Supplementary Information

Peptide Nano 'Beading' for SDT-Facilitated Immune Checkpoints Blocking

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Contents

Materials 4
Method 5
1. Synthesis of the OBOC peptide library 5
2. Solid phase peptide synthesis 5
3. SPRi analysis of the binding between RT and CD47 6
4. Confocal laser scanning microscope (CLSM) imaging 6
5. Competition experiments of RT with SIRPα at the cellular level 7
6. Co-Assembly of RT and QDs 7
7. Circular dichroism (CD) 7
8. Atomic force microscopy (AFM) imaging: 7
9. Cell culture 7
10. Gene knockdown of CD47 in CT26 cells 8
11. In vitro ROS detection 8
12. Intracellular ROS detection 8
13. Live/dead analysis by Calcein AM/PI staining 8
14. Hemolysis assay 9
15. *In vivo* therapy assays 9
16. *In vivo* imaging assays. 10
17. Bilateral tumor treatment 10
18. Transcriptomics analysis 11
19. Statistical analysis. 13
Figure S1. 14
Figure S2. 15
Figure S3. 16
Figure S4. 17
Figure S5. 18
Figure S6. 19
Figure S7. 20
Figure S8. 21

Figure S9.....22
Figure S10.....23
Figure S11.....24
Figure S12.....25
Figure S13.....26
Figure S14.....27
Figure S15.....28
Figure S16.....29
Figure S17.....30
Figure S18.....31
Figure S19.....32
Figure S20.....33
Figure S21.....34
Figure S22.....35

Materials

Tentagel resin was obtained from Rapp Polymere (Germany, loading 0.35 mmol/g). 9-Fluorenylmethoxycarbonyl (Fmoc)-protected amino acids, Wang resin, and 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoro-phosphate (HBTU) were purchased from GL Biochem (China). Fluorescein isothiocyanate (FITC), Hoechst 33342 and streptavidin-coated magnetic beads (1 μm), were brought from Sigma-Aldrich (USA). Nuclear indicator DRAQ5was purchased from Bicostatus (UK). N-methyl morpholine (NMM), piperidine, Tips (Triisopropyl silane) and N, N'-dimethylformamide (DMF) were all obtained from Beijing Chemical Plant (China). SPRi gold chips (Nanocapture, 5 mm×75 mm in size, 47.5 nm-thickness of the gold coating) were purchase from Plexera and used as SPRi chips. CD47 protein was obtained from Sino Biological (Beijing, China). Biotin labeling kit was purchased from SoluLink. 1,3-diphenylisobenzofuran (DPBF) was purchased from Beijing Chemical Plant (China). TUNEL apoptosis detection kit (FITC) was purchased from Yeasen biotechnology. Calcein-AM/PI Kit and CCK-8 Kit were purchased from Solarbio (China). TPE-COOH was from AIEgen Biotech. siRNA-CD47 was purchased from GenePharma (China). DMEM (Dulbecco's modified Eagle's medium)/high glucose medium, MEM (minimum Eagle's medium)/EBSS (Earle's balanced salt solution) and trypsin were purchased from GE Healthcare Life Sciences. Mouse colon adenocarcinoma cell (CT26) and human embryonic kidney cell line (293T) were purchased from Cell Resource Center, Chinese Academy of Medical Sciences (China). All cell lines were supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 U/mL penicillin, and 100 U/mL streptomycin (Gibco). Female C57BL/6 (6-8-week-old) were purchased from Vital River Laboratory Animal Technology (China). Mouse red blood cells were purchased from SenBeiJia Biological Technology Co., Ltd. Ag_2S quantum dot was purchased from Suzhou Yingrui Optical Technology Co., LTD. All reagents were used as received, and solvents were purified according to general procedures before use.

Method

1. Synthesis of the OBOC peptide library

The OBOC library was synthesized by employing the Fmoc SPPS (solid-phase peptide synthesis) strategy. TentaGel resin (loading 0.35 mmol/g) was used as the solid phase support. The whole synthesis process was carried out in anhydrous DMF. During the coupling steps, TentaGel resin was mixed with HBTU (4 mmol), Fmoc-amino acid (4 mmol), and 4 mL of 0.4 M NMM in DMF solution. The coupling time was 40 min. In the deprotection steps, 20 *v/v*% piperidine (dissolved in DMF) was used to remove the Fmoc group and the deprotection time was 10 min. In the coupling step, solid support beads were split equally, and each portion was then coupled with a different amino acid. The amino acid coupling process was carried out in the "split" step, while the deprotection process was carried out in the "pool" step. Upon completion of the synthesis, a cleavage reagent (92.5 *v/v*% TFA, 2.5 *v/v*% water, and 2.5 *v/v*% Tips) was introduced to cleave side chain protecting groups on each residue. The cleavage time was 2 h. All the experiments mentioned above were carried out in solid-phase peptide synthesis vessels with sieves. Finally, a cyanogen bromide (CNBr) solution (30 mg/mL) was reacted with the peptide resin overnight to release the synthesized peptides.

2. Solid phase peptide synthesis

The designed peptides were synthesized using the standard solid-phase Fmoc peptide synthesis strategy. The peptide sequences were prolonged from C-termini to N-termini. Wang resin was used as the solid phase support. 20% v/v piperidine (dissolved in anhydrous DMF) was used to remove the Fmoc group of amino acids. The carboxy group of Fmoc protected amino acid was activated by NMM (0.4 mol/L in anhydrous DMF) and HBTU (equal moles as amino acids). The deprotection or coupling efficiency is measured by using Kaiser reagents ((5% ninhydrin ethanol solution (m/v), 80% phenol ethanol solution (m/v), 2% vitamin C/pyridine solution (m/v) 1:1:1 v/v/v). Finally, the peptide is cleaved from the resin in the acid solution (95% v/v TFA, 2.5%

v/v water, and 2.5% v/v Tips). Acid solution was removed by vacuum rotary evaporation, and the obtained crude product was precipitated in cold ether. The crude peptides were purified by high performance liquid chromatography (HPLC) and characterized by MALDI-TOF (Matrix assisted laser desorption ionization and time of flight) mass spectra (Bruker Daltonics, Germany)

3. SPRi analysis of the binding between RT and CD47

SPRi analysis was performed with a Plexera PlexArray HT system (Plexera LLC, Bothell, WA) using a bare gold SPRi chip (a Nanocapture gold chip with a 47.5-nm-thick gold coating). All the purified peptides were printed onto the surface of the gold chip surface through thiol groups on Cysteine residues. The chip was then incubated overnight in a humid box at 4 °C. The SPRi chip was washed and blocked using 5% (*w/v*) skim milk in PBS overnight before use. The SPRi analysis procedure was carried out with injection cycles. And in each cycle, PBST (phosphate buffered saline with 0.05% Tween-20) buffer (baseline stabilization), protein samples (CD47, six different concentrations, binding), PBST buffer (washing), and 0.5 *v/v*% H_3PO_4 in deionized water (regeneration) were injected in sequence. Protein was diluted with PBST to concentrations of 570 nM, 285 nM, 142 nM, 71 nM, 36 nM and 18 nM. Real-time binding signals were recorded and analyzed by the PlexArray HT software.

4. Confocal laser scanning microscope (CLSM) imaging

For CLSM, about 1×10^5 mL^{-1} of CT26/293T cells were seeded into culture dishes and cultured overnight for cell adherence. For FITC-RT assay, FITC-labeled peptides were dissolved in cold PBS at a concentration of 100 μM. These cells were incubated with 200 μL of FITC-labeled peptide solution and 1 mM of hoechst 33342 in dark at 4 °C for 30 min. Finally, these cells were washed three times with cold PBS and inspected with a ZEISS LSM710 confocal microscope (Japan). A 488 nm laser was used as the excitation source for FITC throughout the experiment and a 525 nm laser was used as the emission source. Hoechst 33342 emitted at 461 nm upon excitation at 405 nm.

5. Competition experiments of RT with SIRPα at the cellular level

The binding behavior of RT and SIRPα toward CD47 were performed by CLSM imaging. First, approximately 1×10^5 mL^{-1} CT26 cells were seeded into culture dishes and cultured overnight for cell adherence. Next, CT26 were incubated with a mixture of RT (100 μM) and Cy5-labeled SIRPα (50 μg/mL) for 1 h at 4 °C. After that, cell nuclei were stained with DRAQ5™ for 15 min at 4 °C. Finally, these cells were washed with cold PBS three times and analyzed by CLSM (ZEISS LSM710, Japan). (DRAQ5™: E_x= 647 nm, Cy5: E_x=635 nm).

6. Co-Assembly of RT and QDs

RT (1 mg/mL) was dissolved in PBS and assembled at room temperature for approximately 6 h, followed by the addition of QDs (50 μg/mL). Co-assembly was carried out at 4 °C for 48 h and then characterized.

7. Circular dichroism (CD)

Secondary structure measurement after 24 h of RT^{QDs} assembly (RT: 1 mg/mL, QDs: 50 μg/mL). CD measurements were performed at room temperature with MOS-450 spectrometer (Bio-logic, France). Measurement range: 190-260 nm; path-length: 0.1 mm.

8. Atomic force microscopy (AFM) imaging:

AFM images were obtained in air conditions using scanning probe microscope (Bruker, dimension fast-scan). For morphology characterization, the sample was dropped onto the mica sheet surface for 10 min, then the excess solution was washed off with water and blown dry with nitrogen. Scanning mode: ScanAsyst; probe type: ScanAsyst-AIR; resolution: 512×512; scanning speed;1.0 Hz.

9. Cell culture

Mouse colon cancer cell line CT26 were employed as CD47 positive cells. Human kidney epithelial cell line (293T) was used as CD47 negative cells. CT26 cells were

cultured in RPMI 1640 medium, while 293T cells were cultured in DMEM medium. All cells were incubated under humidified atmosphere containing 5% CO_2 at 37 ℃.

10. Gene knockdown of CD47 in CT26 cells

CT26 cells were cultured in confocal dish. Then medium was changed into RPMI 1640 medium plus 10% FBS without antibiotics. 200 nM siRNA-CD47 complexed with 2 μg/mL Lipofectamine™ 2000 (Invitrogen, Carlsbad, USA, stock solution is 1 mg/mL) was added in the cells for transfection for 24 hours. For the positive CT26 cells, an uncorrelated siRNA was used for the same procedures.

11. In vitro ROS detection

50 μL of DPBF (1 mg/mL, DMSO) probe was added to 200 μL of RT^{QDs} (QDs: 50 μg/mL, RT:1 mg/mL) and the absorbance values after different times of sonication (0 min, 1 min, 3 min, 5 min, 7 min) were detected at 410 nm, sonication parameters (Sonicator 740, 1.0 MHz, 1.5 W/cm^2, 50% duty cycle).

12. Intracellular ROS detection

CT26 cells were inoculated at 10^5 density and cultured overnight in 96-well plates. The cells were incubated with PBS, QDs (50 μg/mL), RT^{QDs} (QDs: 50 μg/mL, RT:1 mg/mL) for 4 h respectively, then DCFH-DA (1:1000 free medium) probe was added for 30 min, washed 3 times with PBS, cells were irradiated or with ultrasound or without irradiating for 3 min and finally observed using inverted fluorescence microscope. Sonication parameters: Sonicator 740, 1.0 MHz, 1.5 W/cm^2, 50% duty cycle.

13. Live/dead analysis by Calcein AM/PI staining

Calcein AM/PI staining was performed to study the cell viability of CT26 cells from different treatment groups. CT26 cells incubated overnight in 96-well plates at a density of 10^5, then incubated with PBS, QDs (50 μg/mL), RT^{QDs} (QDs: 50 μg/mL, RT:1 mg/mL) for 4h, washed three times with PBS and irradiated with ultrasound for 2 min or without irradiating. 10 μL of Calcein-AM and 15 μL of PI were added to 5 mL of PBS (final concentration: Calcein-AM: 1 μM, PI: 1.5 μM), stained for 30 min, washed

3 times with PBS and observed by inverted fluorescence microscopy. Sonication parameters: Sonicator 740, 1.0 MHz, 1.5 W/cm^2, 50% duty cycle.

14. Hemolysis assay

Red blood cells (RBC) were suspended in PBS at a concentration of 2% (v/v). 500 μL of RBC suspension was added to the samples at different concentrations, incubated at 37°C for 1 h, then centrifuged at 1000 g for 5 min and the absorbance was measured at 540 nm to obtain hemoglobin release. Water and PBS were used as positive and negative controls.

15. *In vivo* therapy assays

Five groups of female C57BL/6 mice (n=6, 5-6-weeks-old) were subcutaneously implanted with CT26 cells. When tumors developed to about 50 mm^3, *in vivo* tumor suppression studies were carried out to investigate the toxicity and tumor inhibition. PBS, QDs (5 mg/kg, ultrasound), RT, RT^{QDs} (RT:6 mg/kg, QDs:5 mg/kg, without ultrasound). RT^{QDs} (RT:6 mg/kg, QDs:5 mg/kg, ultrasound) were intravenously injected into each group of mice, respectively. Sonication parameters: Sonicator 740, 1.0 MHz, 7 min, 1.5 W/cm^2, 50% duty cycle. Administrations were carried out every other day. After the mice sacrifice, tumors and other organs were collected. The toxicity in tumors and organs of each group were determined by H&E. The apoptosis of tumor cells was also detected by TUNEL assay. Tumor weights and sizes were recorded daily. Tumor sizes were measured with a Vernier caliper. Tumor volumes were calculated by the formula: $(L \times W^2)/2$, where L and W were the longest and the shortest tumor diameter (mm), respectively. The experimental data were assessed as mean standard deviations (n=5). Finally, the results were calculated by Student's t-test and P-values of each group. All the animal experiments were conducted in compliance with the guide for the care and use of laboratory animals of the Animal Study Committee of Beijing Institute of Technology's requirements, the approval number for animal experimentation is 'BIT-EC-SCXK2016-0006-M-2021059.

16. *In vivo* imaging assays.

Female BALB/c nude mice of about 18 g (8-week-old on arrival) were purchased from Vital River Laboratory Animal Center (Beijing, China), and kept under specific pathogen-free conditions with free access to standard food and water. All the animal experiments were conducted in compliance with the guide for the care and use of laboratory animals of the Animal Study Committee of Beijing Institute of Technology's requirements, the approval number for animal experimentation is 'BIT-EC-SCXK2016-0006-M-2021059. The xenograft tumors were established by subcutaneously (S.C.) injection of MC38 cells (1×10^7/mL) in PBS (6 μL) to the right hind legs of each mouse. Tumor growth was measured periodically and until the tumors reached to about 7 mm in diameter. Mice were all injected via the caudal vein with QDs and RT^{QDs} (120 μL, 50 μg/mL, RT:1 mg/mL), respectively. Near-infrared imaging was carried out at 0 h, 3 h, 6 h and 10 h and 10 h using a NIRvana640 imaging system with InGaAs camera (Teledyne Princeton Instruments, Trenton, USA). All images were taken with a 0.1-second exposure time to ensure consistency in the data.

17. Bilateral tumor treatment

MC38 cells were injected into the left and right flanks of C57BL/6 mice (n=3, 5-6-weeks-old) to create a bilateral tumor model. The tumor on the right was designated as the primary tumor for local ultrasound irradiation and the left tumor was designated as the distant tumor (without ultrasound). When tumors developed to about 50 mm^3, *in vivo* tumor suppression studies were carried out to investigate the toxicity and tumor inhibition. The mice were randomly divided into 4 groups and intravenously injected with PBS, QDs (5 mg/kg, ultrasound), RT (RT:6 mg/kg) and RT^{QDs} (RT:6 mg/kg, QDs:5 mg/kg, ultrasound), respectively. Injections were given every 2 days for a total of 4 times, and ultrasound irradiation was performed on the primary tumor after 6 h of injection. Sonication parameters: Sonicator 740, 1.0 MHz, 7 min, 1.5 W/cm^2, 50% duty cycle. All the animal experiments were conducted in compliance with the guide for the care and use of laboratory animals of the Animal Study Committee of Beijing Institute of Technology's requirements, the approval number for animal experimentation is

‘BIT-EC-SCXK2016-0006-M-2021059.

18. Transcriptomics analysis

(1) Sample collection and preparation

A. RNA quantification and qualification

①RNA degradation and contamination was monitored on 1% agarose gels.

②RNA purity was checked using the NanoPhotometer® spectrophotometer (IMPLEN, CA, USA).

③RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

B. Library preparation for Transcriptome sequencing

A total amount of 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext® UltraTM RNA Library Prep Kit for Illumina® (NEB, USA) following manufacturer’s recommendations and index codes were added to attribute sequences to each sample. Briefly, tumor samples (PBS, QDs (+), RT^{QDs} (+)) were individually milled to a power in a mortar with liquid nitrogen. mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H). Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities. After adenylation of 3’ ends of DNA fragments, NEBNext Adaptor with hairpin loop structure were ligated to prepare for hybridization. In order to select cDNA fragments of preferentially 250-300 bp in length, the library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37 °C for 15 min in followed by 5 min at 95 °C before PCR. PCR was performed with phusion high fidelity DNA polymerase,

universal PCR primers and Index (X) primer. At last, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bioanalyzer 2100 system.

C. Clustering and sequencing (Novogene Experimental Department)

The clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq PE Cluster Kit v3-cBot-HS (Illumia) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Novaseq platform and 150 bp paired-end reads were generated.

(2) Data Analysis

A. Quality control

Raw data (raw reads) of fastq format were firstly processed through in-house perl scripts. In this step, clean data (clean reads) were obtained by removing reads containing adapter, reads containing ploy-N and low-quality reads from raw data. At the same time, Q20, Q30 and GC content the clean data were calculated. All the downstream analyses were based on the clean data with high quality.

B. Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website directly. Index of the reference genome was built using Hisat2 v2.1.0 and paired-end clean reads were aligned to the reference genome using Hisat2 v2.1.0. We selected Hisat2 as the mapping tool for that Hisat2 can generate a database of splice junctions based on the gene model annotation file and thus a better mapping result than other non-splice mapping tools.

C. Quantification of gene expression level feature

Counts v1.5.0-p3 was used to count the reads numbers mapped to each gene. And then FPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. FPKM, expected number of Fragments Per Kilobase of transcript sequence per Millions base pairs sequenced, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels.

D. Differential expression analysis

Differential expression analysis of two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (1.16.1). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the false discovery rate. Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed.

19. Statistical analysis.

Regular statistical tests were performed in Origin 8.1 and we used one-way ANOVA followed by post hoc Tukey's test for the indicated comparison. Functional enrichment analyses of transcriptomic were performed in GeneCodis 3.0, and Hypergeometric test followed by Permutation correction to high throughput results with FDR (q value) <0.05 were used. The number of animals and cells is included in the text or in figure legends. P-values <0.05 were statistically significant. When a statistical test was used, the P-value is noted either in the manuscript text or depicted in figures and legends as: *P<0.05, **P<0.01, ***P<0.001, n.s., not significant, P≥ 0.05.

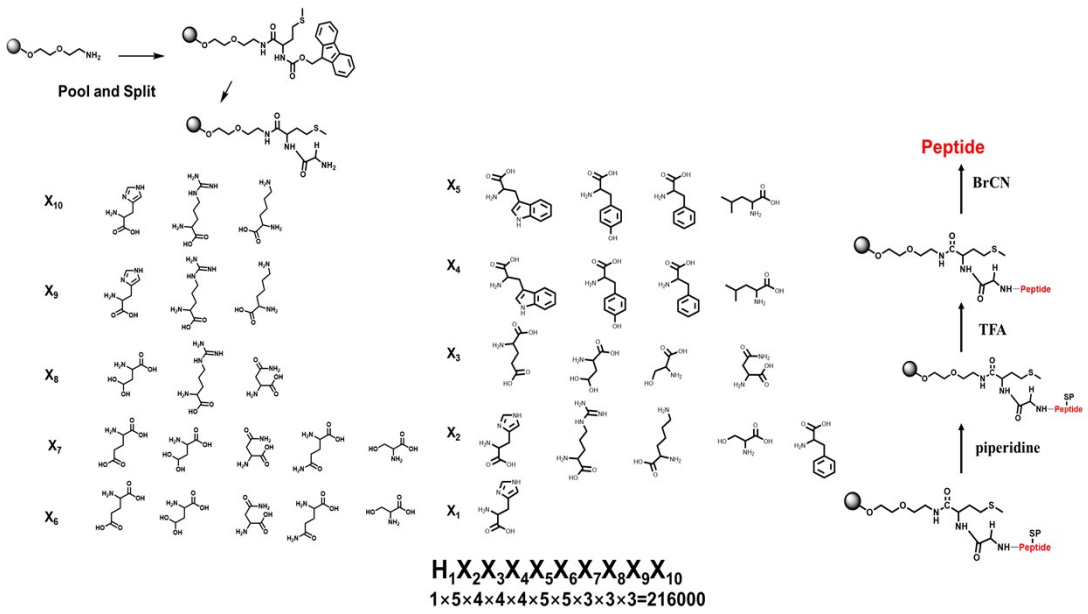

Figure S1. The schematic of the synthesis process of the OBOC peptide library through combinatorial chemistry strategy.

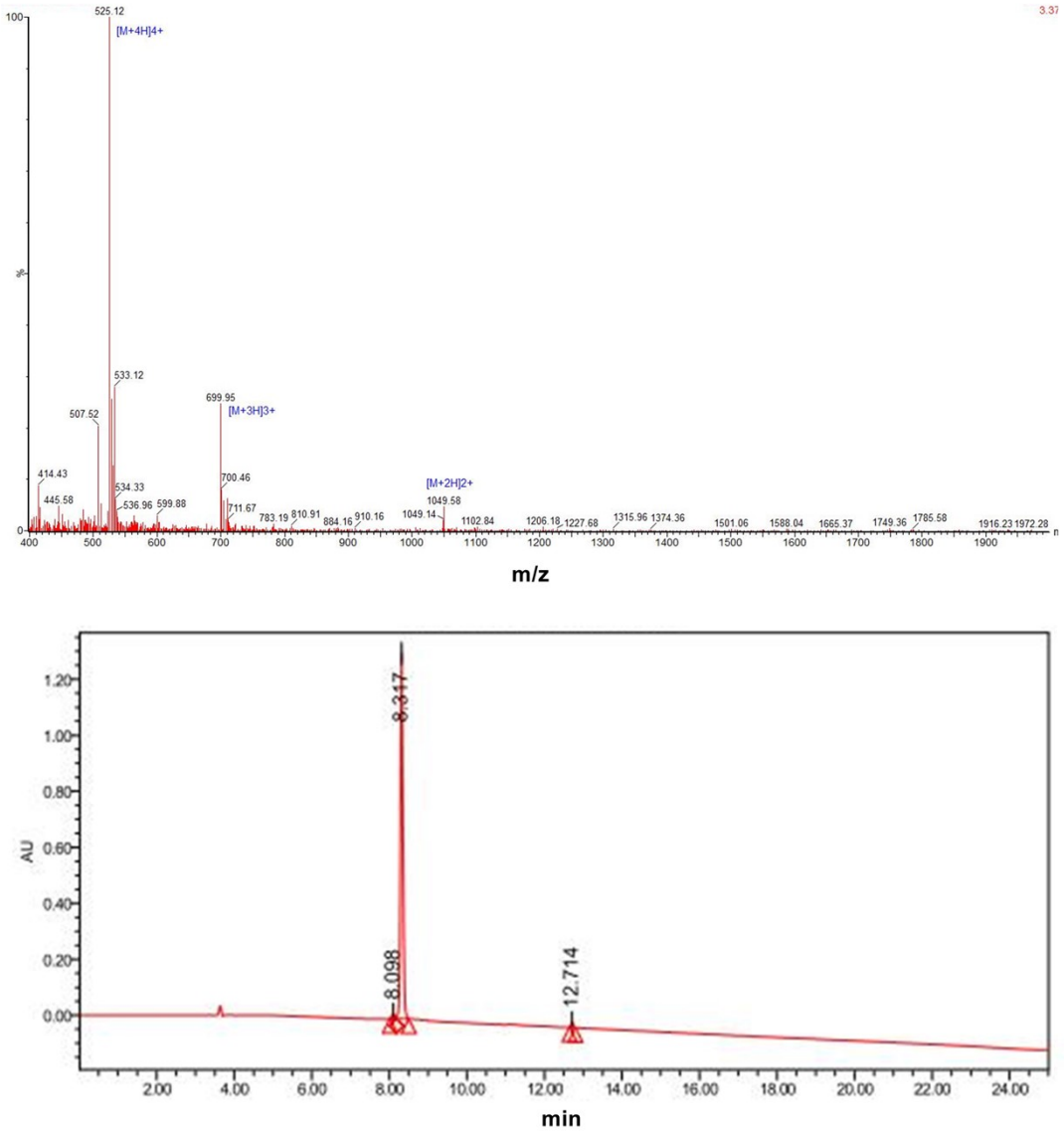

Figure S2. MALDI-TOF MS and HPLC results of RT. (HPLC: Column:GS-120-5-C18-BI0 (4.6×250 mm); Gradient:0-25-25.1 min, 24%-80%-100% aqueous acetonitrile containing 0.1% TFA; Flow rate:1.0 mL/min; UV:220 nm; volume: 5 μL)

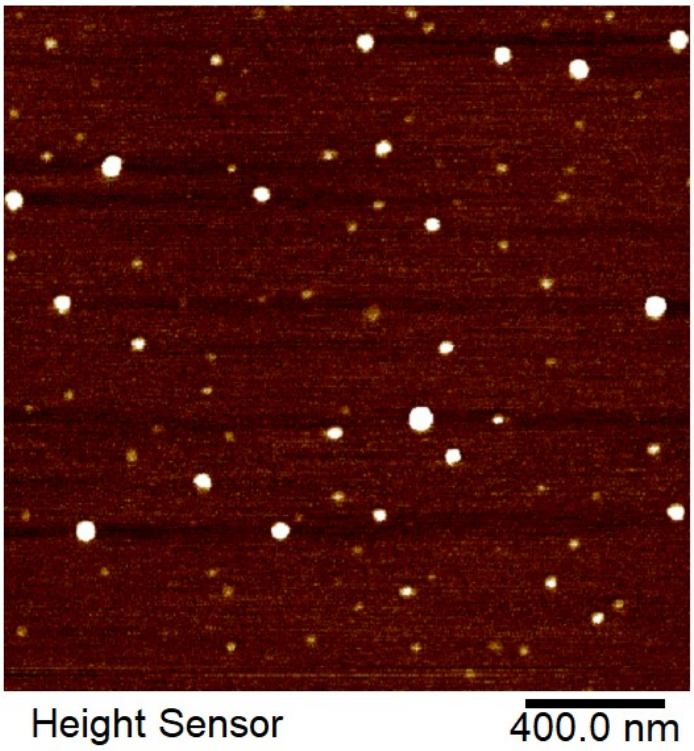

Figure S3. AFM image of QDs.

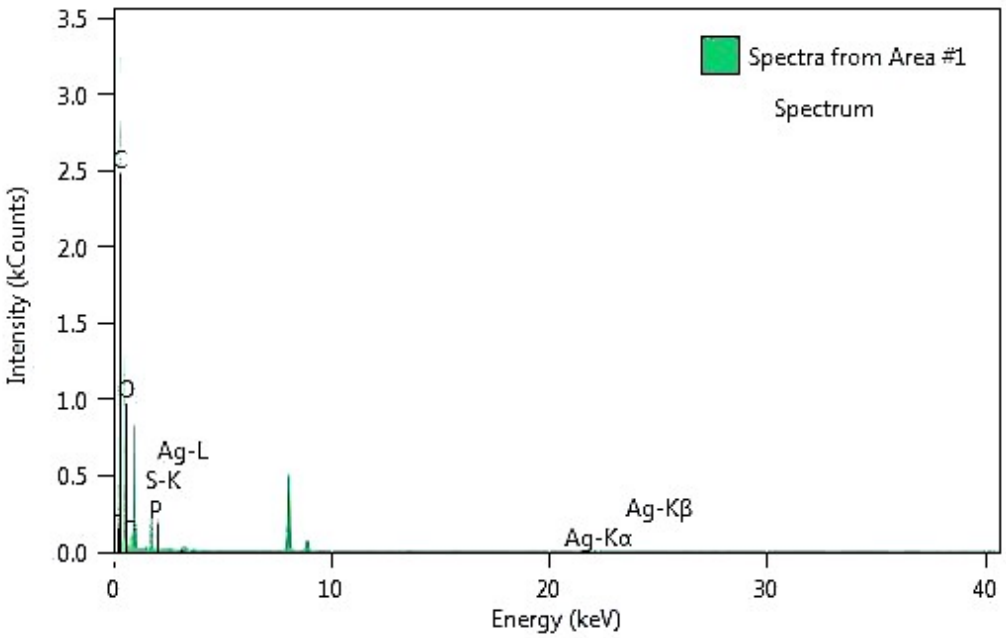

Figure S4. Element mapping results of RT^{QDs}.

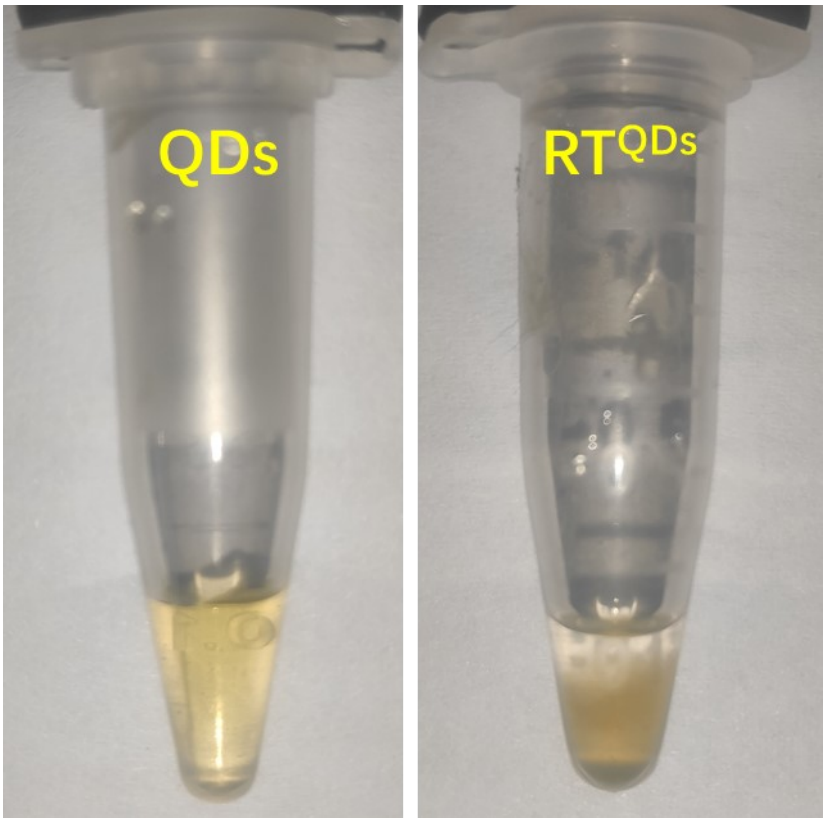

Figure S5. Images of RT^{QDs} and QDs alone after 1 month of room temperature storage.

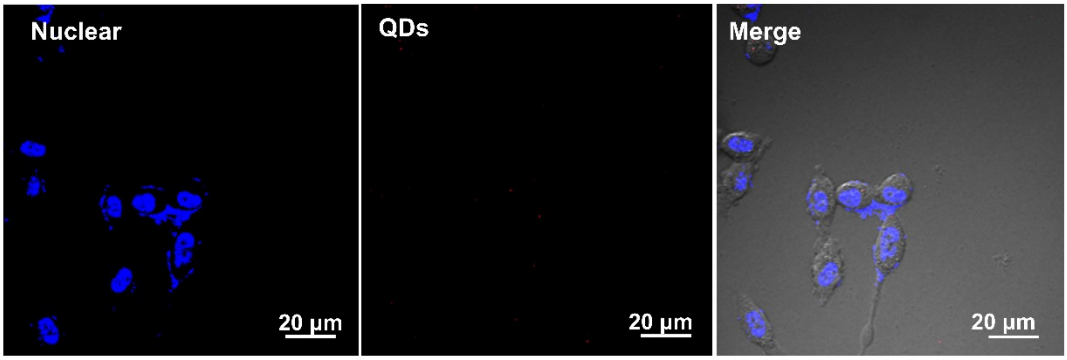

Figure S6. Confocal images of QDs incubated with 293T cells.

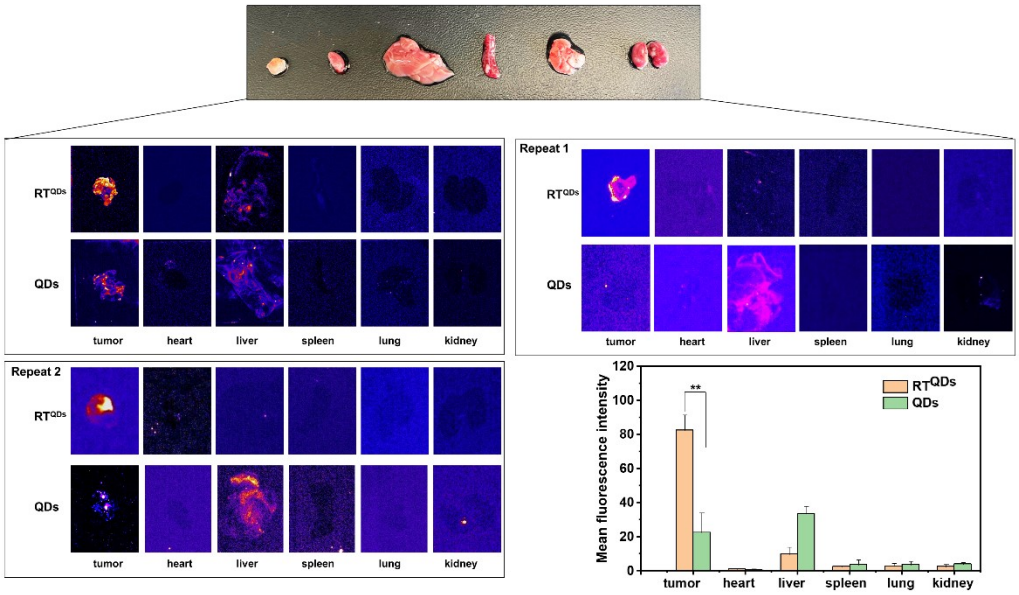

Figure S7. *Ex vivo* images of tumor and other organs after QDs and RT^{QDs} treated. Data are shown as means±s.d. (n=3). **P < 0.01, P values were performed with one-way analysis of variance (ANOVA) followed by post hoc Tukey's test for the indicated comparison.

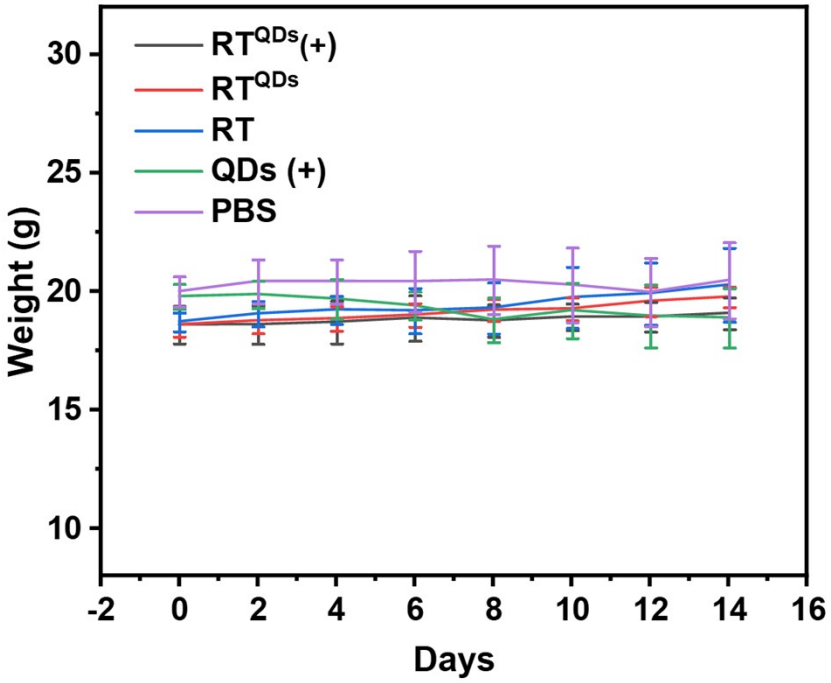

Figure S8. Body weight change curves of mice during treatment in different groups. Data are shown as means±s.d. (n=6).

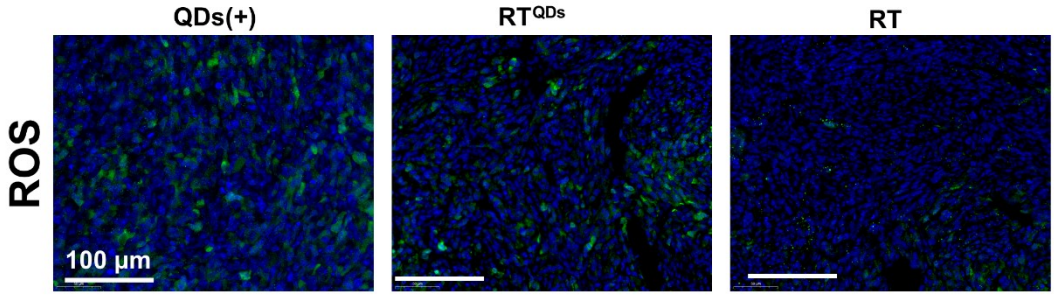

Figure S9. ROS levels in tumor tissues of different treatment groups.

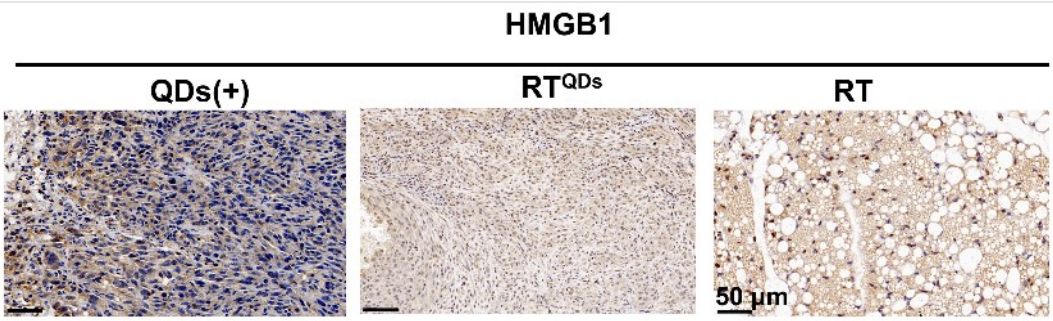

Figure S10. Immunofluorescence staining results of damage-associated model molecules HMGB1 in different tumor tissues.

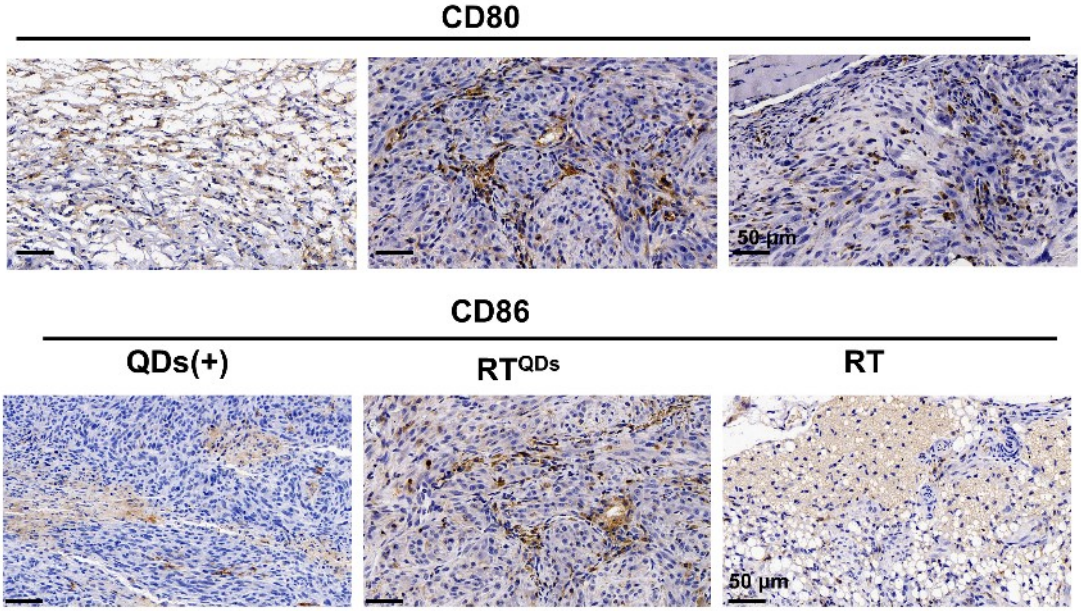

Figure S11. $CD80^{+}/CD86^{+}$ dendritic cells (DC) in tumor tissue from different treatment groups.

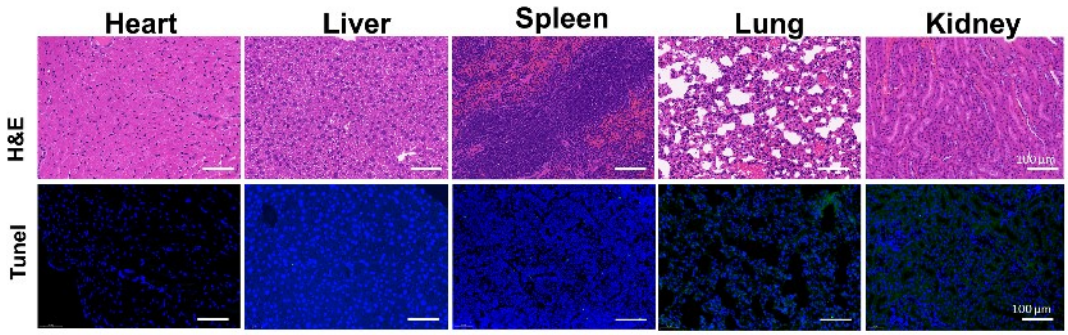

Figure S12. H&E and TUNEL staining of normal organs after receiving RT^{QDs} (+) treatment.

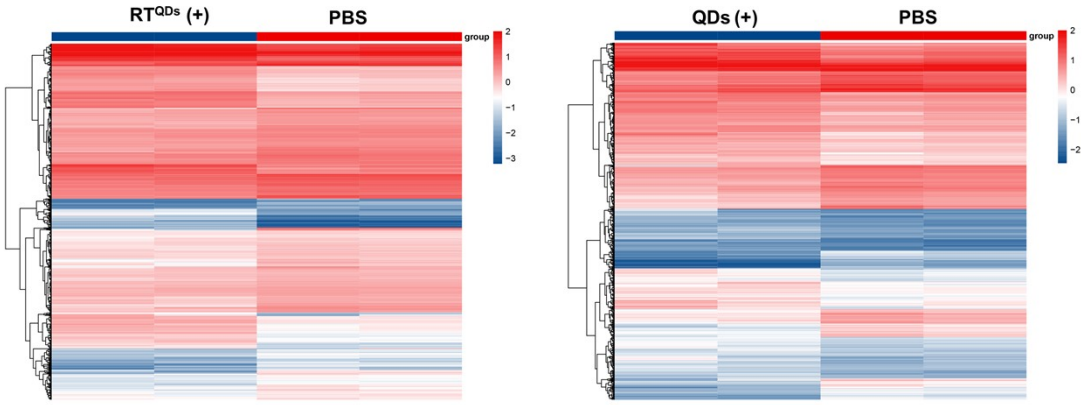

Figure S13. Heat map of differential gene expression in tumors of different treatment groups.

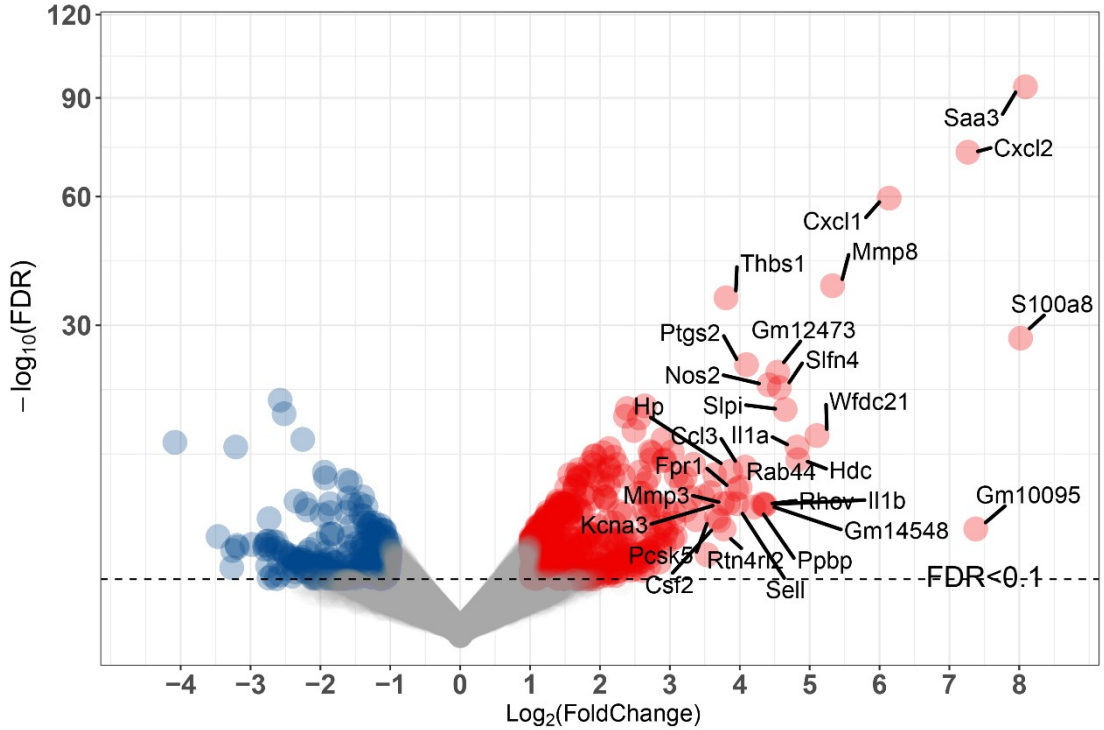

Figure S14. Volcano plots of RT^{QDs} (+) therapy groups. The volcano plots were assembled which represented that the highly deregulated genes marked with red or blue color appear in the left or right sides. They showed the different expression genes for the different therapies.

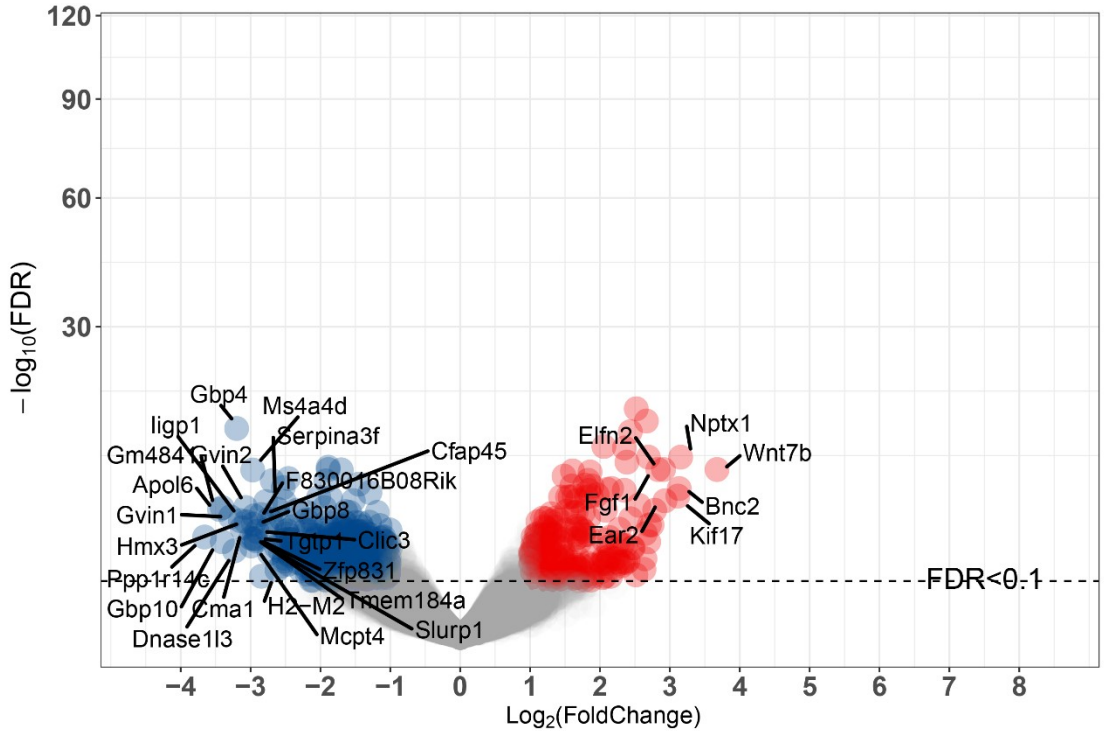

Figure S15. Volcano plots of QDs (+) therapy groups.

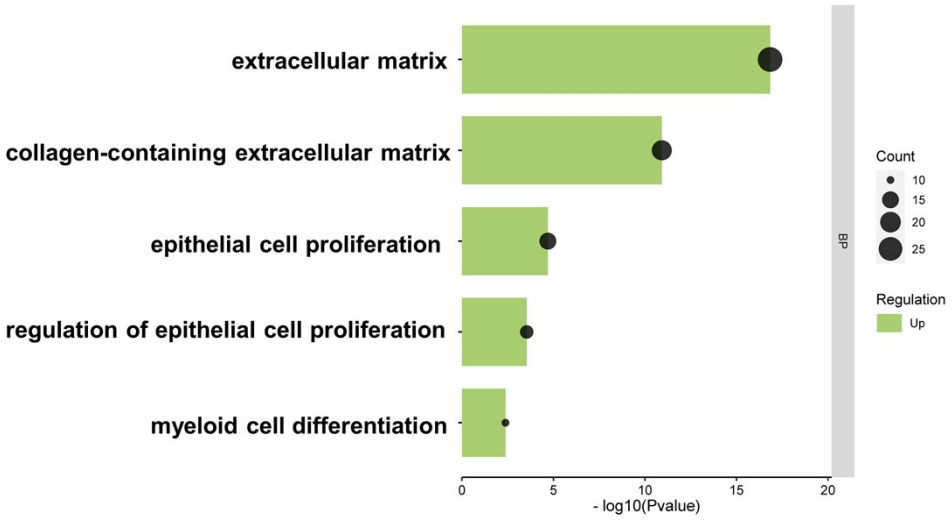

Figure S16. KEGG analysis of altered genes in QDs (+) treated group.

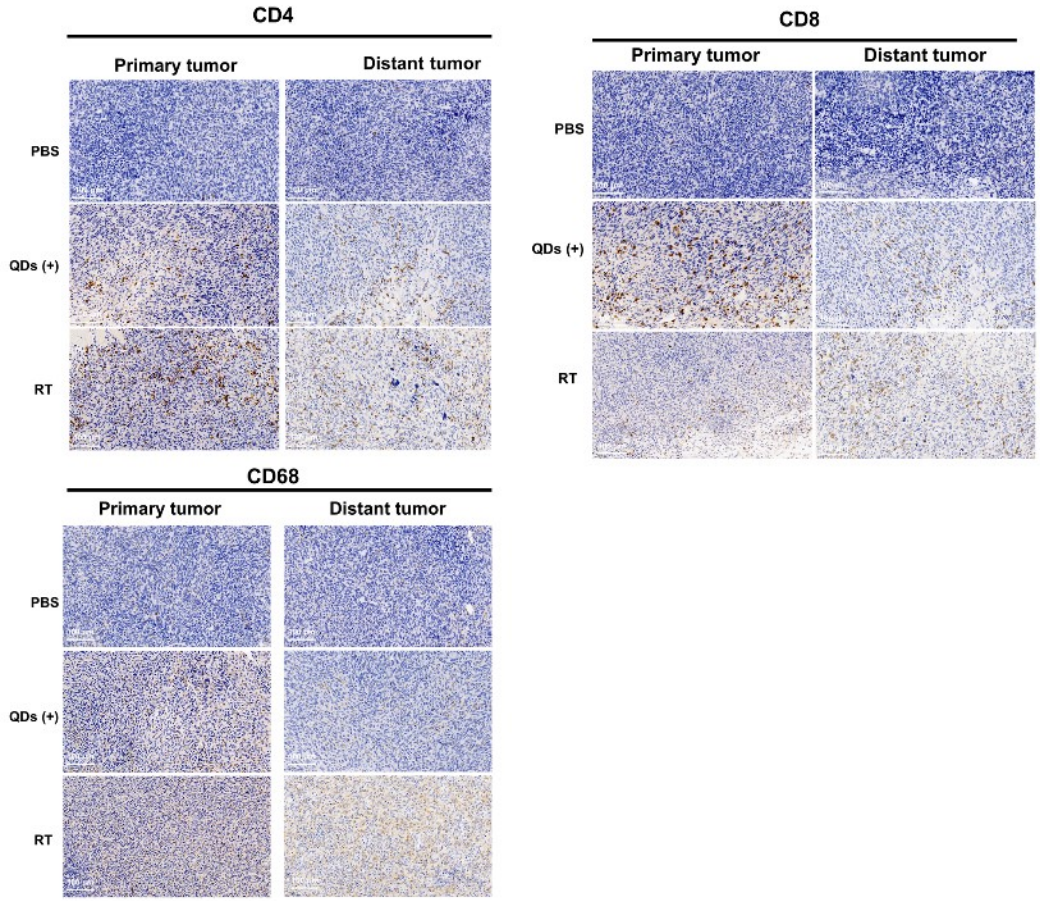

Figure S17. Immunofluorescence staining for CD8, CD4 and CD68 in primary and distal tumors after treatment with PBS, QDs (+) or RT.

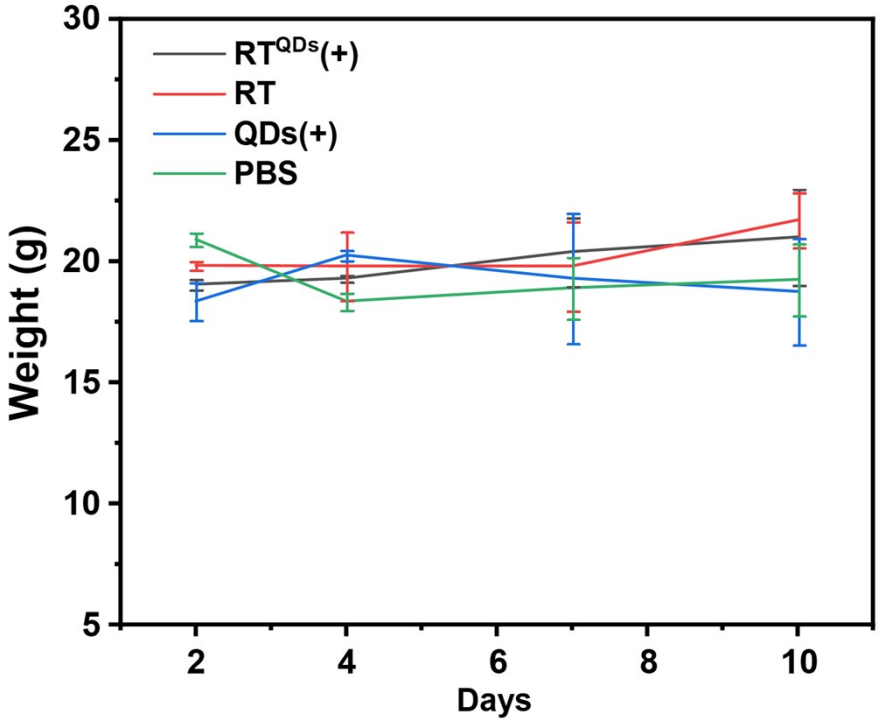

Figure S18. Curves of body weight changes of mice during bilateral tumor treatment. Data are shown as means ± s.d. (n=3).

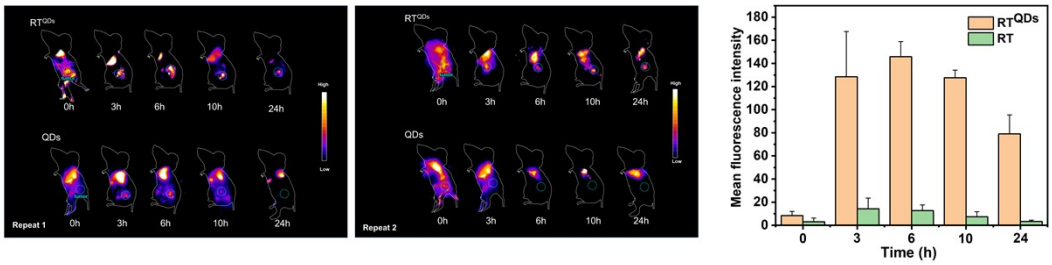

Figure S19. Repeat in vivo fluorescence imaging of living mice at different time points post injection of QDs or RT^{QDs}. Data are shown as means±s.d. (n=3).

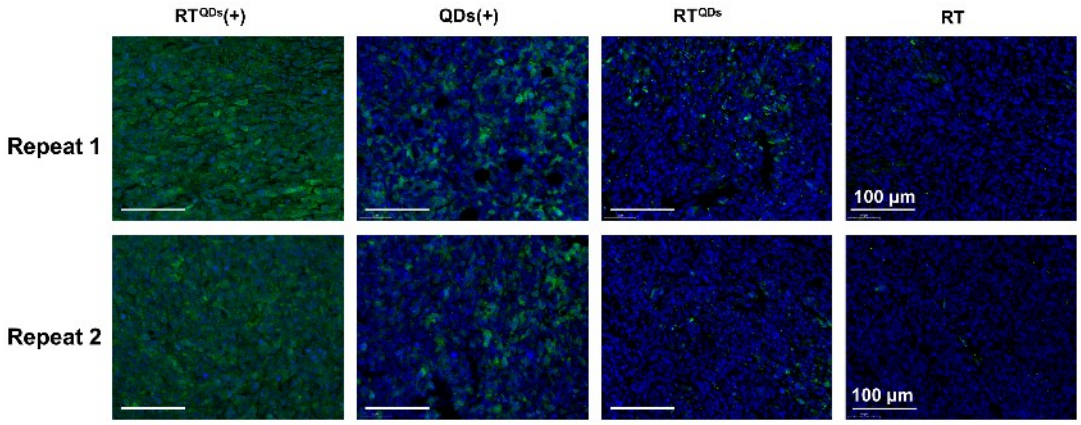

Figure S20. Repeat ROS levels in different treatment groups.

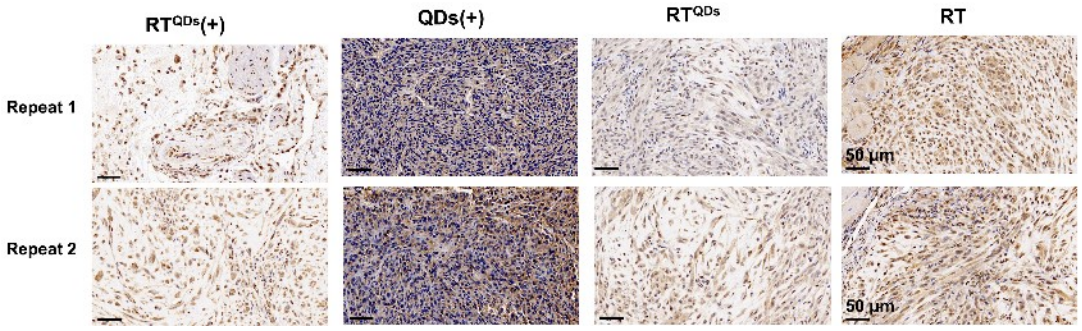

Figure S21. Repeat HMGB1 levels in different treatment groups.

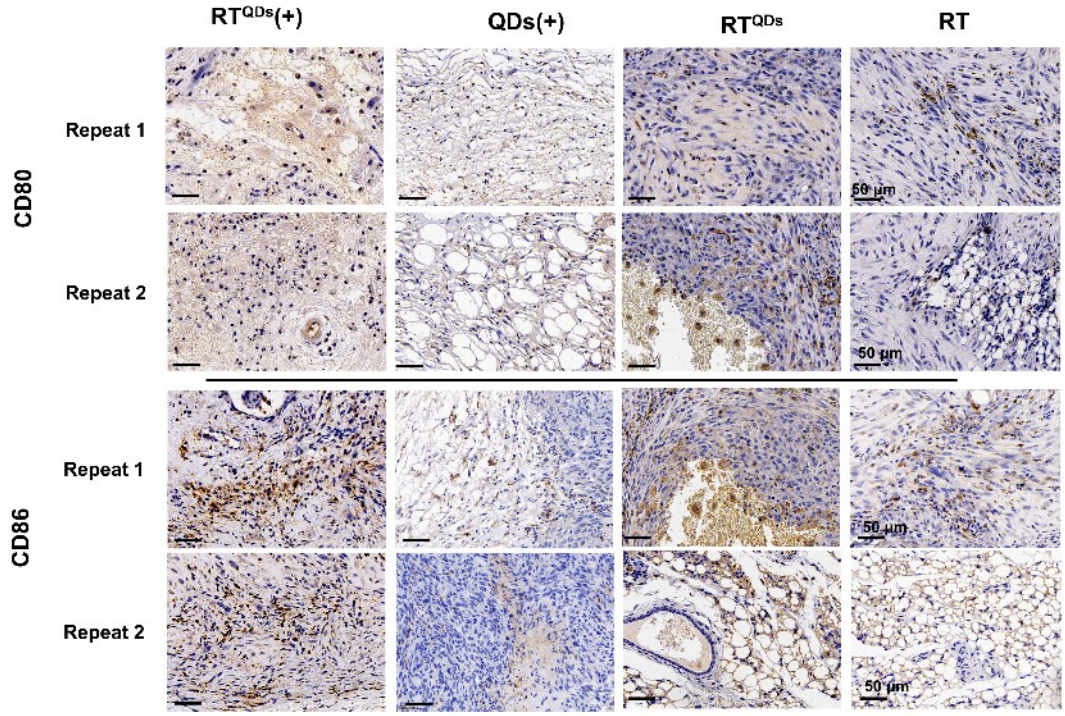

Figure S22. Repeat CD80+/CD86+ dendritic cells (DC) in tumor tissue from different groups.