Supporting Information

Mild-Phototherapy Mediated by Manganese Dioxide-Loaded Mesoporous Polydopamine Enhances Immunotherapy Against Colorectal Cancer

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Materials and Methods

Materials

Pluronic F127 and dopamine hydrochloride (98%, AR) were obtained from Aladdin Industrial Inc.(Shanghai, China). 1,3,5-trimethylbenzene (TMB, AR, 97%) was aquired from Macklin (Shanghai, China), mPEG-NH_2 (Mw=2000) were aquired from Tansh. Anti-mouse PD-L1 (B7-H1) was obtained by BioXcell Co., Ltd., USA. Cell culture medium RPMI 1640 medium and Phosphate buffered saline (PBS) were purchased from GIBCO (Carlsbad, USA). 0.25% Trypsin-EDTA (1x) was obtained from Beyotime (Shanghai, China). Fetal bovine serum (FBS) was obtained from Invitrogen Co. (Carlsbad, USA).

Synthesis of Mesoporous Polydopamine Nanosponges (MPDA NPs)

A one-pot synthesis method was used to prepare the MPDA nanoparticles.[1, 2] Typically, 0.36 g of TMB and 0.36 g of F127 were dissolved in a mixture of ethanol

(60 mL) and H_2O (65 mL). After stirring for 30 min, 90 mg TRIS was dissolved in 10 mL of H_2O and then the solution was introduced to the mixture, followed by addition of 60 mg dopamine hydrochloride. After stirring at room temperature for 24h, the product nanoparticles were separated by centrifugation, and then washed with distilled water and ethanol three times in turn. The final MPDA nanoparticles were suspended in distilled water for further use.

Synthesis of Manganese Dioxide-Modified Mesoporous Polydopamine (MnO_2@MPDA-PEG)

MnO_2@MPDA-PEG NPs were prepared according to the methods previously reported.[3] Firstly, MPDA-PEG was prepared by simply mixing MPDA NPs (2mg) with $mPEG_{2k}$-NH_2 (2 mg) in 10 mM Tris buffer (pH 8.5) under stirring for 24h. The products were then collected after centrifugation and washing for 3 times, followed by suspending into distilled water (10mL). After that, under vigorously stirring, 1 mL of $KMnO_4$ (2 mg/mL) solution was dropped slowly and then stir the mixture at room temperature for 1h. MnO_2 was formed and deposited on the surface of MPDA-PEG by redox reaction. The MnO_2@MPDA-PEG nanoparticles were collected and purified by centrifugation and suspended in distilled water for future use.

Characterization

Morphology and size of samples were characterized by using a Transmission electron microscopy (Tecnai G2 Spirit 120 kV). The elemental mapping images were obtained on highresolution TEM (JEOL-2100F). The zeta potentials and size distribution of the nanoparticles were obtained by Zetasizer Nano ZS instrument

(Malvern Instruments Ltd). UV–vis spectra were measured with UV–vis spectrophotometer (Perkin Elmer-Lambda 950).

Photothermal Properties of MnO_2@MPDA-PEG NPs

Nanoparticle solutions (200 uL) with concentrations of 100 μg/ml and 300 μg/ml were irradiated with the 808 nm laser. To monitor the changes of temperature, an infrared thermal imaging camera was employed to record thermographic maps.

Cell Lines and Animals

The murine CRC cell line of CT26 and dendritic cell line of DC2.4 were purchased from Cobioer (Nanjing, China), and cultured in RPMI-1640 medium (Gibco) supplemented with 1% penicillin/streptomycin and 10% fetal bovine serum (FBS) under a humidified chamber with 5% CO_2 at 37 ◦C. BMDCs were differentiated from bone marrow cells, and cultured in RPMI 1640, supplemented with 10% FBS, 20 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 10 ng/mL interleuk-4 (IL-4).

Female BALB/c mice (6 - 8 weeks) were provided by GuangDong GemPharmatech Co., Ltd. All care and experiments on animal were carried out under the international animal experiment guidelines and maintained under protocols approved by Sixth Affiliated Hospital, Sun Yat-Sen University Laboratory Animal Center (SYXK 2018-0190) and Sun Yat-sen University Laboratory Animal Center (SYXK 2016-0112). Orthotopic animal tumor model was established according to the methods previously reported.[4] Typically, 1×10^6 CT26 cells suspended in 10μL of PBS were injected into 6-week-old mice at the submucosal tissue layer of the rectum.

Cellular Uptake

CT26 and DC2.4 cells were cultured with coumarin6-loaded MPDA-PEG (C6@MPDA-PEG) at different incubated conditions. The cells were later rinsed with PBS, fixed by paraformaldehyde, stained with 4′,6-diamidino-2-phenylindole (DAPI) (C1022, Beyotime Co., Ltd., China) and observed on a CLSM. Besides, the cellular uptake of C6@MPDA-PEG was analyzed quantitatively by flow cytometry.

Cell Viability

To measure cell viability, CT26 cells were plated in a 96-well plate and treated with various concentrations of MPDA-PEG or MnO_2@MPDA-PEG. Cells were treated with/without mild-PTT for 5 min. After cultured for additional 24 hours, medium was replaced with 100ul fresh culture medium containing 10% Cell Counting Kit-8 (CCK8) reagent (Sigma, USA). After incubation at 37°C for 1 hour, the absorbance of each well was measured using a microplate reader (Invitrogen, Carlsbad, CA) at 540 nm.

In Vitro Cellular MRI and in Vivo MRI

To assess the Mn^{2+} accumulation in CT26 cells, *in vitro* cellular MRI were performed. 1×10^6 CT26 cells were incubated with MPDA-PEG and MnO_2@MPDA-PEG NPs at different concentrations from 0 μg/mL to 1000 μg/mL for 24 h, and then harvested for imaging under a clinical 3.0 T MRI unit (Ingenia 3.0T; Philips Medical Systems). The main MRI sequences included T1WI (FOV, 100 mm×100 mm; TR = 400 ms; TE =18 ms; voxel, 0.39 mm×0.39 mm; slice thickness, 2 mm; matrix, 256 mm×251 mm; flip angle, 90°; NSA, 3) and T1-mapping sequence (FOV, 80 mm×80

mm; TR = 1500 ms; TE = 20 ms; voxel, 0.4 mm×0.4 mm; matrix, 228 mm×289 mm; reconstruction matrix, 512; slice thickness, 2 mm; IR delay, 400 ms; NSA, 1).

For *in vivo* MRI imaging, the CT26-bearing mice were scanned on a 3.0T MR unit (GE Discovery MR750w). 100μL of MnO_2@MPDA-PEG suspension (6.0 mg/mL) was injected into the CT26-bearing mice through tail vein injection. The MRI sequence, T1WI (TR/TE=622/mim ms, NSA=8, FOV=80 ×7.5 mm, section thickness=1.0 mm, matrix=320 × 320) was acquired before and after tail vein injection of MnO_2@MPDA-PEG NPs. To further validate the degree of contrast enhancement promoted by the MnO_2@MPDA-PEG NPs, the signal intensity of tumors on T1WI were analyzed by the region of interest (ROI) technique.

Detection of ICD Signaling Molecules Produced by MnO_2@MPDA-PEG NPs in Vitro

CT26 cells were seeded on climbing coverslips (φ20mm, TC, NEST) and treated with PBS, MPDA-PEG, MnO_2@MPDA-PEG NPs for 24 h, followed by 808 nm irradiation for 10 mins. To monitor the changes of temperatures *in vitro*, an infrared thermal imaging camera was employed to record thermographic maps in 24 well plates, whose temperatures were mainted at 43-45 °C by adjusting the laser power manually. After cultured for additional 24 hours, the cells were rinsed with PBS, fixed by paraformaldehyde, stained with anti-rabbit CRT antibody (Abcam, 1:1000 dilution), according to the manufacturer's instructions. The nuclei were stained with DAPI before observation via confocal laser scanning microscopy (Nikon C2). To detect the release of HMGB1, the supernatants of the treatments were collected, and

then the concentration of HMGB1 was directly measured using HMGB1 ELISA kit (MEIMIAN, China), as indicated by manufacturer's instructions.

BMDCs Maturation in Vitro

Bone marrow-derived cells (BMDCs) were differentiated from bone marrow cells. Briefly, female BALB/c mice (4-5 weeks) were anesthetized and sacrificed, then the femur and tibia were collected under sterile conditions. Bone marrow was rinsed with RPMI 1640 medium and filtered through a 200-mesh filter. Cell suspension was centrifuged at 1500 rpm for 10 min and cultured in RPMI 1640, supplemented with 10% FBS, 20 ng/ml granulocyte-macrophage colony stimulating factor (GM-CSF) and 10 ng/mL interleuk-4 (IL-4). After 48 h culture, the suspended cells were taken out and the adherent cells were cultured for another 2 days, the medium was half-replaced every 2 days. In the end, the highly purified BMDCs were obtained and identified by CD11c staining on day 4.

For *in vitro* BMDCs stimulation experiments by nanoparticles, we cocultured MPDA-PEG, MnO_2@MPDA-PEG NPs (50 μg/ml) with BMDCs for 24 h. For *in vitro* BMDCs stimulation experiments by CT26 cells treated with nanoparticles and NIR laser irradiation. MPDA-PEG and MnO_2@MPDA-PEG NPs were co-incubated with CT26 cells for 24 h, and given the mild-photothermal treatment by manually controlled NIR irradiation. The supernatant was then co-incubated with BMDC cells for another 24 h. The maturation of BMDC cells were detected by CD80 and CD86 expression.

In Vivo Antitumor Effect of MnO_2@ MPDA-PEG NPs

Orthotopic animal tumor model was established as described above. When the tumor volume reached approximately 100 mm^3, the mice were randomly assigned to six groups, and received the treatment of PBS, aPD-L1, MPDA-PEG+L, MnO_2@MPDA-PEG, MnO_2@MPDA-PEG+L, MnO_2@MPDA-PEG+L+aPD-L1, respectively. On the third day after nanodrug administration, mice were injected with aPD-L1 at a dose of 50 μg per mouse via tail vein twice a day, for a total of 5 times. The tumors were exposed to 808 nm laser for 10 min at 24h, 48h, 72h post-administration of nanodrug. The temperature was maintained at 43-45 °C by adjusting the laser power manually. The survival rates and body weights of mice were monitored every 2 days and tumor volumes were calculated by: tumor volume (mm^3) = 1/2 × length × $width^2$.

In Vivo Antitumor Immune Response

The *in vivo* ICD signaling molecules induced by MPDA-PEG and MnO_2@MPDA-PEG NPs under near-infrared laser irradiation were investigated. To validate the exposure of CRT *in vivo*, tumor tissues of CT26 tumor-bearing mice were dissected, fixed with 4% paraformaldehyde for 48 hours, dehydrated and embedded in paraffin. Four-micrometer sections of tumor were acquired using a microtome and stained with anti-rabbit CRT antibody (Abcam, 1:1000 dilution). Subsequently, sections were observed under confocal laser scanning microscopy.

For the assessment of DCs maturation in tumor and tumor-draining lymph node as well as $CD8^+$ T-cell infiltration in tumor, mice were sacrificed on day 10 and single-cell suspensions of all tissues were made for flow cytometry (NovoCyte 2060)

analyses. Briefly, tumor-draining lymph nodes and tumors were ground through 70-μm cell strainer to obtain single-cell suspensions. Lymphocytes were further extracted using a Mouse Tumor Infiltrating Tissue Lymphocyte Separation Kit (Sigma, USA), as indicated by manufacturer's instructions.

To analyze the maturation of DCs, single-cell suspensions were stained with anti-mouse CD11c-FITC, CD80-PE and CD86-APC antibodies (Biolegend, USA) before a quantitative analysis by flow cytometry. To analyze the infiltration of T cells, the single-cell suspensions from tumors were stained with anti-mouse CD3-APC, CD4-FITC, CD8a-PE antibodies (Biolegend, USA) before a quantitative analysis by flow cytometry.

Biochemistry Index and Histology Analysis

On day 10 after the first treatment, serums were collected from the mice to assay the biomedical indexes by an autobiochemical analyzer (Rayto Chemray 240).

Western Blot Analysis

STING, pTPK1 and PD-L1 expressions were determined by western blot analysis. Proteins from cells were extracted and separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Sample were then transferred to polyvinylidene difluoride (PVDF) membranes, blocked with 5% BSA and incubated with STING (Cell Signaling, 1:1000 dilution), pTPK1 (Cell Signaling, 1:1000 dilution), PD-L1 (Abcam, 1:1000 dilution), overnight at 4 °C. Signals were detected by using chemi-luminescence system (GE ImageQuant LAS 500).

Statistical Analysis

All analysis was performed in SPSS by using an independent sample t test where $p < 0.05$ is defined as significant. ($p \geq 0.05$ (n.s.), $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***)).

Supplementary Figures

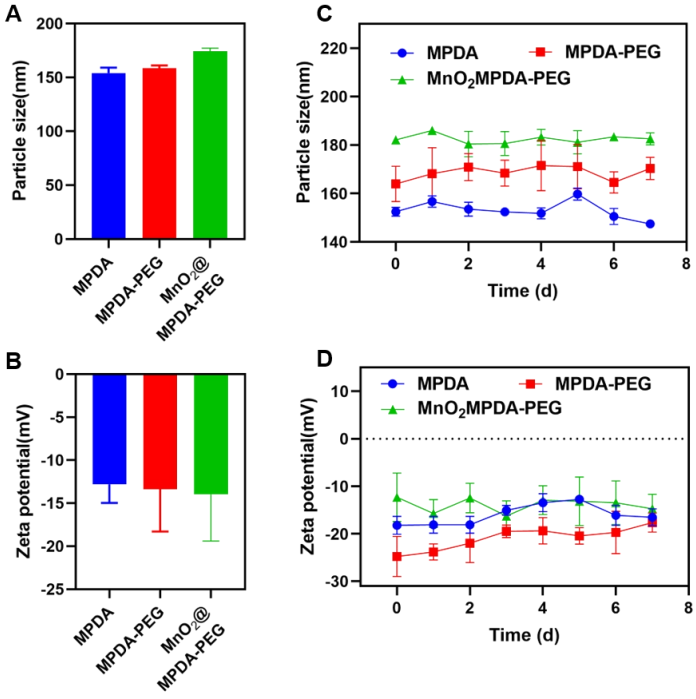

Figure S1. Hydrodynamic sizes (A) and zeta potentials (B) of MPDA NPs, MPDA-PEG and MnO_2@MPDA-PEG NPs solutions. The stability of size (C) and zeta potentials (D) of MPDA NPs, MPDA-PEG and MnO_2@MPDA-PEG NPs within 7 days.

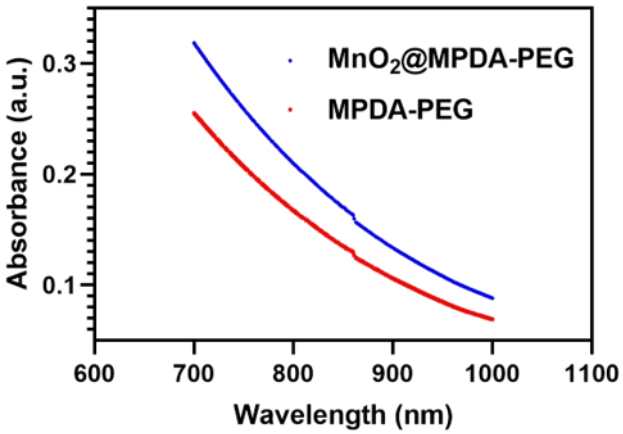

Figure S2. UV-vis absorbance of MPDA-PEG and MnO_2@MPDA-PEG NPs.

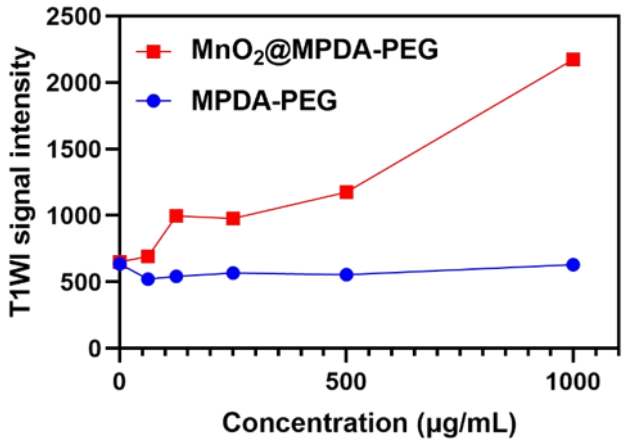

Figure S3. T1WI signal intensity of CT26 cells upon treatment with MPDA-PEG and MnO_2@MPDA-PEG NPs.

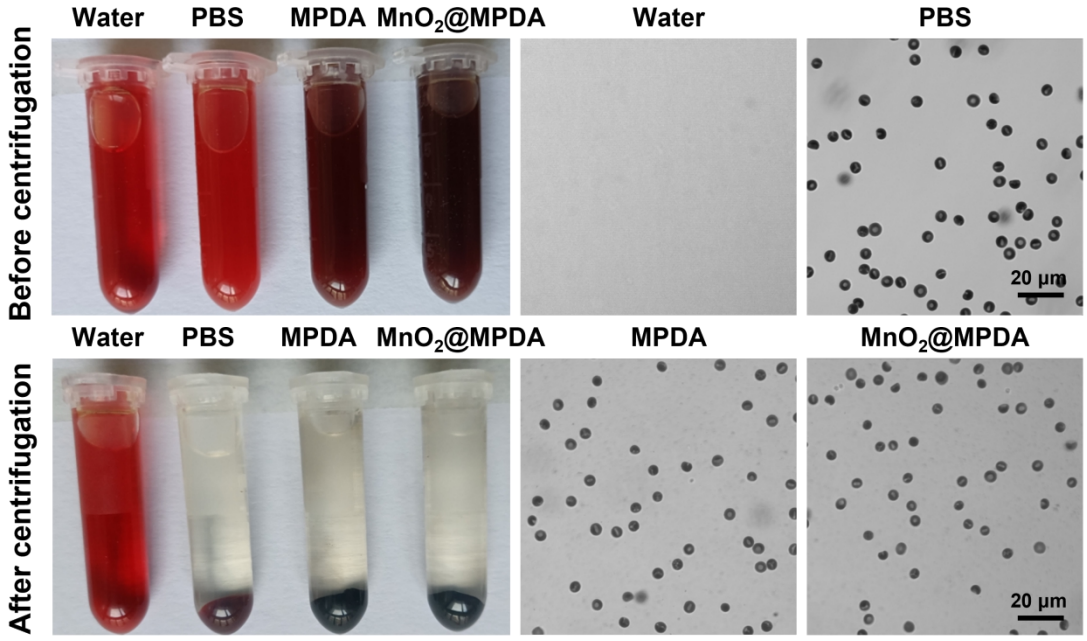

Figure S4. Hemolysis of MPDA-PEG and MnO_2@MPDA-PEG NPs (100μg/mL) and representative images of erythrocytes after different treatments. Scale bar:20 μm.

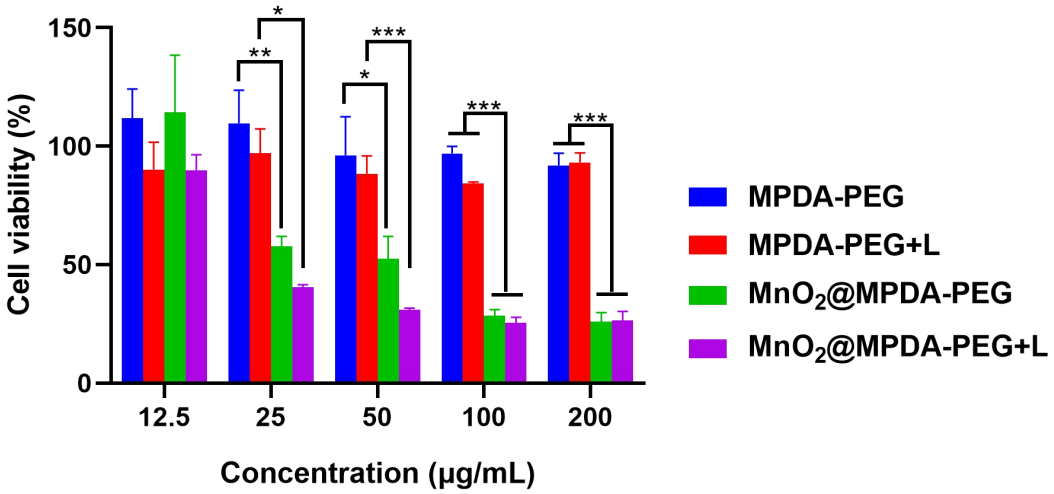

Figure S5. *In vitro* cytotoxicity experiments. Graph showing the relative viabilities in CT26 cells after incubation with different concentrations of MPDA-PEG, MnO_2@MPDA-PEG (μg/mL) with and without laser irradiation.

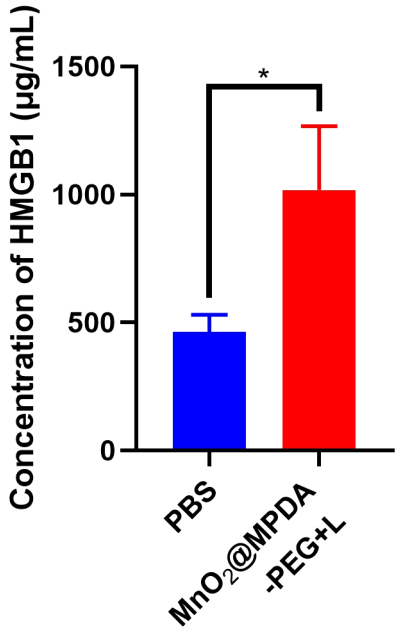

Figure S6. CT26 cells were treated with MnO_2@MPDA-PEG for 24 hours, followed by 808nm NIR irradiation for 10 mins. Supernatants were analyzed for HMGB1 by ELISA.

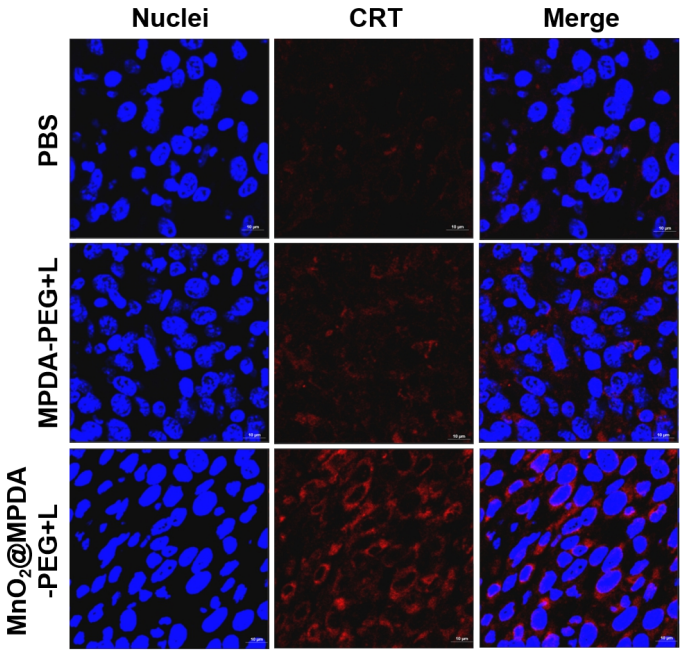

Figure S7. Immunofluorescent staining of CRT exposure in tumor tissues after different treatments. Scale bar:10μm.

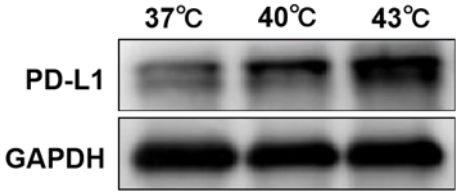

Figure S8. PD-L1 expression on CT26 cells after different treatments.

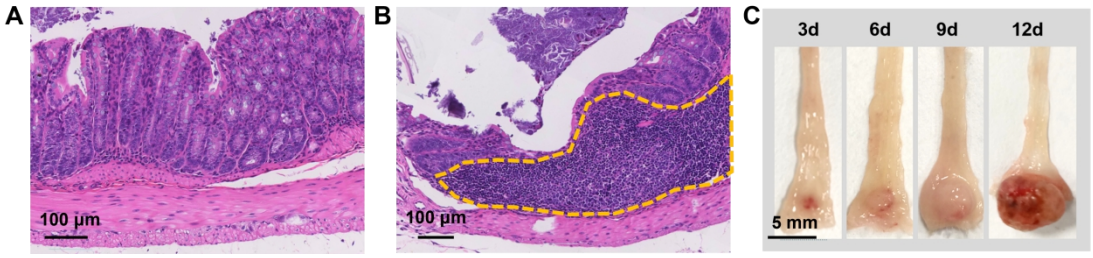

Figure S9. Establishment of orthotopic colorectal tumor models. H&E staining of normal gut tissue (A) and tumors (B) isolated from mice. Scale bar: 100 μm. (C).Gross photos of resection specimens of mice. Scale bar: 5 mm.

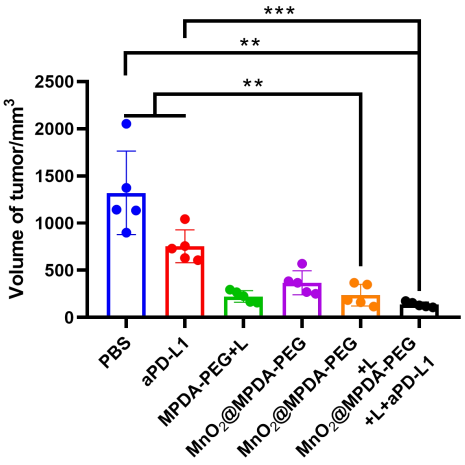

Figure S10. Tumor growth of the mice on day 9 after different treatments of PBS, aPD-L1, MPDA-PEG + L, MnO_2@MPDA-PEG, MnO_2@MPDA-PEG + L, and MnO_2@MPDA-PEG + L + aPD-L1.

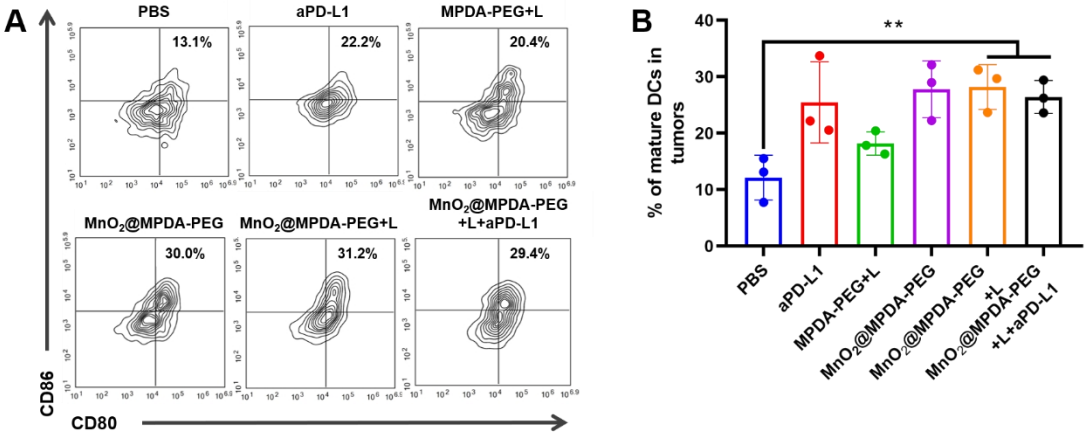

Figure S11. (A) Flow cytometry assay of DC cells maturation ($CD11c^+CD80^+CD86^+$) in tumors and (B) the corresponding percentages. Cells collected from mice after different treatments at day 10.

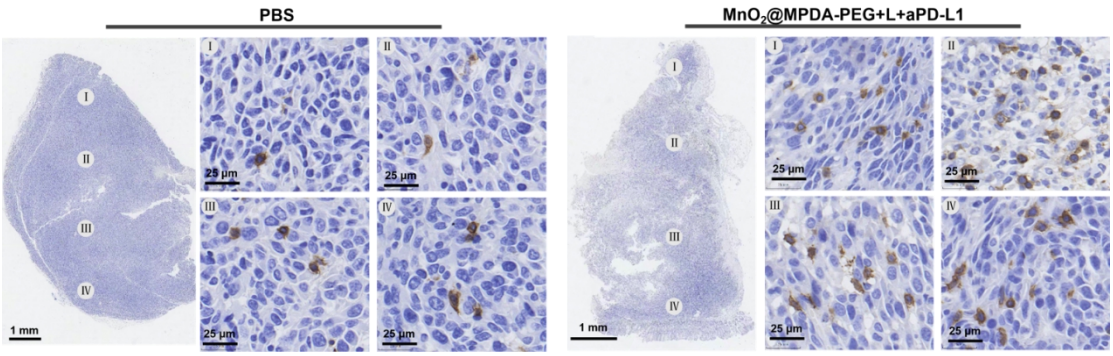

Figure S12. Immunohistochemical staining of $CD8^+$ T cells in CT26 tumor sections at 10 days. Scale bar, 25 μm.

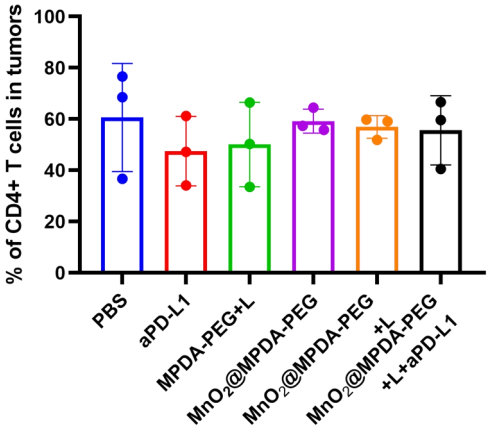

Figure S13. Quantitative analysis of $CD4^+$ T cells in CT26 tumors induced by different treatments. (gated on live $CD3^+$ T cells).

1. F. Chen, Y. Xing, Z. Wang, X. Zheng, J. Zhang and K. Cai, *Langmuir*, 2016, **32**, 12119-12128.
2. Y. Xing, J. Zhang, F. Chen, J. Liu and K. Cai, *Nanoscale*, 2017, **9**, 8781-8790.
3. Y. Wang, S. Song, T. Lu, Y. Cheng, Y. Song, S. Wang, F. Tan, J. Li and N. Li, *Biomaterials*, 2019, **220**, 119405.
4. N. Hite, A. Klinger, L. Hellmers, G. A. Maresh, P. E. Miller, X. Zhang, L. Li and D. A. Margolin, *Dis Colon Rectum*, 2018, **61**, 698-705.