three mouse models under GDNPs/PD-1 Ab/Vehicle treatment regimen assay. (B) Tumor volume of B16-F10, CT26, 4T1 three murine tumor models under PD-1 mAb/Vehicle treatment. (n = 5 for each group, **p < 0.01) (C) Ratio of $F4/80^+/CD45^+$ in tumor immune microenvironment for B16-F10 melanoma, 4T1 breast cancer, CT26 colon cancer three murine tumor models (n = 5 for each group, ***p < 0.001). (D) Correlation analyses between ratio of $CD8^+/CD45^+$ and $CD206^+/F4/80^+$ for CT26 murine colon tumor model under GDNPs/Vehicle treatment. (for CT26 murine colon tumor model: n = 17, p = 0.0256, R = 0.2905) (E) Tumor volume of CT26 murine colon cancer, 4T1 murine breast cancer two murine models under GDNPs/PBS treatment. (n = 4 for each group, ****p < 0.0001) (F) Survival curve of mice treated with GDNPs or Vehicle controls. (n = 5 for each group, **p < 0.01) Data are presented as mean ± SEM and analyzed using student t test, Two-way ANOVA and Mantel-Cox test.

Supplementary Figure 2

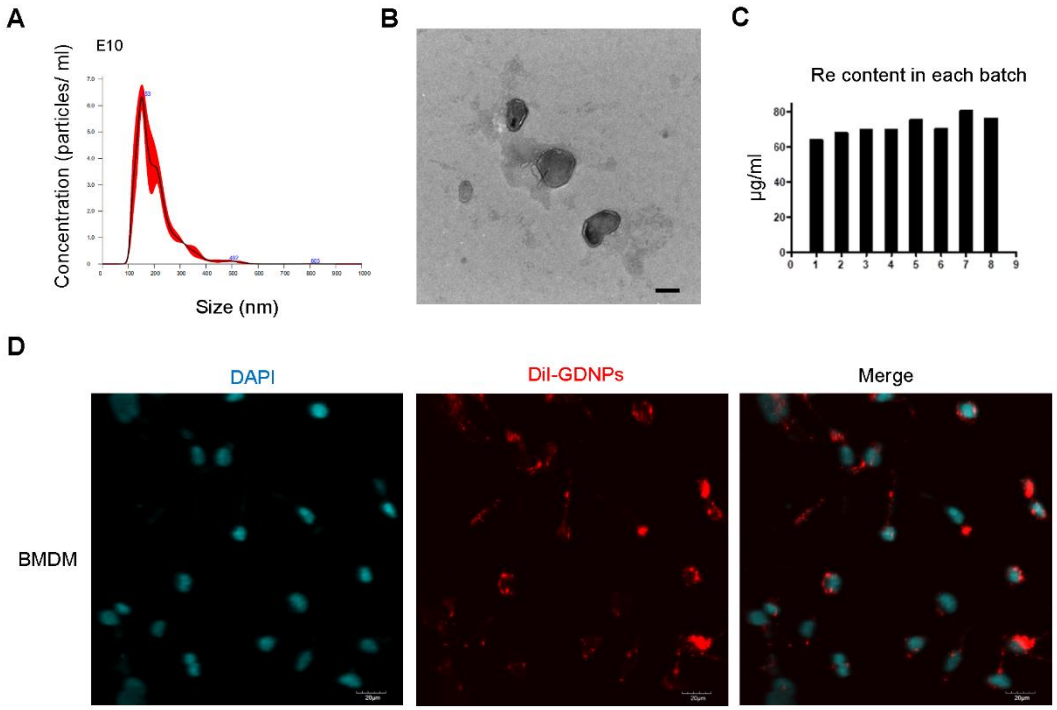

Figure S2. Characterization of ginseng-derived nanoparticles (GDNPs) prepared from fresh ginseng roots. (A) GDNPs were characterized by nanoparticle tracking system (NTA). (B) GDNPs from sucrose density gradient (45%) were characterized by transmission electron microscopy (TEM) (Scale bar = 200 nm). (C) Re ginsenoside content in each batch of GDNPs performed by HPLC-MS. (D) Immunofluorescent images for DiI-labelled GDNPs cocultured with BMDM (10 μg/ml). BMDMs were incubated with DiI-labelled GDNPs for 12 h. (scale bar = 20 μm)

Supplementary Figure 3

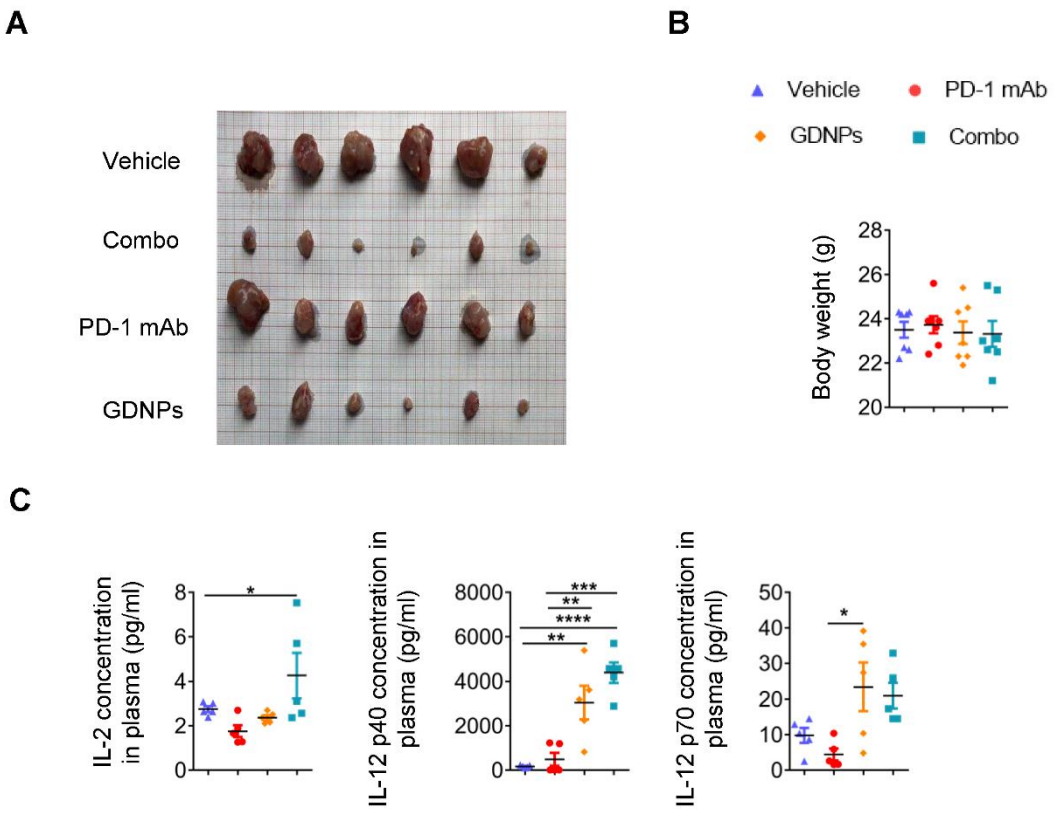

Figure S3. GDNPs combined with PD-1 mAb depresses CT26 murine colon tumor progression. (A) Tumor pictures for Vehicle, PD-1 mAb, GDNPs, Combo treatment (*n* = 6 for each group). (B) Body wight of CT26 tumor bearing mice under Vehicle, PD-1 mAb, GDNPs, and Combo treatment on day 21 before sacrifice. (n = 7 for each group) (C) IL-2, IL-12 p40, IL-12 p70 concentration in mice plasma by Milliplex Luminex assay. (n = 5 for each group, *p < 0.05, **p <0.01, ***p < 0.001, ****p < 0.0001). Data are presented as mean ± SEM and analyzed using One-way ANOVA.

Supplementary Figure 4

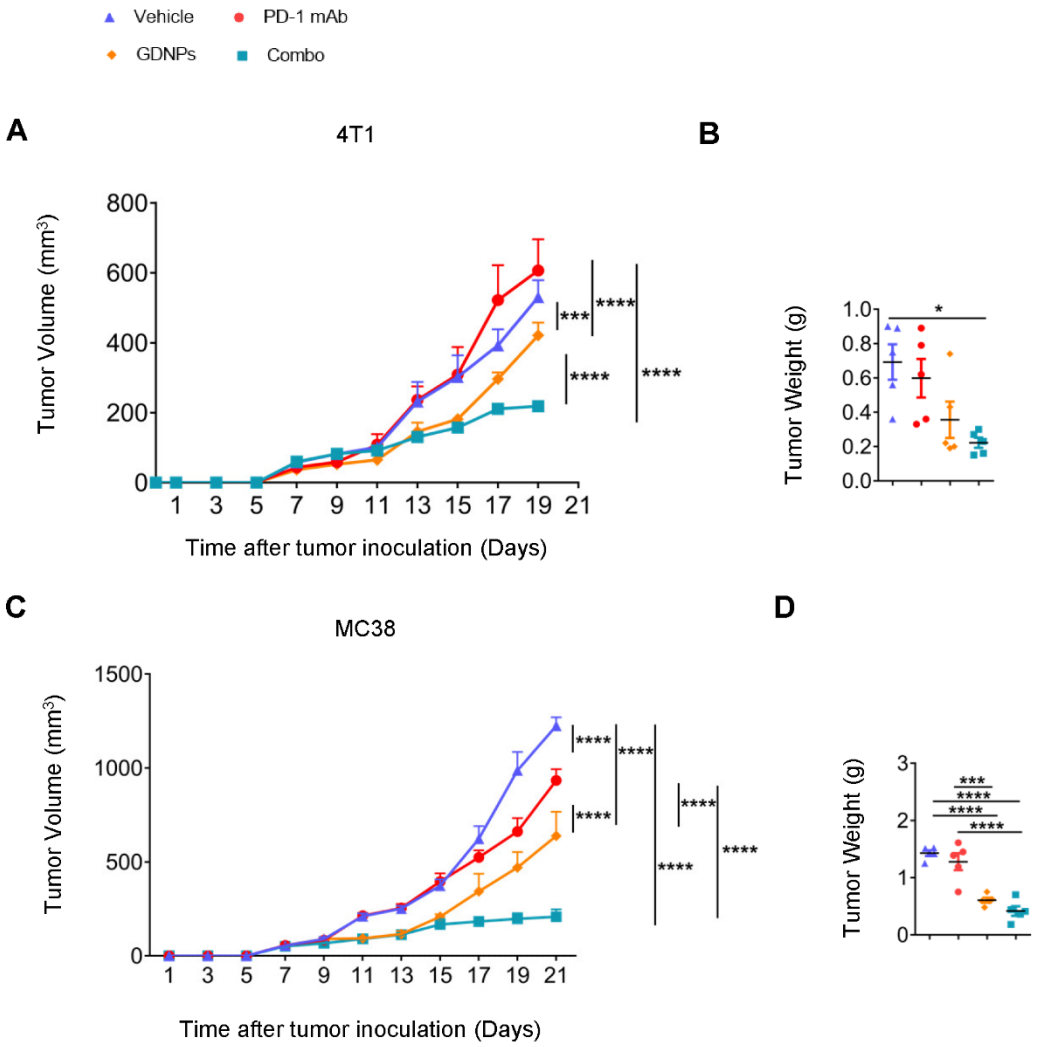

Figure S4. GDNPs combined with PD-1 mAb depresses 4T1 and MC38 murine tumor progression. (A) Tumor volume and (B) Day 21 Tumor weight for 4T1 murine breast cancer mouse model (n = 5 for each group, *p < 0.05, ****p < 0.0001). (C) Tumor volume and (D) Day 21 Tumor weight for MC38 murine colon tumor model (n = 5 for each group, ****p < 0.0001). Data are presented as mean ± SEM. Analyzed by One-way ANOVA or Two-way ANOVA.

Supplementary Figure 5

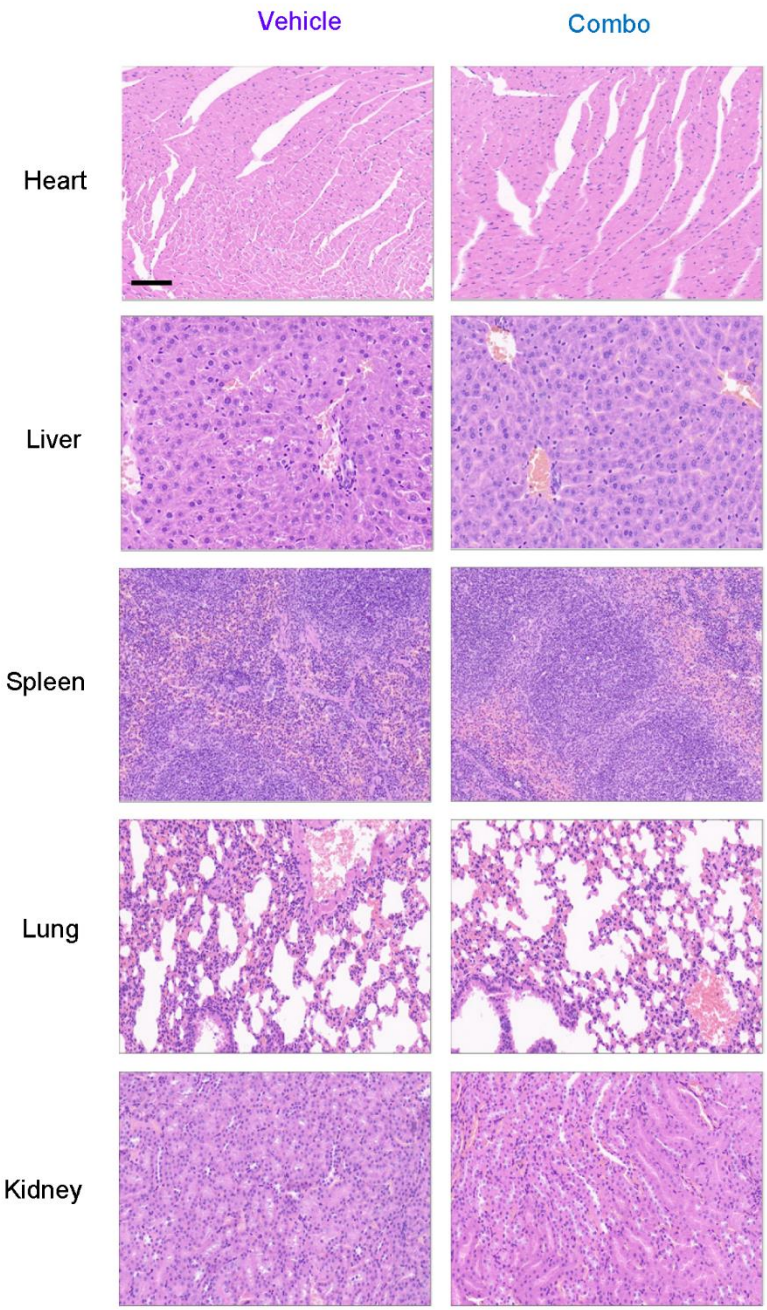

Figure S5. Safety evaluation assay. Analyses of microscopic pathological changes in murine main organs. Representative picture for HE staining for heart, liver, spleen, lung, kidney in Vehicle or Combo treatment group. (Scale bar = 50 μm).

Supplementary Figure 6

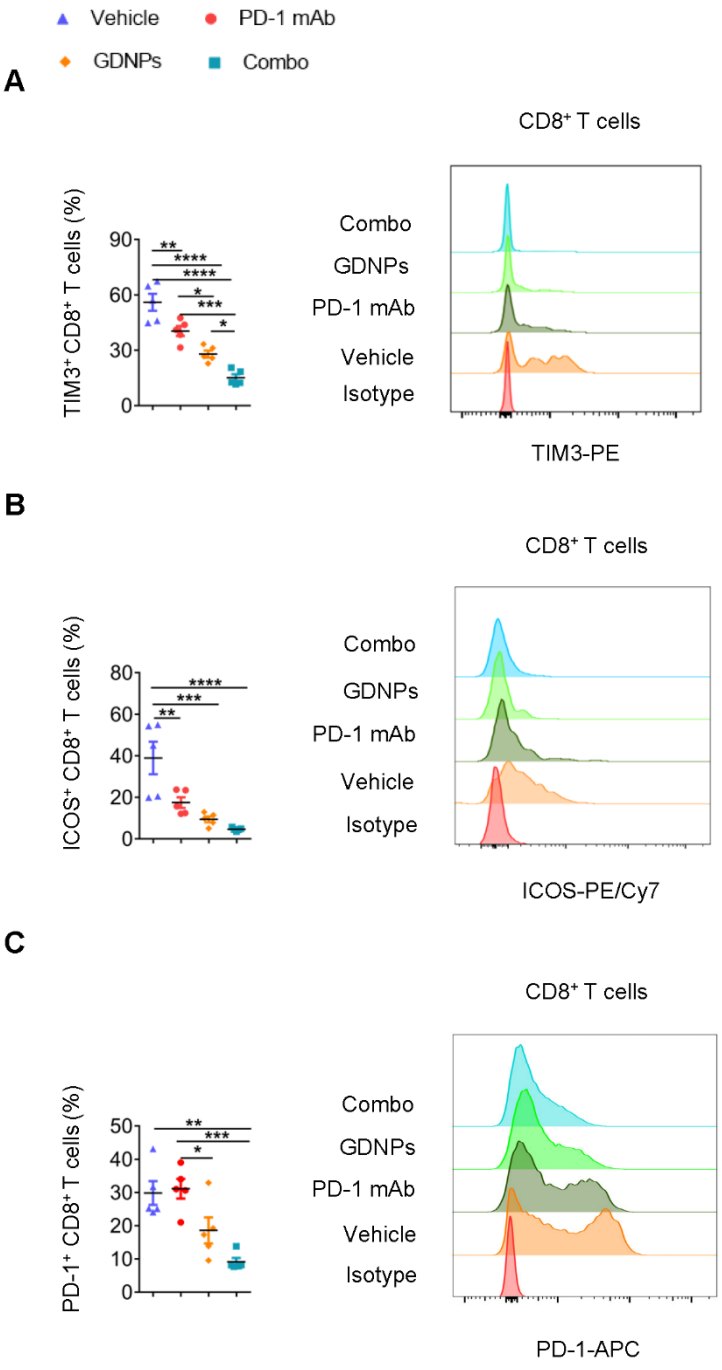

Figure S6. GDNPs combined with PD-1 mAb decreases immune checkpoint expression in T cell in CT26 murine colon tumor bearing mice. Fractions of (A) $TIM3^+$, (B) $ICOS^+$, (C) $PD\text{-}1^+$ in $CD8^+$ T cells and the representative flowcytometry

pictures in Vehicle, PD-1 mAb, GDNPs, and Combo groups. Data are presented as mean ± SEM. n = 5 for each group, *p < 0.05, **p < 0.01, ***p <0.001, ****p < 0.0001. Analyzed by One-way ANOVA.

Supplementary Figure 7

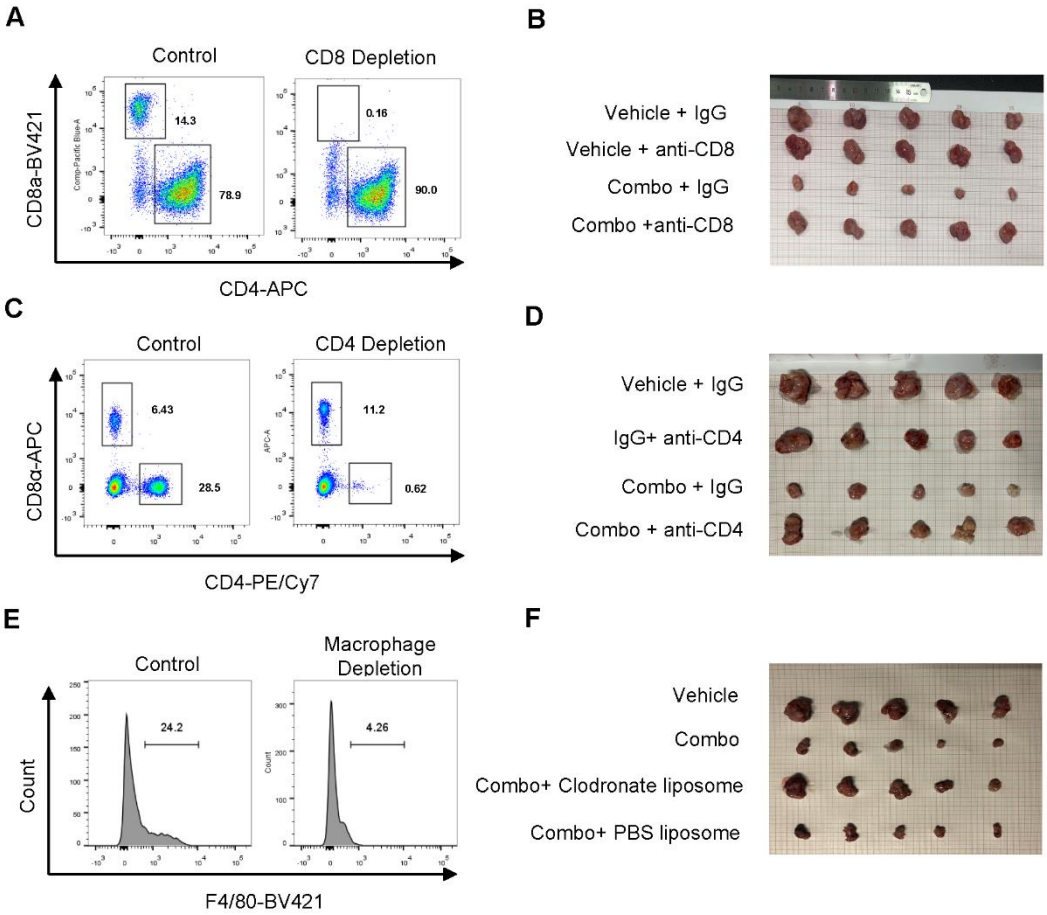

Figure S7. CD4$^+$, CD8$^+$ and macrophage *in vivo* depletion in CT26 murine colon tumor bearing mice. (A) Representative flow cytometric picture for CD8$^+$ T cell depletion verification in peripheral blood. (B) Tumor pictures for Vehicle + IgG, Combo + IgG, Vehicle + anti-CD8, Combo + anti-CD8. (C) Representative flow cytometric picture for CD4$^+$ T cell depletion verification in peripheral blood. (D) Tumor pictures for Vehicle + IgG, Combo + IgG, Vehicle + anti-CD4, Combo + anti-CD4. (E) Representative flow cytometric picture for macrophage depletion verification in peripheral blood. (F) Tumor pictures for Vehicle, Combo, Combo + Clodronate liposome, Combo + PBS liposome.

Supplementary Figure 8

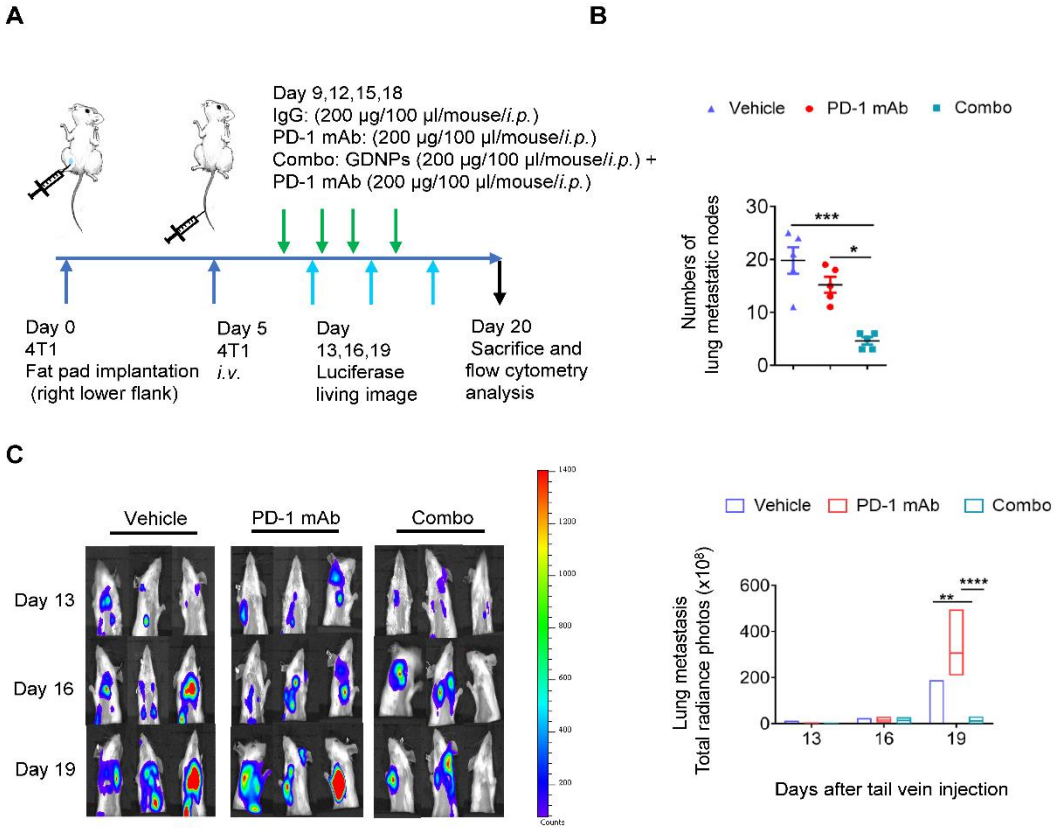

Figure S8. GDNPs combined with PD-1 mAb effectively inhibits 4T1 murine breast cancer lung metastasis. (A) Schematic diagram and administration methods for 4T1 murine breast cancer lung metastasis. (B) Quantification of 4T1 murine lung metastatic nodes in Vehicle, PD-1 mAb, and Combo groups ($n = 5$ per group, $^{*}p < 0.05$, $^{***}p < 0.001$). (C) Luciferase bioluminescent images of 4T1-Luc murine breast cancer lung metastasis in Vehicle, PD-1 mAb, and Combo groups. Results were calculated from three independent experiments. ($n = 3$ per group, $^{**}p < 0.01$, $^{****}p < 0.0001$) Data are presented as mean ± SEM. Analyzed by One-way ANOVA.

Supplementary Figure 9

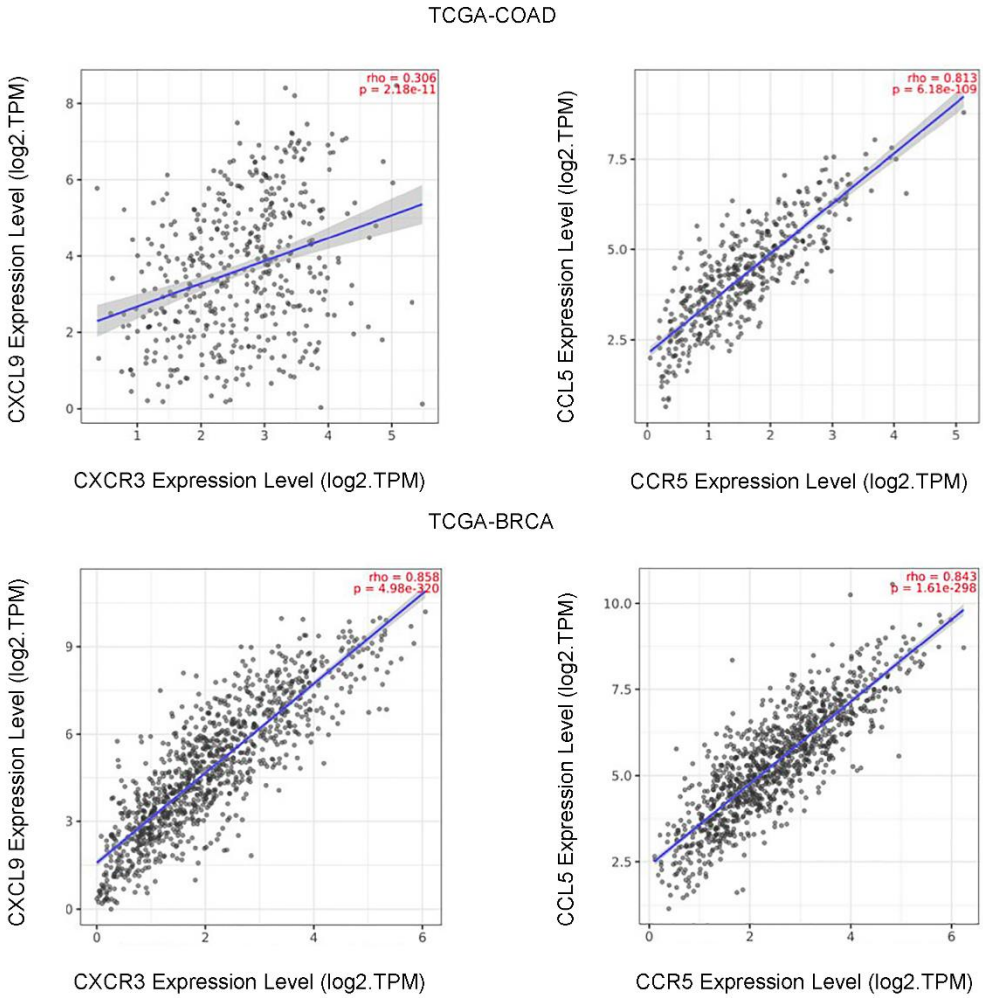

Figure S9. Correlation analyses between gene transcriptomes, such as CXCR3 and CCL9, CCR5 and CCL5 in COAD and BRCA patients in TCGA by using TIMER2.0 (http://timer.cistrome.org/).

Supplementary Figure 10

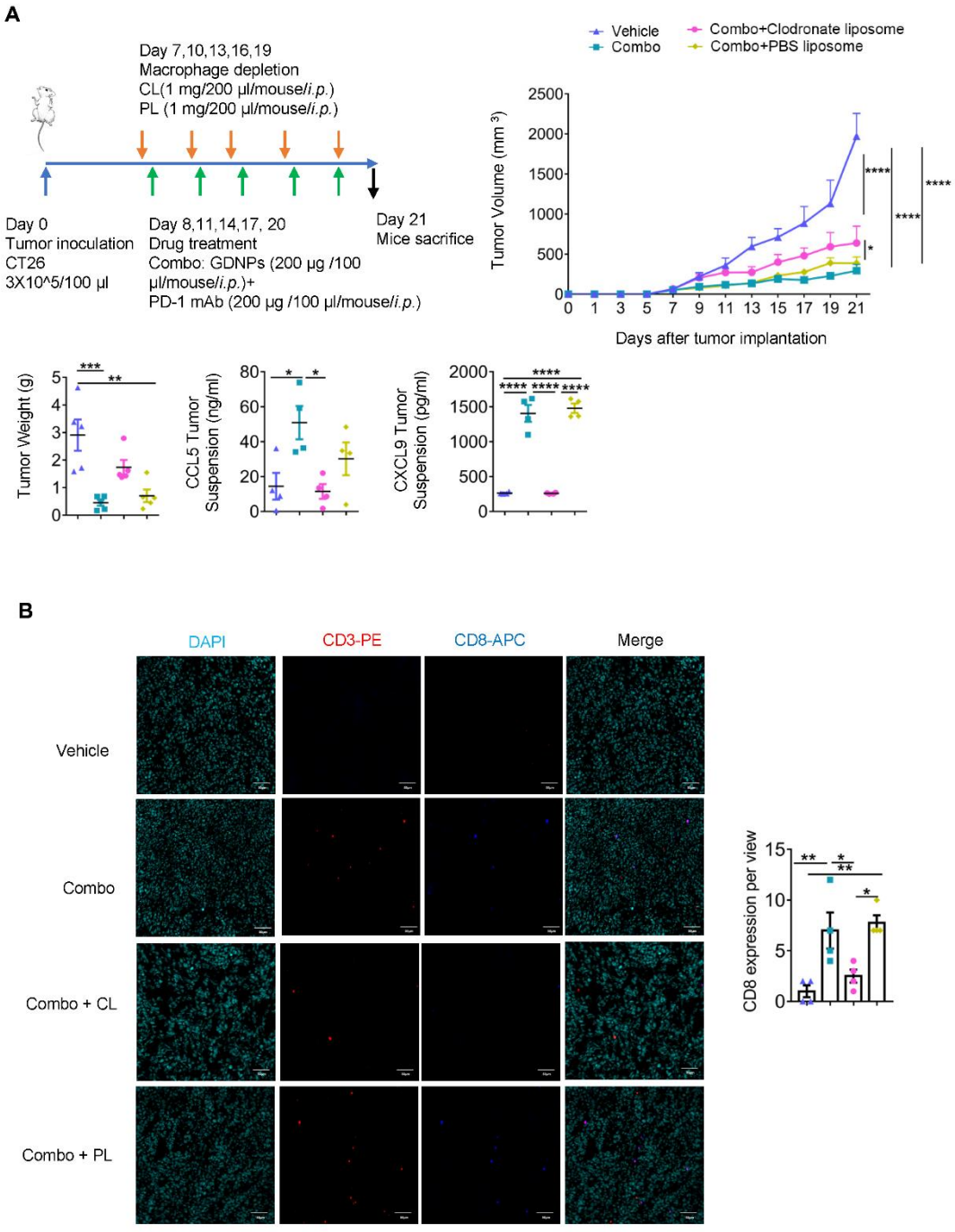

Figure S10. GDNPs activated macrophage recruited CD8$^+$ T lymphocytes into TME in CT26 murine colon tumor model. (A) Paradigm of tumor implantation, macrophage depletion assay by clodronate liposome (CL) or negative control PBS liposome (PL), drug treatment time schedule in CT26 murine colon tumor model.

Tumor volume and tumor weight for Combo, Combo + CL, Combo + PL, Vehicle four groups. CXCL9 and CCL5 concentration in tumor suspension in the four groups ($n = 5$ per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$). (B) Immunofluorescent staining of tumor sections in Combo, Combo + CL, Combo + PL, Vehicle groups ($n = 4$ per group, $^{*}p < 0.05$, $^{**}p < 0.01$). Data are presented as mean ± SEM. Analyzed by One-way ANOVA and Two-way AVONA.

Supplementary Figure 11

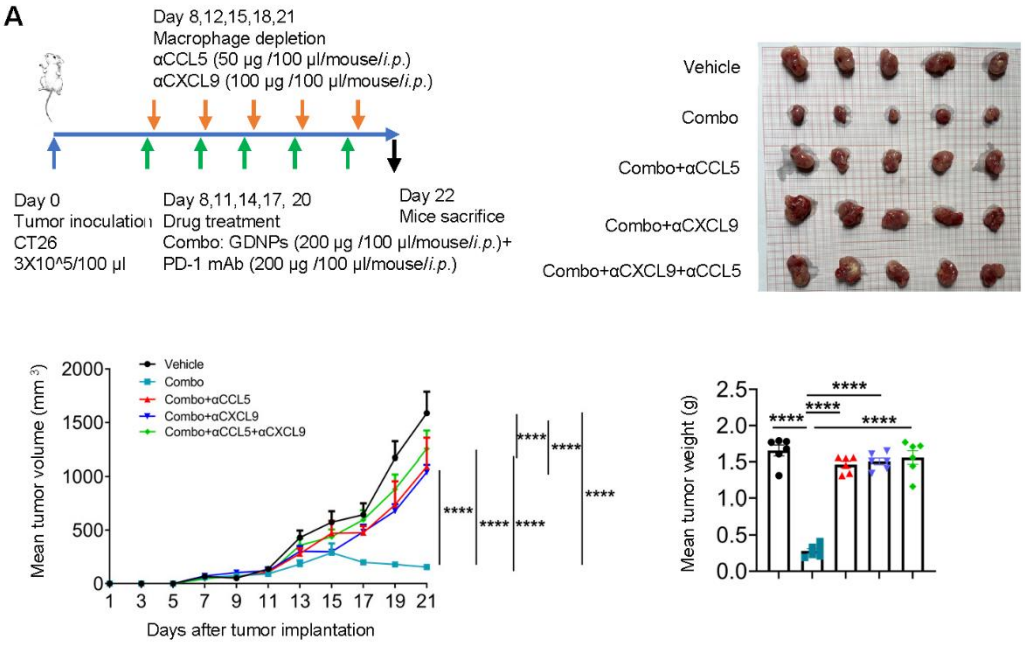

Figure S11. CCL5 and CXCL9 neutronization in CT26 murine colon model. (A) The time schedule, tumor picture, mean tumor volumes and tumor weight for chemokines *in vivo* neutralization assay (n = 5~6, Two-way ANOVA or One-way ANOVA).

Supplementary Figure 12

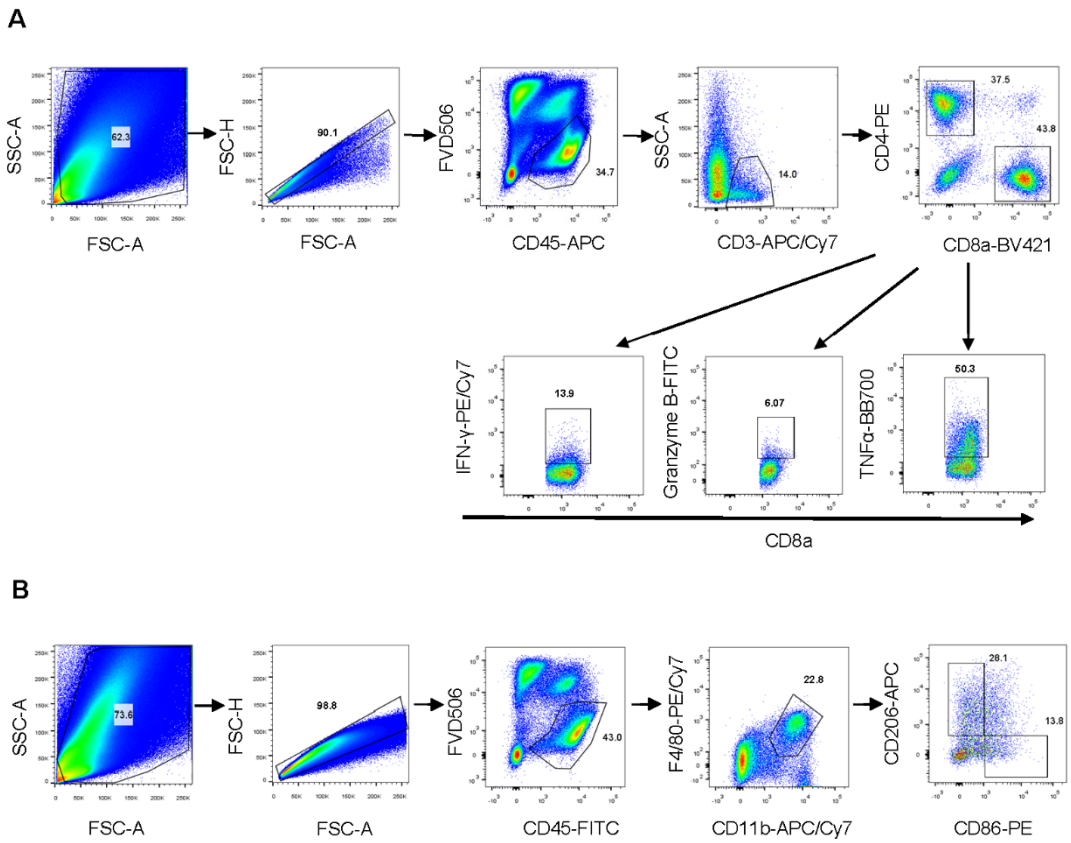

Figure S12. Flow cytometry gating strategy for tumor infiltrated (A)T lymphocytes and (B) macrophage polarization.