PT-112 induces immunogenic cell death and synergizes with immune checkpoint blockers in mouse tumor models

Takahiro Yamazaki, Aitziber Buqué, Tyler D. Ames, and Lorenzo Galluzzi

ABSTRACT
PT-112 is a novel platinum-pyrophosphate conjugate under clinical development for cancer therapy. PT-112 mediates cytostatic and cytotoxic effects against a variety of human and mouse cancer cell lines in vitro. The cytotoxic response to PT-112 is associated with the emission of danger signals underpinning the initiation of anticancer immunity, including calreticulin exposure on the surface of dying cells, as well as ATP and HMGB1 secretion. Consistently, mouse cancer cells succumbing to PT-112 in vitro can be used to provide syngeneic, immunocompetent mice with immunological protection against a subsequent challenge with living tumor cells of the same type. Moreover, PT-112 administration synergizes with PD-1 or PD-L1 blockade in the control of mouse cancers in immunologically competent settings, as it simultaneously recruits immune effector cells and depletes immunosuppressive cells in the tumor microenvironment. Finally, PT-112 employed intratumorally in the context of immune checkpoint inhibition initiates a robust immune response that has systemic outreach and limits the growth of untreated, distant lesions. Thus, PT-112 induces the immunogenic demise of cancer cells, and hence stands out as a promising combinatorial partner of immune checkpoint blockers, especially for the treatment of otherwise immunologically cold tumors.

Introduction

Platinum-based chemotherapeutics such as cisplatin (CDDP), carboplatin and oxaliplatin have extensively been used for the clinical management of numerous neoplasms, including (but not limited to) pulmonary, ovarian and colorectal tumors. However, platinum derivatives are associated with considerable toxicity and a high incidence of acquired resistance, calling for the identification of improved chemical entities. R,R,1,2 cyclohexanediimine-pyrophosphato-platinum(II) (PT-112, Figure 1a) has been developed in this setting, with the specific aim of altering the cellular mechanisms of action of the drug to improve its efficacy and at the same limit its toxicity. From a chemical perspective, PT-112 differs from other platinum derivatives as Pt\(^{2+}\) ions are chelated by diaminocyclohexane and pyrophosphate moieties. Pyrophosphate exists in the plasma in a di-anionic state, providing PT-112 with improved pharmacokinetic and pharmacodynamic properties, including a considerable tendency to accumulate in the lung, liver and bones (in mice). In line with this notion, multiple individuals with primary or metastatic lesions in these organs, who failed several lines of conventional and/or experimental therapy, have experienced robust and durable responses upon systemic administration of PT-112 in the context of ongoing, dose-escalation, Phase I clinical trials (NCT02266745, NCT03409458). In particular, PT-112 monotherapy enabled durable responses in three patients with solid tumors, including two individuals who progressed on immune check-point blocker (ICB)-based immunotherapy. Moreover, heavily pretreated men with castration resistant prostate cancer exhibited serologic and radiographic responses to PT-112, employed as standalone therapeutic agent or combined with avelumab, an ICB specific for CD274 (best known as PD-L1) that is poorly active in such patients. Together with existing preclinical data, these observations suggest that PT-112 treatment may elicit, or at least be compatible with, a tumor-targeting immune response that can be potentiated by ICBs.

Although conventional platinum derivatives share the ability to cause DNA lesions with some degree of specificity for highly-proliferating cells, including (but not limited to) cancer cells, the immunomodulatory profile of these drugs exhibits considerable variability. In particular, CDDP, carboplatin and oxaliplatin differ in their capacity to elicit bona fide immunogenic cell death (ICD), a specific form of regulated cell death (RCD) that is sufficient (in immunocompetent, syngeneic settings) for the initiation of adaptive immunity against dead cell-associated antigens. At least in part, this reflects the proficient activation of intracellular stress responses culminating with the emission of adjuvant signals commonly known as damage-associated molecular patterns (DAMPs) by oxaliplatin (which is...
largely considered as a *bona fide* ICD inducer), but less so by CDDP and carboplatin (whose immunogenicity remains a matter of debate).

Based on these premises, we set out to investigate the emission of ICD-associated DAMPs including calreticulin (CALR), ATP and high mobility group box 1 (HMGB1) by cancer cells responding to PT-112, as well as the ability of PT-112 to (1) drive *bona fide* ICD in gold standard vaccination and abscopal models, and (2) synergize with ICBs in the eradication of established mouse tumors. Here, we report that PT-112 causes a form of cancer cell death that is immunogenic per se. ICD induction by PT-112 potentially explains durable responses to the drug observed in the context of ongoing Phase I/II clinical trials, and suggests that PT-112 can be successfully combined with ICBs for superior therapeutic activity.

**Materials and methods**

**Chemicals and cell culture**

Media and supplements for cell culture were obtained from Invitrogen™-Thermo Fisher, unless otherwise noted. All cells were maintained according to ATCC recommendations, and cells between passage 2 and 10 were employed for experimental determinations.

**Cell number**

Residual number of living cells upon exposure of human cancer cell lines to increasing doses of PT-112 for 72 hours was assessed with the CyQUANT Proliferation Assay (Thermo Fisher), as per the manufacturer’s recommendations.

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**Figure 1.** Cell death driven by PT-112 is associated with DAMP emission. (a). Chemical structure of *R,R*-1,2-cyclohexanediamine-pyrophosphato-platinum(II) (PT-112). (b). IC_{50} values associated with exposure of 121 human cancer cell lines to PT-112 for 72 hours. Results are means ± SEM, based on cancer cell histology. Mean IC_{50} ± SEM for all cells is reported in red. **p < .01 (one-way ANOVA), as compared to untreated cells.***< .01 (one-way ANOVA), as compared to untreated cells at the same time point. (d). Percentage of DAPI (dead) TSA cells upon treatment with 50 µg/mL PT-112, 15 µM cisplatin (CDDP), or 2.5 µM mitoxantrone (MTX) for 24 hours. Representative histograms (isotype staining is reported as dashed profile) and quantitative results (mean MFI ± SEM) are reported. n = 2–3 independent experiments; *p < .05, **p < .01, ***p < .001 (one-way ANOVA), as compared to untreated cells. (f,g). ATP (f) and HMGB1 (g) amounts in the supernatant of TSA cells treated as in panel d. Quantitative results (means ± SEM) are reported. n = 2–3 independent experiments; *p < .05, **p < .01, ***p < .001 (one-way ANOVA), as compared to untreated cells.
**Cell death**

Cell death was assessed by flow cytometry upon co-staining cells with the mitochondrial transmembrane potential-sensitive dye 3,3'-dihexyloxacarbocyanine iodide (DiOC₆(3), from Invitrogen®-Thermo Fisher) (40 nM) and either of the vital dyes 4',6-diamidino-2-phenylindole (DAPI, from Sigma-Aldrich) (25 ng/mL) and propidium iodide (PI, from Sigma-Aldrich) (0.5 μg/mL), as per standard protocols.²⁹ Stained samples were acquired on a MACSQuant® Analyzer 10 (Miltenyi Biotech) and data were analyzed with FlowJo v. 10.6 (FlowJo LLC).

**DAMP emission**

CALR exposure on the cell surface was measured by flow cytometry upon staining cells with a rabbit antibody specific for CALR (Abcam, #AB2907) at 4°C for 1 hour, followed by incubation with anti-rabbit IgG Alexa Fluor488® conjugates (Invitrogen, #A11070) plus 0.5 μg/mL PI for 30 min. As per gold-standard recommendations,³⁰ PI⁺ cells were excluded from the analysis. Extracellular ATP and HMGB1 levels were quantified with the luciferase-based Enliten ATP Assay (Promega) and the HMGB1 ELISA Kit (Tecan), respectively, as per manufacturer’s recommendations.

**Animal experiments**

Mice were maintained in specific pathogen-free conditions, and experiments followed the Guidelines for the Care and Use of Laboratory Animals guidelines. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Weill Cornell Medical College (n° 2018–0002). Wild-type BALB/c or C57BL/6j mice (4–6 weeks old) were obtained from Taconic Bioscience.

**Tumor growth**

Female BALB/c or C57BL/6j mice were inoculated s.c. with 0.25 × 10⁶ CT26 or 0.5 × 10⁶ MC38 cells, respectively, in the right flank, and monitored routinely for tumor growth with a common lab caliper. When tumors reached an area of 15–25 mm² (day 0), mice were randomly allocated to the following treatment groups (n = 7–8 per group): (1) vehicle i.p biweekly (5 doses for CT26 tumors, 7 doses for MC38 tumors); (2) 90 mg/Kg PT-112 in 50 μL in phosphate buffer i.v. weekly (5 doses); (3) a programmed cell death 1 (PDCD1)-specific antibody (Bio X Cell, clone #RMP1-14), 10 mg/Kg i.p biweekly (5 doses for CT26 tumors, 7 doses for MC38 tumors); (4) a CD274-specific antibody (Bio X Cell, clone #FJK-16s), and a live/dead (L/D) stain (BioLegend, #423101). Stained samples were acquired on a FACSCalibur (Becton Dickinson) and data were analyzed with FlowJo v. 10.6 (FlowJo LLC).

**Vaccination assays**

One x 10⁶ TSA cells treated in vitro with 150 μM CDDP, 2.5 μM mitoxantrone (MTX), or 150 μg/mL PT-112 for 24 hours were washed once and resuspended in 100 μL PBS for subcutaneous inoculation into the lower flank of 7 weeks old female BALB/c mice (vaccination). One week later, mice received 0.1 × 10⁶ untreated TSA cells s.c. into the contralateral flank (challenge). Tumor incidence and growth were monitored routinely with a common lab caliper, and mice were euthanized when tumor size exceeded ethical limits or with manifestations of systemic disease (such as hunched posture, anorexia, and weight loss). Mice rejecting the challenge injection were re-challenged 60 days later with 0.1 × 10⁶ untreated TSA cells in one flank, as a control for vaccination durability.

**Abscopal assays**

Two neoplastic lesions were established by inoculating 0.1 × 10⁶ TSA cells s.c. into either lower flanks of female 4–9 weeks old BALB/c mice three days apart. Mice were routinely monitored for tumor growth at both sites by means of common lab caliper, and once primary tumors reached a surface area of 15–25 mm² (day 0), mice were allocated to either of the following treatment groups: (1) 100 μL vehicle i.p. on days 2, 5 and 8; (2) a cytotoxic T lymphocyte associated protein 4 (CTLA4)-specific antibody (Bio X Cell, clone #9H10), 200 μg/mouse in 100 μL i.p., on days 2, 5 and 8; (3) 150 mg/Kg PT-112 in 50 μL phosphate buffer i.t. on day 0; and (4) 150 mg/Kg PT-112 in 50 μL phosphate buffer i.t. on day 0 plus 9H10, 200 μg/mouse in 100 μL PBS i.p., on days 2, 5 and 8. Mice were monitored for tumor growth at both disease sites and signs of systemic toxicity as above.

**Immune infiltration**

CT26 and MC38 tumors treated as above were harvested on day 12 and dissociated according to standard procedures for the assessment of immune cell infiltration,³¹ upon staining with cocktails of fluorescent antibodies specific for CD3 (BioLegend, clone #17A2), CD4 (BioLegend, clone #GK1-5), CD8 (BioLegend, clone #53-6.7), CD11b (BioLegend, clone #M1/70), CD11c (BioLegend, clone #N418), CD25 (BioLegend, clone #PC61), CD45 (BioLegend, clone #30-F11), F4/80 (BioLegend, clone #BM8), FOXP3 (eBioscience, clone #FJK-16s), and a live/dead (L/D) stain (BioLegend, #423101). Stained samples were acquired on a FACScalibur (Becton Dickinson) and data were analyzed with FlowJo v. 10.6 (FlowJo LLC).

**Statistical analyses**

Statistical significance on cell death, DAMP emission and immune infiltration was assessed by one-way ANOVA. Tumor surface was calculated as $S = (\pi \times A \times B)/4$, where A and B are the longest and shortest lesion diameter, respectively. Statistical significance on growth curves was assessed by two-way ANOVA, while statistical significance on Kaplan-Meier curves was assessed by hazard ratio (Log-rank) and Mantel-Cox tests.
**Results**

**PT-112 exerts cytotoxic effects that are accompanied by the emission of immunostimulatory DAMPs**

To characterize the cytostatic and cytotoxic activity of PT-112, we harnessed a commercial DNA-based test to estimate residual cell number upon exposing a large panel of 121 human cancer cell lines of various histological derivation to increasing concentrations of the drug for 72 hours. As expected, we identified a spectrum of sensitivities to PT-112, with IC\(_{50}\) values ranging from 0.287 µM (for human gastric adenocarcinoma AGS cells) to 222.14 µM (for human breast carcinoma MDAMB415 cells) (Table 1). Interestingly, mean IC\(_{50}\) values for cell lines of different histological derivation exhibited limited variation as compared to mean IC\(_{50}\) value for all other cell lines confounded (Figure 1b), with the sole exception of breast carcinoma cells, largely due to the extraordinary resistance of MDAMB415 cells. Since human cancer cells are intrinsically incompatible with *in vivo* immunoncology studies, we decided to switch to utilize murine systems.

The proliferation of mouse colorectal carcinoma CT26 cells was virtually arrested upon exposure to PT-112 *in vitro* (Figure 1c), mostly in the absence of overt cytotoxicity (data Table 1. Cytostatic and cytotoxic effects of PT-112 against human cancer cell lines (IC\(_{50}\), µM).

| Blood          | IC\(_{50}\) | Bone IC\(_{50}\) | Breast IC\(_{50}\) | Cologectum IC\(_{50}\) | Esophagus IC\(_{50}\) | Pancreas IC\(_{50}\) |
|----------------|------------|-----------------|-------------------|------------------------|-----------------------|---------------------|
| AMO1           | 0.387      | HOS 3.953       | MCF7 2.623        | LS513 0.825            | KYSE270 1.186         |                     |
| MOLP8          | 3.121      | U2OS 6.06       | DU447S 3.495      | T84 0.909              | KYSE270 6.428         |                     |
| L363           | 3.391      | CADOC5 6.879    | MADBAM468 4.613   | SW948 1.089            | T84 8.155             |                     |
| RPM8226        | 4.075      | A673 7.411      | BT549 6.341       | SW937 1.117            | KYSE410 8.715         |                     |
| KMS11          | 6.546      | SAOS2 37.74     | T47D 10.114       | LOVO 1.146             | OE19 27.192           |                     |
| U266B1         | 18.137     |                 |                   |                       |                       |                     |
| LP1            | 20.263     |                 |                   |                       |                       |                     |

| Kidney IC\(_{50}\) | Liver IC\(_{50}\) | Lung IC\(_{50}\) | Ovary IC\(_{50}\) | Pancreas IC\(_{50}\) |
|--------------------|-------------------|-----------------|------------------|---------------------|
| A498               | 0.922             | HUCC7 5.073     | CALU6 0.675      | A2780 1.248          |                     |
| OSRC2              | 2.201             | HEPG2 1.668     | NC1H460 1.662    | ES2 2.238            | KP4 3.381            |                     |
| CAM2               | 4.314             | NCGH2OS2 3.926  | AS54 2.301       | IGROV1 2.36           | CAPAN1 3.758         |                     |
| 786O               | 6.334             | HLF 13.711      | NC1H526 4.322    | OVCA8 6.249           | MIAAPC2 4.56         |                     |
|                   |                   |                 | NC1H520 5.042    | CAOV3 14.731          | SW1990 4.727         |                     |
|                   |                   |                 | NC1H69 5.304     | NIHOVCAR3 20.333      | ASPC1 8.235          |                     |
|                   |                   |                 | DMS53 5.835      | SKOV3 73.75           | CFPCA1 16.033        |                     |
|                   |                   |                 | NC1H23 6.184     |                    |                     |                     |
|                   |                   |                 | NC1H358 7.197    |                    |                     |                     |
|                   |                   |                 | NC1H7192 7.441   |                    |                     |                     |
|                   |                   |                 | SKME51 9.8       |                    |                     |                     |
|                   |                   |                 | NC1H701 10.225   |                    |                     |                     |
|                   |                   |                 | NC1H299 10.252   |                    |                     |                     |
|                   |                   |                 | EBC1 11.634      |                    |                     |                     |
|                   |                   |                 | NC1H522 14.095   |                    |                     |                     |
|                   |                   |                 | NC1H373 15.418   |                    |                     |                     |
|                   |                   |                 | SKLU1 17.054     |                    |                     |                     |
|                   |                   |                 | HC4006 25.101    |                    |                     |                     |
|                   |                   |                 | NC1H1048 35.987  |                    |                     |                     |
|                   |                   |                 | NC1H441 49.533   |                    |                     |                     |
|                   |                   |                 | NC1H648 59.687   |                    |                     |                     |

| Mean IC\(_{50}\) | Prostate IC\(_{50}\) | Salivary glands IC\(_{50}\) | Skin IC\(_{50}\) | Soft tissue IC\(_{50}\) |
|------------------|---------------------|-----------------------------|-----------------|------------------------|
| DETROIT562       | 5.849               | LNCAPCLONEFGC 1.72          | A253 3.877      | SKME5 1.614             | HT1080 2.563         |                     |
| FADU             | 7.005               | PC3 5.247                    | A253 3.877      | SKME28 3.503            | SKUT1 4.716          |                     |
|                  |                     | 22RV1 6.565                 | A2058 7.49      | A204 7.2               |                     |                     |
| Mean IC\(_{50}\) | Thyroid IC\(_{50}\) | Tongue IC\(_{50}\) | Urethra IC\(_{50}\) | Uterus IC\(_{50}\) |
|-----------------|-------------------|-----------------|------------------|--------------------|
| A51G             | 0.287             | TTTHY 7.816     | CAL27 2.714      | RT4 0.519           | AN3CA 1.031          |                     |
| NUGC4            | 1.99              | SW579 19.97     | SCC25 16.844     | SW780 2.596         |                     |                     |
| SNU1             | 2.112             |                 | SCC4 25.945      |                   |                     |                     |
| SNU601           | 2.172             |                 | SCC9 35.39       | RT11284 10.092      |                     |                     |
| SNU668           | 2.343             |                 | SCC15 77.209     | HT1376 18.702       |                     |                     |
| HGC77            | 2.891             |                 |                  |                    |                     |                     |
| SNJ72            | 3.384             |                 |                  |                    |                     |                     |
| MKN1             | 3.584             |                 |                  |                    |                     |                     |
| SNU5             | 3.784             |                 |                  |                    |                     |                     |
| OCUM1            | 6.832             |                 |                  |                    |                     |                     |
| SNU216           | 7.426             |                 |                  |                    |                     |                     |
| Mean IC\(_{50}\) | 3.346             | Mean 13.893      | Mean 31.62       | Mean 2.438           | Mean 1.031          |                     |

Abbreviations: IC\(_{50}\), inhibitory concentration 50%.
not shown). Conversely, PT-112 efficiently killed mouse mammary carcinoma TSA cells in a dose- and time-dependent manner, as determined by flow cytometry upon co-staining with fluorescent probes for mitochondrial outer membrane permeabilization and plasma membrane rupture (Figure 1d). We therefore selected mouse TSA cells to investigate the ability of PT-112 to cause the emission of DAMPs that have been mechanistically linked to the activation of anticancer immunity by RCD.\(^\text{21}\) We employed CDDP (which in our hands is unable to cause bona fide ICD)\(^\text{24}\) as a negative control, and MTX (a potent ICD inducer)\(^\text{32}\) as a positive control.

In line with previous findings from the Kroemer laboratory,\(^\text{34}\) TSA cells responding to MTX exposed CALR on the outer leaflet of the plasma, and secreted abundant amounts of HMGB1 (Figure 1e–g). Unexpectedly, exposure of TSA cells to CDDP also caused CALR exposure and ATP release, but poor HMGB1 secretion (Figure 1e–g). Of note, CALR exposure by cancer cells exposed to CDDP has not been observed with mouse colorectal carcinoma CT26 cells,\(^\text{74}\) but reportedly occurs in mouse ovarian carcinoma 2F8 cells,\(^\text{35}\) pointing to some degree of variability across different cell types. Irrespective of this partially unexpected finding, PT-112 was highly efficient at causing the emission of ICD-associated DAMPs from TSA cells (Figure 1e–g). Similar results have previously been obtained with human colorectal carcinoma HCT 116 cells,\(^\text{7}\) suggesting that PT-112 may constitute a novel bona fide inducer of ICD in both mouse and human tumor models.

**PT-112 causes bona fide ICD in vivo**

As surface CALR exposure, ATP release and HMGB1 secretion are all required, but not sufficient, for cancer cell death to be perceived as immunogenic,\(^\text{36}\) we next set to evaluate the immunogenicity of PT-112-driven RCD in gold-standard vaccination assays.\(^\text{30}\) To this aim, fully immunocompetent, wild-type BALB/c mice were vaccinated by subcutaneous inoculation of PBS (negative control) or TSA cells pre-exposed in vitro to a cytotoxic dose of CDDP, MTX, or PT-112. One week later, all mice were challenged contralaterally with living TSA cells and monitored over time for the ability of the latter to form progressing tumors. Neither PBS nor CDDP-treated TSA cells conferred a significant degree of immunological protection against the challenge injection (Figure 2a). In this specific sets of experiments, MTX-treated cells exhibited partial (but statistically significant) immunogenicity, as they enabled 40% tumor-free survival 35 days after challenge with living TSA cells, as well as with a reduction in the growth rate of tumors evolving despite vaccination (Figure 2a). Conversely, TSA cells succumbing in vitro to PT-112 conferred 100% immunological protection against the subsequent injection of living TSA cells (Figure 2a). Nine out of ten mice rejecting a first challenge with TSA cells were subcutaneously rechallenged 60 days later with TSA cells to check for the durability of protection. Such living TSA cells failed to generate progressing tumors in 5/9 mice (Suppl. Figure 1), suggesting that the immunological protection conferred by PT-112-treated cells is durable.

These findings demonstrate that the demise of cancer cells driven by PT-112 administration is sufficiently immunogenic to protect tumor-naive mice from a challenge with living cancer cells of the same type.

**PT-112 causes systemic immune outreach in abscopal tumor models**

Since vaccination assays are exquisitely sensitive, we decided to investigate the immunogenicity of PT-112 in abscopal settings, adapting a model and procedures that are generally employed for radiation oncology studies.\(^\text{37}\) To this aim, BALB/c mice were used as hosts for the establishment of two slightly asynchronous TSA lesions (one on each flank), followed by the intratumoral administration of a systemically inactive dose of PT-112 to a single lesion (primary tumor) in the context of systemic CTLA4 blockade (which per se is also inactive in this model). In this setting, the growth of untreated (abscopal) lesions can be influenced only by the activation of robust immunity with systemic outreach. As intended, PT-112 exhibited limited activity upon intratumoral inoculation into TSA lesions, but such an effect was considerably potentiated by whole-body CTLA4 blockade, resulting in disease eradication at the primary site in 5 out of 7 mice (Figure 2b). Moreover, the growth of PT-112-naive (secondary) tumors was reduced in mice receiving intratumoral PT-112 (at a systematically inactive dose) to the primary site plus a CTLA4-blocking antibody i.p. (Figure 2b).

These data demonstrate that local PT-112 therapy can synergize with CTLA4 blockade at the reversion of intratumoral immunosuppression and the activation of a potent immune response with systemic outreach affecting untreated disease sites.

**PT-112 synergizes with ICBs to eradicate established mouse tumors**

Reassured by the ability of PT-112 to trigger bona fide ICD, and to synergize with CTLA4 blockers in the initiation of systemic anticancer immunity (abscopal responses) in the TSA model, we next set to assess the synergism between PT-112 and ICBs targeting programmed cell death 1 (PDCD1, best known PD-1) or its main ligand PD-L1, and hence operating at the effector (rather than priming) phase. To this aim, we selected two mouse cancer cell lines that exhibit incomplete sensitivity to PT-112 and are syngeneic to different mouse strains, namely CT26 cells (syngeneic to BALB/c mice), and mouse colorectal carcinoma MC38 cells (syngeneic to C57BL/6j mice). PT-112 monotherapy reduced the growth of CT26 tumors established in immunocompetent BALB/c mice but was unable to extend overall survival to a statistically significant degree (Figure 2c). PD-L1 blockers synergized with PT-112 at extending the survival of CT26-bearing mice, although PD-L1 blockade mediated some anticancer activity (but virtually no effects on survival) per se (Figure 2c). Conversely, PD-1 blockers had limited therapeutic activity against CT26 lesions when employed as standalone agents (Figure 2c). However, therapeutic effects were pronounced when PT-112 was combined with PD-1 blockade, resulting in significant extensions in overall survival as compared to either therapy alone (Figure 2c). Of note, 5/7
Figure 2. PT-112 induces bona fide ICD and can be combined with ICIs in vivo. (a) Tumor-free survival (TFS) and tumor area in BALB/c mice vaccinated with PBS or TSA cells exposed in vitro to 150 μg/ml PT-112, 150 μM cisplatin (CDDP), or 2.5 μM mitoxantrone (MTX) for 24 hours, and (one week later) challenged contralaterally with living TSA cells. Number of mice is indicated. Tumor areas are reported as means ± SEM. ns: not significant. **p < .01 (Log-rank for TFS, two-way ANOVA for tumor area), as compared to PBS-vaccinated mice. See also Suppl. Figure 1. (b) Growth of primary and abscopal TSA lesions established in immunocompetent, syngeneic BALB/c mice that were optionally allocated to receive 150 mg/Kg PT-112 i.t. in the context of optional, systemic CTLA4 blockage. Number of mice and incidence of disease eradication are indicated. Tumor growth data are reported as means ± SEM. ***p < .001 (two-way ANOVA), as compared to untreated mice; *p < .05, **p < .01 (two-way ANOVA), as compared to mice treated with CTLA4 blockers. (c,d) Growth of CT26 (c) or MC38 (d) tumors established in immunocompetent, syngeneic BALB/c or C57BL/6J mice, respectively, that were allocated to receive 90 mg/Kg PT-112 weekly i.v. in the context of optional, biweekly systemic (i.p.) PD-1 or PD-L1 blockage (or PD-1 or PD-L1 blockage alone). Number of mice, incidence of disease eradication, overall survival (OS), hazard ratio (HR) and p values (two-way ANOVA for tumor growth, Mantel-Cox for OS) are reported. *compared to untreated mice; †compared to mice treated with PD-1; ‡compared to mice treated with PD-1 and PD-L1 blockers.
Figure 3. Immune infiltration of CT26 and MC38 responding to PT-112 plus ICBs. (a,b). Percentage of CD45+ (over total live), CD11b+ (over CD45+), F4/80+ (over CD11b+), and CD11c+ (over CD11b+) cells, and CD11c+ to F4/80+ cell ratio, in CT26 (a) and MC38 (b) tumors treated with PT-112 in the context of optional PD-1 (a) or PD-L1 blockage. *p < .05, **p < .01, ***p < .001 (one-way ANOVA), as compared to untreated tumors; †p < .05, ††p < .01, †††p < .001 (one-way ANOVA), as compared to tumors treated with PT-112 only; ‡p < .05, ‡‡p < .01, ‡‡‡p < .001 (one-way ANOVA), as compared to tumors treated with PD-1 or PD-L1 blockers only, as relevant. See also Suppl. Figure 2a. (c,d). Percentage of CD3+ (over CD45+), CD8+ (over CD3+), CD4+ (over CD3+), and CD25+FOXP3+ (over CD4+) cells, and CD8+ to CD25+FOXP3+ cell ratio, in CT26 (c) and MC38 (d) tumors treated with PT-112 in the context of optional PD-1 (c) or PD-L1 blockage. *p < .05, **p < .01, ***p < .001 (one-way ANOVA), as compared to untreated tumors; p < .05 (one-way ANOVA), as compared to tumors treated with PT-112 only; #p < .05, ##p < .01, ###p < .001 (one-way ANOVA), as compared to tumors treated with PD-1 or PD-L1 blockers only, as relevant. See also Suppl. Figure 2b.
mice receiving PT-112 plus PD-1 blockade achieved complete tumor eradication, four of which occurring within 35 days after initiation of treatment. These four mice rejected a novel challenge with a tumorigenic dose of CT26 cells, suggesting the activation of long-term immunological protection. Similar results were obtained in the MC38 model (Figure 2d). In this setting, however, PT-112 monotherapy was associated with a statistically significant increase in survival that was further enhanced by combination with PD-L1 (but not PD-1) blockers (Figure 2d). Of note, we were unable to document overt signs of toxicity (e.g., anorexia, hunched posture, weight loss) in any treatment group (data not shown).

Of note, both CT26 and MC38 tumors receiving PT-112 monotherapy exhibited a considerable decrease in the relative abundance of CD45<sup>+</sup> cells, which was largely accounted for by a reduction in CD11b<sup>+</sup> myeloid cells and was not altered by PD-1 or PD-L1 blockade (Figure 3a,b). In the CT26, (but not in the MC38) model, such a loss of CD11b<sup>+</sup> was largely confined to immunosuppressive F4/80<sup>+</sup> tumor-associated macrophages (TAMs), while the relative proportion of CD11c<sup>+</sup>F4/80<sup>-</sup> dendritic cells (DCs) increased at least to some degree (Figure 3a,b). Consistent with these findings, the microenvironment of PT-112-treated CT26 and MC38 tumors was enriched for CD3<sup>+</sup> T cells, with a predominance of CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs) over CD4<sup>+</sup> helper T cells (Figure 3c,d). Moreover, the CD4<sup>+</sup> compartment of CT26 and MC38 tumors exposed to PT-112 exhibited reduced polarization toward an immunosuppressive CD25<sup>+</sup>FOXP3<sup>+</sup> regulatory T (T<sub>REG</sub>) phenotype, in particular when PT-112 was combined with PD-1 or PD-L1 blockers (Figure 3c,d).

Altogether, these findings indicate that PT-112 favors the establishment of an immunostimulatory tumor microenvironment characterized by increased CD8<sup>+</sup> CTL infiltration and reduced TAM- and T<sub>REG</sub> cell-dependent immunosuppression, and that some of these beneficial alterations can be boosted by PD-1 or PD-L1 blockade along with the activation of therapeutically relevant anticancer immunity.

**Discussion**

This is the first demonstration that PT-112, a novel platinum-pyrophosphate conjugate under clinical development, causes the emission of immunostimulatory DAMPs by dying cancer cells (Figure 1), drives bona fide ICD and hence can initiate anticancer immunity per se (Figure 2), synergizing with ICBs in the context of superior immune infiltration (Figures 2 and 3). These findings are in line with preliminary clinical evidence on the use of PT-112 in patients with solid tumors, either as a standalone therapeutic agent (NCT02266745),<sup>11,12</sup> or in combination with the PD-L1 blocker avelumab (NCT03409458).<sup>13</sup>

While platinum derivatives such as CDDP, carboplatin and oxaliplatin have been extensively employed for the treatment of multiple solid tumors,<sup>14-16</sup> they (1) are frequently associated with toxicities and relatively prone to cause acquired resistance,<sup>4,5</sup> and (2) have limited activity in bone lesions.<sup>38,39</sup> Moreover, the actual value of platinum derivatives in the context of ICB-based immunotherapy remains to be determined. Indeed, ICBs have been successfully combined with standard-of-care platinum-based chemotherapy in patients with a variety of ICB-sensitive tumors, such as non-small cell lung carcinoma.<sup>40</sup> However, little benefit has been documented from the addition of ICBs to chemotherapy with current platinum derivatives in ICB-resistant tumors, such as microsatellite stable colorectal tumors.<sup>41</sup>

Conversely, PT-112 appears to possess a unique combination of factors, including (1) safety in heavily pretreated patients,<sup>11,12</sup> (2) an improved pharmacokinetic and pharmacodynamic profile including (but not limited to) a prominent osteotropism,<sup>13</sup> (3) monotherapy efficacy in patients with pulmonary tumors, prostate cancer and thymoma,<sup>11,12</sup> (4) combinatorial efficacy in the context of PD-L1 blockade in men with castration resistant prostate cancer,<sup>14</sup> and (5) activity in immunocompetent mouse models of breast and colorectal cancer linked to the initiation of ICD, as demonstrated in this paper.

Thus, PT-112 stands out as a promising agent for the treatment of solid neoplasms that display limited sensitivity to ICBs and/or originate or tend to metastasize to the bone.<sup>42-45</sup> Based on our preclinical findings, it is tempting to speculate that PT-112 may cause a robust wave of ICD associated with an increased abundance of antigenic material from malignant cells as well as with the emission of chemotactic and immunostimulatory signals that altogether (re)activate anticancer immunity, de facto setting the stage for efficacious ICB-based immunotherapy. Additional experiments are required to validate this working model. Irrespective of unknowns related to the molecular mechanism of action of PT-112 and its potential ICD-independent immunomodulatory activity, this novel platinum-pyrophosphate conjugate stands out as a promising agent for the implementation of innovative ICB-based immunochemotherapeutic regimens.

**Disclosure of Potential Conflicts of Interest**

TDA is a full-time employee of and owns equity in Phosplatin Therapeutics. LG provides remunerated consulting to Astra Zeneca, Boehringer Ingelheim, Inzen and the Luke Heller TECPR2 Foundation, and he is member of Scientific Advisory Committees for Boehringer Ingelheim and OmniSEQ. As per standard operations at Oncoimmunology, LG has been excluded from all steps of editorial evaluation of the present article.

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**ORCID**

Takahiro Yamazaki <http://orcid.org/0000-0002-7420-4394>

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