Supplementary Information for:

Manganese-mineralized cancer cells as immunogenic cancer vaccines for tumor immunotherapy

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Experimental section

1. Materials

All chemicals and reagents used were at least of analytical grade. Ultrapure water was obtained using Heal Force Smart N-II Water Purification Systems. $KMnO_4$, nitric acid, hydrochloric acid, glycine, glutaraldehyde, ethanol, agar, acetone, and lead citrate were purchased from Hushi (Shanghai). Osmic acid, Spurr resin, and uranyl acetate were purchased from SPI Supplies. Dulbecco's modified Eagle's medium (DMEM), RPMI1640 medium, and Fetal bovine serum (FBS) were purchased from Gibco. Phosphate-buffered saline (PBS), Penicillin-streptomycin solution, and 0.05% Trypsin-EDTA solution were purchased from Genom (Hangzhou). LPS, CCK8, 4% paraformaldehyde solution, Hoechst 33342, ACK lysis buffer, and western/IP cell lysis buffer were purchased from Beyotime (Shanghai). MnO_2 nanoparticle was purchased from DekeDaojin (Beijing). Cytokine GM-CSF and IL4 were purchased from PeproTech. CellTracker Green CMFDA and Dylight594 NHS Ester were purchased from ThermoFisher. β-mercaptoethanol, collagenase, and DNase I were purchased from Sigma-Aldrich. Total Cellular RNA Extraction Kit was purchased from TIANGEN (Beijing). One Step TB Green PrimeScript RT-PCR Kit II was purchased from TaKaRa. Percoll was purchased from Cytiva. Mouse lymphocyte separation medium was purchased from Dakewe (Shenzhen).

2. Antibody

TruStain FcX PLUS anti-mouse CD16/32, Clone:S17011E (BioLegend); APC/Cyanine7 anti-mouse/human CD11b, Clone:M1/70 (BioLegend); PE-Cyanine7 anti-Mo CD11c, Clone:N418 (eBioscience); PE anti-Mouse CD40, Clone:1C10 (eBioscience); FITC anti-mouse CD40, Clone:HM-40-3 (BioLegend); APC anti-mouse CD80, Clone:16-10A1 (BioLegend); APC/Cyanine7 anti-mouse CD86, Clone:GL-1 (BioLegend); PE/Cyanine7 anti-mouse CD86, Clone:GL-1 (BioLegend); FITC Anti-Mouse CD45, Clone:30-F11 (BD Pharmingen); APC/Cyanine7 anti-mouse CD3,

Clone:17A2 (BioLegend); PerCP-Cy5.5 Anti-Mouse CD4, Clone:Rm4-5 (BD Pharmingen); PE-Cy7 Anti-Mouse CD8a, Clone:53-6.7 (BD Pharmingen); PE anti-Mo MHC Class I (H-2Kb), Clone:AF6-88.5.5.3 (eBioscience); FITC anti-mouse I-A/I-E, Clone:M5/114.15.2 (BioLegend); T-Select H-2Kb OVA Tetramer-SIINFEKL-APC (MBL); APC anti-mouse H-2Kb bound to SIINFEKL, Clone:25-D1.16 (BioLegend).

3. Cell Culture

Mouse melanoma cell line B16F10 and macrophage cell line RAW264.7 were provided by the National Collection of Authenticated Cell Cultures (Shanghai). Stable B16F10 cell line overexpressing ovalbumin was constructed by Genomeditech (Shanghai) via lentivirus transfection. Cell lines were cultured in complete DMEM containing 10% FBS and 100 U/mL penicillin 100 ug/mL streptomycin at 37 °C and 5% CO_2. Trypsin-EDTA was used to collect cells. Bone marrow-derived dendritic cells (BMDCs) were differentiated from primary bone marrow cells. 8-week-old C57BL/6 mice were sacrificed by cervical dislocation, soaked in 75% alcohol for 10 minutes, and then placed on a clean bench. The femur and tibia were extracted and the attached tissue was removed and both ends of the bones were sheared after LPS cleaning. Bone marrow cells were rinsed from the bone canal using the RPMI1640 medium. The collected cells were treated with ACK lysis buffer and cultured in complete RPMI1640 medium containing 10 ug/mL IL-4, 20 ug/mL GM-CSF, 10% FBS, and 100 U/mL penicillin 100 ug/mL streptomycin at 37 °C and 5% CO_2. Bone marrow cells extracted from each mouse were cultured in a six-well cell culture plate with 3 mL medium added to each well. After 2 days, half the medium in every well was replaced. After another 2 days, the entire medium was replaced and the suspended cells were discarded. Then replace the medium every two days. The adherent cells after day 7 were BMDCs.

4. Preparation of B16F10@MnO_2

$KMnO_4$ was dissolved in ultrapure water to form a 10 mM solution. The collected suspended B16F10 cells were washed twice with PBS and counted. The $KMnO_4$

solution was diluted to the desired concentration with PBS. Cells were added to the diluted $KMnO_4$ solution in quantities of 5×10^6/mL and fully suspended. The B16F10@MnO_2 was generated after leaving for 30 minutes at room temperature. The generated B16F10@MnO_2 was centrifuged at 1000 g for 3 minutes, washed twice with PBS, and finally suspended in PBS or DMEM.

5. Cell viability test

To test the cell's proliferation viability, B16F10 or B16F10@MnO_2 were added to 96-well cell culture plates in 1×10^6/well. CCK8 was used immediately and cells were cultured for 2 hours. The absorption value of the sample at 450 nm wavelength for each well was measured using a BioTek Synergy H1 microplate reader. The cell viability of the sample in each well was calculated by the equation: Viability = $[(OD_{sample} - OD_{blank}) / (OD_{control} - OD_{blank})]$.

6. XPS and XRD

B16F10@MnO_2 was lysed in Western/IP cell lysis buffer and centrifuged at 1000 g for 3 minutes. The sediment was washed three times with ultra-pure water and frozen in liquid nitrogen, then dried under vacuum for 48 hours. Untreated B16F10 cells used as the control group were directly frozen in liquid nitrogen and dried under vacuum for 24 hours. The remaining dried samples were ground into a uniform powder using a smooth ceramic mortar. ESCALAB 250Xi X-ray Photoelectron Spectrometer Microprobe was used to determine XPS data of C and Mn for samples. Bruker D8 ADVANCE was used to determine the XRD data. The samples were scanned in the instrument from 3 ° to 70 °. The XPS result was compared with the NIST database (https://icsd.nist.gov).

7. TGA

The B16F10@MnO_2 or blank control B16F10 cells were washed twice with ultrapure water and then centrifuged. The cell suspensions were frozen in liquid nitrogen and dried under vacuum for 48 hours. The dried samples were ground into a uniform powder

using a smooth ceramic mortar. Mettler Toledo TGA2 Thermogravimetric Analyzer was used to determine the thermogravimetric analysis data. The samples were heated from room temperature to 800 °C in nitrogen at a rate of 10 °C per minute.

8. Critical point drying and SEM

The cell samples were first fixed with 2.5% glutaraldehyde in phosphate buffer for 12 hours and washed three times in the phosphate buffer, then fixed with 1% osmic acid in phosphate buffer for 2 hours and washed three times in the phosphate buffer and washed three times in the phosphate buffer for 15 minutes at each step. The samples were first dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%, 90%, and 95%) for about 15 minutes at each step, then dehydrated two times by alcohol for 20 minutes at each step. The samples in alcohol were dehydrated in a Hitachi Model HCP-2 critical point dryer. The dehydrated samples were coated with gold-palladium in Hitachi Model E-1010 ion sputter for 5 minutes, then observed in GeminiSEM300.

9. Cell slicing and TEM

The cell samples were first fixed with 2.5% glutaraldehyde in phosphate buffer for 4 hours and washed three times in the phosphate buffer, then embedded in hot agar. The cooled solidified agars the cells embedded in were cut into 2~5 mm pieces. The agar pieces with cells were fixed with 1% osmic acid in phosphate buffer for 2 hours and washed three times in the phosphate buffer and washed three times in the phosphate buffer for 15 minutes at each step. The agar pieces were first dehydrated by a graded series of ethanol (30%, 50%, 70%, 80%) and then dehydrated by a graded series of acetone (90%, 95%) for about 15 minutes at each step. In the end, they were dehydrated twice by absolute acetone for 20 minutes respectively. The agar pieces with cells were placed in a 1:1 mixture of absolute acetone and the final Spurr resin mixture for 1 hour at room temperature, then transferred to a 1:3 mixture of absolute acetone and the final resin mixture for 3 hours and to the final Spurr resin mixture for overnight. The agar pieces with cells in Spurr resin were heated at 70 °C for 12 hours. The samples were

sliced in LEICA UC7 ultramicrotome and sections were stained by uranyl acetate and alkaline lead citrate for 10 minutes respectively and observed in JEM-1400Flash TEM. Untreated cells were fixed with 2.5% glutaraldehyde and washed in water, then settled on carbon films supported copper grid and observed in the TEM.

10. *In vitro* cells co-culture

2 mL complete medium containing 2×10^6 RAW264.7 was added to each well of 6-well cell culture plates. After two hours of culture, the cells were attached to the wall. Tumor cells or B16F10@MnO_2 were added to the cell culture plate of RAW264.7 or BMDCs. The fixed tumor cells were obtained by being treated with 4% paraformaldehyde fixative solution for 30 min and then washed twice with PBS solution added with 3 mM glycine. Then the cells were cultured in complete DMEM at 37 °C and 5% CO_2. After 24 hours, cells were collected for flow cytometry or mRNA extraction.

11. Flow cytometry

Co-culture cells were rinsed with PBS from the plate and were divided into several parts as needed. FcX was added to each part of the cells, followed by the required fluorescent antibody. The sample was placed in a dark place for 15 minutes and then washed twice with PBS, and then measured by CytoFLEX LX. In experiments with fluorescently labeled cells, stain RAW264.7 or BMDCs with CellTracker Green CMFDA and stain B16F10@MnO_2 with Dylight594 NHS Ester and Hoechst 33342 before co-culture.

12. mRNA analysis

The co-cultured adherent macrophage was washed with PBS in the plate to remove the remaining dead cells. The mRNA was exacted using the Total Cellular RNA Extraction Kit according to the manufacturer's protocol. The obtained mRNA solution was stored at -80 °C. The qPCR reaction solution was prepared using One Step TB Green PrimeScript RT-PCR Kit II according to the manufacturer's protocol and determined

using Bio-Rad CFX96 according to the recommended reaction procedure. The relative abundance of mRNA was normalized to GAPDH. Primers for qPCR are included in [Table. S1].

13. Lymph node immune cell analysis

1×10^6 B16F10@MnO_2 or formaldehyde-fixed cells were injected subcutaneously into the right side of the abdomen of each 8-week-old C57BL/6 mouse. After 7 days, the mice were killed by cervical dislocation, and lymph nodes on the right side of the abdomen were removed. Lymph nodes were cut and placed in DMEM with 2% FBS, collagenase IV, and DNase I, and then digested at 37 °C for 30 minutes. The lymph node fragments were ground on 40 μm cell sieves. The cell suspension obtained by filtration was collected and centrifuged, and then the cells were washed with PBS. The cells were labeled with fluorescent antibodies and detected by flow cytometry.

14. Anti-tumor efficacy in mouse tumor models

Tumor models were constructed by subcutaneous injection of 5×10^5 B16F10 cells into each 8-week-old C57BL/6 mouse. Then the mice were randomly divided into four groups. The four groups were respectively treated by injection of PBS, formaldehyde-fixed B16F10 cells, and B16F10@MnO_2 prepared with 0.5 mM or 1.0 mM $KMnO_4$. We injected 1×10^6 treated cells at the tumor site 5 times according to the schedule as intratumoral injection treatment. The formaldehyde-fixed B16F10 cells were suspended in PBS and the B16F10@MnO_2 were suspended in the reaction supernatant of their preparation process. The longest diameter (L) and the shortest vertical transverse diameter (W) of solid tumors were measured with a vernier caliper, and the tumor volume was calculated by the equation: $\text{Volume} = L \times W^2 \times 0.5$. Record the time of death of the mice as the date of their natural death or tumor growth to a volume of more than 2 cm^3. For photographs of excised tumor and organ toxicity, on the 20th day after the injection of tumor cells, the mice were sacrificed by cervical dislocation. The tumors were photographed and organs were obtained for H&E staining. For cell

analysis, on the 30th day, the mice that had not died naturally were sacrificed, and the tumors and spleens were obtained. Tumors were cut and placed in DMEM with 2% FBS, collagenase IV, and DNase I, and then digested at 37 °C for 60 minutes. The Tumor fragments were ground on 40 μm cell sieves and the cell suspension obtained by filtration was collected and centrifuged. Then the cells were suspended in PBS containing 70% percoll and centrifuged at 500 g for 5 minutes. cells floating on top of the liquid and supernatant were discarded after centrifugation. The pelleted cells were treated with ACK lysis buffer, and centrifuged again to discard the supernatant. Spleens were cut and placed in DMEM with 2% FBS, then ground on 40 μm cell sieves. The cell suspension obtained by filtration was collected and centrifuged. The cells obtained by centrifugation were treated with the mouse lymphocyte separation medium, and then the separated lymphocytes were washed with DMEM. Then the obtained cells were treated with ACK lysis buffer, and centrifuged again to discard the supernatant. The cells were suspended in PBS and labeled with fluorescent antibodies, then detected by flow cytometry.

15. Tissue paraffin section staining

The organs were fixed with 4% paraformaldehyde for 3 days, then transferred to 75% and 95% ethanol, and finally dehydrated using Leica ASP300S Fully Enclosed Tissue Processor. The dehydrated samples were embedded in paraffin using Modular Tissue Embedding Center EC 350 and the sample paraffin blocks were sliced using Leica RM2235 rotary microtome. and organ samples were stained using hematoxylin and eosin. The stained samples were photographed using OLYMPUS SLIDEVIEW VS200.

SUPPLEMENTARY FIGURES

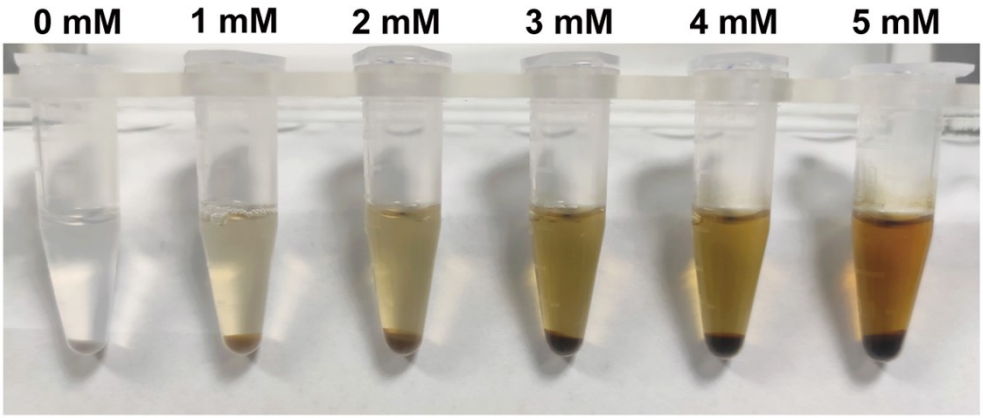

Fig. S1 Products of the reaction of B16F10 cells with $KMnO_4$ solution of different concentrations.

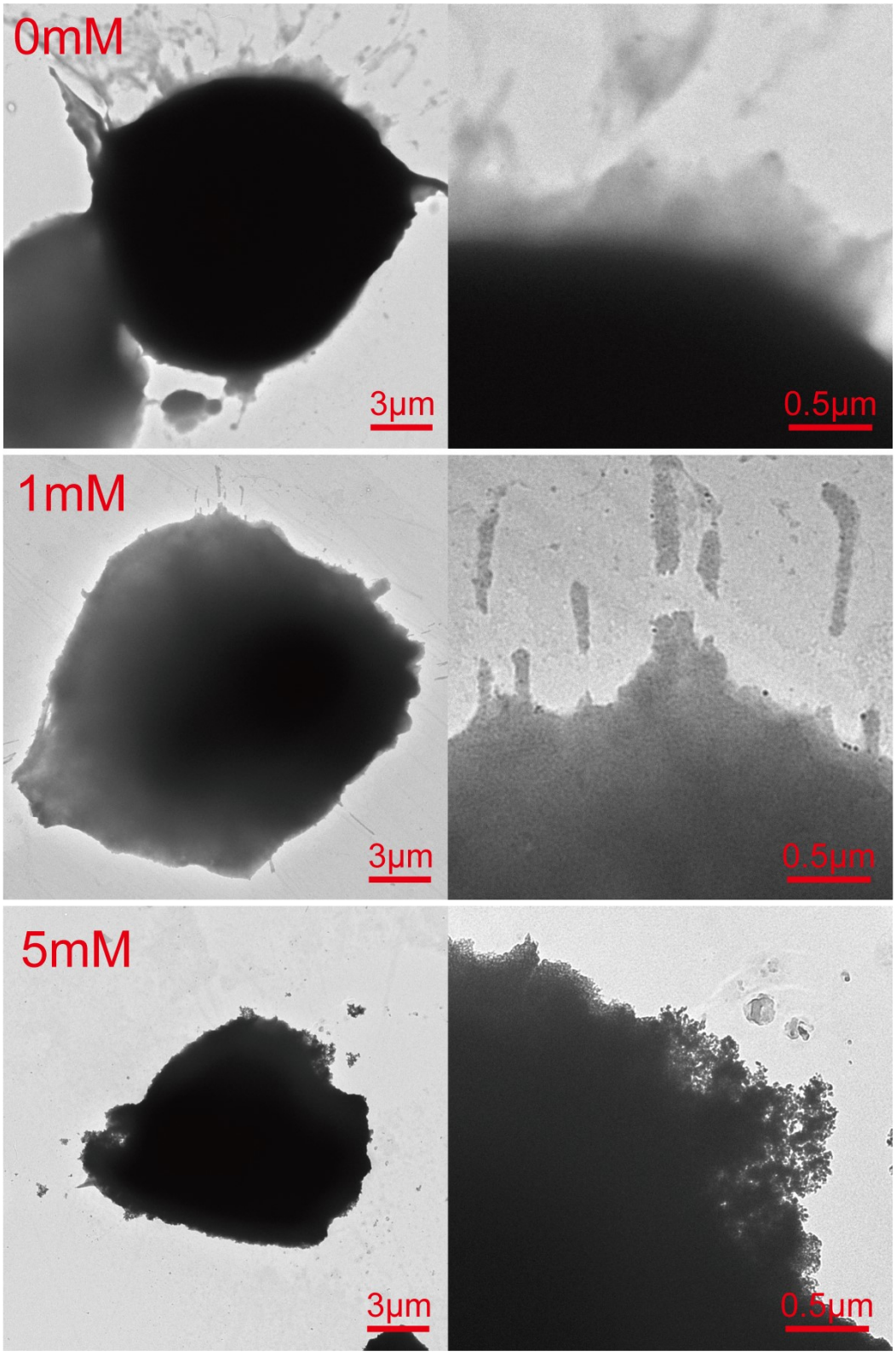

Fig. S2 TEM images of B16F10 cells treated with solutions of different concentrations.

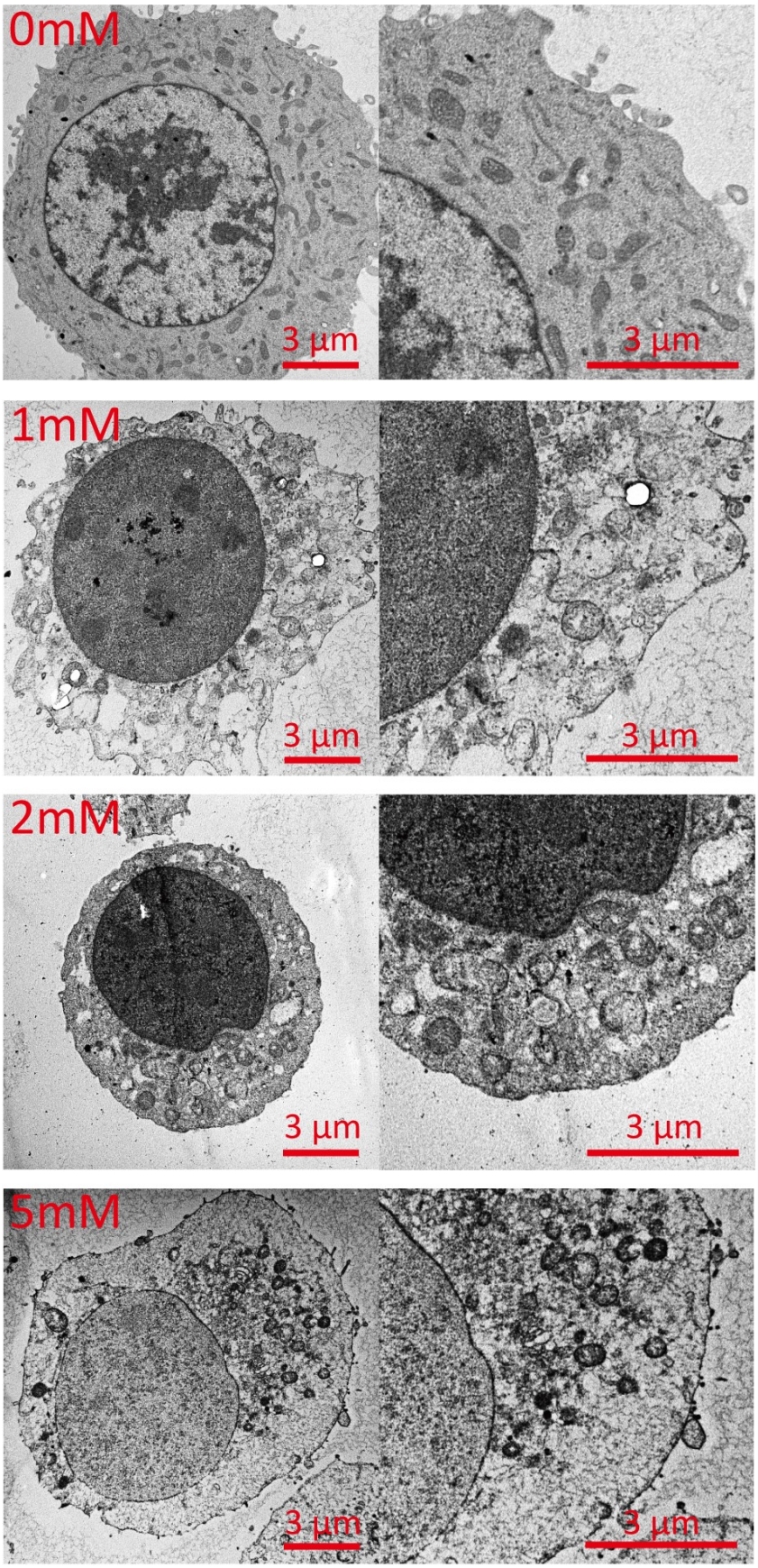

Fig. S3 TEM images of slices B16F10 cells treated with solutions of different concentrations.

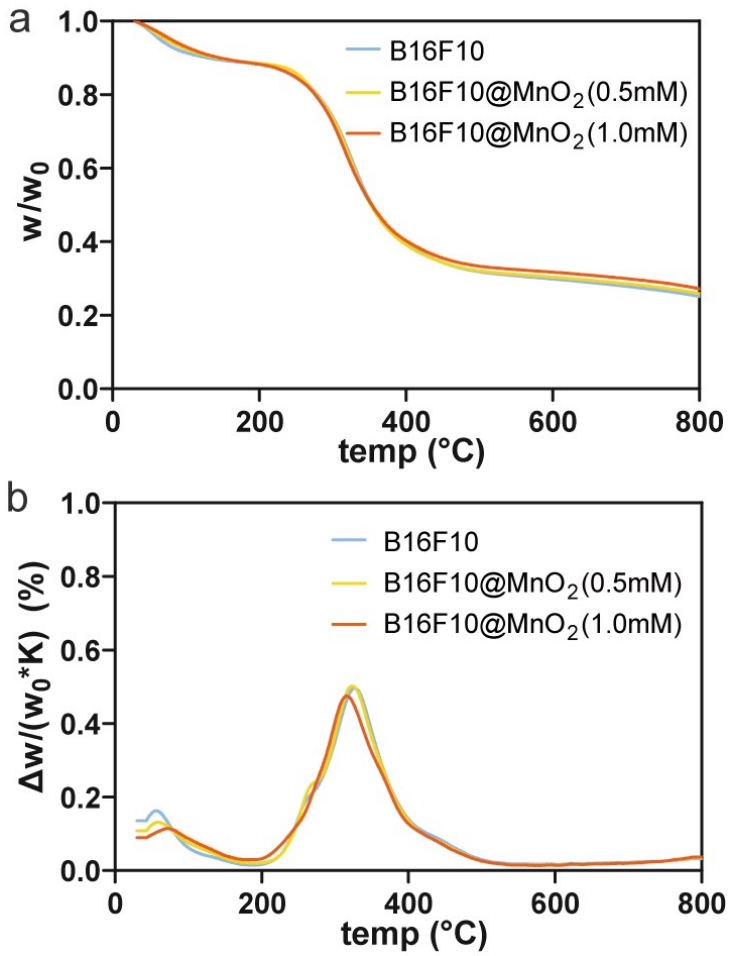

Fig. S4 (a) TG curve and (b) TGA curve of lyophilized B16F10 and B16F10@MnO_2 powder, and MnO_2 particles.

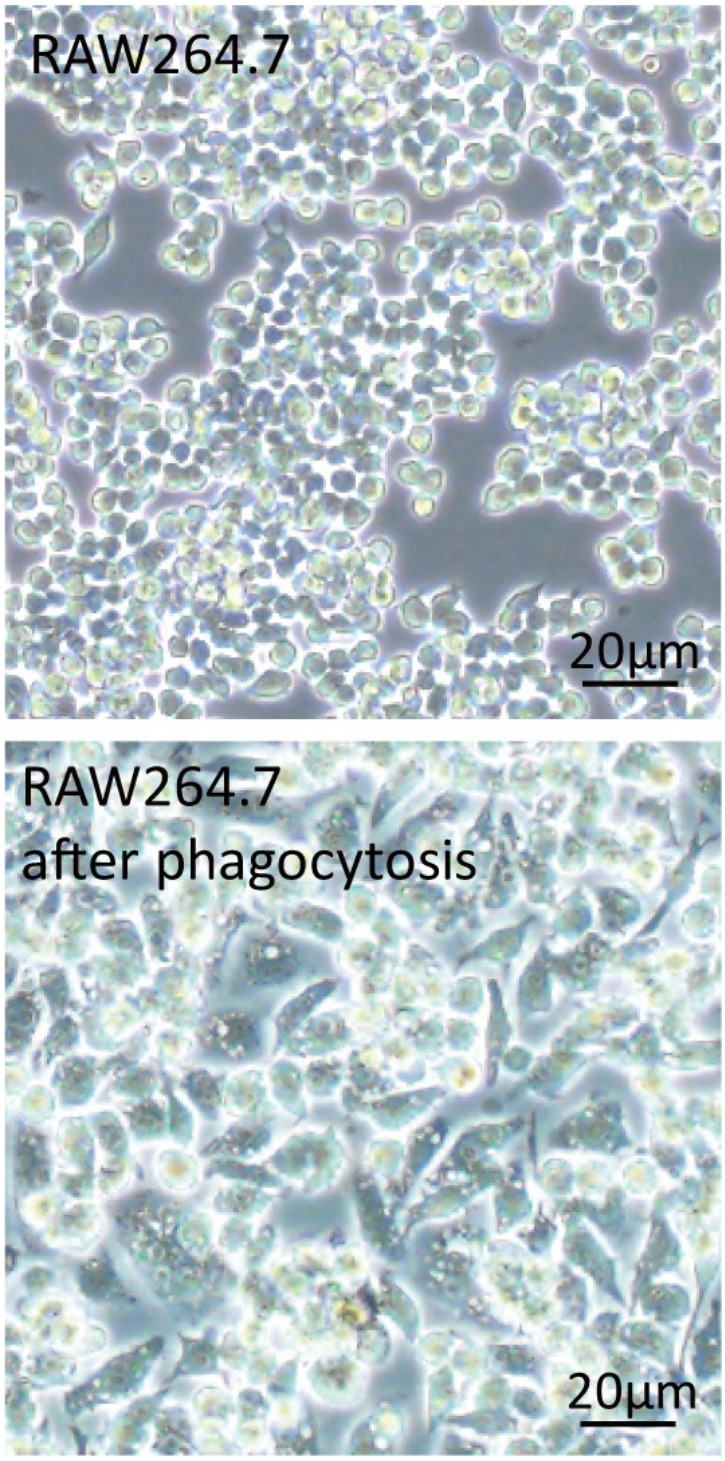

Fig. S5 Microscope photographs of normal RAW264.7 and RAW264.7 phagocytosed B16F10@MnO_2.

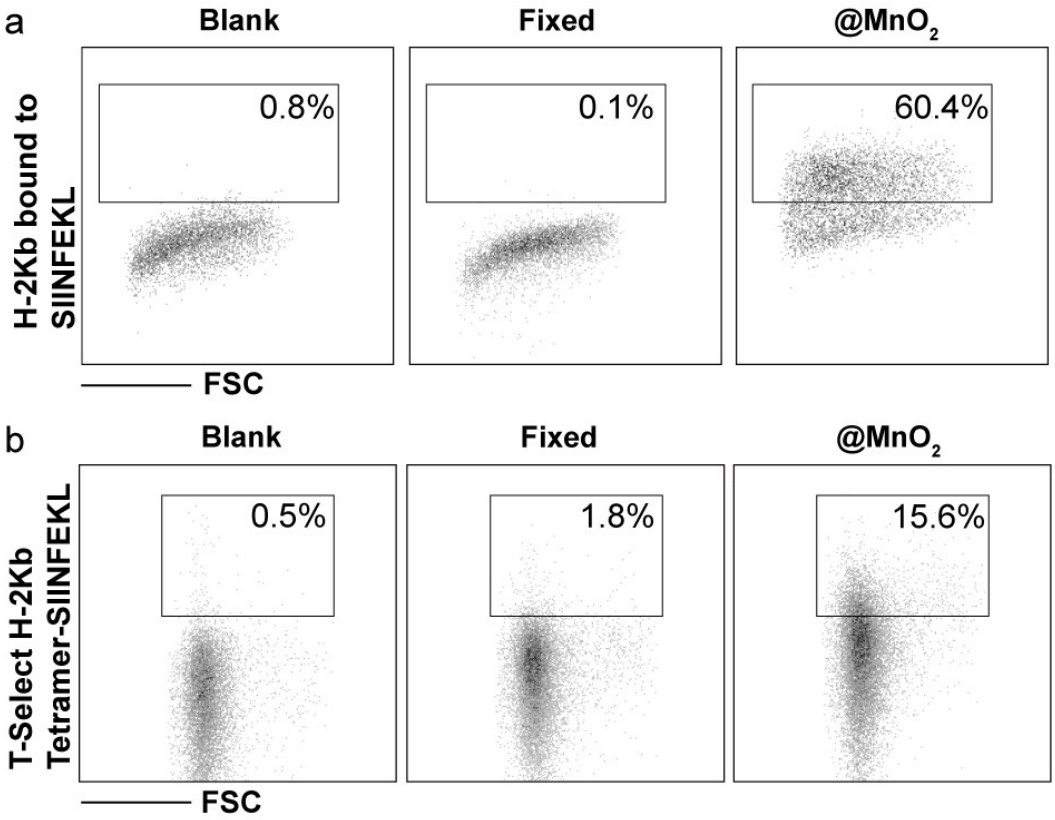

Fig. S6 Representative flow cytometry dot-plot data of (a) H-2Kb bound to SIINFEKL^{+} in CD11c^{+} BMDCs and (b) H-2Kb Tetramer-SIINFEKL^{+} in CD3^{+}CD8^{+} T cells.

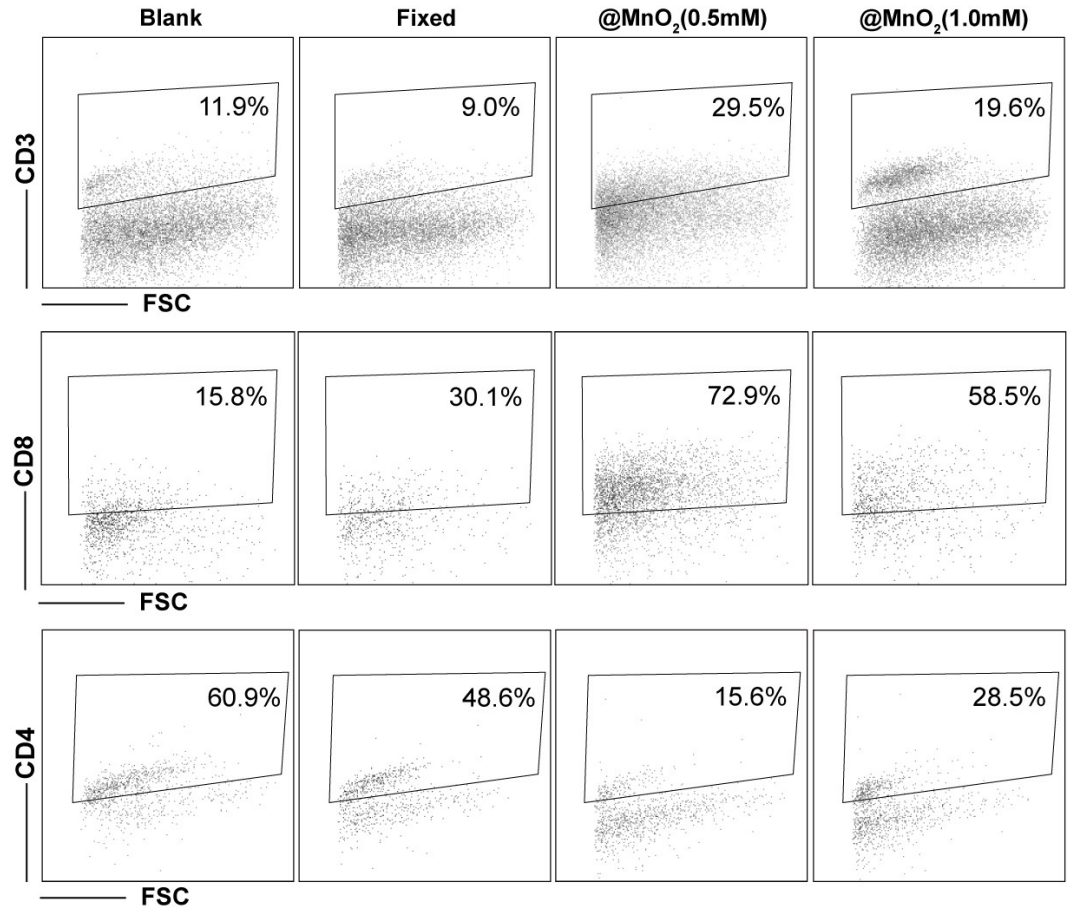

Fig. S7 Representative flow cytometry dot-plot data of $CD3^+$ T cells in $CD45^+$ infiltrating lymphocytes and $CD4^+$ T cells and $CD8^+$ T cells in $CD3^+CD45^+$ infiltrating T cells.

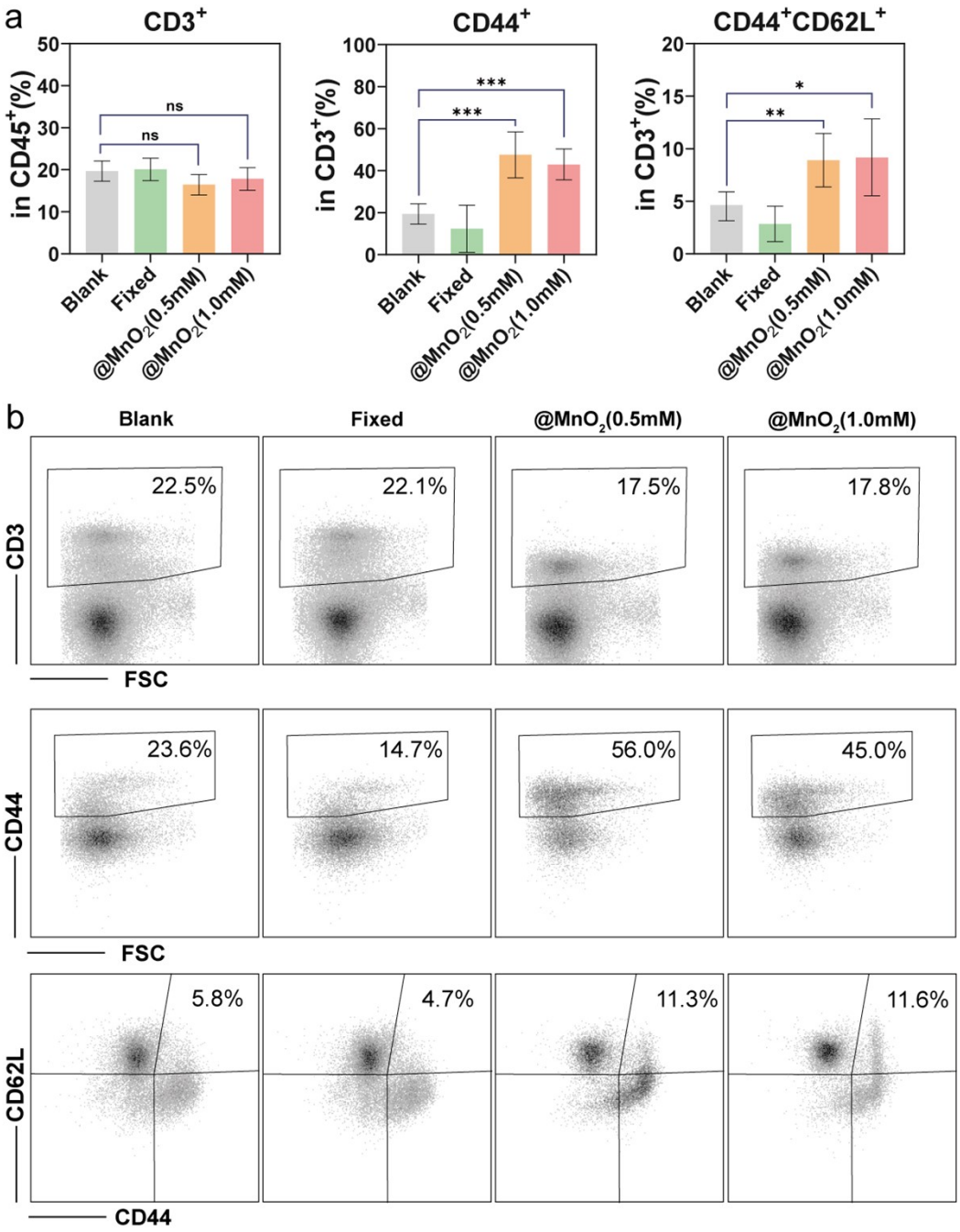

Fig. S8 (a) $CD3^+$ T cells in $CD45^+$ spleen lymphocytes and $CD44^+$ memory T cells and $CD44^+$ $CD62L^+$ central memory cells in $CD3^+CD45^+$ spleen T cells after different treatments and (b) representative flow cytometry dot-plot data.

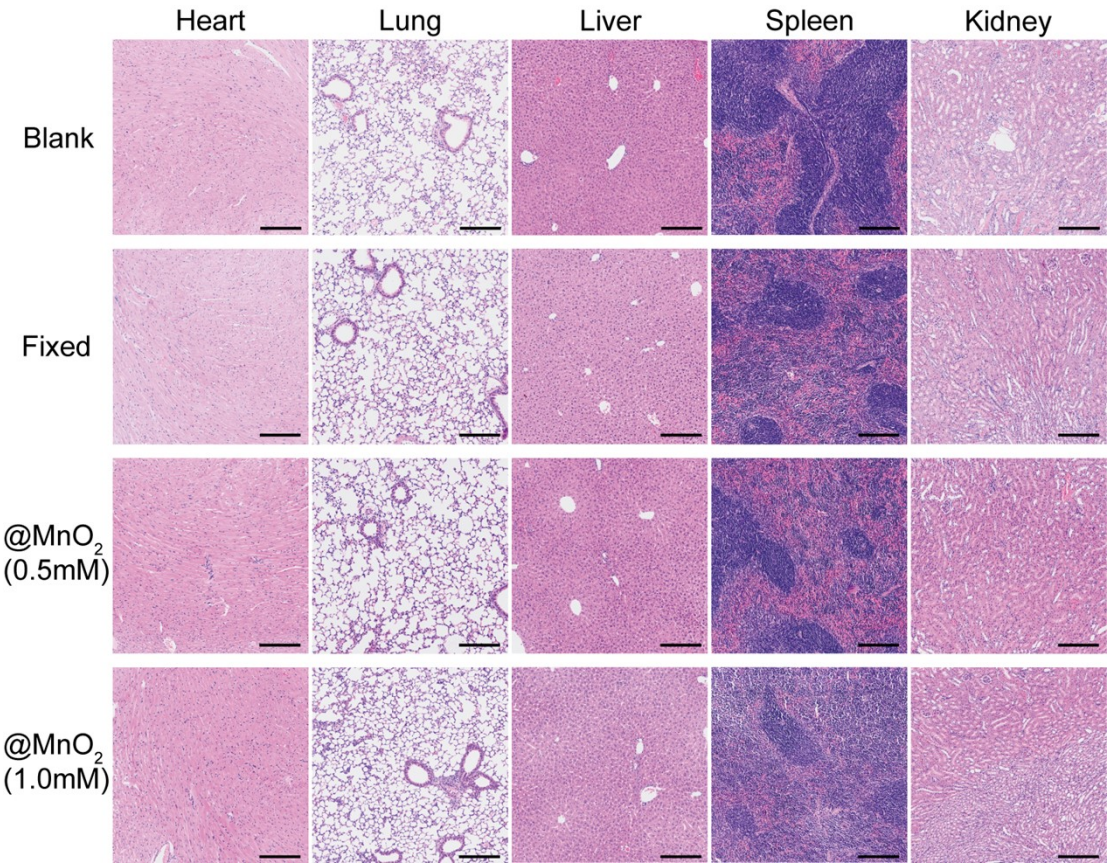

Fig. S9 Histopathological images of the major organs in mice with various treatments at day 20. The scale bars are 200 μm.

Table. S1 List of RNA primers used in the experiment.

Primer	Base sequence
GAPDH(F)	5’-GAAGGGCTCATGACCACAGT-3’
GAPDH(R)	5’-GGATGCAGGGATGATGTTCT-3’
TNFα (F)	5’-CCTGTAGCCCACGTCGTAGC-3’
TNFα (R)	5’-AGCAATGACTCCAAAGTAGACC-3’
IL6 (F)	5’-ACAAAGCCAGAGTCCTTCAGAGA-3’
IL6 (R)	5’-CTGTTAGGAGAGCATTGGAAATTG-3
IL10 (F)	5’-ACTGGCATGAGGATCAGCAG-3
IL10 (R)	5’-CTCCTTGATTTCTGGGCCAT-3
TGFβ (F)	5’- CTCCCGTGGCTTCTAGTGC-3
TGFβ (R)	5’- GCCTTAGTTTGGACAGGATCTG-3
IDO(F)	5’-TGGCCAGCTTCGAGAAAGAG-3
IDO(R)	5’-TGGCAAGACCTTACGGACATC-3
CTLA4(F)	5’-AGAACCATGCCCGGATTCTG-3
CTLA4(R)	5’-CATCTTGCTCAAAGAAACAGCAG-3