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Supporting Information

Patient-derived Immunocompetent Tumor Organoids: A Platform for Chemotherapy Evaluation in the Context of T-cell Recognition

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SUPPORTING INFORMATION

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Experimental Procedures

Human specimens and patient characteristics
Fresh bladder tumor samples and matched blood samples were obtained from patients undergoing surgical treatment at the Nanjing Drum Tower Hospital. All patients provided informed consent and this study was approved by the Ethics Committee of Nanjing Drum Tower Hospital in accordance with the relevant ethical regulations (No. 2021-394-01). Detailed patient characteristics were shown in **Table S1**.

Tumor samples collection
Fresh tumor samples were obtained by cystectomy and preserved in sample preservation medium (RPMI-1640 medium supplemented with 5% fetal bovine serum ([FBS], 10μM Y-27632 [STEMCELL], and 100 μg/mL Primocin [InvivoGen]). Tissues were shipped on ice directly to the laboratory on ice within 4 hours and used for organoid culture and parental tumor analysis.

Immunocompetent bladder cancer organoid culture
After the tumor tissue was obtained, the sample together with the sample preservation medium was centrifuged at 450 g for 3 minutes at 4°C to remove the supernatant. Under sterile conditions, tumors were cut into 2-3 mm small pieces and digested in RPMI-1640 medium supplemented with collagenase I, II, and IV at 1 mg/mL (Sigma), DNase at 30 U/mL (Sigma) at 37°C for 30 minutes. The digested tissue suspensions were filtered through a 70 μm filter, centrifuged at 400 g for 5 minutes, and washed once with RPMI-1640 medium + 5% FBS + DNase (15 U/mL). After centrifugation, 1mL of red blood cell lysate was added for 5 minutes and washed twice with DPBS. The pellet after centrifugation was resuspended in organoid medium (Adv DMEM/F-12, B27 [2%], A83-01 [5 μM], N-acetylcysteine [1.25 mM], nicotinamide [10 mM], FGF10 [100 ng/ml], FGF7 [25 ng/ml], FGF2 [12.5 ng/ml], Y-27632 [10μM], and IL-2 [500 IU/mL]) in a 24-well ultra-low attachment plate (Corning) at 37°C and 5 % CO^2. Organoid medium was replaced every 2-3 days and IL-7 (10 ng/mL) and IL-15 (10 ng/mL) were added in the medium.

Histological analysis and immunostaining
The tumor tissues and organoids were processed for histologic analysis and immunostaining. The tumor tissues and organoids were fixed with 4% paraformaldehyde overnight. The samples were then washed and embedded in paraffin blocks. H&E staining was performed on paraffin sections (4-5 μm) according to standard protocols. For immunofluorescence (IF), the CK5 (Abcam, ab52635) and Ki67 (Abcam, ab15580) were used. For immunohistochemistry (IHC), a primary antibody against CD3 (Abcam, ab16669) was used.

Flow cytometry analysis
Single cells suspensions from tumor tissues and organoids were collected for analysis of immune cell populations and phenotypes by flow cytometry (Agilent). The suspension was suspended in FACS buffer (DPBS supplemented with 2% FBS) and incubated with Human TruStain FcX™ (BioLegend, 422302) for 5 minutes. 50 μL diluted antibodies solution was used to suspend cells and incubated at 4°C for 30 minutes in the dark. After washing twice with FACS buffer, the resuspended cells were prepared for analysis. The antibodies, including PE/Cyanine7 anti-human CD45, PE anti-human CD3, Pacific blue anti-human CD4, APC anti-human CD8, FITC anti-human PD-1, BV605 anti-human CD39, AF700 anti-human CD69, APC/Cy7 anti-human CD137, and PE/Cy5 anti-human CD25 were used in this study and all antibodies were purchased from BioLegend, Inc.

Bulk RNA-seq and TCR-seq analysis
Gene expression profiling analyses were performed using 3 individual organoids and parental tumor tissues. Total RNA from tumor tissues or organoids was isolated using the FastPure Cell/Tissue Total RNA Isolation Kit V2 (Vazyme) according to the manufacturer's recommendations. Library concentration was measured using the Qubit® RNA Assay Kit in Qubit® 3.0 for preliminary quantification. Insert size was assessed using the Agilent Bioanalyzer 2100 system. Sequencing was performed by the NovaSeq6000 sequencing platform, and raw data was filtered to obtain high-quality sequences (clean data) for further analysis. The Sangerbox tools, a free online data analysis platform (www.sangerbox.com), was used to generate the heatmap. RNA-seq and TCR-

seq analysis of drug treated BCOs were performed using AccuraCode® HTP OneStep RNAseq Kit (1071066) and AccuraCode® TCR Library Construction Kit (13630104) (Singleron Biotechnologies, Nanjing, China).

Whole-exome sequencing

Whole-exome sequencing (WES) libraries were prepared according to the manufacturer's recommendations using the TruSeq DNA PCR-free prep kit (Illumina). The library was quality checked using the Agilent High Sensitivity DNA Kit (Agilent), and 2x 150bp paired-end sequencing was performed on the NovaSeq sequencer. Sequence reads were aligned to the human reference genome (UCSC hg19) using the Burrows-Wheeler Aligner (BWA) software. Copy number variations (CNVs) were detected using the Control-FREEC software on BAM files. Sequencing services were provided by Personal Biotechnology Co., Ltd. Shanghai China.

Synthesis and characterization of Pt^{IV} compounds

Oxoplatin was synthesized in a easy way. Hydrogen peroxide (30 wt %, 20 mL) was added dropwise to the suspension of cisplatin (400 mg, 1.33 mmol) in H_2O (12 mL) at 60 °C. After 4 hours, the bright yellow solution was cooled at room temperature overnight to afford yellow crystals. The crystals were filtered and washed with cold water. Yield: 90%.

Pt-18: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with Epalrestat (64 mg, 0.20 mmol), triethylamine (28 µL, 0.20 mmol), and TBTU (64 mg, 0.20 mmol) at ambient temperature for 24 hours. The obtained solution was concentrated by rotary evaporator to 1 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a yellow precipitate. The precipitate was washed with methanol and diethyl ether twice, respectively, and dried under vacuum. Pt-18 was obtained as solid with a yield of 41%.^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 7.37-7.58 (m, 7H), 5.80-6.06 (m, 6H), 4.70 (s, 2H),2.23 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d6): δ (ppm) 193.54, 173.02, 167.04, 144.64, 139.83, 136.34, 133.55, 130.08, 129.19, 129.10, 121.16, 46.64, 16.31. ^{195}Pt-NMR (69 MHz): 1054.27 ppm. ESI-HRMS result: calculated, 632.9758 {[M-H]$^-$}; found, 632.9780. Elemental analysis found (calcd) for $C_{15}H_{19}Cl_2N_3O_4PtS_2$: C, 28.51 (28.35); H, 3.02 (3.01); N, 6.59 (6.61).

Pt-23: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with 3-Nitrocinnamic acid (174 mg, 0.90 mmol), triethylamine (125 µL, 0.90 mmol), and TBTU (290 mg, 0.90 mmol) at ambient temperature for 48 hours. The obtained yellow solution was concentrated by rotary evaporator to 1 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a whilte precipitate. The precipitate was washed with methanol and diethyl ether thrice, respectively, and dried under vacuum. Pt-23 was obtained as solid with a yield of 62%. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 8.46 (d, J = 1.6 Hz, 1H), 8.30–8.06 (m, 2H), 7.70 (t, J = 8.0 Hz, 1H), 7.54 (d, J = 15.9 Hz, 1H), 6.81 (d, J = 16.0 Hz, 4H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ (ppm) 173.61, 148.81, 139.26, 136.88, 134.10, 130.87, 124.62, 124.46, 123.07 ppm. ^{195}Pt-NMR (69 MHz, DMSO-d_6): 1207.43 ppm. ESI-HRMS result: calculated, 682.0066 {[M-H]$^-$}; found, 682.0067. Elemental analysis found (calcd) for $C_{18}H_{18}Cl_2N_4O_8Pt$: C, 31.80 (31.59); H, 2.67 (2.65); N, 8.17 (8.19).

Pt-24: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with 3,4,5-Trimethoxycinnamic acid (214 mg, 0.90 mmol), triethylamine (125 µL, 0.90 mmol), and TBTU (290 mg, 0.90 mmol) at ambient temperature for 72 hours. The obtained solution was concentrated by rotary evaporator to 3-5 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a yellow precipitate. The precipitate was washed with methanol and diethyl ether thrice, respectively, and dried under vacuum. Pt-24 was obtained as solid with a yield of 56%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ (ppm) 7.34 (d, J = 15.8 Hz, 1H), 6.97 (s, 2H), 6.72 (s, 2H), 6.60 (d, J = 15.8 Hz, 2H), 3.82 (s, 6H), 3.69 (s, 3H). ^{13}C-NMR (101 MHz, DMSO-d_6) δ (ppm) 174.48, 153.55, 141.80, 139.23, 121.16, 105.69, 60.52, 56.44. ^{195}Pt-NMR (69 MHz, DMSO-d_6) δ 1212.94 ppm. ESI-HRMS result: calculated, 772.0998 {[M-H]$^-$}; found, 772.1028. Elemental analysis found (calcd) for $C_{24}H_{32}Cl_2N_2O_{10}Pt$: C, 37.40 (37.22); H, 4.18 (4.16); N, 3.59 (3.62).

Pt-25: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with Epalrestat (287 mg, 0.90 mmol), triethylamine (125 µL, 0.90 mmol), and TBTU (290 mg, 0.90 mmol) at ambient temperature for 48 hours. The obtained solution was concentrated by rotary evaporator to 1 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a yellow precipitate. The precipitate was washed with methanol and diethyl ether twice, respectively, and dried under vacuum. Pt-25 was obtained as solid with a yield of 53%. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 7.37-7.59 (m, 14H), 6.50 (s, 6H), 4.81 (s, 2H), 2.22 (s, 3H). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 193.40, 173.25, 166.90, 162.77, 144.80, 140.05, 136.32, 133.52, 130.08, 129.21, 129.10,120.97, 45.21, 16.30. ^{195}Pt-NMR (69 MHz): 1253.33 ppm. ESI-HRMS result: calculated, 933.9989 {[M-H]$^-$}; found, 934.0005. Elemental analysis found (calcd) for $C_{30}H_{30}Cl_2N_4O_6PtS_4$: C, 38.65 (38.46); H, 3.24 (3.23); N, 5.96 (5.98).

Pt-27: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with Ataluren (57 mg, 0.20 mmol), triethylamine (28 µL, 0.20 mmol), and TBTU (64 mg, 0.20 mmol) at ambient temperature for 24 hours. The obtained solution was concentrated by rotary evaporator to 1 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a whilte precipitate. The precipitate was washed with methanol and diethyl ether twice, respectively, and dried under vacuum. Pt-27 was obtained as solid with a yield of 32%. ^{1}H-NMR (400 MHz, DMSO-d_6) δ 8.61 (t, J = 1.7 Hz, 1H), 8.26 (td, J = 7.6, 1.8 Hz, 1H), 8.22 (dt, J = 7.7, 1.5 Hz, 1H), 8.11 (dt, J = 7.8, 1.5 Hz, 1H), 7.85-7.78 (m, 1H), 7.66 (t, J = 7.8 Hz, 1H), 7.57 (ddd, J = 11.1, 8.5, 1.0 Hz, 1H), 7.51 (td, J = 7.6, 1.1 Hz, 1H), 6.33 -5.86 (m, 6H). ^{13}C-NMR (100 MHz, DMSO-d_6) δ (ppm) 173.13, 173.09, 172.88, 168.31, 161.75, 159.19, 136.29, 136.21, 132.96, 131.42, 130.12, 129.47, 128.48, 126.07, 126.03, 117.92, 117.72, 112.31, 112.20. ^{195}Pt-NMR (69 MHz): 1035.62 ppm. ESI-HRMS result: calculated, 598.0018 {[M-H]$^-$}; found, 598.0030. Elemental analysis found (calcd) for $C_{15}H_{15}Cl_2FN_4O_4Pt$: C, 30.40 (30.01); H, 2.53 (2.52); N, 9.34 (9.33).

Pt-28: Oxoplatin (100 mg, 0.30 mmol) was stirred in dry DMF (10 mL) with Nateglinide (63 mg, 0.20 mmol), triethylamine (28 µL, 0.20 mmol), and TBTU (64 mg, 0.20 mmol) at ambient temperature for 24 hours. The obtained solution was concentrated by rotary evaporator to 1 mL, and then was added to a mixture of H_2O and EtOH (20 mL, 1:1) to get a whilte precipitate. The precipitate was washed with methanol, acetone and diethyl ether thrice, respectively, and dried under vacuum. Pt-28 was obtained as solid with a yield of 38%. ^{1}H-NMR (400 MHz, DMSO-d_6): δ (ppm) 7.57 (d, 1 H, J = 8.3 Hz), 7.25-7.14 (m, 5 H), 5.59 (s,6 H), 4.42-4.35 (m,1 H), 3.09 (dd,

1 H, J = 13.9, 4.4 Hz), 2.78 (dd, 2 H, J = 13.9, 9.3 Hz), 2.00 (t, 1 H, J = 12.0 Hz), 1.74-1.52 (m, 4 H), 1.40-1.08 (m, 4 H), 0.94-0.88 (m, 2 H), 0.82 (d, 6 H, J = 6.8 Hz). ^{13}C-NMR (100 MHz, DMSO-d_6): δ (ppm) 179.29, 174.73, 138.46, 129.22, 127.87, 125.94, 54.25, 43.29, 42.84, 32.29, 29.32, 29.16, 28.50, 19.61. ^{195}Pt-NMR (69 MHz):1045.69 ppm. ESI-HRMS result: calculated, 631.1412 {[M-H]$^-$}; found, 631.1441. Elemental analysis found (calcd) for $C_{19}H_{33}Cl_2N_3O_4Pt$: C, 36.57 (36.02); H, 5.31 (5.25); N, 6.60 (6.63).

Drug treatment and screening

The organoids were plated and cultured in 96-well ultra-low attachment plates (CORNING, 3474) for 24 hours, and the Pt drugs were added to the culture medium at a final concentration of 2 μM for primary drug screening. Toripalimab, BGB-A317, nivolumab, durvalumab, atezolizumab, and KN035 were added to the combination therapy group at 10 μg/mL. After 72 hours of drug incubation, organoid viability was assessed using the CellCounting-Lite 3D Luminescent Cell Viability Assay (Vazyme, DD1102) according to the manufacturer's instructions.

ELISA analysis of secreted cytokines

ELISA kits for human PF ELISA (RX104195H) and human IFN-γ ELISA (RX106205H) were purchased from RUIXIN BIOTECH. ELISA assays were performed in accordance with the manufacturer's instructions.

Statistical analysis

All statistical analyses were performed using GraphPad Prism software (version 8.0). Student's t test was performed to analyze the significance of different treatment groups. $p < 0.05$ were considered statistically significant and in all figures, ns, $p > 0.05$; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Results and Discussion

Supplementary Figures and Tables

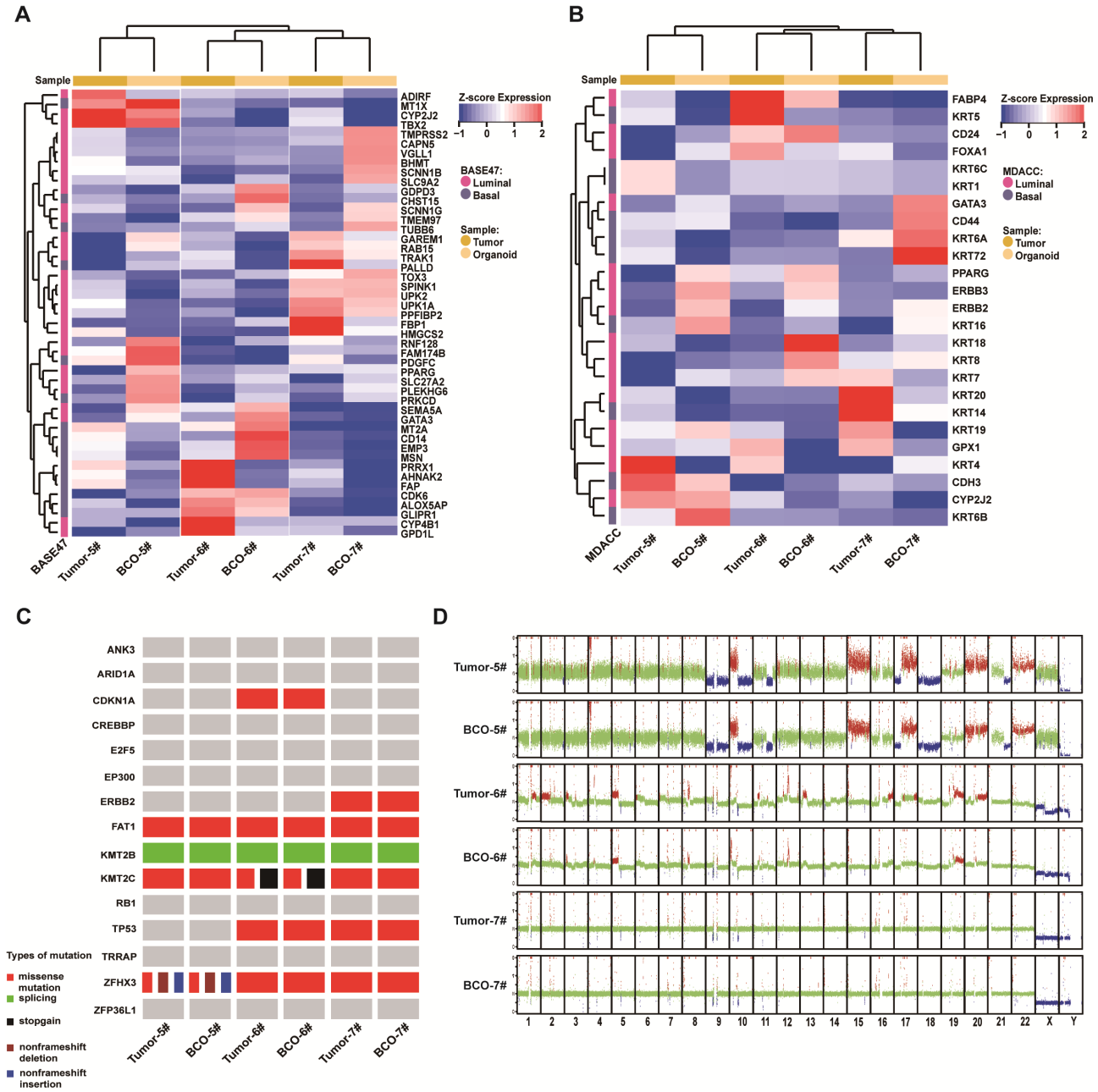


Figure S1. Repertoire of transcriptional expression and genetic mutations in patient-derived bladder cancer organoids and parental tumors. (A, B) Molecular subtypes of organoids and corresponding tumors based on the BASE47 and the MDACC classifiers. (C) Somatic genomic landscape of bladder cancer organoids and the parental tumors. (D) Genome-wide CNVs of bladder cancer organoids and the parental tumors.

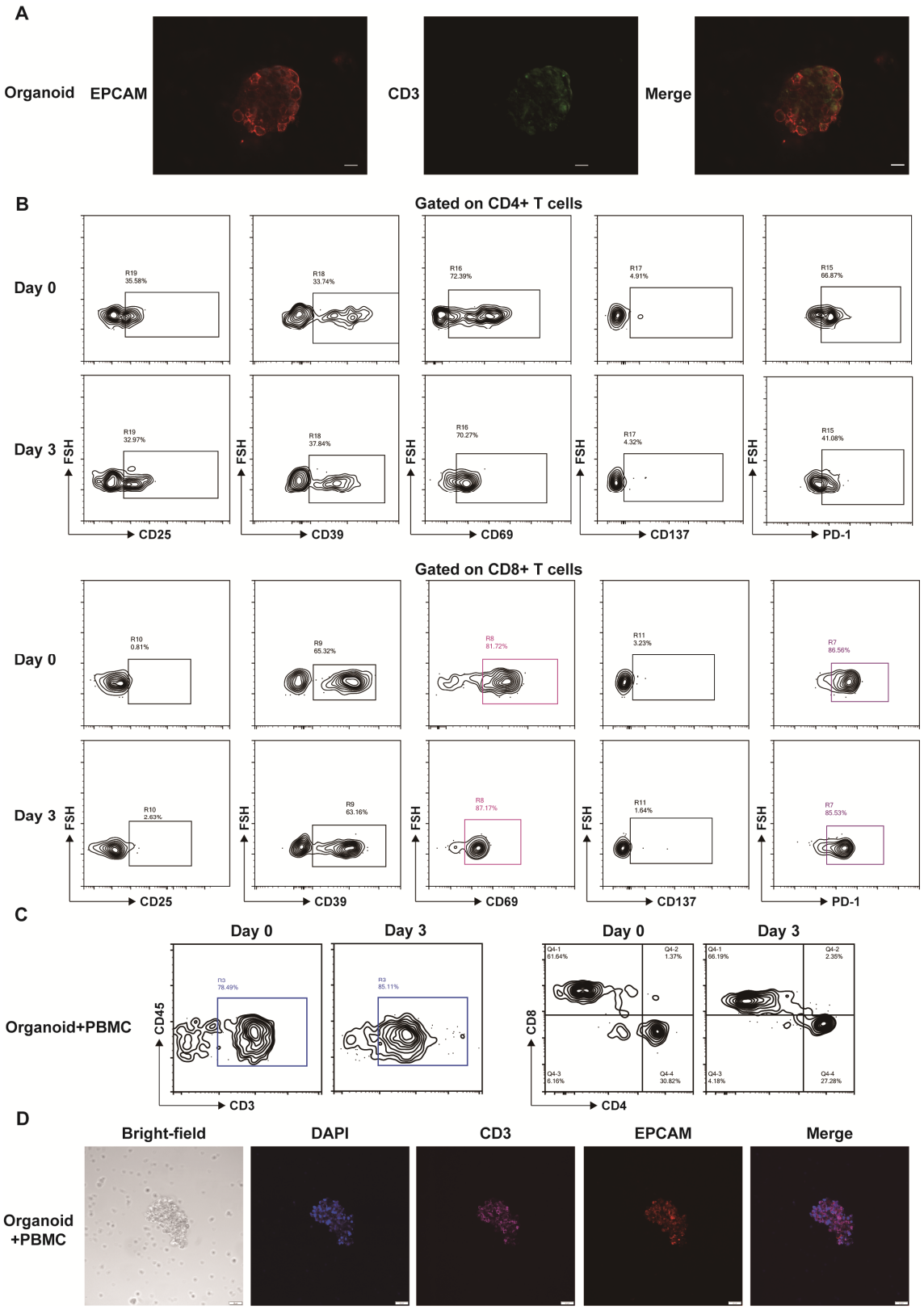
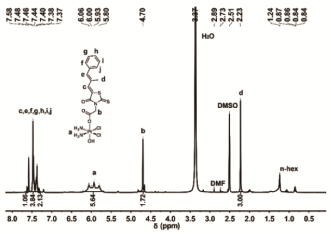


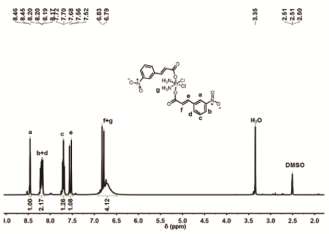
Figure S2. Validation of immunocompetent bladder cancer organoids. (A) Confocal microscopy images show the cellular composition of BCO. (B) Flow cytometric analysis of biomarkers expressed in T cells in bladder cancer organoid and corresponding tumor. (C) Flow cytometry analysis of $CD3^+$ T cells, $CD4^+$ of $CD3^+$ T cells, and $CD8^+$ of $CD3^+$ T cells in bladder cancer organoids containing PBMCs. (D) Confocal microscopy images show the cellular composition of immune desert-type organoids containing PBMCs.

SUPPORTING INFORMATION

^{1}H-NMR spectrum



Pt-18



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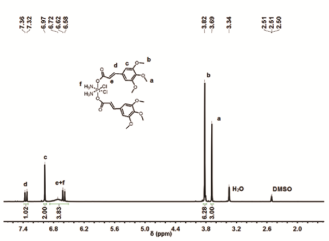

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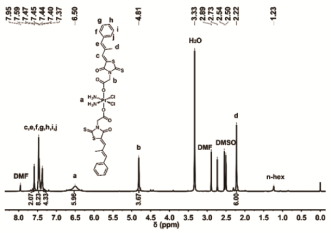

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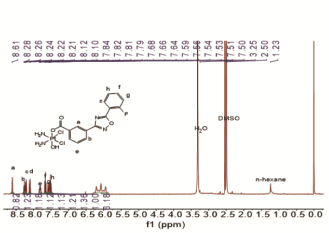

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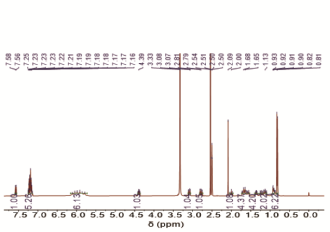
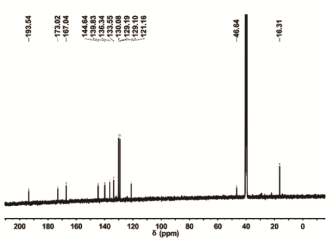


Pt-28

^{13}C-NMR spectrum



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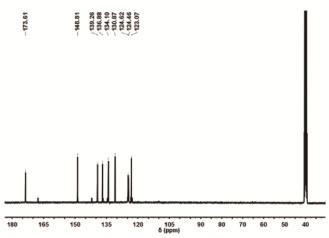

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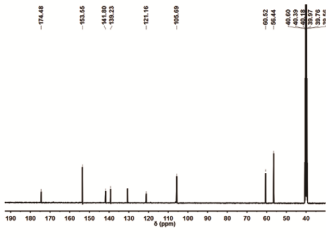

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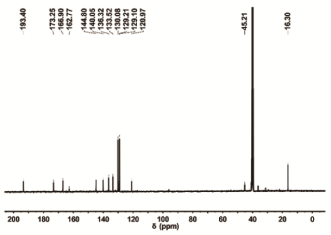

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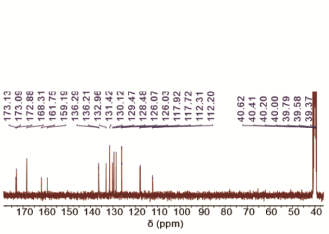

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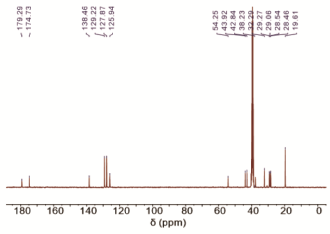
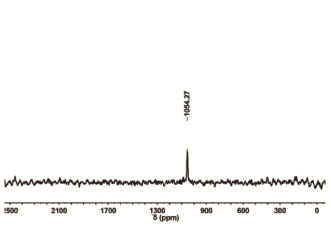


Pt-28

^{195}Pt-NMR spectrum



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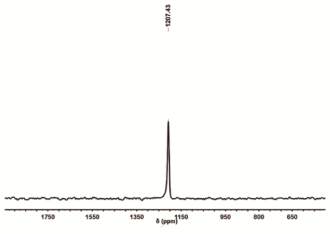

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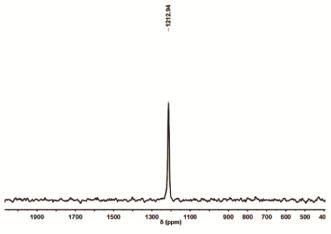

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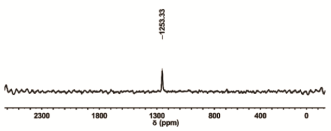

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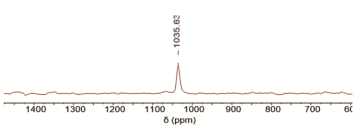

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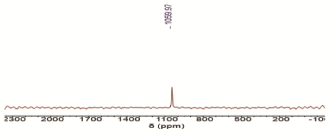

Pt-28

Figure S3. ^{1}H-, ^{13}C-, and ^{195}Pt-nuclear magnetic resonance (NMR) spectrum of the newly reported Pt^{IV} compounds.

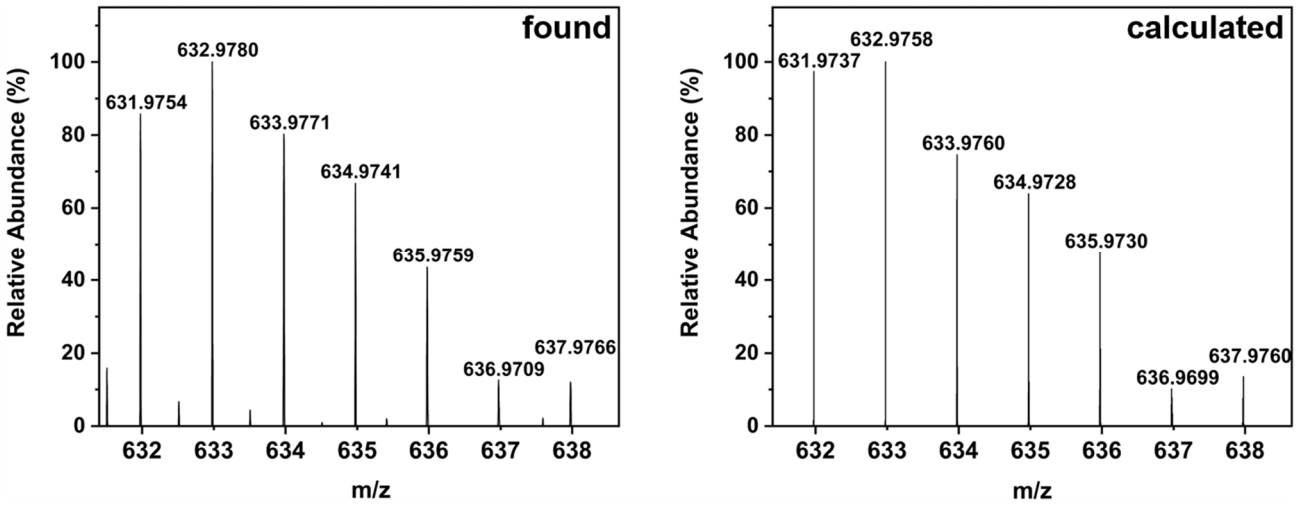

Figure S4. High resolution mass spectra of Pt-18.

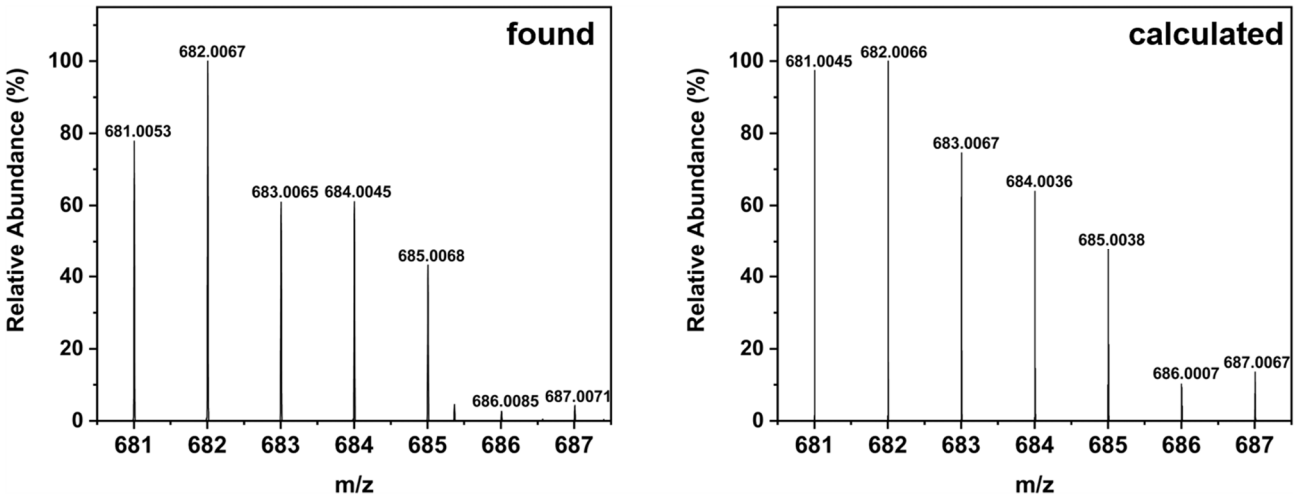

Figure S5. High resolution mass spectra of Pt-23.

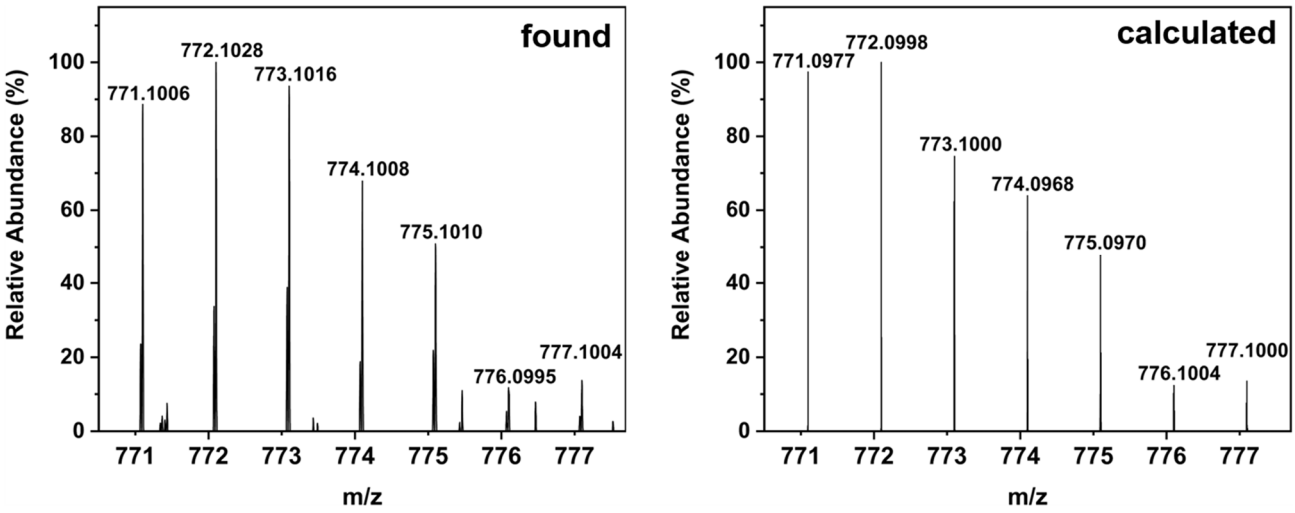

Figure S6. High resolution mass spectra of Pt-24.

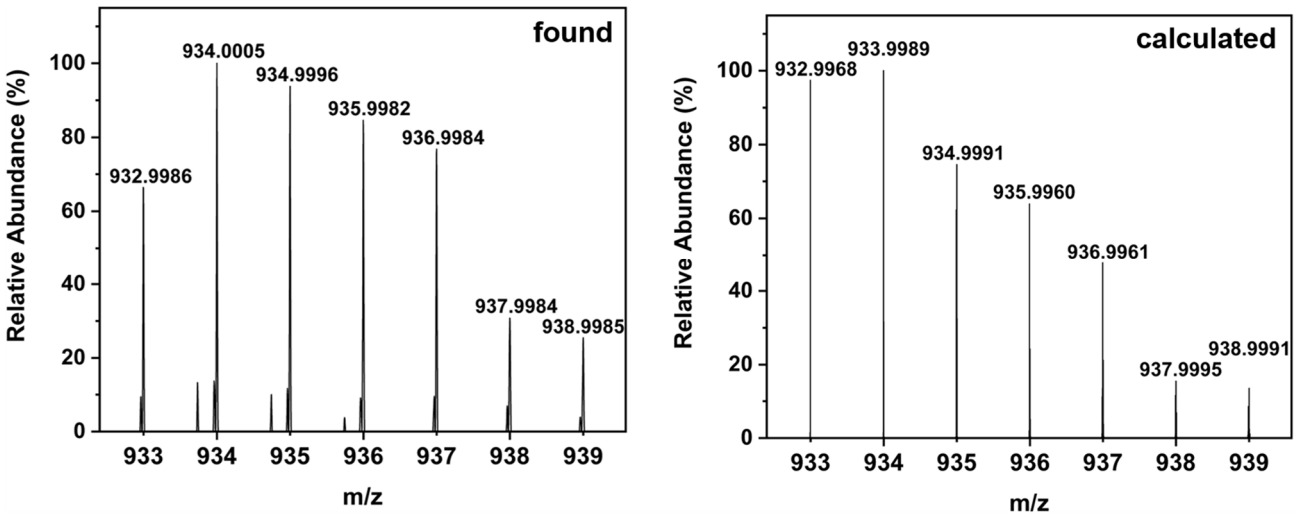

Figure S7. High resolution mass spectra of Pt-25.

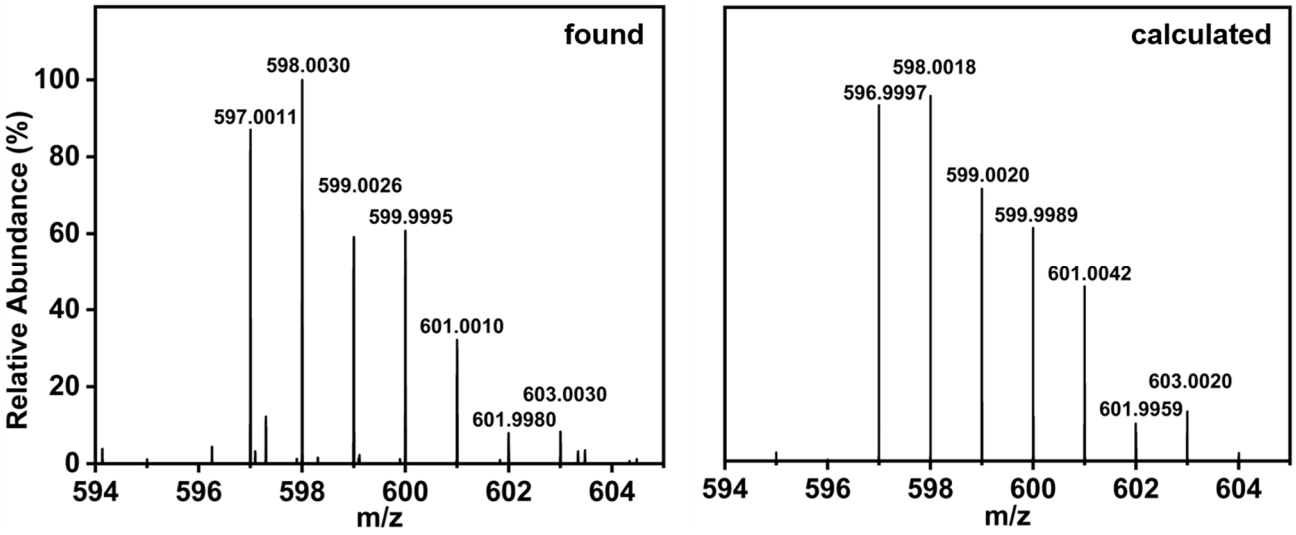

Figure S8. High resolution mass spectra of Pt-27.

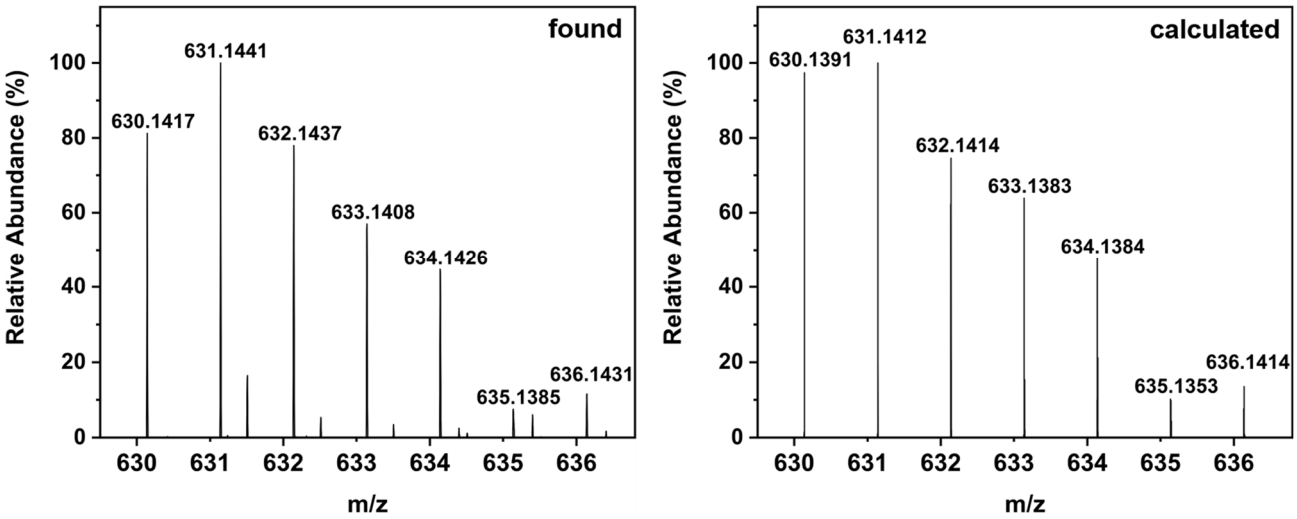

Figure S9. High resolution mass spectra of Pt-28.

SUPPORTING INFORMATION

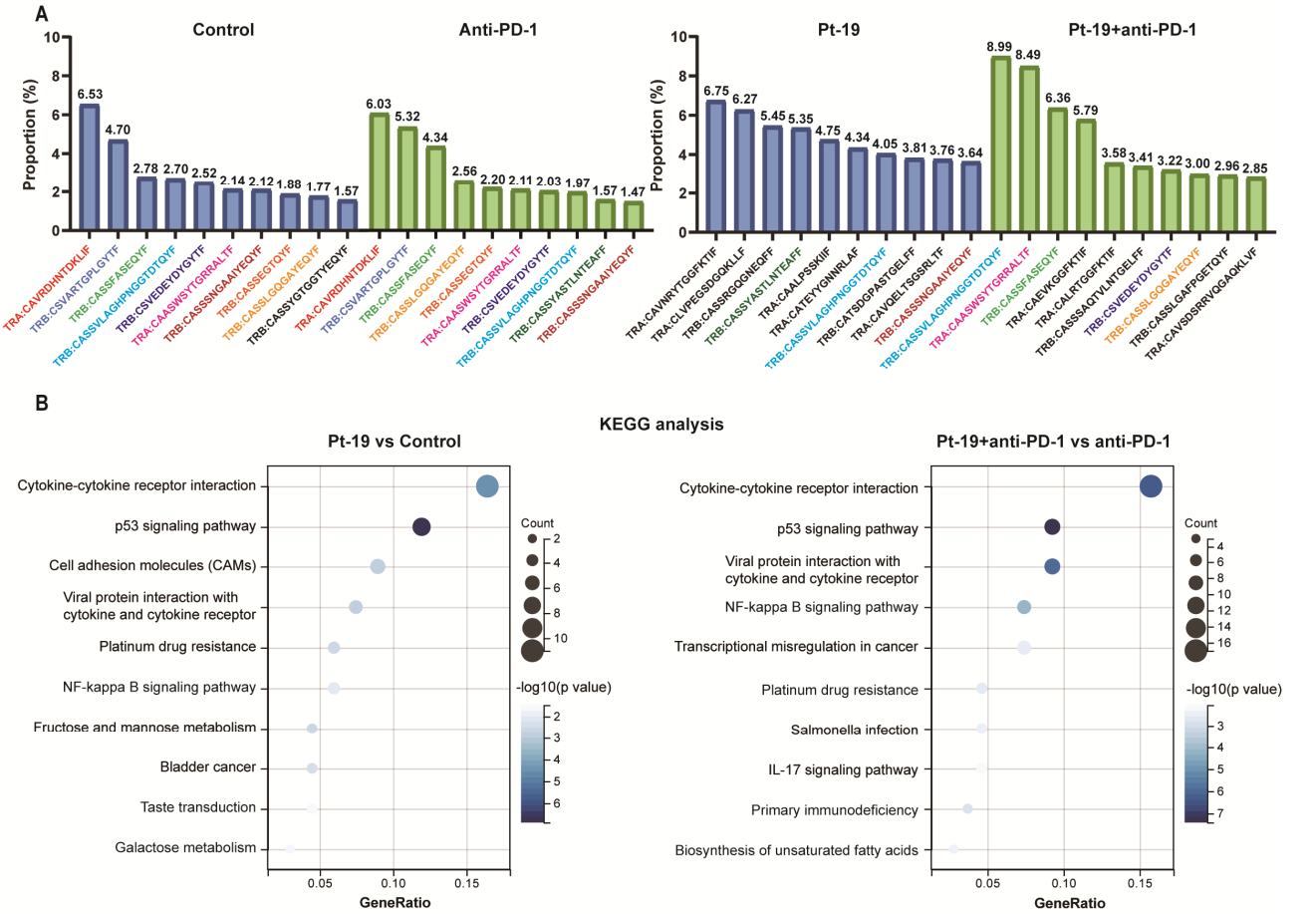

Figure S10. Immune synergy mechanism of Pt-19. (A) The top 10 TCR clones in different treatment group. Sequences labeled with the same color indicate the identical TCR clone sequences, except those labeled in black. (B) KEGG pathway analysis for the differentially expressed genes.

SUPPORTING INFORMATION

Table S1. Summary of patient-derived bladder tumor organoid lines and corresponding clinical data.

Patient ID	Age	Gender	T-stage	N-stage	M-stage	Grade	Histology
Patient 1	69	Male	a	0	0	Low	Papillary
Patient 2	65	Male	1	0	0	High	Papillary
Patient 3	80	Male	a	0	0	High	Papillary
Patient 4	61	Female	2	0	0	High	Papillary
Patient 5	90	Female	a	0	0	High	Papillary
Patient 6	72	Male	2	0	0	High	Non-papillary
Patient 7	64	Male	a	0	0	High	Papillary
Patient 8	67	Male	a	0	0	Low	Papillary

Author Contributions

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