Electronic Supplementary Material (ESI) for Nanoscale Horizons

Non-Depleting Reformation of the Immunosuppressive Myeloid Cell to Broaden Application of anti-PD Therapy

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Experimental Section

Materials. Tetraethyl orthosilicate (TEOS), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- (methoxy(polyethylene glycol)-2000) (PEG–DSPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- (amino(polyethylene glycol)-2000) (DSPE-PEG2000-NH2) and all-trans retinoic acid (ATRA) were purchased from Sigma-Aldrich. 3-aminopropyltriethoxysilane (APTES), lecithin and cholesterol were purchased from Aladin Co., Ltd.. N-fluorenyl-9-methoxycarbonyl (Fmoc) protected amino acids, O-benzotriazole-N,N,N',N',-tertamethyluroniumhexafluorophosphate (HBTU), N-hydroxybenzotriazole (HOBt), Rink-Amide resin were purchased from GL Biochem Ltd. (Shanghai China). Diisopropylethylamine (DIEA), Trifluoroacetic acid (TFA) and Piperidine were purchased from Energy-Chemical. Recombinant human MMP-2 protease was purchased from R&D Systems (catalogue no: 902-MP). Anti-mouse PD-1 antibody was purchased from BioXCell (West Lebanon, NH). Enzyme-linked immunosorbent assay (ELISA) test kits of TNF-α, IFN-γ and granzyme-B were purchased from 4A Biotech Co., Ltd.. Enzyme-linked immunosorbent assay test kits of anti-PD-1 antibody was purchased from KANGLANG Biological Technology Co, Ltd. (Shanghai). The following antibodies were used in flow cytometry: APC anti-Ly6C (HK1.4, 17-5932-82), PE anti-Ly6G (1A8-Ly6g, 12-9668-80), PE anti-F4/80 (BM8, 12-4801-82), APC anti-CD86 (GL1, 17-0862-81), PE anti-CD80 (16-10A1, 12-0801-81), FITC anti-CD11c (N418, 11-0114-81) and FITC anti-CD4 (GK1.5, 11-0041-82) were purchased from eBioscience; APC anti-CD206 (C068C2, 141708), APC anti-CD8a (53-6.7, 100712) and FITC anti-CD11b (M1/70, 101206) were from BioLegend. Antibodies for immunohistochemistry and immunofluorescence: anti-Ly6G, anti-CD4 and anti-CD8 were purchased from Servicebio, anti-Argnase-1 was purchased from Cell Signal Technology.

Cell Culture. CT26 murine colon cancer cells (ATCC-CRL-2639), 4T1 murine breast cancer cells (ATCC-CRL-2539) and B16F10 murine melanoma cancer cells (ATCC-CRL-6475)

were was cultured in RPMI-1640 medium; RAW264.7 macrophage cell line (ATCC-TIB-71) was maintained in DMEM/high glucose medium. The above medium was supplemented with 10% FBS and 1% penicillin /streptomycin. And all cells were cultured at 37 °C in a humidified incubator containing 5% CO_2.

Animal Experiment. All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Experiment Center of Wuhan University (Wuhan, China). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of People's Republic of China. 6–8 weeks old female BALB/C or C57BL/6 mice were used in this study. 2×10^5 B16-F10 cells were s.c. (subcutaneously injected) into the right flank of C57BL/6 mice. Similarly, 2×10^5 4T1 or CT26 cells were s.c. into the right flank of BALB/C mice. When tumors reached 100 mm^3, mice were randomized and treated separately with PBS (100 μL, i.v., intravenous injection), mouse PD-1 antibody (2.5 mg kg^{-1}, i.p., intraperitoneal injection), ATRA@MSN@Lipo (75 mg kg^{-1}, i.v.,), MSN-aPD-1@Lipo (75 mg kg^{-1}, i.v.) or ATRA@MSN-aPD-1@Lipo (75 mg kg^{-1}, i.v.) nanoparticles every three days for a total of five times. 15 days after treatment, the mice were sacrificed and tumors were collected. Tumor size was measured with digital caliper every two days and the volume was calculated as (length × $width^2$)/2.

Synthesis of MMP-2-cleavable Peptide (sequence: DSK(C18)DSGPLGIAGQDSK (C18)DS). The peptides were synthesized using standard solid phase peptide synthesis method. The peptide was linked to a rink amide resin and HOBT/HBTU/DIEA were used to promote coupling efficiency of the Fmoc-protected amino acids. Fmoc protecting group was deprotected by 30% piperdine/dimethyl formamide (DMF) (v/v). The peptide was cleaved from resin with a cleavage mixture of trifluoroacetic acid (TFA) and deionized (DI) water (95:5, v/v) for 1 h. Subsequently, the filtrate was concentrated and participated in cold ether

(−20℃) overnight. The white solid was collected by centrifugation, and washed with cold ether for three times. Finally, the peptide got dried under vacuum. The low solubility of peptide caused by dual-C18 group leaded to the difficulty of proteolysis. Thus, for the convenience of proteolysis and detection in vitro, we synthesized the peptide not containing the C18 group (sequence: DSKDSGPLGIAGQDSKDS). The molecular weights were determined by Matrix-Assisted Laser Desorption/ Ionization Time of Flight Mass Spectrometry (MALDI-TOF-MS).

Preparation of MMP-2-responsive Liposome. MMP-2-cleavable peptide was doped to compose the corresponding liposome. Tersely, 3 mg lecithin, 0.5 mg cholesterol, 0.1mg 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- (methoxy(polyethylene glycol)-2000) (DSPE-PEG_{2000}), and 0.1 mg peptide-2 were firstly dissolved in 3 mL dichloromethane (DCM). Then, the solvent was removed by rotary evaporator to form phospholipid film. After that, 4 mL DI water was added into the film and the mixture was sonicated for about 20 mins to obtain a clear solution of liposome. For fluorescence visualization of the nanoparticle, 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N- (amino(polyethylene glycol)-2000) (DSPE-PEG_{2000}-NH_2) was doped to substitute part of DSPE-PEG_{2000}(1:1, m/m). Besides, Cy5.5-NHS ester was added to obtain Liposome-Cy5.5.

Preparation of MSN. To prepare MSN around the size of 100 nm, the procedure from the literature was adopted with slight modification. 20 g hexadecyl trimethyl ammonium chloride (CTAC) and 0.2 g triethanolamine (TEA) were dissolved in 200 mL deionized water for 1 h under intensive stirring at 90 ℃. Then, 15 mL precursor, TEOS, was added dropwise and the solution was stirred for another 1 h. The MSNs were collected with centrifugation, and the nanoparticles were washed with DI water and ethanol for 3 times, respectively. Finally, the surfactant was removed by refluxing the nanoparticles with a mixture of methanol/HCl (500:19, v/v) for 24 h. MSNs were collected by centrifugation and washed with ethanol for three times and kept in ethanol for later use.

Preparation of Amino-functionalized MSN (MSN-NH_2). The amino-functionalization was realized via APTES treatment. Briefly, MSNs were dispersed in ethanol at the concentration of 1 mg mL^{-1}, followed by the addition of APTES (1μL mL^{-1}), and the solution was refluxed for 48 h. Finally, the nanoparticles were centrifugated, washed with ethanol for three times and stored in ethanol at room temperature.

Preparation of ATRA@MSN. For ATRA loading, 40 mg ATRA was first dissolved in 2 mL of ethanol, and 200 mg MSN-NH_2 were dispersed in 8 mL ethanol, then the above-mentioned solutions were mixed and stirred for 48 h in dark. After that, the ATRA-loaded nanoparticles were collected by centrifugation, and the ATRA loading efficiency was confirmed by measuring the UV-vis absorption spectrum of the residual ATRA of the supernatant solution at the wavelength of 340 nm.

Preparation of MSN-aPD-1/ATRA@MSN-aPD-1. For aPD-1 loading, before adding aPD-1, the MSN-NH_2/ATRA@MSN was first dispersed in deionized water and cooled to 0 ℃, then different weight ratios of nanoparticle to aPD-1 were adopted (1:0.025/0.050/0.075) and the mixture was stirred gently for 6 h at 0 ℃. Finally, the nanoparticles were collected by centrifugation and redispersed in DI water. The aPD-1 loading efficiency was confirmed via aPD-1 ELISA kit. For fluorescence visualization, BSA-FITC (FTIC labeled bovine serum albumin) was substituted for aPD-1 to obtain the corresponding nanoparticles, and the nanoparticles were treated the same.

Detection of PD1 antibody loading efficiency on Nanoparticles. We quantitatively detected the PD1 antibody loading rate of nanoparticles by calculating the difference between the total added amount and the supernatant amount of PD1 antibody. The concentration of remaining PD1 antibodies in the supernatant was measured by a commercial ELISA kit (KALANG) according to the manufacturer's instructions.

Preparation of Liposome Coated MSN (MSN@Lipo). Corresponding MSN (ATRA@MSN-aPD-1/MSN-aPD-1/ATRA@MSN) was firstly dispersed in DI water or PBS at the

concentration of 10 mg mL^{-1}, and the solution was mixed with the liposome solution (10 mg mL^{-1}). The mixture was coextruded for 11 passes through a 200 nm porous polycarbonate membrane to obtain MSN@Lipo.

In Vitro Proteolysis of MMP-2-cleavable Peptide. Given that the hydrophobicity of the peptide containing C18 group, the hydrophilic one (peptide-1) was used to testify the proteolysis reaction. In brief, 2 mg peptide-1 was dissolved in 2 mL DI water, and 2 mL PBS containing 0.5 μg MMP-2 was added. The mixture was transferred into dialysis bag (MWCO=1000 Da), and incubated with shaking at 37 ℃. After 12 h the dialysate was collected for Liquid Chromatography tandem-Mass Spectrometry (LC-MS/MS) analysis.

In Vitro Drug Release Studies. To simulate the behavior of drug release of the nanoparticles in vitro, two different protocols were adopted. For the simulation of macromolecular drug (aPD-1) release, BSA-FITC was used as the model drug for the convenience of the detection. Concisely, 2 mg MSN-BSA-FITC was dispersed in 2 mL PBS (pH 7.4 or 6.5, respectively). At pre-determined time intervals, 1.5 mL supernate was collected for analysis after centrifugation and equivalent corresponding buffers were supplemented. This process continued for totally 72 h. The amount of BAS-FITC released was monitored by fluorescence measurement (λ_{ex} 488 nm, λ_{em} 520 nm). For the simulation of the release of small molecular drug (ATRA), two experimental group was added, namely, the group pH 7.4+MMP-2 and pH 6.5+MMP-2. Specifically, 5 mg ATRA@MSN-BSA-FITC@Lipo was dispersed in corresponding buffers containing 1% Tween 80 and transferred into dialysis bags (MWCO=1000 Da). At predetermined time intervals, the dialysate was replaced with fresh PBS (pH 7.4 or 6.5) containing 1% Tween 80. The amount of ATRA released was monitored by US-vis spectrometry at 340 nm.

Testification of the biosafety of the MSN@Lipo. To verify the biocompatibility of the nanoparticles, 100 μL PBS or solution of MSN@Lipo (15 mg mL^{-1}) in PBS was intravenously injected into health mice, respectively. This process was conducted every 3

days, for totally 15 days (5 times of injections). After 27 days, the mice were sacrificed. The blood was collected for blood biochemistry and hematological parameter analysis, and the major organs (heart, liver, spleen, lung, and kidney) were excised and fixed in formalin for H&E staining and analysis.

In Vitro Assay of ATRA on MDSC Differentiation. Tumor-bearing mice were euthanized, and the spleen tissues were immediately harvested in a sterile condition and prepared into single cell suspensions, followed by Percoll (GE Healthcare) gradient centrifugation to isolate monocytes. $Ly6G^+$ cell populations were then isolated by myeloid-derived suppressor cell isolation kit and a LS MACS column according to the manufacturer's instructions (Miltenyi Biotec). Purified MDSC cells were used for *in vitro* experiments. Briefly, cells were inoculated in 12-well plates at a density of 2×10^6/well, and cultured in RPMI 1640 (10% FBS) medium contained 20 ng/mL recombinant murine GM-CSF (PeproTech), then ATRA or DMSO control was added to the cells, and after 48 hours, cells were collected for flow cytometry staining and analysis.

In Vitro assay for ATRA on Macrophage Polarization. RAW264.7 cells were inoculated in 12-well plates at a density of 2×10^6/well, and cultured in DMEM medium (10% FBS) contained recombinant murine IL-4 (20 ng mL^{-1}, PeproTech) or LPS (100 ng mL^{-1}, Sigma), and treated with 1 μmol/L ATRA or DMSO control for 48 hours. Cells were then collected for flow cytometry staining and analysis.

Flow Cytometry. Freshly isolated 4T1 and CT26 tumor tissues were immediately prepared into single cell suspensions: tumor samples were digested in a combined digestive solution (1 mg/ml collagenase D (Roche) + 0.1 mg ml^{-1} hyaluronidase (Biosharp) + 0.2 mg ml^{-1} DNase I (BIOFROXX) in RPMI 1640) at 37°C for 30 min after dissociation (gentleMACS Dissociator, Miltenyi Biotec). (B16F10 tumor does not need to go through the above steps for digestion). After filtration in a 70-μm strainer, the TILs (Tumor infiltrating lymphocytes) were then separated with Lymphoprep™ reagent (STEMCELL Technologies) according to the

manufacturer's protocol. TILs were incubated with cell surface antibodies in PBS buffer with 2% FBS for 60 min on ice after Fc receptors blocking. For intracellular staining, samples were first stained for surface markers as described above, then cells were stained with corresponding intracellular antibodies after the treatment with the Fixation & Permeabilization Kit (MULTI SCINENCE). Fixable Viability Dye eFluor™ 780 (eBioscience, 65-0865-14) was used to exclude dead cells. The stained cells were run on a flow cytometer (CytoFLEX S, Beckman Coulter), and data analysis were performed on FlowJo 10 (V10.0.6, Treestar).

ELISA. To quantify cytokines, fresh tumor tissues were added to PBS (1mg tissue/100 uL PBS) and gently ground into tissue homogenate, after concentration, the supernatants were collected and then quantified protein concentration for each specimen by BCA protein assay kit (Beyotime). Concentration of different cytokines (INF-γ, TNF-α and Granzyme-B) was assessed by ELISA kits (4A Biotech) and the absorbance was measured at 450 nm by BioTek plate reader (BioTek).

Statistics. Statistical analyses were performed using GraphPad Prism v.7.0. Two-tailed unpaired Student's t-test or one-way ANOVA test was carried out to determine the statistical significance of difference between two groups or multiple-group comparisons. Unless specifically noted, all data are shown as mean ± SEM, and $P < 0.05$ was considered statistically significant.

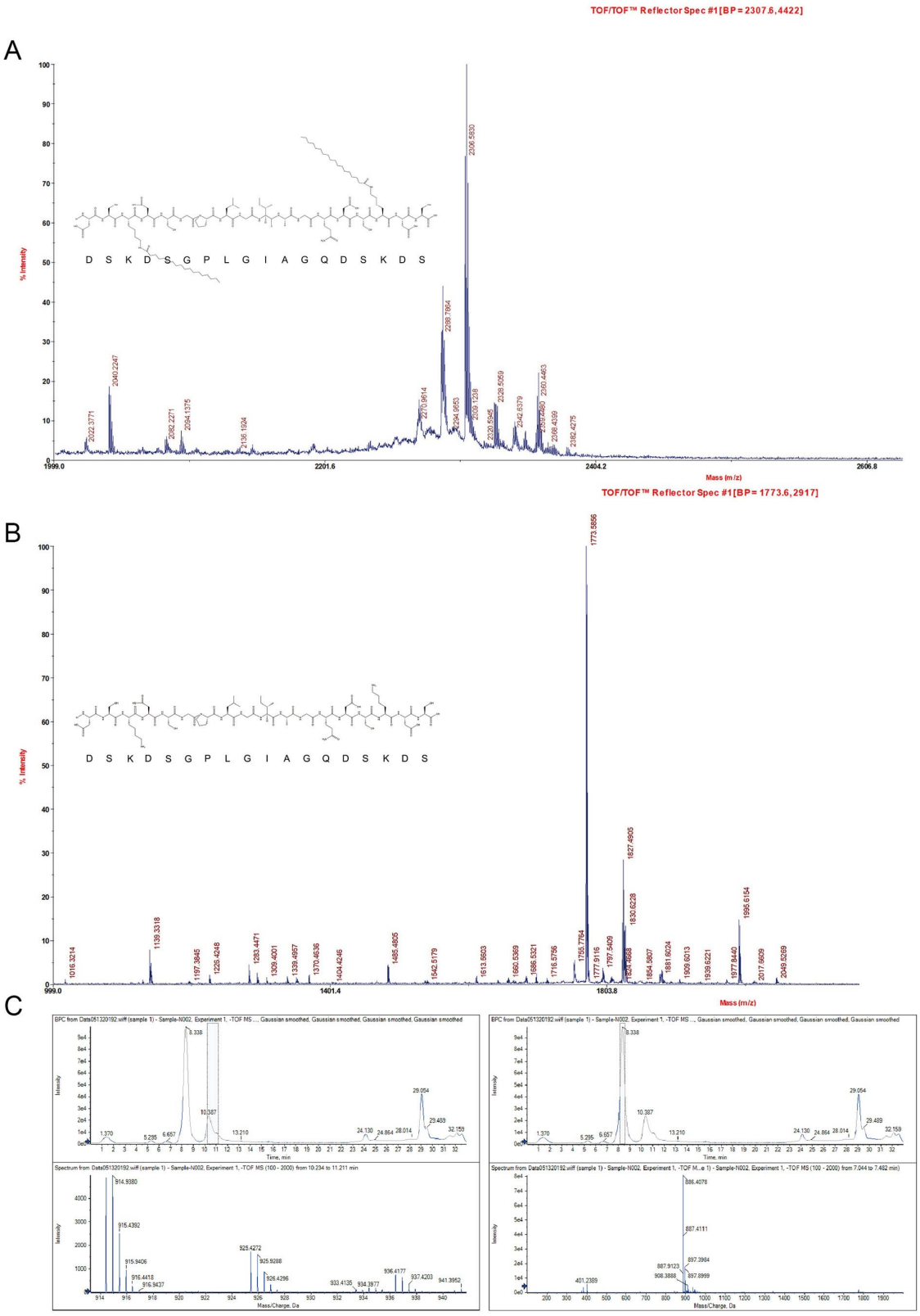

Fig. S1. Mass spectra of peptides before and after proteolysis by MMP-2. (A) Representative MALDI-TOF-MS result of peptide containing C18 groups, peptide-1. (B)

Representative MALDI-TOF-MS result of peptide not containing C18 groups, peptide-2. (C and D) LC-MSMS results of proteolysis of peptide-2 by MMP-2.

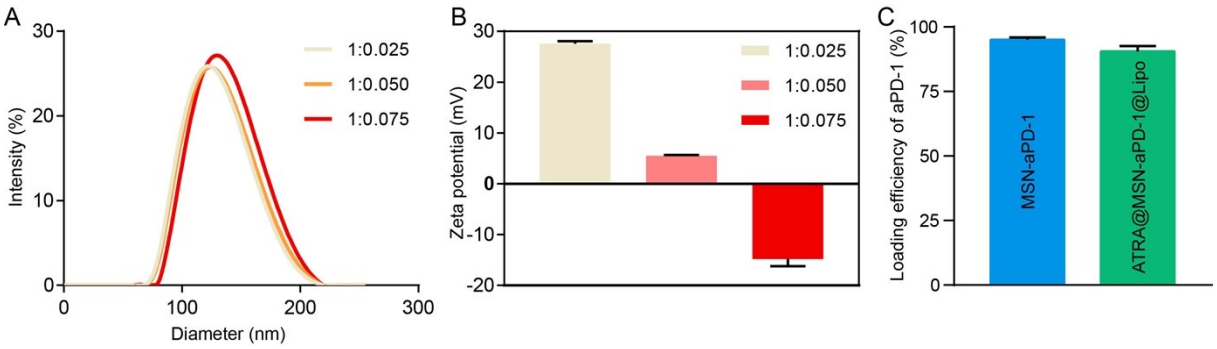

Fig. S2. Effects of weight ratios of MSN/aPD-1 on nanoparticle size distribution and zeta potential. (A) DLS size data of different nanoparticles. (B) Zeta potential data of corresponding nanoparticles. (C) Absorption efficiency of the aPD-1 onto the MSN core nanoparticles and the adsorption efficiency of the aPD-1 loaded into ATRA@MSN-aPD-1@Lipo. Datas of absorption efficiency were assessed by ELISA.

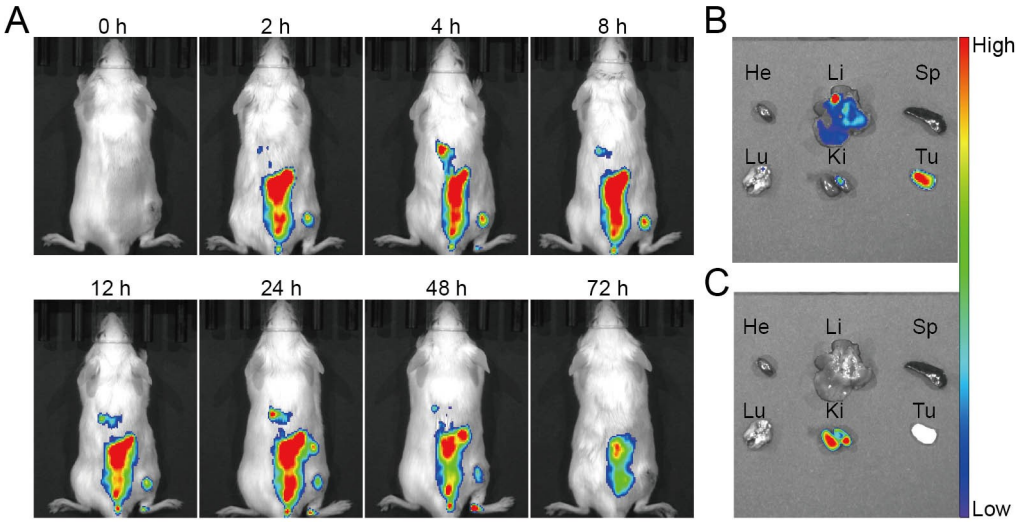

Fig. S3. In vivo and ex vivo fluorescence images of 4T1 tumor model. (A) Representative in vivo fluorescence images of 4T1 tumor-bearing mice treated with MSN-BSA-FITC@Lipo-Cy5.5 at different time points. (B and C) Representative ex vivo fluorescence images of tumors and other major organs from CT26 tumor-bearing mice treated with MSN-BSA-

FITC@Lipo-Cy5.5 after 72 h of injection. (B) Fluorescence images of FITC. (C) Fluorescence images of Cy5.5.

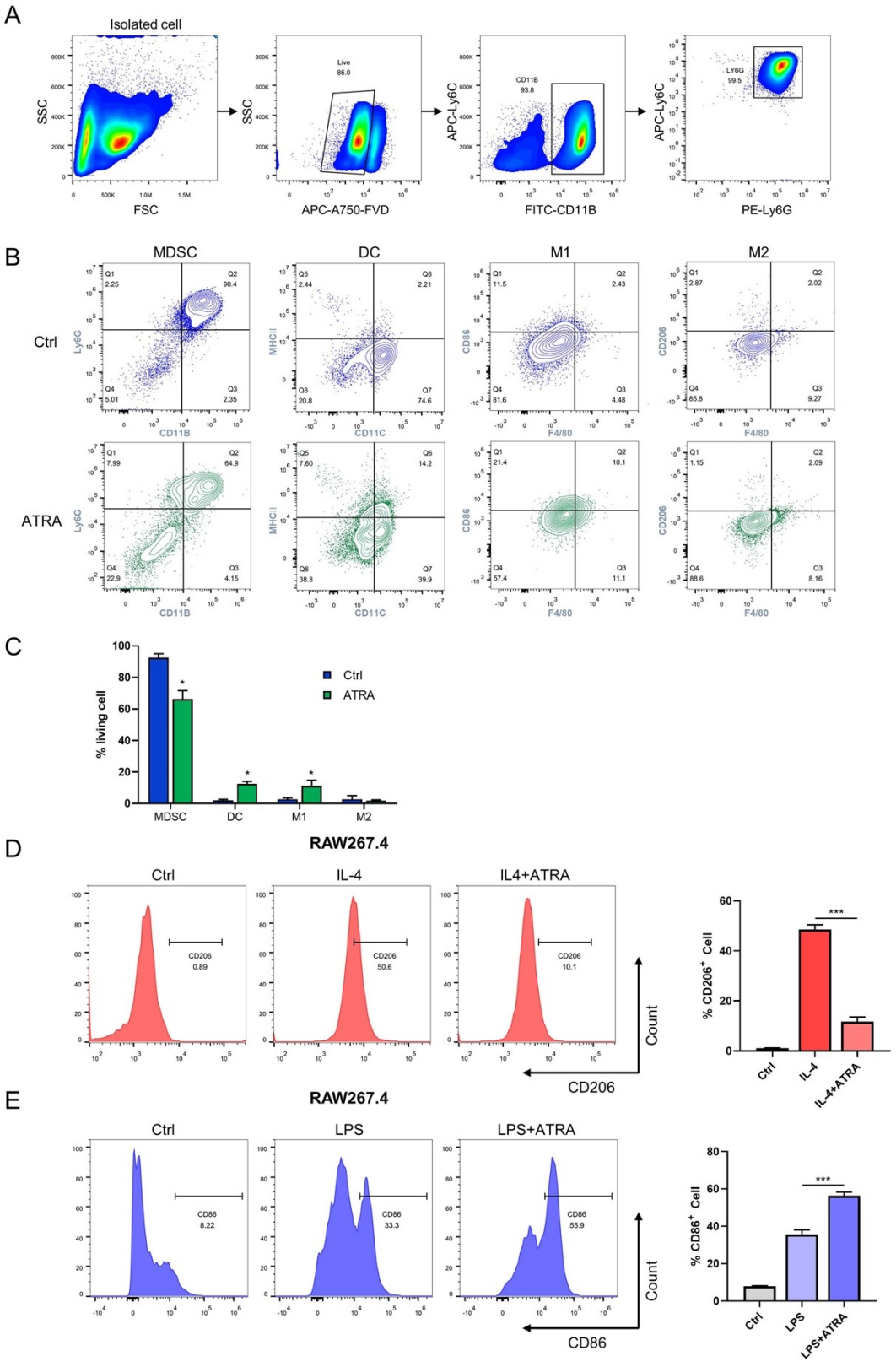

Fig. S4. The effect of ATRA on MDSCs differentiation and M1 and M2 macrophages polarization. (A) Flow cytometry dot plots show the purity of isolated MDSCs from tumor bearing mice spleen. (B and C) Representative FACS plots and quantification analysis show the population change of MDSC, DC, and macrophages after ATRA treatment. (D) Flow cytometry analysis show ATRA inhibited IL-4 induced M2 polarization determined by $CD206^+$ cells percentage. n=6, ***$p < 0.001$. (E) Flow cytometry analysis show ATRA improved LPS induced M1 polarization determined by $CD86^+$ cells percentage. n=6, ***$p < 0.001$.

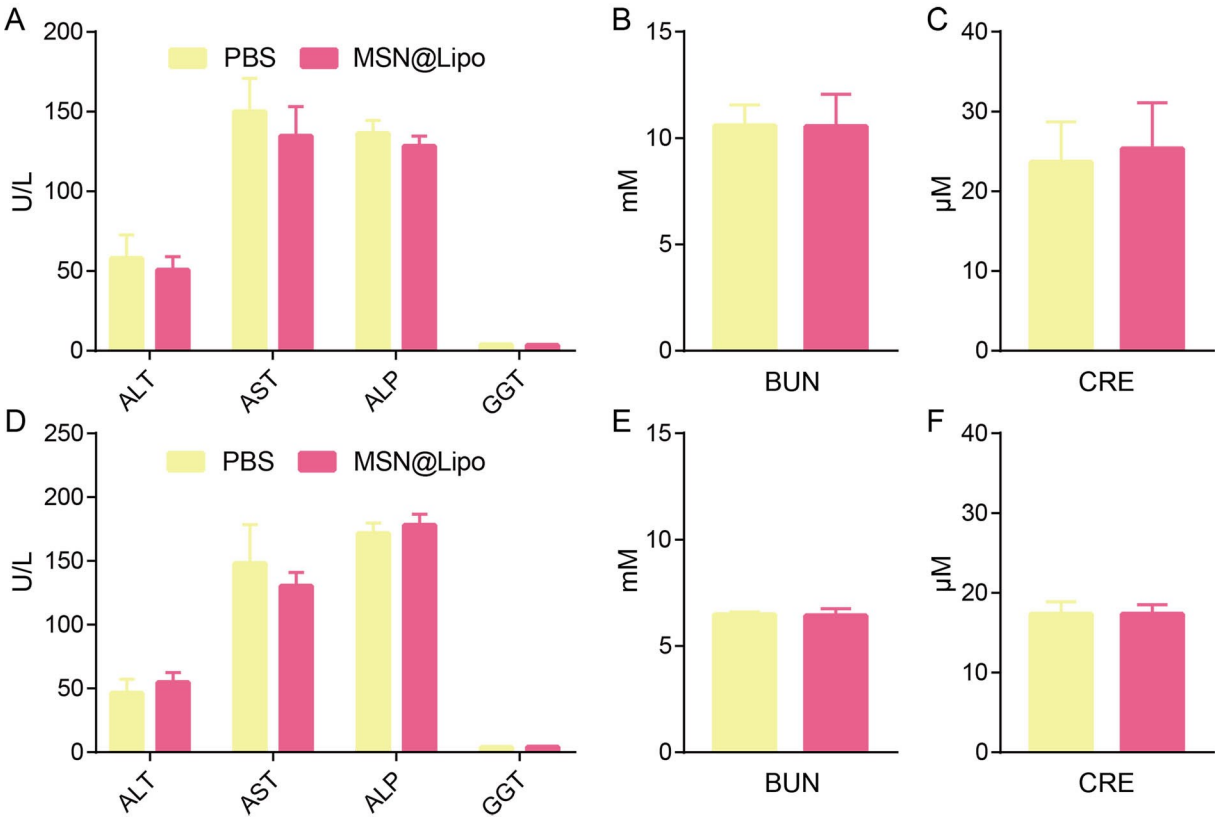

Fig. S5. Blood biochemistry data of liver and kidney function markers. Alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (ALP), glutamyl transpeptidase (GGT), blood urea nitrogen (BUN) and creatine (CRE). (A to C) Blood biochemistry data of BALB/c in different groups. (D to F) Blood biochemistry data of C57BL/6 in different groups.

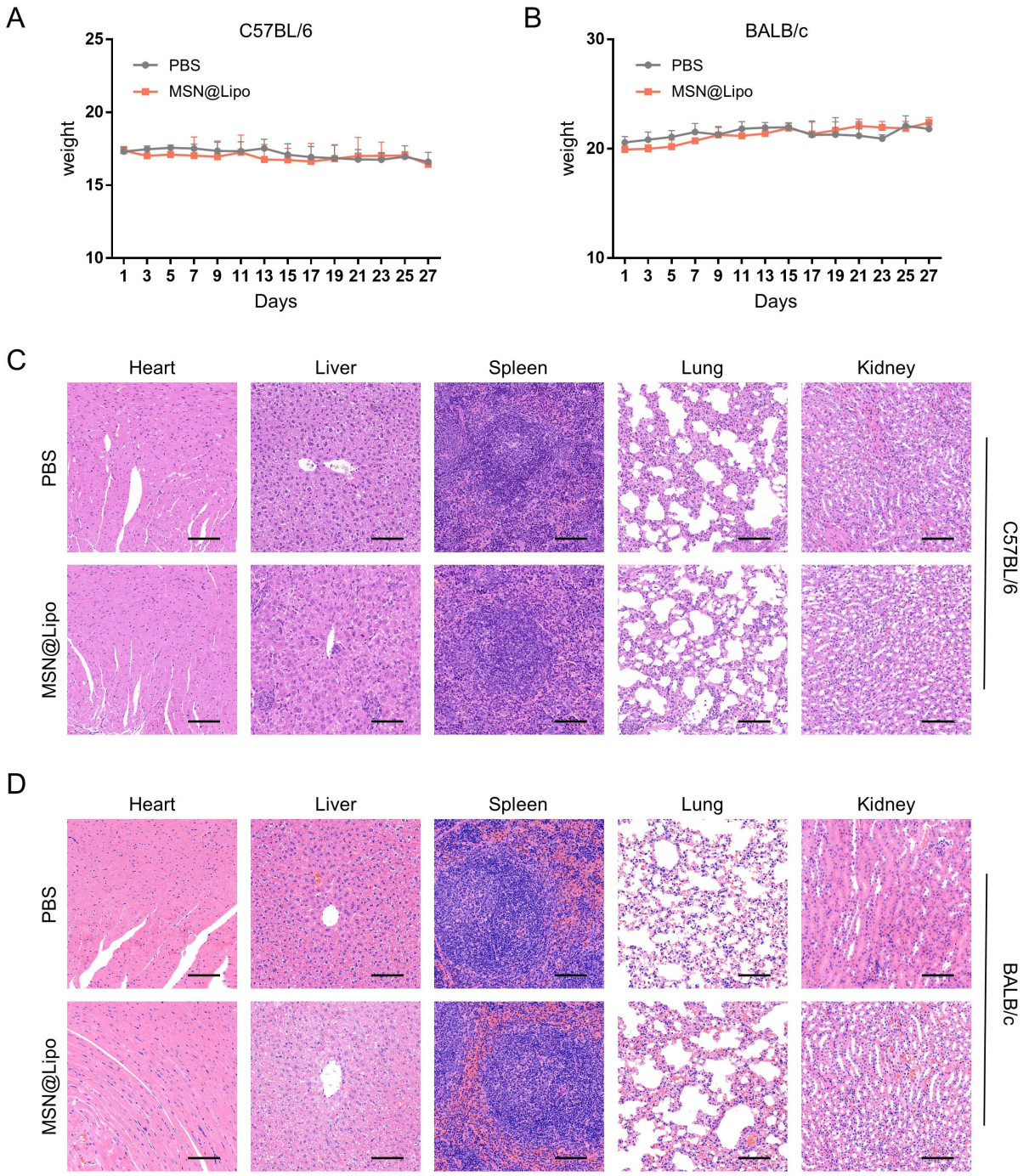

Fig. S6. Body weight curves and H&E staining of major organs. (A) Body weight curves of C57BL/6 in different groups. (B) Body weight curves of BALB/c in different groups. (C) H&E staining of hearts, livers, spleens, lungs and kidneys of C57BL/6 in different groups. Scale bar = 50 μm. (D) H&E staining of hearts, livers, spleens, lungs and kidneys of BALB/c in different groups. Scale bar: 50 μm.

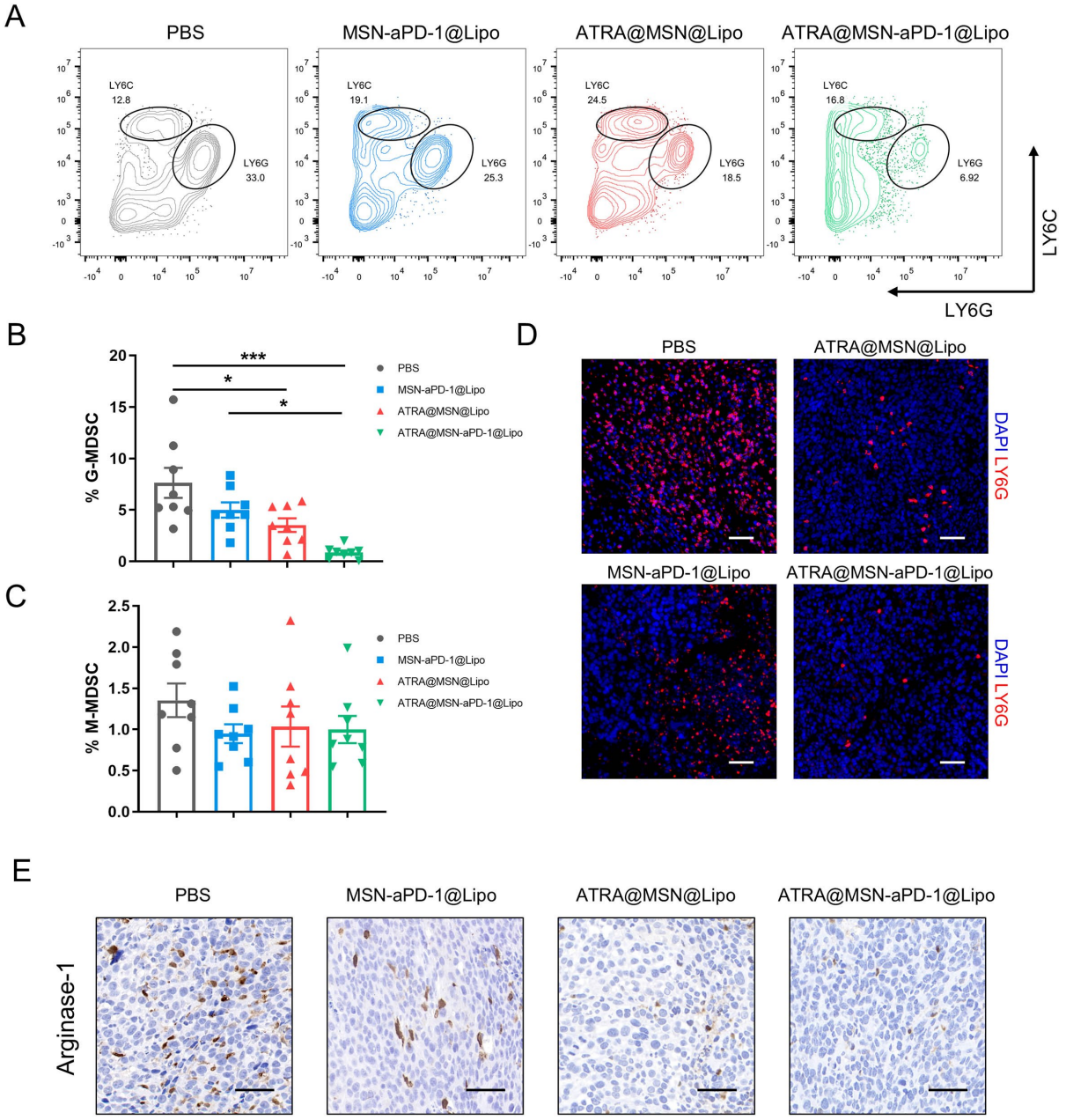

Fig. S7. ATRA@MSN-aPD-1@Lipo treatment significantly reduced the recruitment of MDSCs and suppressed the expression of arginase-1 in 4T1 tumor. (A to C) Representative FACS plots and quantification analysis for $LY6G^{-}LY6C^{high}$ (M-MDSC) and $LY6G^{+}LY6C^{low}$ (G-MDSC) cells on $CD11b^{+}$ gated cells in the 4T1 TILs (n=8, $*p < 0.05$, $***p < 0.001$). (D) Representative immunofluorescence staining images of Ly6G (red) and DAPI (blue) in four groups of tumor tissue. (E) Representative IHC staining images of Arginase-1 in tumor tissue. Scale bar: 50 μm.

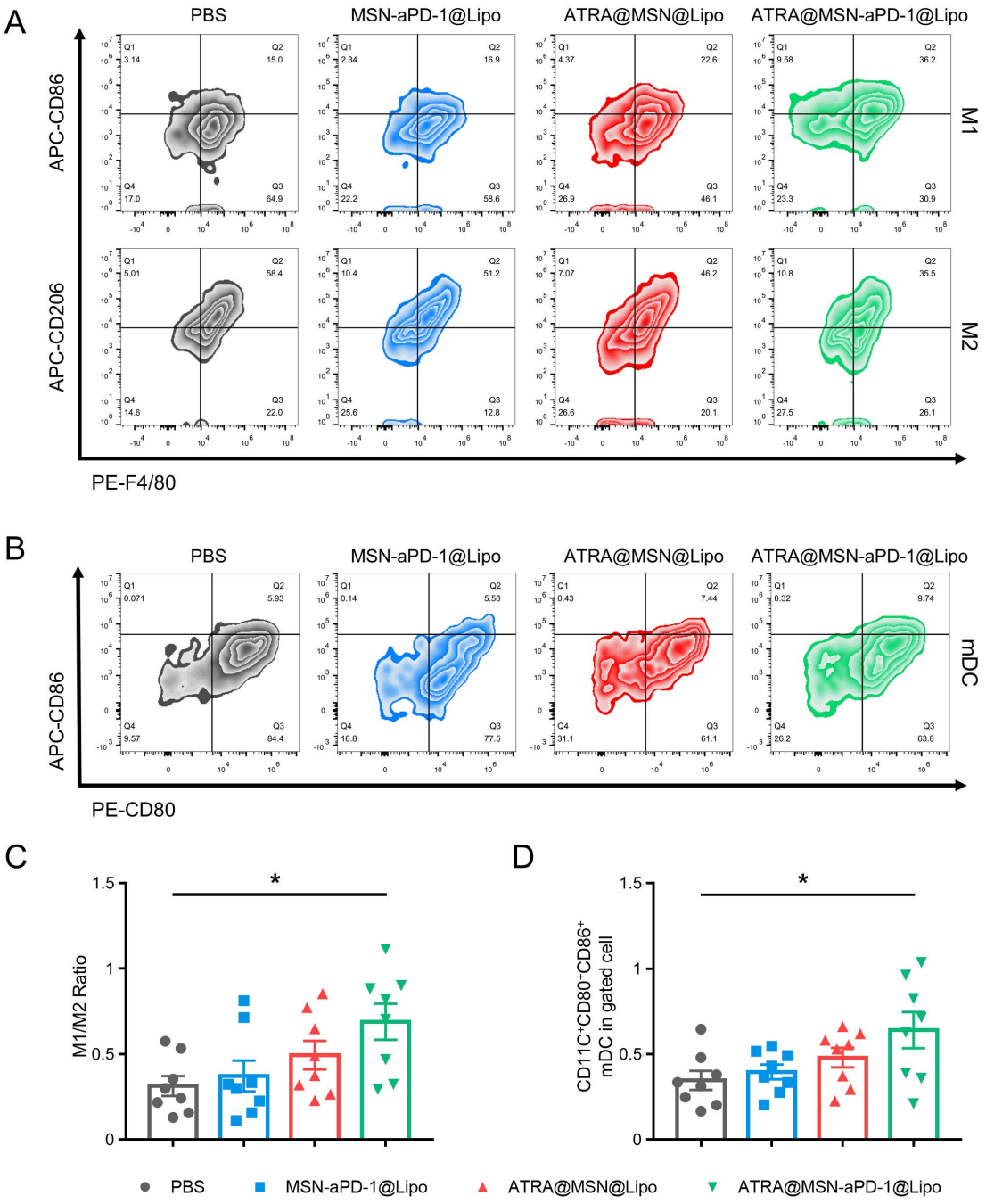

Fig. S8. The effects of ATRA@MSN-aPD-1@Lipo treatment on M1, M2 macrophage population and DC cell maturation in 4T1 tumor. (A) Representative FACS plots of $F4/80^+CD86^+$ M1-like macrophages and $F4/80^+CD206^+$ M2-like macrophages populations on $CD11b^+$ gated cells in 4T1 TILs. (B) Representative flow cytometry dot plots for $CD80^+CD86^+$ matured DCs on $CD11c^+$ gated cells in 4T1 tumor. (C and D) Quantitative analysis of the ratio of M1/M2 macrophages and $CD11C^+CD80^+CD86^+$ matured DCs populations in 4T1 TILs (n=8, $*p < 0.05$).

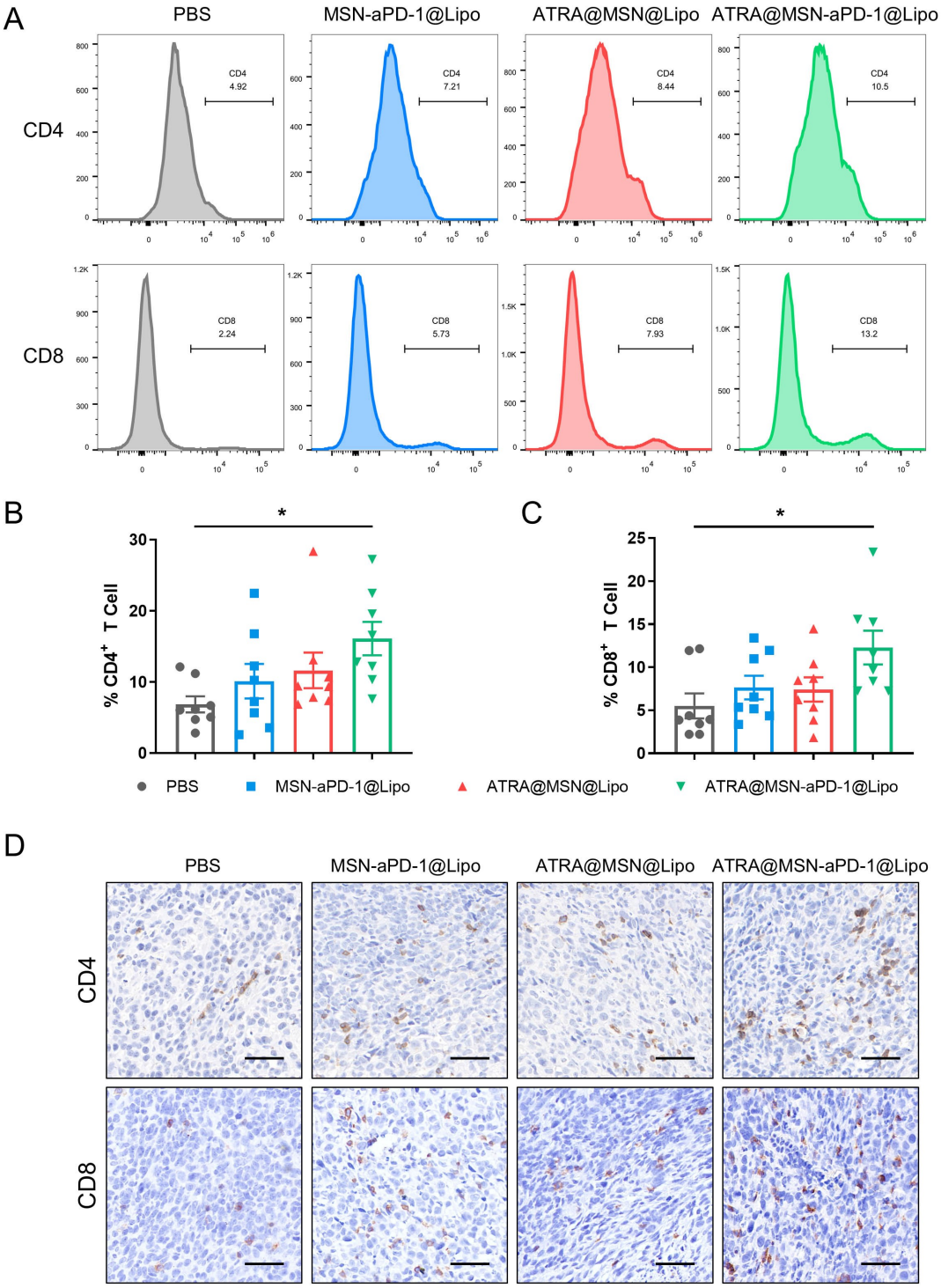

Fig. S9. ATRA@MSN-aPD-1@Lipo treatment enhanced the tumor infiltration of both CD4$^+$ and CD8$^+$ T cells in 4T1 tumor. (A) Flow cytometry analysis of CD4$^+$ and CD8$^+$ T cells populations in 4T1 TILs. (B and C) Quantification analysis of CD4$^+$ and CD8$^+$ T cells in TILs (*$p < 0.05$). (D) Representative IHC staining images of CD4 and CD8 in four groups of tumor tissue. Scale bar: 50 μm.

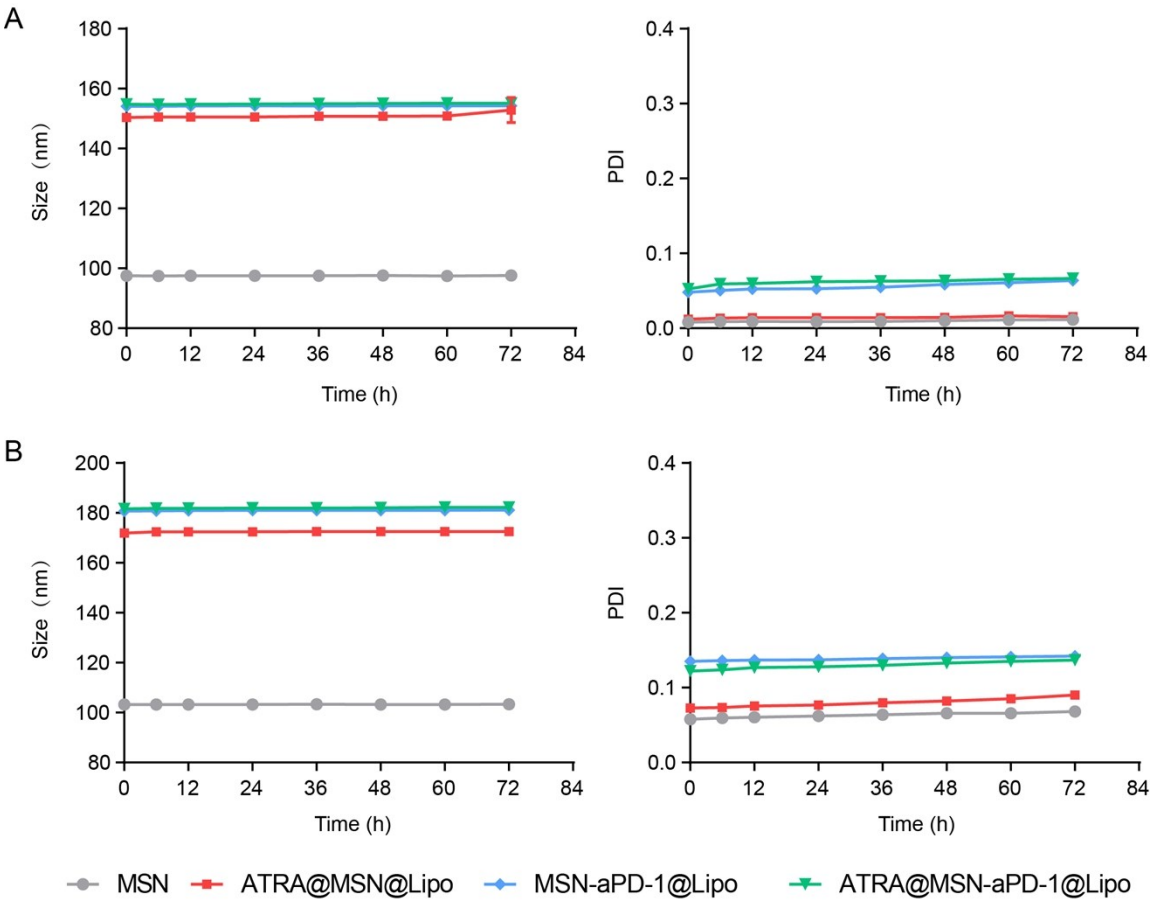

Fig. S10. Characterization of stability. The size and PDI of different nanoparticles in (A) DI water, (B) PBS.

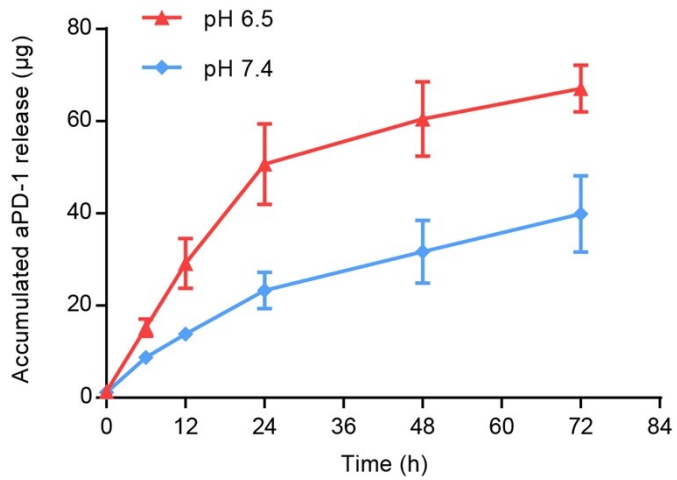

Fig. S11. The release behavior of aPD-1 from MSN-aPD-1. The release of aPD-1 was detected with aPD-1 ELISA kit.

Table. S1. The hematological parameters of BALB/c (up) and C57BL/6 (down) mice

Markers	PBS	MSN@Lipo
WBC(10^9/L)	5.7 ± 0.4	5.4 ± 0.4
RBC(10^{12}/L)	8.6 ± 0.4	7.5 ± 0.7
HGB(g/L)	126 ± 4	119 ± 2.1
HCT(%)	47.8 ± 3.6	43.8 ± 1.0
MCHC(g/L)	265 ± 12	278 ± 5
PLT(10^9/L)	678 ± 87	625 ± 190

Markers	PBS	MSN@Lipo
WBC(10^9/L)	7.6 ± 1.6	7.5 ± 0.8
RBC(10^{12}/L)	9.9 ± 1.3	8.3 ± 0.1
HGB(g/L)	134 ± 17	117 ± 13
HCT(%)	50.4 ± 8.2	38.6 ± 0.5
MCHC(g/L)	267 ± 13	277 ± 4
PLT(10^9/L)	720 ± 109	766 ± 108