Supplementary Information (SI) for Materials Chemistry Frontiers.

Supporting Information

Sono-catalytic Nanorod-Adjuvanted In Situ Cancer Vaccine Augments Antitumor T Cell Immunity Through Potentiated Immunomodulation

Qingying Wu,[ab] Hongze Ren,[*ab] Bincheng Xi,[a] Jiayi Zhang,[a] Yu Chen[*ab] and Meihua Yu[*a]

[a] Materdicine Lab, School of Life Sciences, Shanghai University, Shanghai 200444, China.

[b] School of Medicine, Shanghai University, Shanghai 200444, China.

*Corresponding author E-mail address: rhz19980401@shu.edu.cn (H. Ren); myu@shu.edu.cn (M. Yu); chenyuedu@shu.edu.cn (Y. Chen)

Experimental Details

Chemicals and biological agents:

Manganese (II) acetylacetonate ($C_{10}H_{14}MnO_4$), potassium tellurite (K_2TeO_3, 99.5%), MB, DPBF and TEMP were purchased from Shanghai Macklin Biochemical Co.,Ltd. Sodium hexachloroiridate hydrate ($Na_3IrCl_6 \cdot xH_2O$) were purchased from Sigma-Aldrich. polyvinylpyrrolidone (PVP, Mw 58000), saturated ammonia solution ($NH_3 \cdot H_2O$), hydrazine hydrate aqueous solution ($N_2H_4 \cdot H_2O$), ethanol (C_2H_5OH, 99.9%), and acetone (CH_3COCH_3, ≥ 99.5%) were purchased from Sinopharm Chemical Reagent Co., Ltd.

Mice and cell lines:

Female C57BL/6J mice aged 6-8 weeks were obtained from Shanghai Model Organisms Centre Inc. All animal experiments were conducted in accordance with the guidelines of the National Ministry of Health and approved by the Animal Ethics Committee of Shanghai University (Approved ID: YS 2023-025). TC-1 cells were cultured under a humidified atmosphere at 37 °C with 5% CO_2.

Synthesis of $IrTe_2$ and $IrTe_x$-Mn:

$IrTe_2$ and $IrTe_x$-Mn nanoparticles were synthesized via a hydrothermal method. Briefly, for $IrTe_2$: 28 mg $Na_3IrCl_6 \cdot xH_2O$, 30 mg K_2TeO_3, and 60 mg PVP were dissolved in a mixed solvent system containing 20 mL deionized water, 20 mL saturated ammonia solution, and 10 mL hydrazine hydrate solution; the mixture was transferred to an autoclave and reacted at 180°C for 3 h. For $IrTe_x$-Mn: 10.4 mg manganese (II) acetylacetonate, 14 mg $Na_3IrCl_6 \cdot xH_2O$, 30 mg K_2TeO_3, and 60 mg PVP were dissolved in an identical solvent system (20 mL deionized water, 20 mL saturated ammonia solution and 10 mL hydrazine hydrate solution) and reacted under identical conditions. After completion, the system was cooled naturally to room temperature. The products were collected by centrifugation at 12,000 rpm for 10 min, the supernatant was discarded, and the precipitate was thoroughly washed three times with an anhydrous ethanol-acetone mixed solution (1:7, v/v), followed by drying in an oven at 40°C for subsequent use.

Characterization of $IrTe_x$-Mn:

$IrTe_2/IrTe_x$-Mn nanoparticles were dispersed in anhydrous ethanol and sonicated to form a homogeneous suspension, which was drop-cast onto copper grids and dried at room temperature in a fume hood for TEM imaging and EDS mapping analysis (JEOL JEM-F200). Dried $IrTe_2/IrTe_x$-Mn nanoparticles were prepared for X-ray photoelectron spectroscopy (XPS) analysis (Escalab 250Xi, Thermo Scientific). Quantitative elemental analysis was performed by inductively coupled plasma optical emission spectrometry (ICP-OES, 7300DV, PerkinElmer), where aliquots of $IrTe_2/IrTe_x$-Mn were digested in aqua regia (concentrated nitric acid: concentrated hydrochloric acid = 1:3, v/v) for 24 h prior to measuring Ir, Te and Mn content. The nanoparticles were dispersed in ultrapure water (50 μg/mL) for zeta potential measurements using a Malvern Zetasizer Nano ZS. To evaluate physical stability, $IrTe_x$-Mn nanoparticles were separately dispersed in deionized water, PBS buffer, and RPMI-1640 medium (200 μg/mL) in sealed transparent glass vials and stored at room temperature for 7 days.

ROS production evaluation by $IrTe_x$-Mn:

IrTe2/IrTex-Mn nanorods were dispersed in ultrapure water (50 μg/mL) under ultrasonication. Subsequently, the generation of $^1O_2$ was detected using the DPBF probe, while ·OH was assessed with the MB probe. The absorbance changes between US-treated groups (Ctrl + US, IrTe2 + US, IrTex-Mn + US) and non-US groups (Ctrl, IrTe2, IrTex-Mn) were monitored at 300-600 nm using UV-vis spectrophotometry to evaluate US-mediated ROS generation kinetics.

Electron paramagnetic resonance (EPR) spectroscopy (EMX PLUS, Bruker) was employed to analyze characteristic radical peak signals before and after US exposure. $IrTe_2/IrTe_x$-Mn nanorods were dispersed in PBS buffer (50 μg/mL) with or without US treatment (50% duty cycle, 2 W/cm², 1.0 MHz, 4 min). TEMP was applied as the $^1O_2$-trapping agent for EPR detection.

In vitro detection of intracellular ROS:

To visualize intracellular ROS production, TC-1 cells (5×10^4 cells) were seeded into confocal dishes and cultured in an incubator for 24 h to allow adherence and growth. After removing culture medium, cells were treated with fresh complete DMEM (cDMEM) medium supplemented with 10% FBS and 1% containing either $IrTe_2$ or $IrTe_x$-Mn nanomaterials (both at a final concentration of 50 μg/mL), while the blank control group received an equal volume of cDMEM medium. The cells

were further incubated for 6h to facilitate the uptake of nanomaterials. Subsequently, the medium was removed, and the cells were washed three times with PBS. Serum-free DMEM medium containing the DCFH-DA probe was added to each dish, followed by incubation in the dark for 20 min to allow probe uptake. After incubation, the cells were washed three times with PBS buffer. The experimental groups were then subjected to ultrasound irradiation (50% duty cycle, 0.8 W/cm^2, 1.0 MHz, 2 min), while the blank control group received no ultrasound treatment. Afterwards, serum-free DMEM medium containing Hoechst 33342 was added to all dishes, and the cells were incubated in the dark for 15 min for nuclear staining. After staining, the cells were washed three times with PBS, and imaging was performed using a laser scanning confocal microscope (CLSM).

TC-1 cells were seeded in 6-well plates at a density of 2×10^5 cells/mL. After cell adherence, $IrTe_2$ or $IrTe_x$-Mn was added at a concentration of 50 μg/mL, with groups (Ctrl + US, $IrTe_2$ + US, $IrTe_x$-Mn + US) subjected to US treatment (0.8 W/cm², 50% duty cycle, 1.0 MHz, 2 min) or maintained as non-US control groups (Ctrl, $IrTe_2$, $IrTe_x$-Mn). Following 6 h incubation (37 °C, 5% CO_2), medium was removed and cells were stained with DCFH-DA probe for 20 min in dark. Next, cells were trypsinized and collected. The cells were washed with PBS and then resuspended in 2% FACS buffer for flow cytometry analysis to quantify intracellular ROS production.

Cell Viability Validation:

TC-1 tumor cells were seeded in 96-well plates and divided into six groups: Ctrl, Ctrl + US, $IrTe_2$, $IrTe_x$-Mn, $IrTe_2$ + US, and $IrTe_x$-Mn + US. After adding corresponding nanorods (100 μg/mL) and incubating for 24 h, designated groups received US treatment for 2 min (50% duty cycle, 0.8 W/cm², 1.0 MHz) followed by additional 12-h incubation. Cell viability was quantitatively assessed using CCK-8 assay kit (Beyotime Biotech Inc). Absorbance at 450 nm (OD value) was measured by a microplate reader, with parallel measurements of cell-free medium wells (negative control) and untreated TC-1 cell wells (blank control) for baseline correction. The relative cell viability was calculated to evaluate the in vitro cytotoxicity of $IrTe_x$-Mn nanorods.

Cell Cytotoxicity Validation:

TC-1 cells were seeded in 96-well plates at a density of 5×10^3 cells per well and co-incubated with gradient concentrations of $IrTe_x$-Mn nanoparticles (0-200 μg/mL) for 24 h. Following

incubation, the culture medium was removed and cell viability was quantitatively assessed using the CCK-8 assay kit (Beyotime Biotech Inc).

Validation of Cell Apoptosis:

TC-1 cells were seeded in 6-well plates at 2×10^5 cells/mL. After cell adherence, nanorods were added at a final concentration of 50 μg/mL (PBS group as negative control). Following 12-h incubation (37°C, 5% CO_2, designated groups received US treatment, 0.8 W/cm², 50% duty cycle, 1.0 MHz, 2 min). After additional 6-h incubation, cells were collected and stained with PI and Annexin V-FITC in dark for 20 min. The cells were then immediately detected by flow cytometer.

BMDC culture:

Bone marrow cells were isolated from the femurs and tibias of 6-8-week-old C57BL/6J mice and treated with red blood cell lysis buffer (ACK buffer), followed by seeding at a density of 1×10^6 cells per 100 mm culture dish in RPMI-1640 complete medium supplemented with murine IL-4 (100 ng/mL) and GM-CSF (200 ng/mL). The cells were cultured at 37°C under 5% CO_2 for differentiation induction, with complete medium changes performed on days 3 and 6 using fresh cytokine-containing medium. On day 7, immature BMDCs were collected for subsequent experiments.

Evaluation of the in vitro adjuvant effect of $IrTe_x$-Mn nanorods:

Immature BMDCs were seeded in 6-well plates at 1×10^5 cells/mL and divided into Ctrl, $IrTe_2$, and $IrTe_x$-Mn groups. The experimental groups were treated with 50 μg/mL suspensions of $IrTe_2$ or $IrTe_x$-Mn, then incubated for 24 h in a 37°C humidified incubator with 5% CO_2. Cells were then centrifuged at 350×g for 5 min, washed twice with PBS, and transferred to flow cytometry tubes for staining. After incubation with the mixed antibody containing Fc block and Aqua viability dye (Thermo Fisher Scientific) for 20 min in PBS, cells were washed with FACS buffer and stained with cocktail antibody including anti-mouse CD80 (FITC, Biolegend) and anti-mouse CD86 (Pacific Blue, Biolegend) on ice for 30 min (in dark). Labeled cells were finally resuspended in FACS buffer and detected by flow cytometry.

Assessment of $IrTe_x$-Mn induced ICD effect of TC-1 cells:

TC-1 cells were seeded in 6-well plates and divided into six groups (Ctrl, Ctrl + US, $IrTe_2$, $IrTe_2$ + US, $IrTe_x$-Mn, and $IrTe_x$-Mn + US). Experimental groups were treated with 50 μg/mL of nanorods for 6 h, followed by US treatment (50% duty cycle, 0.8 W/cm², 1.0 MHz, 2 min) for designated groups and additional 12-h incubation. Culture supernatants were then collected by centrifugation at 1000 × g for 5 min. Immature BMDCs were suspended at 1×10^5 cells/mL in tubes, mixed with 100 μL of the cell supernatant, and incubated at 37°C in a 5% CO_2 incubator for 24 h. BMDCs were collected and stained by the standard procedure as aboved. Then, the maturation of BMDCs was analyzed by flow cytometer.

TC-1 cells were seeded into confocal dishes at a density of 5×10^4 cells per dish and incubated at 37°C in a 5% CO_2 incubator for 24 h to allow adherence and growth to an appropriate density. Subsequently, either $IrTe_2$ or $IrTe_x$-Mn nanomaterials (both at a final concentration of 50 μg/mL) were added, and the cells were further cultured for 6 h. Cells in the ultrasound irradiation group were then subjected to ultrasound treatment (50% duty cycle, 0.8 W/cm^2, 1.0 MHz, 2 min), followed by an additional 2h incubation. After treatment, the cells were washed three times with PBS and fixed with 4% paraformaldehyde at room temperature for 15 min. Following fixation, the cells were washed three times with PBS for 5 min each. For HMGB1 staining, cells were permeabilized with 0.1% Triton X-100 at room temperature for 10 min, permeabilization was not required for CRT staining. Next, the cells were blocked with 5% BSA at room temperature for 1 h, followed by incubation with diluted primary antibodies against CRT and HMGB1 (Cell Signaling Technology) (1:200 dilution) on a shaker at 4°C overnight. After removing the primary antibodies, the cells were washed three times with PBS for 5 min each. Alexa Fluor 594-conjugated secondary antibody (1:500 dilution) was then added and incubated in the dark at room temperature for 1 h. After discarding the secondary antibody, the cells were washed three times with PBS and mounted with an antifade mounting medium containing DAPI. Finally, the fluorescence signal of Alexa Fluor 594 was observed using a CLSM to visually analyze the exposure level of CRT on the cell membrane surface and the translocation dynamics of HMGB1 from the nucleus to the cytoplasm, thereby assessing the occurrence of ICD.

Anti-tumor evaluation:

Female C57BL/6J mice aged 6-8 weeks were subcutaneously injected with 100 μL TC-1 tumor cell suspension (2×10^5 cells per mice) and randomly divided into six groups when tumor volume reached 30 mm^3: Ctrl, Ctrl + US, $IrTe_2$, $IrTe_2$ + US, $IrTe_x$-Mn, and $IrTe_x$-Mn + US groups. Nanorods were intratumoral injected according to the experiment timeline. The indicated groups were treated with US (50% duty cycle, 0.8 W/cm^2, 1.0 MHz, 3 min) on day 11 and 13. Tumor volume were measured daily using digital calipers to calculate volume ($V = 0.5 \times length \times width^2$), with body weight monitoring. When the tumor volume reached 1500 mm^3, the animals were euthanized. At the experimental endpoint, animals were euthanized and tumor tissues were surgically excised for pathological analysis, including H&E staining, TUNEL staining and Ki-67 staining and CD8 staining.

Biosafety evaluation of $IrTe_x$-Mn:

Blood samples were collected via orbital bleeding from $IrTe_x$-Mn + US group mice on day 0 and day 20. Whole blood samples were used for blood routine test containing RBCs, WBCs, PLTs, and HGB levels. Serum samples were obtained for biochemical analysis, including ALT, AST, CREA, and urea levels. The major organ tissues (heart, liver, spleen, lung, and kidney) of tumor-bearing mice were collected on day 0 and day 20 which were used for histology studies.

Statistical analysis:

Statistical analysis data were analyzed using GraphPad Prism 9.5.1 software. All experiments were performed with at least three independent biological replicates ($n \geq 3$), and data are presented as mean ± standard deviation (SD) unless otherwise specified. For comparisons between two groups, an unpaired two-tailed Student's t-test was used. For multiple group comparisons, one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was performed. Statistical significance was defined as *$P < 0.05$, **$P < 0.01$,***$P < 0.001$, and ****$P < 0.0001$. Detailed analysis methods are described in figure captions.

Supplementary Figures

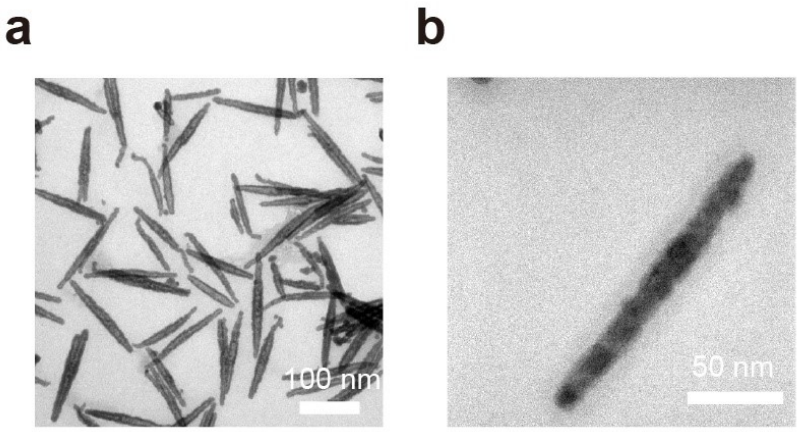

Figure S1. TEM images of $IrTe_2$ (a-b).

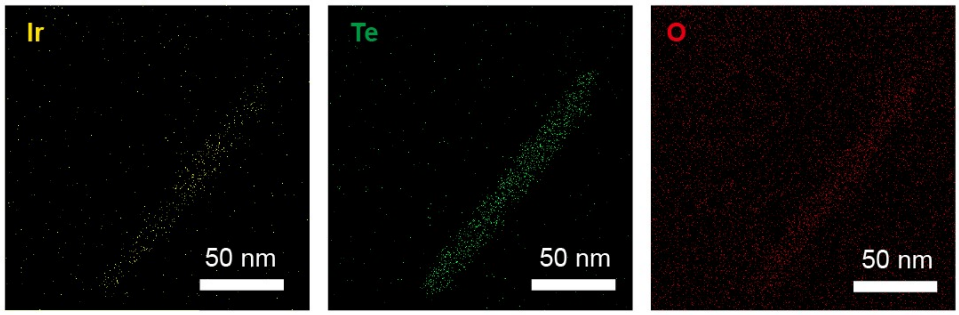

Figure S2. Elemental mapping images of $IrTe_2$.

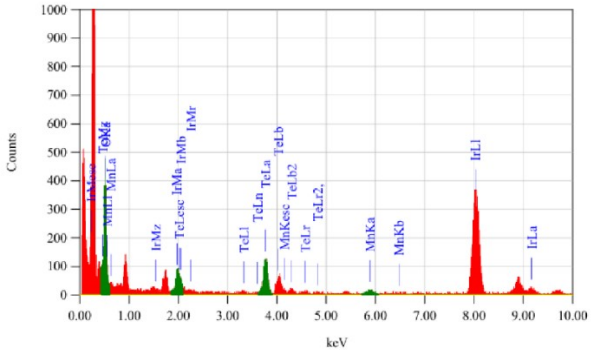

Figure S3. EDS mapping element contents and relative atomic ratios of Ir, Te and Mn in $IrTe_x$-Mn.

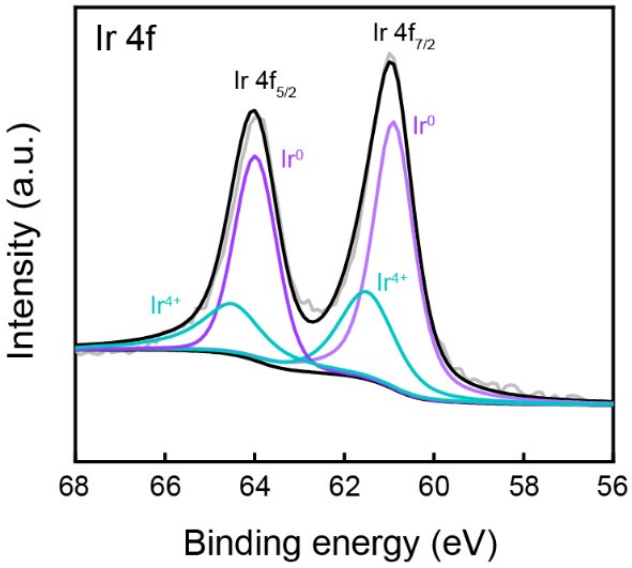

Figure S4. XPS spectra of the $IrTe_2$ showing the Ir 4f bands.

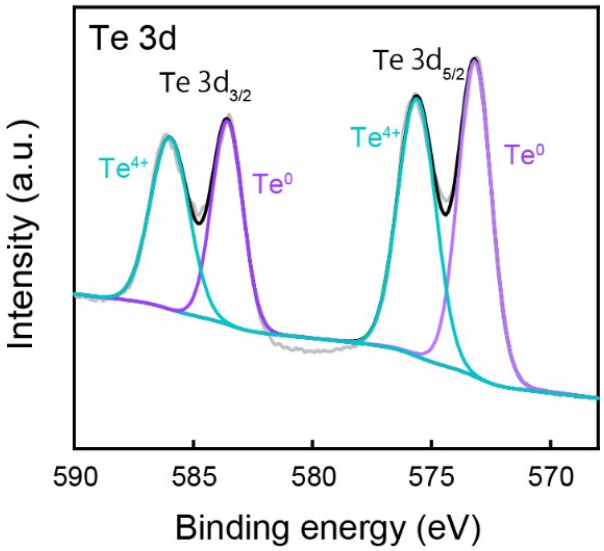

Figure S5. XPS spectra of the $IrTe_2$ showing the Te 3d bands.

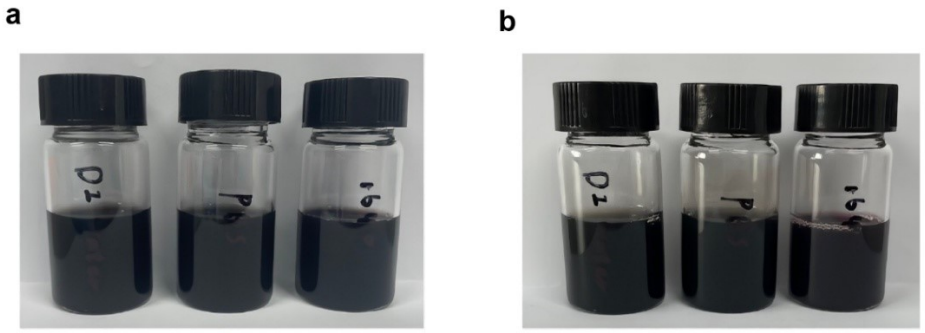

Figure S6. Digital images of $IrTe_x$-Mn dispersed in deionized water, PBS, and RPMI-1640 medium (from left to right) on (a) day 0 and (b) day 7.

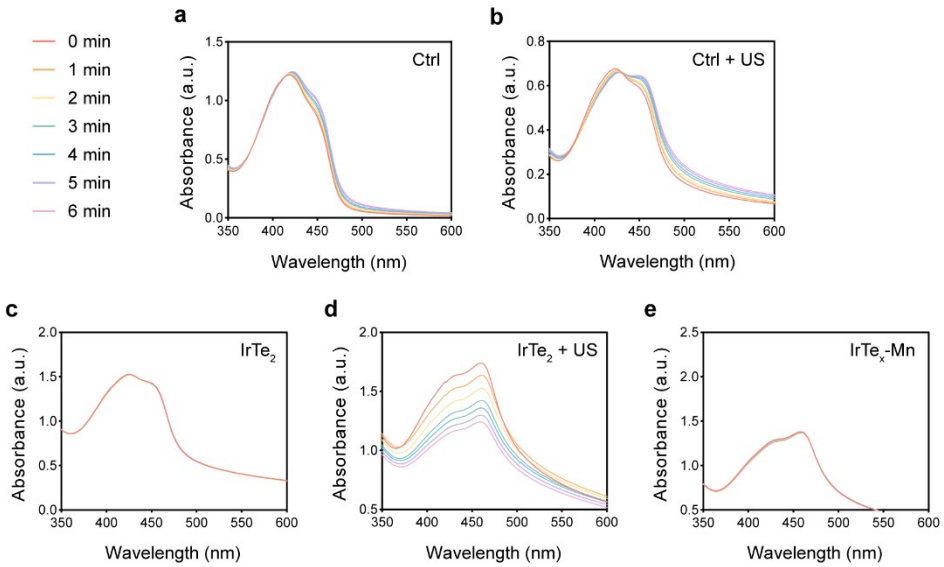

Figure S7. UV-Vis absorption spectra of DPBF under different treatment groups at varying time intervals (0, 1, 2, 3, 4, 5, and 6 min).

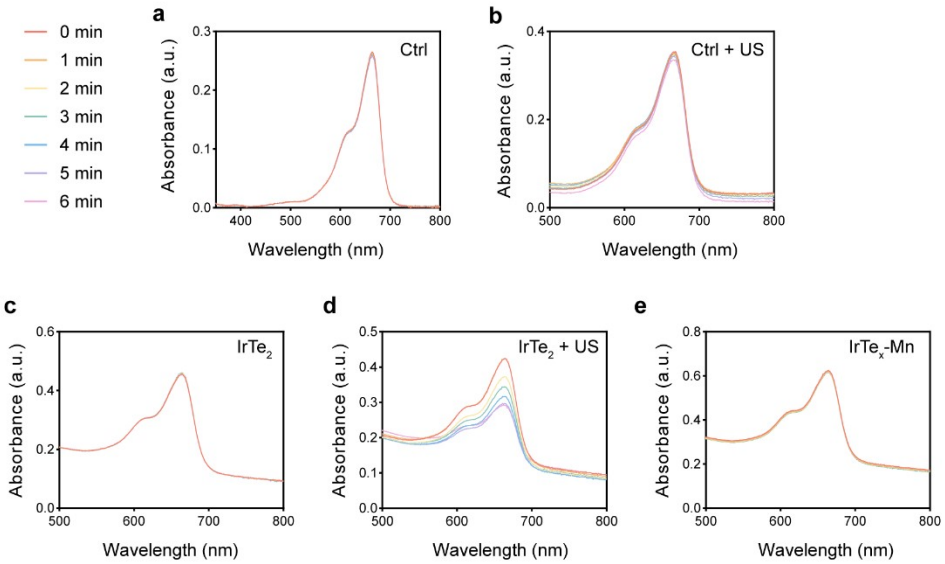

Figure S8. UV-Vis absorption spectra of MB under different treatment groups at varying time intervals (0, 1, 2, 3, 4, 5, and 6 min).

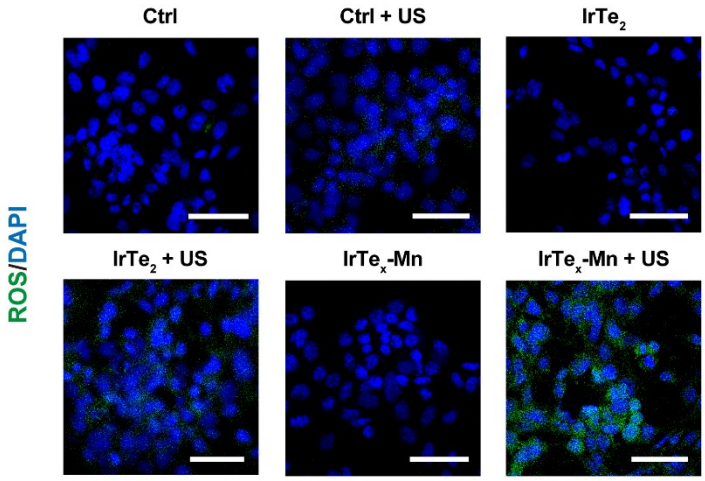

Figure S9. Confocal microscopy images of TC-1 cells post different treatments and incubation with ROS probe DAFH-DA. Scale bar = 50 μm.

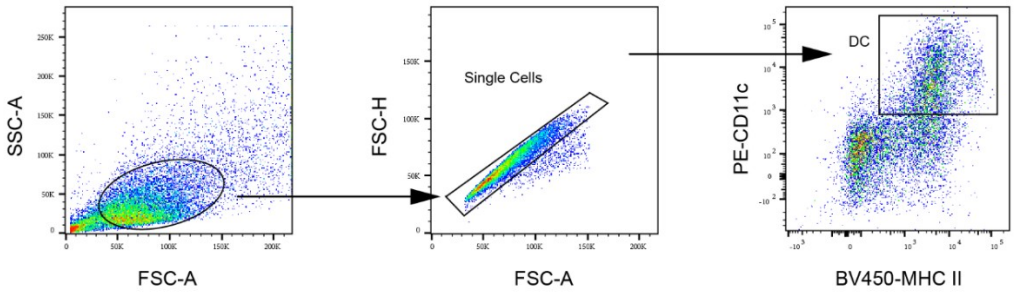

Figure S10. Flow cytometry gating strategy for BMDCs after co-incubation with $IrTe_2$ or $IrTe_x$-Mn for 24 h.

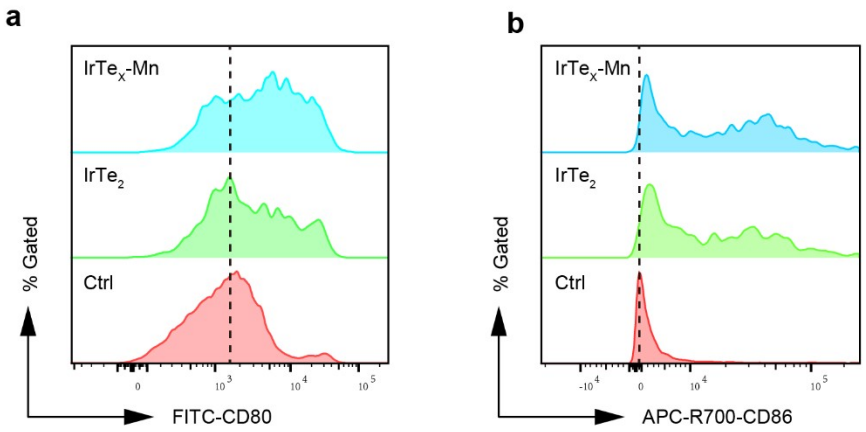

Figure S11. Representative overlaid histograms of (a) CD80 and (b) CD86 expression on BMDCs after 24 h co-incubation of $IrTe_2$ and $IrTe_x$-Mn.

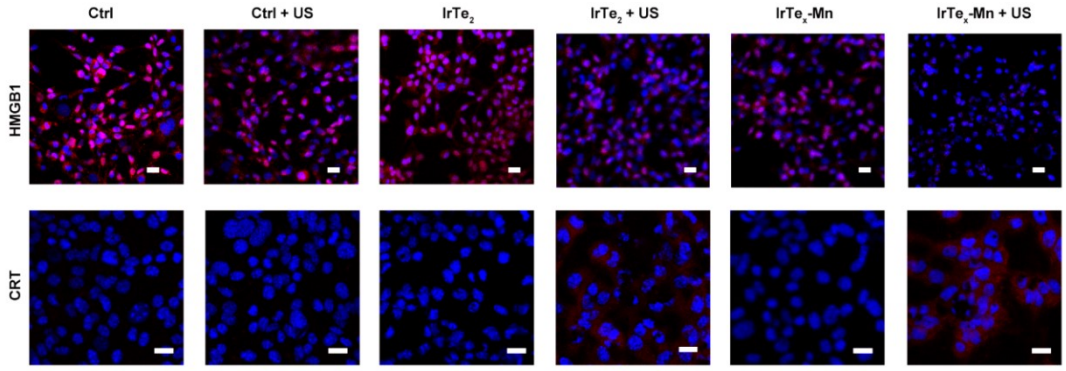

Figure S12. Confocal images of CRT and HMGB1 expressed in TC-1 cells with different treatments as indicated. Scale bar = 20 μm.

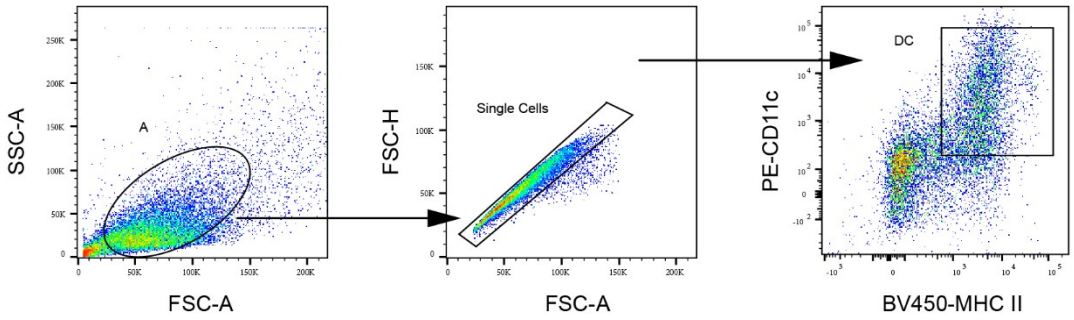

Figure S13. Flow cytometry gating strategy for BMDCs after 24 h co-incubation of BMDCs with supernatants of TC-1 cells subjected to different treatments.

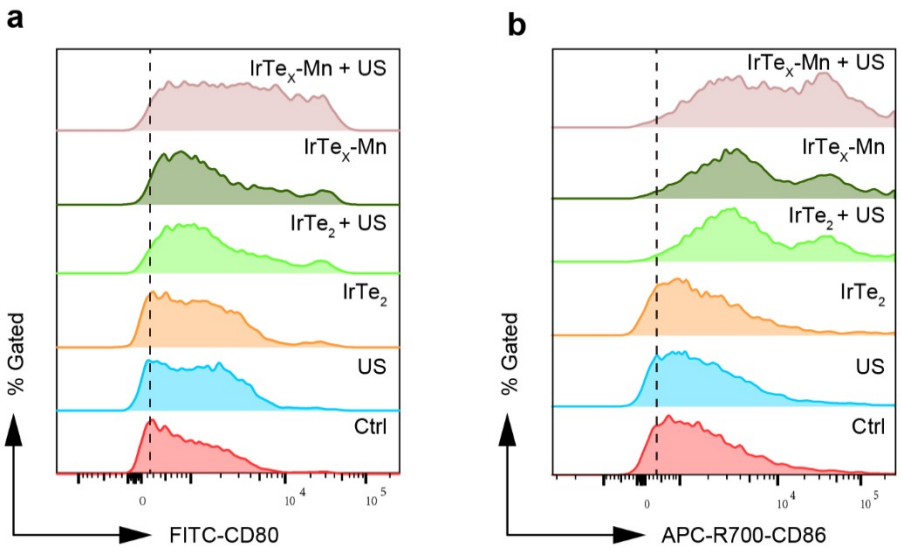

Figure S14. Representative overlaid histograms of (a) CD80 and (b) CD86 expression on BMDCs after 24 h coincubation of BMDCs with supernatants from TC-1 cells treated under different conditions.