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

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Abstract:

Most of anticancer compounds synthesized by chemists are primarily evaluated for their direct cytotoxic effects at the cellular level, often overlooking the critical role of the immune system. In this study, we have developed a patient-derived, T-cell-retaining tumor organoid model that allows us to evaluate the anticancer efficacy of chemical drugs under the synergistic paradigm of antigen-specific T-cell-dependent killing, which may reveal the missed drug hits in the simple cytotoxic assay. We evaluated clinically approved platinum (Pt) drugs and a custom library of twenty-eight Pt^{IV} compounds in this platform. We observed low direct cytotoxicity of clinically used Pt drugs, but variable synergistic effects in combination with immune checkpoint inhibitors (ICIs) in different patients. In contrast, the majority of Pt^{IV} compounds exhibited potent tumor killing capabilities. Interestingly, several Pt^{IV}

compounds went beyond direct tumor killing and showed significant immunosynergistic effects with ICIs, especially being outstanding at sub-micromolar concentrations. Among these, Pt-19, Pt^{IV} compounds with cinnamate axial ligands, emerged as the most therapeutically potent, showing pronounced immunosynergistic effects by promoting the release of cytotoxic cytokines, activating immune-related pathways and enhancing TCR clonal expansion. Overall, this initiative marks the first use of patient-derived immunocompetent tumor organoids to explore and study chemotherapy, advancing their path toward more effective small molecule drug discovery.

Introduction

Chemists have generated a plethora of compounds aimed at combating cancer, yet the majority of these substances have only been assessed for their cytotoxicity against cancer cells at the cellular level, disregarding the immune system present within the human body, especially T-cells[1-3]. These T-cells offer alternative mechanisms for targeting cancer cells beyond the direct cytotoxic effects of the compounds. As a result, many compounds are discarded post-synthesis without being evaluated through more complex models that take the immune system into account, primarily due to their insufficient direct cytotoxic effects on cancer cells. This conventional approach overlooks the intricate interplay between cancer cells and the immune system, which is crucial for a comprehensive understanding of drug efficacy.

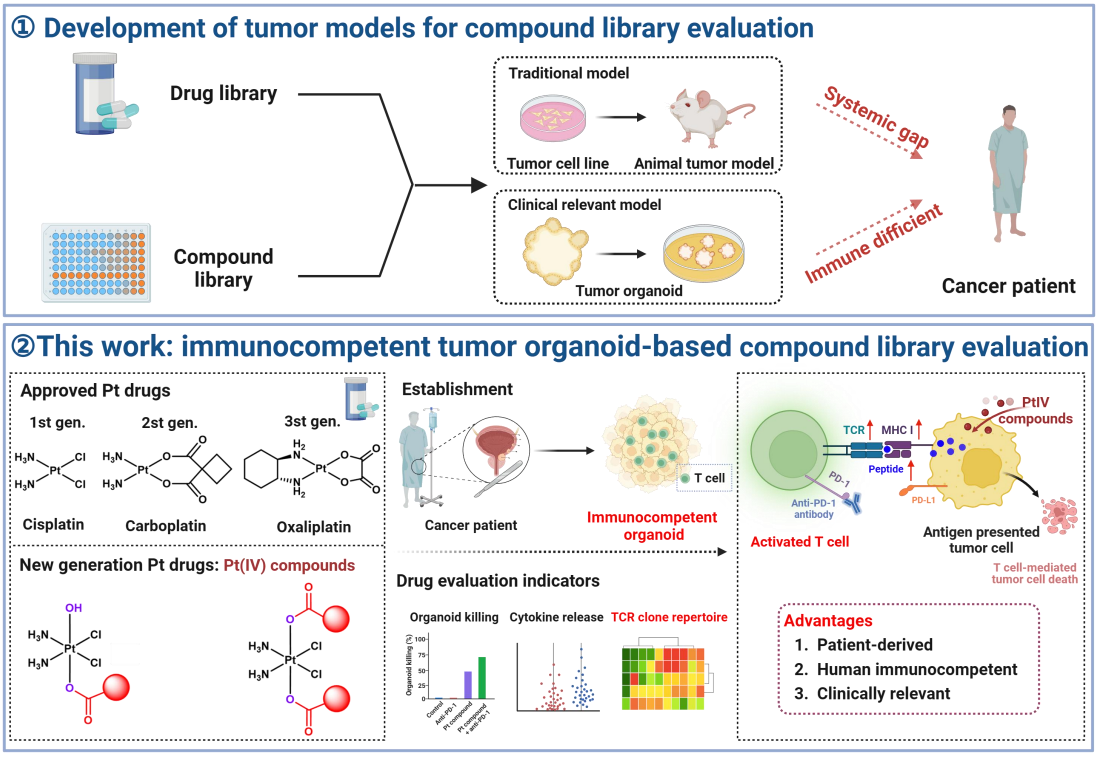
Clinically, there are examples that highlight the potential contribution of T cells to the overall efficacy of chemotherapy[4]. In particular, platinum (Pt) drugs, which are among the most widely used anti-tumor agents, have received accelerated approval from the U.S. Food and Drug Administration (FDA) for use in combination with immune checkpoint inhibitors (ICIs) for the treatment of various solid tumors[3]. This accelerated approval highlights the clinical recognition of the synergistic effects of T cells, as ICIs primarily interact with the PD-1 axis on T cell surfaces. Indeed, T cells, with their unique capacity for antigen-directed cytotoxicity, have emerged as a focal

point for harnessing the immune system to fight cancer[5]. However, the killing functionality and immune mechanisms orchestrated by T cells have often been overshadowed in the evaluation of chemical drugs due to the challenges of maintaining T cells in the *ex vivo* screening system.

In the traditional paradigm of chemotherapy drug screening, libraries of small chemical molecules are rigorously screened against a wide variety of cancer cell lines. The goal is to identify potential drug molecules that exhibit high cytotoxicity against a variety of cancer cells, while maintaining a lower toxicity profile towards normal cells. Following this preliminary screening, these promising compounds are then evaluated in mouse models where their *in vivo* efficacy, safety profile and pharmacokinetic behavior are carefully assessed. However, it has been reported that approximately 90% of preclinical drug candidates, despite showing promising activity in mouse models, fail to progress to human clinical trials[6, 7]. This high attrition rate highlights the systemic gap between mouse and human models. The recently developed patient-derived tumor organoid (PDTO) faithfully recapitulates the intricate parental structures, gene expression signatures and molecular profiles of the parental tumor[8, 9]. Previous studies have shown that the PDTO accurately predicts the patient's response to cancer treatment, including chemotherapy, targeted agents and combination drug strategies[10, 11]. However, due to the absence of T cells, the conventional Matrigel-based PDTOs have difficulties in evaluating the ICIs commonly used in clinical practice, thereby hindering the use of PDTOs for combination immunotherapy screening[12].

In this study, we first developed a T cell-containing bladder cancer patient-derived organoid (BCO) using a Matrigel-free culture system and autologous immune cells derived from tumor infiltrating lymphocytes (TILs) or peripheral blood mononuclear cells (PBMCs) to evaluate the immunosynergistic effect of Pt compounds in Pt-ICI combination therapy. We systematically evaluated the immunosynergistic effect between PD-1 inhibitors and these Pt^{IV} compounds, and

several clinically approved Pt drugs, including cisplatin, carboplatin, oxaliplatin, nedaplatin, lobaplatin as controls, taking advantage of the rapid establishment of the high-throughput screening platform based on the BCO model. Interestingly, several Pt^{IV} compounds showed the ability to enhance the therapeutic efficacy of PD-1 inhibitors. Further mechanistic studies demonstrated that Pt^{IV} complexes can promote the secretion of cytotoxic cytokines, the activation of immune-related pathways, and the expansion of T cell receptor (TCR) clones, suggesting a critical role of T cells in Pt-ICI combination therapy. Furthermore, these Pt compounds exhibited varying immunosynergistic effects when combined with different brands of PD-1/PD-L1 antibodies. In summary, the unique BCO screening platform provided a novel tool for the discovery and investigation of the immune-related mechanisms of next-generation metalloimmunotherapeutics, potentially accelerating their clinical translation (**Scheme 1**).



Scheme 1. T cell-containing organoid from bladder cancer patient for evaluation of immunosynergistic effect of Pt drugs.

Results and Discussion

Establishment and validation of T cell-maintained bladder cancer organoid

Traditionally, Matrigel was used to culture the 3D *in vitro* patient-derived organoid as extracellular matrix to mimic the tissue microenvironment *in vivo*, but this model typically contains only tumor epithelial cells and it is difficult to generate enough organoids for drug testing within 2 to 3 weeks[13]. The efficacy of clinical immunotherapies has fostered an exponential interest in reconstituting the tumor immune microenvironment (TME) of organoids, but the maintenance of immune components in organoids is still a challenge[12]. One method of an air-liquid interface (ALI) culture method preserved immune components by mincing primary tissue fragments and embedding them in Matrigel, and another method to maintain autologous lymphocytes in organoids was used the 3D microfluidic culture devices[14, 15], which were relatively complicated and difficult to apply in the clinic. A previous study described the formation of patient-derived tumor-like cell clusters (PTCs) under Matrigel-free conditions and maintained the integrity of the tumor epithelial cells, macrophages, and fibroblasts. However, the PTCs also cannot test the response of immunotherapy due to the absence of T cells[10]. When we went back and rethought the construction of immune-maintaining organoids, it's interesting that the advent of organoids actually solved the big problem of constructing patient-derived *in vitro* tumor models, and since T cells could be maintained, expanded and even infused back into the body to treat cancer about thirty-five years ago, it shouldn't be that difficult to maintain immune cells in organoids. Based on this, we optimized the culture media and conditions to maintain the T cells from parental TILs or PBMCs by adding a variety of nutrients needed for T cell growth as well as a combination of cytokines at appropriate concentrations. And thus, short-term established Matrigel-free and T cell-maintained bladder cancer organoid, which is highly suitable for evaluating T cell-dependent antitumor immunotherapies. The entire process from patient sample

collection to completion of drug evaluation takes approximately 6-7 days and the workflow is shown in **Figure 1A**.

In total, we established 8 independent BCO lines from consenting patients who underwent transurethral resection of bladder tumors at the Nanjing Drum Hospital, and the detailed clinicopathological data of the patients are summarized in **Table S1.** As shown in **Figure 1B,** a single-cell suspension derived from tumor samples can rapidly generate sufficient organoids in as little as three days, making it suitable for efficient drug screening. A series of characterization data indicated that the identified BCOs were consistent with the parental tumor at the pathohistological level. Specifically, hematoxylin and eosin (H&E) staining assay showed that the morphology of the BCOs was dense and had typical tumor features such as disorganized cell arrangement, which was similar to bladder cancer tissue **(Figure 1C)**. Immunofluorescence (IF) analysis also showed that the organoids retained the expression of the bladder epithelial marker CK5 and the proliferation marker Ki67, consistent with the parental tumor **(Figure 1D)**. Transcriptional analysis of three BCO lines and corresponding tumor tissues revealed that the phenotypes were stable in organoid culture. Both the BASE47 gene classifier and the MDACC gene classifier successfully classified the BCO lines into basal or luminal subtypes, which were similar to the parental tumor samples **(Figure S1A, S1B)**[16]. We also performed whole-exome sequencing (WES) to test whether the BCO lines retained the genetic mutations of their parental tumors. As expected, there was a strong concordance between organoids and tumor tissues with respect to somatic genomic mutations and copy number variations (CNVs). All oncogenic mutations of tumor samples were conserved in corresponding BCO lines, such as mutations of FAT1, KMT2B, KMT2C, ERBB2, TP53, and ZFHX3, which are frequently observed in human bladder cancer **(Figure S1C)**[17]. In addition, multiple chromosomal aberrations consisting of gains or losses were also highly conserved in the tissue-organoid pairs **(Figure S1D)**.

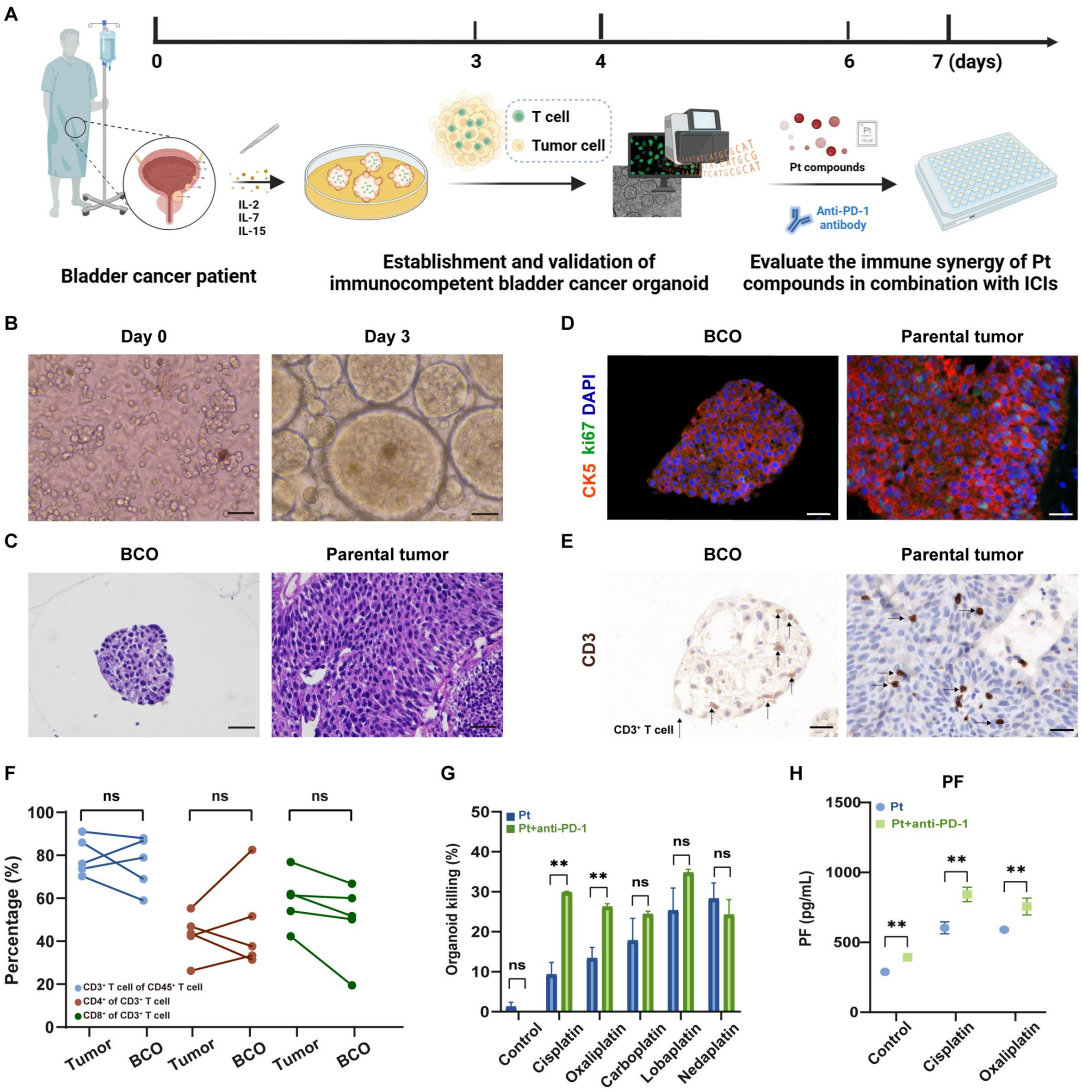


Figure 1. Establishment and validation of immunocompetent organoids derived from bladder cancer patients and evaluation of the immunosynergistic effect of clinically proved Pt drugs. (A) Timeline of BCO culture and Pt compounds combined with ICIs testing. (B) Representative brightfield microscopy images of organoids on day 0 and day 3. (C) Representative H&E staining of BCO and parental tumor. (D) Immunofluorescence staining of BCO and parental tumor for epithelial marker CK5 (red), proliferation marker Ki67 (green) and nuclear stain DAPI (blue). (E) Immunohistochemical staining of BCO and parental tumor for the T-cell marker CD3. (F) The percentage of $CD3^+$ of $CD45^+$ T cells, $CD4^+$ of $CD3^+$ T cells, and $CD8^+$ of $CD3^+$ T cells in BCOs and parental tumors by flow cytometry. (ns, $p > 0.05$. Scale bars: 50 μm). (G) Quantitative analysis of the clinically proved Pt drugs screening results (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 72 hours). (H) Quantitative analysis of the secretion of cytotoxic cytokine perforin (PF) (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 72 hours).

More importantly, immunohistochemistry (IHC) and flow cytometry analysis

showed that the BCOs successfully preserved the T cells, and the percentages of $CD3^+$ T cells, $CD4^+$ T cells, and $CD8^+$ T cells were similar in BCOs and parental tumors **(Figure 1E and 1F)**. We further demonstrated that $CD3^+$ T cells intermixture in organoid were preserved by IF imaging **(Figure S2A)**. There is no doubt that the composition and concentration of cytokines are essential to maintain the balance between the presence of T cells and the interaction between T cells and tumor cells. The cytokine IL-2 has been found to stimulate the growth of T cells, and high-dose (6000IU) IL-2 has been used to rapidly expand T cells from tumor tissue fragments in tumor infiltrating lymphocyte (TIL) therapy[18]. Recent studies have shown that there is a synergistic interaction of IL-2, IL-7 and IL-15 in promoting optimal proliferation and survival of the T cells[19, 20]. Therefore, the optimized combination of these three cytokines maintains the presence and functional phenotypes of T cells in BCOs. Immune cell phenotype profiling including the expression of CD25, CD39, CD69, CD137 and PD-1 of $CD4^+$ T cells and $CD8^+$ T cells in BCOs was highly consistent with that in parental tumors **(Figure S2B)**. Given to the individual differences among patients, not all bladder tumor tissues contain T cells, such as the immune desert type of bladder cancer[21]. For this type of bladder cancer without T cell infiltration, we attempted to co-culture autologous PBMCs with organoids to generate immunocompetent BCOs, and the corresponding data showed that these organoids successfully maintained immune cells **(Figure S2C and S2D)**.

Next, we tested the feasibility of BCO to evaluate immunotherapy using clinically approved Pt drugs in combination with PD-1 inhibitors (toripalimab). Among the five Pt drugs, cisplatin and oxaliplatin have a certain immunosynergistic killing effect **(Figure 1G)**, and the addition of toripalimab promoted the release of the cytotoxic cytokine perforin (PF) **(Figure 1H)**, which is consistent with the success of coadministration of cisplatin or oxaliplatin and ICIs in the clinic[22, 23]. To the best of our knowledge, this is the first time that the

combined tumor killing effect of platinum compound and ICI has been successfully reproduced in an *ex vivo* assay, and it suggests a direct killing role for the T cells of our constructed BCO. Because, the elevated killing effect of PD-1 antibody could only originate from the overcome of T cell exhaustion. At the same time, the facilitating effect of the Pt drug on T-cell recognition is evident here, as the PD-1 antibody alone was not effective **(Figure 1G)**.

Taken together, the above results indicated that we have successfully generated patient-derived immunocompetent BCOs that were Matrigel-free and well prepared for drug evaluation of combination immunotherapies in the context of T-cell recognition.

Construction of a Pt compound library

As mentioned above, the Pt^{IV} prodrug strategy has been widely used for the construction of multifunctional Pt compounds as illustrated in many studies[24]. Our team has long focused on the design of novel Pt^{IV} compounds and has accumulated a small library of Pt^{IV} compounds[25-27]. However, these Pt^{IV} compounds have never been evaluated in clinically relevant models such as organoids. These Pt^{IV} compounds are synthesized in a similar manner[28]. The intermediate *cis*-diamminedichloro-*trans*-dihydroxyplatinum (IV) (oxoplatin) was prepared by oxidation of cisplatin with hydrogen peroxide (30%). Axial ligands are divided into different types, including immunoactivators (MSA-2, artesunate etc.), non-steroidal anti-inflammatory drugs (such as ketoprofen, ibuprofen, naproxen, etc.), oncology drugs (such as lonidamine, bexarotene, etc.), enzyme inhibitors (Mcl-1 inhibitors, PARP inhibitors, etc.), antidiabetic drugs (such as epalrestat, nateglinide, etc.), and other non-oncology drugs (such as seratrodast, ataluren, etc.). The monocarboxylated Pt^{IV} compounds are synthesized by reacting oxoplatin with axial ligands in a molar ratio of 1:0.6 using uranium salt as a coupling agent. The bicarboxylated Pt^{IV} compounds are synthesized by reacting oxoplatin with

axial ligands in a molar ratio of 1:3 using uranium salt as a coupling agent. A total of twenty-eight different Pt^{IV} compounds have been synthesized. Some of them have been reported in previous studies, while others are reported for the first time, including Pt-2, Pt-18, Pt-23, Pt-24, Pt-25, Pt-27, and Pt-28 **(Figure 2)**. The structures and purities of the newly reported compounds were confirmed by ^{1}H-, ^{13}C-, and ^{195}Pt-nuclear magnetic resonance (NMR) **(Figure S3)**, which supported the proposed structures of the conjugates. The synergy of these compounds with ICIs has never been tested, especially in patient-derived organoids.

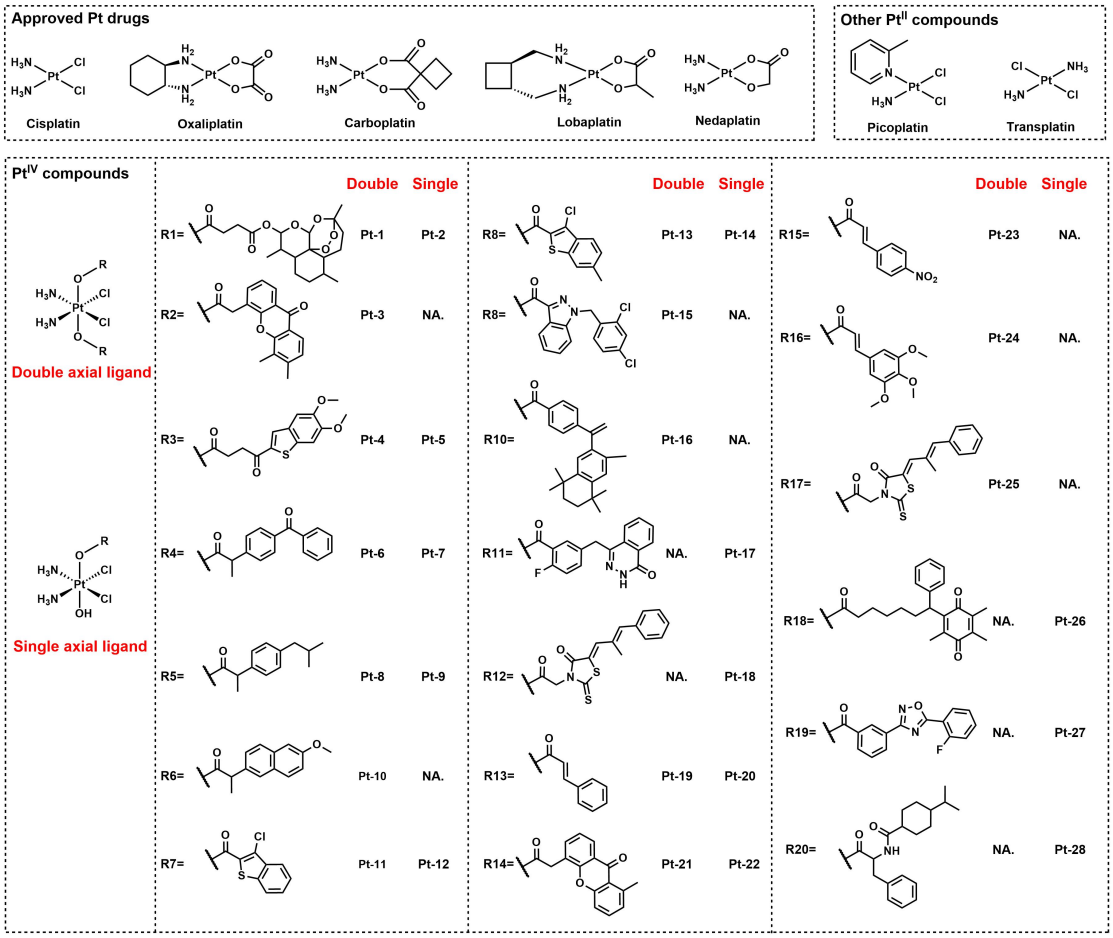


Figure 2. Chemical structures of the Pt agents used in this study.

Immune synergistic screening of Pt compounds and ICIs

To evaluate the immune synergy effect of Pt compounds and anti-PD-1

antibody, we selected two BCO lines containing T cells (from two patients). The percentage of T cells in the two BCOs is different, but which is consistent with their respective parental tumors **(Figure 3A and 3B)**. After the organoids were cultured, they were plated in low adherent 96-well plates to test the killing effect of single Pt compounds and their combination with toripalimab. Cytotoxicity was detected by measuring ATP release from BCOs in biological triplicates. As shown in **Figure 3C and 3D**, all Pt^{II} compounds failed to kill half of the organoids after 72 hours of incubation, either alone or in combination with ICIs. In contrast, these BCOs were highly sensitive to many synthesized Pt^{IV} compounds. In terms of combination immunotherapy, among the clinically approved Pt drugs, only oxaliplatin again demonstrated a synergistic killing effect with PD-1 inhibitors, which is also consistent with clinical practice. In fact, oxaliplatin, has been reported to be an immunogenic death (ICD) inducer to enhance the anticancer immunity[29]. Our previous study also demonstrated that the combination of oxaliplatin and anti-PD-1 antibody can promote immune cells to infiltrate the solid tumor and enhance the antitumor effect of PD-1 blockade [30]. These suggest the efficacy of the established BCO platform for screening combination immunotherapeutic drugs. As for Pt^{IV} compounds, Pt-9, Pt-12, Pt-18, Pt-20, Pt-21, and Pt-23 have also shown the potential to act synergistically with PD-1 inhibitors in various patient-derived BCOs ($p < 0.05$, **Figure 3E**). It is worth noting that these synergistic effects did not follow the same trend between patients, highlighting

the complexity of all immune-related therapies.

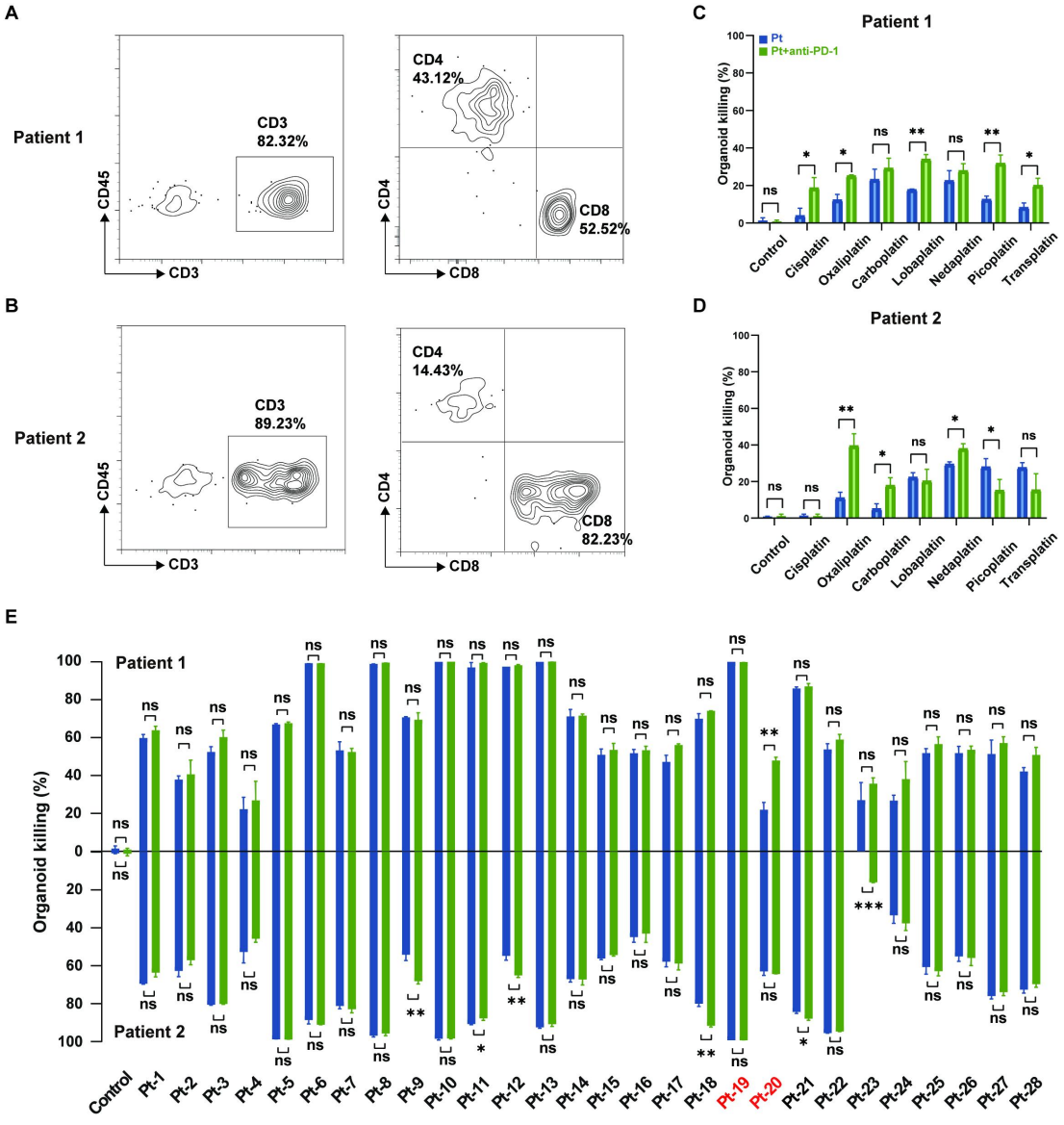


Figure 3. Systematic screening of immunosynergistic Pt compounds using patient-derived immunocompetent organoids. (A) Flow cytometric analysis of T cells for patient 1. (B) Flow cytometric analysis of T cells for patient 2. (C) Quantitative analysis of the Pt^{II} compounds screening results for patient 1. (D) Quantitative analysis of the Pt^{II} compounds screening results for patient 2. (E) Quantitative analysis of the Pt compounds screening results for patient 1 and patient 2. (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 72 hours). (ns, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Interestingly, among these Pt^{IV} compounds, two Pt^{IV} derivatives of cisplatin with axial cinnamato ligand(s) (cinn), cis,trans,cis-$[Pt(NH_3)_2(cinn)_2Cl_2]$ (Pt-19)

and cis,trans,cis-[$Pt(NH_3)_2(OH)(cinn)Cl_2$] (Pt-20), showed different organoid killing ability **(Figure 4A)**. Pt-19 completely killed the cancer organoids, while Pt-20 did not have strong organoid killing ability, but showed an immune synergy of PD-1 inhibitors **(Figure 4B and 4C)**. We therefore speculated that there was a possibility that Pt-19 might kill T cells while killing cancer cells, thereby interfering with the enhanced therapeutic effect of Pt-19 over PD-1 inhibitors. To explore this possibility, we decided to carefully investigate the immunosynergistic potential of Pt-19 with ICIs by optimizing the drug concentration. Surprisingly, with decreasing of drug concentration, Pt-19 also exerted a significant immunosynergistic effect in both patient 1 and patient 2 **(Figure 4D)**. When the dose of Pt-19 was reduced to 0.1 μM, the combined effect of Pt-19 and PD-1 inhibitors was most evident ($p < 0.01$). Therefore, we further tested the immunosynergistic therapeutic effect of Pt compounds and various ICIs, including BGB-A317 (anti-PD-1), nivolumab (anti-PD-1), durvalumab (anti-PD-L1), atezolizumab (anti-PD-L1), KN035 (anti-PD-L1) **(Figure 4E).** We found that not only the response of the organoids to different ICIs was different, but also the immune synergism produced by different combinations of Pt compounds and ICIs was different. The organoids failed to respond to most of the ICIs, while the combination of Pt compounds with ICIs was more potent in killing organoids compared to single drugs ($p < 0.05$). Although almost all ICIs showed some degree of enhancement when combined with the Pt compounds, the degree of enhancement is different. It appeared that oxaliplatin worked best in combination with toripalimab, and Pt-19 worked best in combination with BGB-A317 or durvalumab. Overall, the Pt compounds screened in combination with toripalimab still have immunostimulatory effects in combination with other ICIs, but the optimal combination of Pt compounds and ICIs still needs to be explored.

Due to the individual variability of bladder cancer patients, parts of tumor samples lack TILs[31], which limits the generation of BCOs for immunotherapy

evaluation. Previous studies have shown that immune cells derived from PBMCs play an essential role in immunotherapy responses[32, 33]. Therefore, we established the BCO by adding patients' own PBMCs for immunodeficient tumors **(Figure S2C and S2D)**. In this case, when the dose of Pt-19 was decreased, the immunosynergistic effect of Pt-19 was improved, which was similar to the trends in the BCOs containing TILs without PBMCs **(Figure 4F)**. Therefore, the present results demonstrate that autologous PBMCs from patients can be used to construct the immunocompetent organoids. Such a strong synergistic effect also suggests that PBMC-derived T cells have established antigen-specific clones that recognize cancer cells during BCO construction. This case demonstrates a possible way to construct immunocompetent BCOs for immune desert cancers. Taken together, the immune synergy of low-concentration Pt-19 suggests that the concentration effect of Pt compounds should be fully considered, as high-concentration drug treatment may mask the activation of immunity.

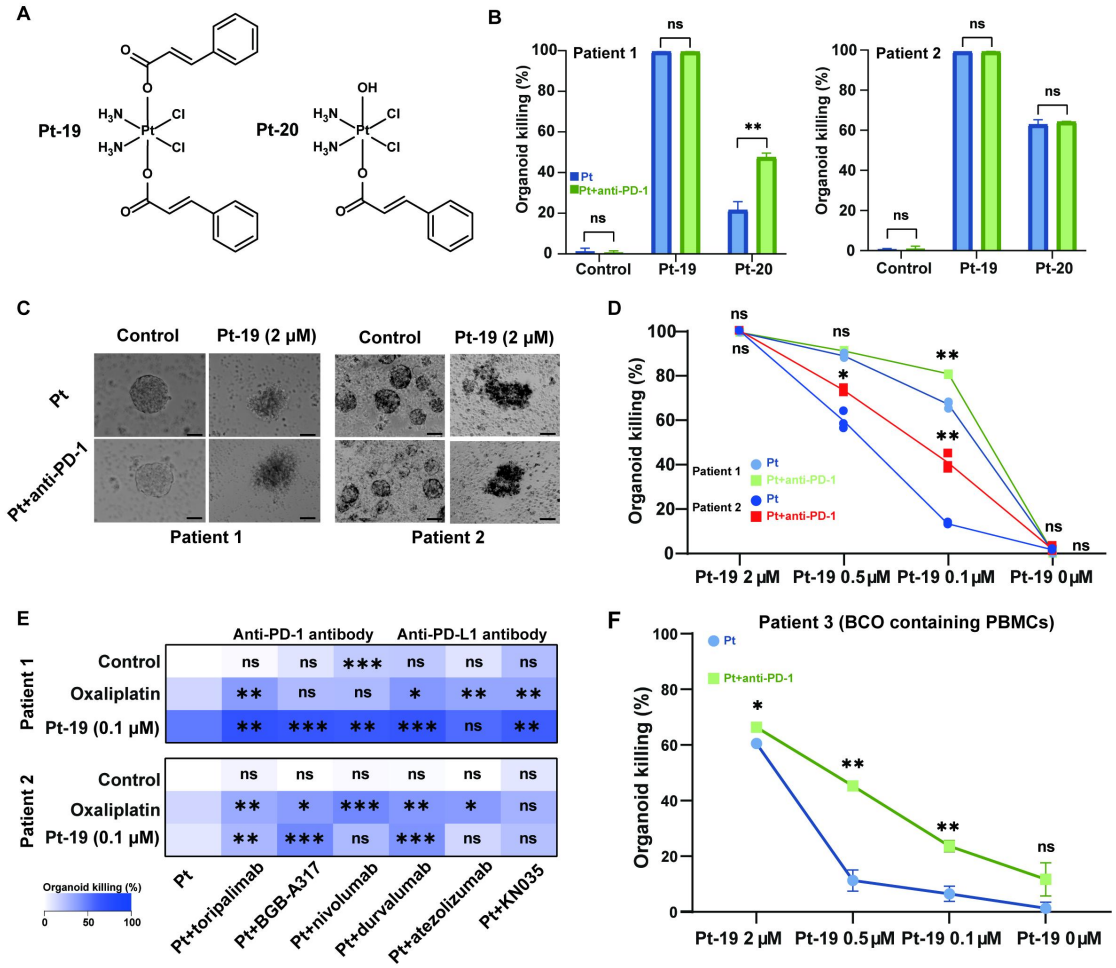


Figure 4. Evaluation of immunosynergistic effects of Pt-19 compounds using patient-derived immunocompetent organoids. (A) Chemical structures of the Pt-19 and Pt-20. (B) Quantitative analysis of the immune synergy of Pt-19 and Pt-20 in killing organoids. (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 72 hours). (C) Bright-field microscopy images of Pt-19-treated BCOs. Drug treatment period: 72 hours. Scale bars: 50 μm. (D) Quantitative analysis of Pt-19-treated BCOs at different doses (2, 0.5, 0.1 μM), drug treatment period: 72 hours. (E) Heatmap representation of oxaliplatin (2 μM) and Pt-19 (0.1 μM), in combination with different ICIs (10 μg/mL) for BCOs. (n = 3, drug treatment period: 72 hours) (F) Quantitative analysis of Pt-19-treated BCOs containing PBMCs at different doses (2, 0.5, 0.1 μM). (n = 3, drug treatment period: 72 hours). (ns, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Immune synergy mechanism of Pt compounds and ICIs revealed by omics analysis

PD-1/PD-L1 inhibitor works by blocking the interaction between PD-1 and PD-L1, thereby overcoming the immune suppression induced by tumor cells and

unleashing the T cell-mediated anti-tumor immune response[34]. Therefore, we next investigated the mechanism of Pt-19 that enhances the efficacy of PD-1 inhibitor from the perspective of T cells **(Figure 5A)**. To further explore the T cell-related mechanisms of these agents, we performed bulk TCR-seq and RNA-seq for BCOs treated with the Pt-19 and PD-1 inhibitor**.** The diversity of TCR clonotypes of BCOs treated with PD-1 inhibitors and Pt-19 was shown in **Figure 5B**. After treatment with Pt-19, the TCR clones of BCOs were significantly amplified compared to the control group and anti-PD-1 group, and the combination of Pt-19 and ICIs further improved the enrichment of TCR clones. The top 10 and top 3 TCR clonotypes of the Pt-19 group and the Pt-19+anti-PD-1 group were more abundant than the control group and the anti-PD-1 group, especially in Top 3 TCR clonotypes, indicating the activation of tumor antigen-reactive T cells under the treatment of Pt-19 and PD-1 inhibitors **(Figure 5C, Figure S4A)**. Interestingly, not all of the significantly enriched TCR clones in the drug-treated group came from the abundant TCR clones in the control group; for example, none of the top 3 TCR clones in the Pt-19 group matched the top 10 TCR clones in the control group, suggesting that the amplification of TCR clones after drug treatment was selective, and that this selectivity most likely represents the specificity of the TCRs for tumor antigens. The secretion of IFN-γ tested by Enzyme linked immunosorbent assay (ELISA) was also consistent with the immunosynergistic effects. Pt-19 significantly promoted the T cells in BCOs to secrete IFN-γ, which plays a key role in T cell-mediated tumor killing, and the addition of anti-PD-1 antibody further enhanced its release ($p < 0.001$, **Figure 5D**).

Transcriptome analysis suggested that Pt-19 combined with PD-1 inhibitors not only promoted T-cell activation, but also improved antigen presentation **(Figure 5E)**. Gene Ontology (GO) enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis were performed based on the significantly differentially expressed genes in different treatment groups **(Figure 5F and S5B)**.

The GO analysis showed that Pt-19 led to the activation of several immune pathways, including regulation of immune system process, regulation of T-cell activation, positive regulation of cytokine biosynthetic process, compared with the control group. The GO analysis of "Pt-19 + anti-PD-1 vs anti-PD-1" enriched several pathways related to programmed cell death, indicating that Pt-19 may enhance the function of PD-1 inhibitor. In addition, the pathway of regulation of IFN-γ production were also enriched, which was consistent with the results of ELISA detection. The KEGG analysis showed that the cytokine-cytokine receptor interaction was enriched in the comparison groups. Interestingly, we also found that the platinum resistance pathway was enriched, suggesting that Pt-19 may overcome platinum resistance in bladder cancer patients. These omics data demonstrated that Pt-19 can effectively activate T-cell immunity to enhance the therapeutic effect of ICIs, and the BCO platform provides a novel tool to discover and investigate of the immune-related mechanisms of the Pt-ICI combination strategy.

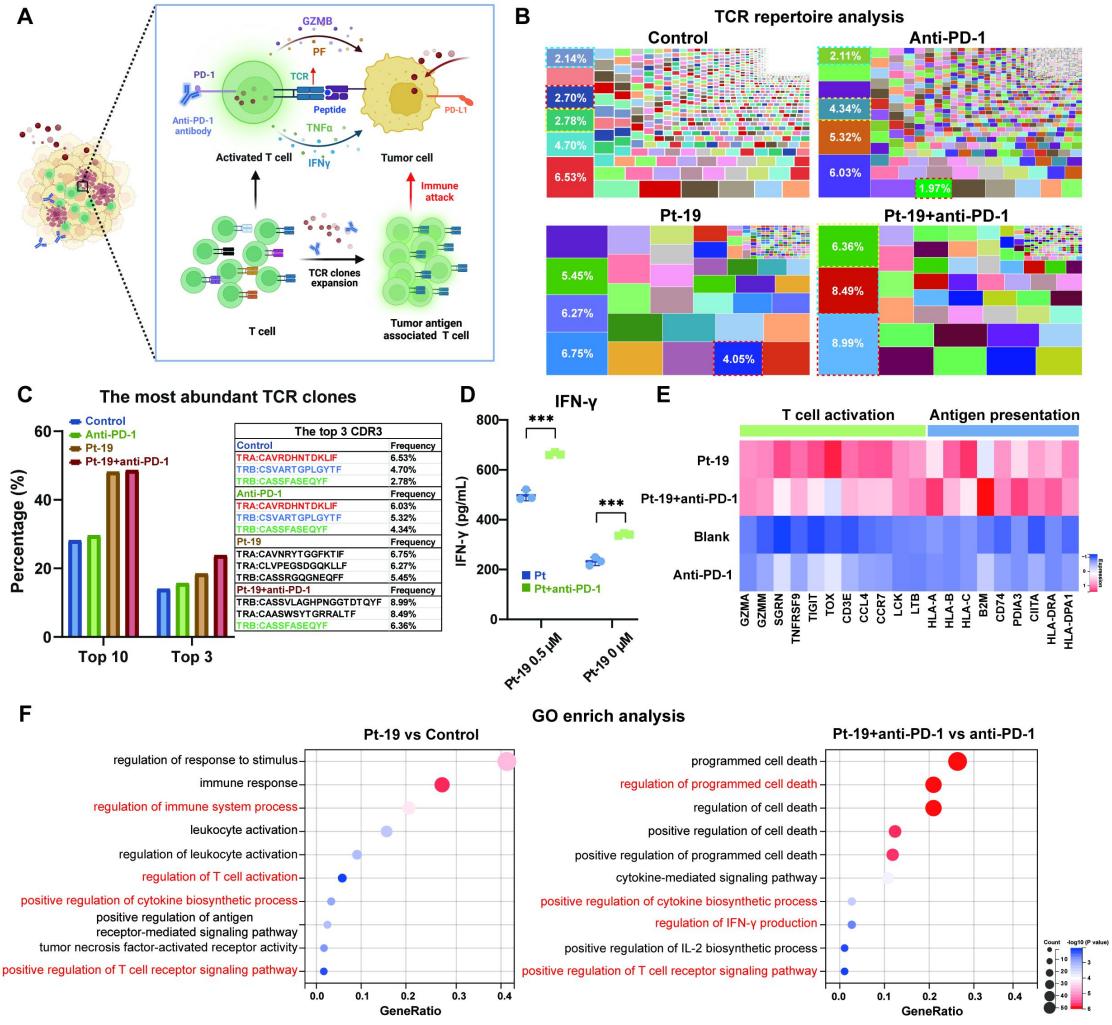


Figure 5. Immune synergy mechanism of Pt-19 and ICIs. (A) Schematic representation of the possible therapeutic mechanism for the combination of Pt compounds and PD-1 inhibitors. (B) Tree maps of TCR clonotypes of different treated BCOs. (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 24 hours) (C) Proportion of the top 10 and top 3 TCR clones in the TCR repertoire. (D) Quantitative analysis of the IFN-γ secretion at the different doses (2, 0.5, 0.1 μM) of Pt-19. (n = 3, ICI: 10 μg/mL, drug treatment period: 72 hours) (E) Heatmap showing the relative expression of selected genes in different treated groups. (n = 3, Pt drug: 2 μM, ICI: 10 μg/mL, drug treatment period: 24 hours). (F) GO terms for the differentially expressed genes of different treated groups. (*** $p < 0.001$).

Conclusion

For a long time, the effects of anticancer compounds synthesized by chemists on the immune system were either ignored or considered as immunosuppressive because of their cytotoxicity against cells, such as Pt drugs[35, 36]. Although Pt drugs

are currently the most widely used anti-cancer drugs in clinical practice, the clinical application of Pt drugs has been further restricted with the advancement of precision medicine and immunotherapy[37]. However, ICIs therapy also faces major challenges in resistance to PD-1/PD-L1 blockade, mainly due to TCR-pMHC (peptide-major histocompatibility complex) dysregulation, T cell exhaustion and resistance to IFN-γ signaling[38, 39].

The strategies of combining immunotherapy with other therapies offer a viable solution to the aforementioned challenges and attract enormous attention, while most clinical combination immunotherapy regimens are based on the direct combination of approved drugs and most cancer combination therapies are often derived from clinician practice based on treatment experience, which is labor-intensive, time-consuming, inefficient, and omits a vast number of promising preclinical drug candidates, especially metal compounds[40-47]. With the rise of metalloimmunology, the potential of metal drugs in cancer combination immunotherapy has been recognized in the clinic[35, 48, 49]. However, a highly efficient, systematic, and clinically predictive platform for the screening and discovery of novel metallodrugs for combination immunotherapy is lacking. Almost all newly reported metallodrugs claiming immune-mediated activity have been evaluated in the mouse model, which lacks clinical relevance[50].

In this study, we successfully established an immunocompetent bladder cancer patient-derived organoid platform suitable for testing immunotherapeutics using a Matrigel-free culture system and autologous immune cells derived from TILs or PBMCs. Subsequently, a Pt compound library comprising seven Pt^{II} compounds and twenty-eight Pt^{IV} compounds was screened for the tumor killing activity and synergy with ICIs according to the BCOs. We found that the clinically approved Pt drugs, cisplatin and oxaliplatin were not highly cytotoxic to organoids, but had a certain immune synergy in combination with ICIs. Fortunately, we found several Pt^{IV} compounds with high tumor killing activity and strong

immunoactivating property. In particular, Pt-19 at a low dose combined with PD-1 inhibitor promotes T-cell activation, thereby increasing cytotoxic cytokine secretion, activating the immune-related pathway, and amplifying TCR clones to enhance the therapeutic effect of PD-1 inhibitor. This result suggests that through immunosynergistic strategies, Pt drugs may be able to act at low doses, reducing the neurotoxicity that may be associated with the use of metal drugs in large quantities. In view of the remarkable antitumor activity of Pt compounds in the BCO model, these lead Pt compounds will be further evaluated in the future research, including pharmacodynamic, pharmacokinetic testing and so on.

To the best of our knowledge, this is the first example of using patient-derived immunocompetent tumor organoids to rapidly, precisely, and individually test the synergistic response of metallodrugs and ICIs, bridging the gap between clinical translation and basic research in metalloimmunology, accelerating drug discovery for cancer immunotherapy and facilitating individualized precision medicine. The unique BCO screening platform not only provides a novel tool for the discovery of novel metalloimmunotherapeutic drugs, but also provides a preclinical model to study the immune-related mechanisms of metallodrugs and exemplifies how to promote the clinical translation of metallodrugs using patient-derived tumor organoids.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Reference

1. Anand, U. *et al.* Cancer chemotherapy and beyond: Current status, drug candidates, associated risks and progress in targeted therapeutics. *Genes & Diseases* **10**, 1367-1401 (2023).
2. Bracci, L., Schiavoni, G., Sistigu, A. & Belardelli, F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death & Differentiation* **21**, 15-25 (2014).
3. Galluzzi, L., Humeau, J., Buqué, A., Zitvogel, L. & Kroemer, G. Immunostimulation with chemotherapy in the era of immune checkpoint inhibitors. *Nature Reviews Clinical Oncology* **17**, 725-741 (2020).
4. Meric-Bernstam, F., Larkin, J., Tabernero, J. & Bonini, C. Enhancing anti-tumour efficacy with immunotherapy combinations. *The Lancet* **397**, 1010-1022 (2021).
5. Waldman, A.D., Fritz, J.M. & Lenardo, M.J. A guide to cancer immunotherapy: from T cell basic science to clinical practice. *Nature Reviews Immunology* **20**, 651-668 (2020).
6. Sharpless, N.E. & Depinho, R.A. The mighty mouse: genetically engineered mouse models in cancer drug development. *Nat Rev Drug Discov* **5**, 741-754 (2006).
7. Lee, H. Genetically engineered mouse models for drug development and preclinical trials. *Biomol Ther (Seoul)* **22**, 267-274 (2014).
8. Lee, S.H. *et al.* Tumor Evolution and Drug Response in Patient-Derived Organoid Models of Bladder Cancer. *Cell* **173**, 515-528.e517 (2018).
9. Drost, J. & Clevers, H. Organoids in cancer research. *Nature Reviews Cancer* **18**, 407-418 (2018).
10. Yin, S. *et al.* Patient-derived tumor-like cell clusters for drug testing in cancer therapy. *Sci Transl Med* **12** (2020).
11. Xu, H., Jiao, D., Liu, A. & Wu, K. Tumor organoids: applications in cancer modeling and potentials in precision medicine. *J Hematol Oncol* **15**, 58 (2022).
12. Yuki, K., Cheng, N., Nakano, M. & Kuo, C.J. Organoid Models of Tumor Immunology. *Trends Immunol* **41**, 652-664 (2020).
13. Schnalzger, T.E. *et al.* 3D model for CAR-mediated cytotoxicity using patient-derived colorectal cancer organoids. *Embo j* **38** (2019).
14. Neal, J.T. *et al.* Organoid Modeling of the Tumor Immune Microenvironment. *Cell* **175**, 1972-1988.e1916 (2018).
15. Jenkins, R.W. *et al.* Ex Vivo Profiling of PD-1 Blockade Using Organotypic Tumor Spheroids. *Cancer Discov* **8**, 196-215 (2018).
16. Robertson, A.G. *et al.* Comprehensive Molecular Characterization of Muscle-

Invasive Bladder Cancer. *Cell* **171**, 540-556.e525 (2017).
17. Comprehensive molecular characterization of urothelial bladder carcinoma. *Nature* **507**, 315-322 (2014).
18. Rosenberg, S.A. & Restifo, N.P. Adoptive cell transfer as personalized immunotherapy for human cancer. *Science* **348**, 62-68 (2015).
19. Coppola, C. *et al.* Investigation of the Impact from IL-2, IL-7, and IL-15 on the Growth and Signaling of Activated CD4+ T Cells. *International Journal of Molecular Sciences* **21**, 7814 (2020).
20. Clénet, M.L., Gagnon, F., Moratalla, A.C., Viel, E.C. & Arbour, N. Peripheral human CD4(+)CD8(+) T lymphocytes exhibit a memory phenotype and enhanced responses to IL-2, IL-7 and IL-15. *Sci Rep* **7**, 11612 (2017).
21. Lee, Y.-C. *et al.* The dynamic roles of the bladder tumour microenvironment. *Nature Reviews Urology* **19**, 515-533 (2022).
22. Rose, T.L. *et al.* Phase II Study of Gemcitabine and Split-Dose Cisplatin Plus Pembrolizumab as Neoadjuvant Therapy Before Radical Cystectomy in Patients With Muscle-Invasive Bladder Cancer. *J Clin Oncol* **39**, 3140-3148 (2021).
23. Xiaofeng, C. *et al.* Camrelizumab plus gemcitabine and oxaliplatin (GEMOX) in patients with advanced biliary tract cancer: a single-arm, open-label, phase II trial. *Journal for ImmunoTherapy of Cancer* **8**, e001240 (2020).
24. Wang, Q. *et al.* In Situ Supramolecular Self-Assembly of Pt(IV) Prodrug to Conquer Cisplatin Resistance. *Advanced Functional Materials* **31**, 2101826 (2021).
25. Zhang, S. *et al.* Interfering in apoptosis and DNA repair of cancer cells to conquer cisplatin resistance by platinum(iv) prodrugs. *Chemical Science* **11**, 3829-3835 (2020).
26. Guo, Y. *et al.* A platinum(iv) prodrug to defeat breast cancer through disrupting vasculature and inhibiting metastasis. *Dalton Transactions* **48**, 3571-3575 (2019).
27. Yang, T. *et al.* Platinum-Based TREM2 Inhibitor Suppresses Tumors by Remodeling the Immunosuppressive Microenvironment. *Angewandte Chemie International Edition* **62**, e202213337 (2023).
28. Chen, C.K., Zhang, J.Z., Aitken, J.B. & Hambley, T.W. Influence of equatorial and axial carboxylato ligands on the kinetic inertness of platinum(IV) complexes in the presence of ascorbate and cysteine and within DLD-1 cancer cells. *J Med Chem* **56**, 8757-8764 (2013).
29. Kroemer, G., Galassi, C., Zitvogel, L. & Galluzzi, L. Immunogenic cell stress and death. *Nature Immunology* **23**, 487-500 (2022).
30. Zhao, Z. *et al.* The combination of oxaliplatin and anti-PD-1 inhibitor promotes immune cells infiltration and enhances anti-tumor effect of PD-1 blockade in bladder cancer. *Frontiers in Immunology* **14** (2023).
31. Liakou, C.I., Narayanan, S., Ng Tang, D., Logothetis, C.J. & Sharma, P. Focus

on TILs: Prognostic significance of tumor infiltrating lymphocytes in human bladder cancer. *Cancer Immunity* **7** (2007).
32. Wu, T.D. *et al.* Peripheral T cell expansion predicts tumour infiltration and clinical response. *Nature* **579**, 274-278 (2020).
33. Fairfax, B.P. *et al.* Peripheral CD8+ T cell characteristics associated with durable responses to immune checkpoint blockade in patients with metastatic melanoma. *Nature Medicine* **26**, 193-199 (2020).
34. Kubli, S.P., Berger, T., Araujo, D.V., Siu, L.L. & Mak, T.W. Beyond immune checkpoint blockade: emerging immunological strategies. *Nature Reviews Drug Discovery* **20**, 899-919 (2021).
35. Rottenberg, S., Disler, C. & Perego, P. The rediscovery of platinum-based cancer therapy. *Nature Reviews Cancer* **21**, 37-50 (2021).
36. Galluzzi, L., Buqué, A., Kepp, O., Zitvogel, L. & Kroemer, G. Immunological Effects of Conventional Chemotherapy and Targeted Anticancer Agents. *Cancer Cell* **28**, 690-714 (2015).
37. Liu, J. *et al.* Older but Stronger: Development of Platinum-Based Antitumor Agents and Research Advances in Tumor Immunity. *Inorganics* **11**, 145 (2023).
38. Sun, J.Y. *et al.* Resistance to PD-1/PD-L1 blockade cancer immunotherapy: mechanisms, predictive factors, and future perspectives. *Biomark Res* **8**, 35 (2020).
39. Lei, Q., Wang, D., Sun, K., Wang, L. & Zhang, Y. Resistance Mechanisms of Anti-PD1/PDL1 Therapy in Solid Tumors. *Front Cell Dev Biol* **8**, 672 (2020).
40. Ni, J.-j., Zhang, Z.-z., Ge, M.-j., Chen, J.-y. & Zhuo, W. Immune-based combination therapy to convert immunologically cold tumors into hot tumors: an update and new insights. *Acta Pharmacologica Sinica* **44**, 288-307 (2023).
41. Yap, T.A. *et al.* Development of Immunotherapy Combination Strategies in Cancer. *Cancer Discov* **11**, 1368-1397 (2021).
42. Cao, Q. *et al.* CAIXplatins: Highly Potent Platinum(IV) Prodrugs Selective Against Carbonic Anhydrase IX for the Treatment of Hypoxic Tumors. *Angew Chem Int Ed Engl* **59**, 18556-18562 (2020).
43. Su, X. *et al.* Disruption of Zinc Homeostasis by a Novel Platinum(IV)-Terthiophene Complex for Antitumor Immunity. *Angew Chem Int Ed Engl* **62**, e202216917 (2023).
44. Deng, Z. *et al.* Near-infrared-activated anticancer platinum(IV) complexes directly photooxidize biomolecules in an oxygen-independent manner. *Nat Chem* **15**, 930-939 (2023).
45. Wang, Z. *et al.* Phorbiplatin, a Highly Potent Pt(IV) Antitumor Prodrug That Can Be Controllably Activated by Red Light. *Chem* **5**, 3151-3165 (2019).
46. Huang, Y. *et al.* A bimetallic nanoplatform for STING activation and CRISPR/Cas mediated depletion of the methionine transporter in cancer cells restores anti-tumor immune responses. *Nature Communications* **14**, 4647 (2023).

47. Zhang, L. *et al.* Liquid Metal as Bioinspired and Unusual Modulator in Bioorthogonal Catalysis for Tumor Inhibition Therapy. *Angew Chem Int Ed Engl* **62**, e202218159 (2023).
48. Wang, C., Zhang, R., Wei, X., Lv, M. & Jiang, Z. Metalloimmunology: The metal ion-controlled immunity. *Adv Immunol* **145**, 187-241 (2020).
49. Li, J., Zheng, P., Zhao, J., Chen, P.R. & Guo, Z. Metal-mediated immune regulations and interventions: prospects of the emerging field of metalloimmunology. *SCIENTIA SINICA Chimica* (2019).
50. Anthony, E.J. *et al.* Metallodrugs are unique: opportunities and challenges of discovery and development. *Chem Sci* **11**, 12888-12917 (2020).