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Assessment of Therapeutic Effects of Combined Treatment With Cisplatin and Anti-mouse Programmed Death (PD)-1 Antibody in a Mouse Urothelial Cancer Model

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Assessment of Therapeutic Effects of Combined Treatment With Cisplatin and Anti-mouse Programmed Death (PD)-1 Antibody in a Mouse Urothelial Cancer Model

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Abstract. *Background/Aim:* The therapeutic impact of combination treatment with an immune checkpoint inhibitor (ICI) and chemotherapeutic agent on patients with urothelial cancer (UC) remains controversial. Therefore, the present study investigated differences in the therapeutic effects of combination therapy with cisplatin plus anti-mouse programmed death (PD)-1 antibody according to the dose of cisplatin using the mouse bladder tumor model MBT2. *Materials and Methods:* The effects of treatment with two different doses cisplatin and/or anti-mouse PD-1 antibody on tumor growth after the subcutaneous injection of MBT2 cells were compared. Infiltrating patterns of lymphocytes into tumors after treatment were assessed using immunohistochemical staining. *Results:* MBT2 tumor volumes were significantly larger in mice receiving high-dose cisplatin alone than in those receiving low-dose cisplatin alone. Combination treatment with cisplatin plus anti-mouse PD-1 antibody exerted significantly stronger growth inhibitory effects on MBT2 tumors than treatment with either agent alone, irrespective of cisplatin doses; however, no significant differences were observed in MBT2 tumor volumes between mice receiving anti-mouse PD-1 antibody plus high-dose cisplatin and those receiving anti-mouse PD-1 antibody plus low-dose cisplatin. Furthermore, CD8+ to CD3+ and CD8+ to CD11b+ T-lymphocyte ratios in MBT2 tumors were both significantly higher in the low-dose cisplatin alone group than in the high-dose cisplatin alone group, whereas no

significant differences were noted in either ratio between the two different combination treatment regimens. *Conclusion:* When combined with ICI, a lower dose of cisplatin may achieve favorable antitumor effects in UC patients by preventing lymphocyte exhaustion.

Urothelial carcinoma (UC) encompasses carcinomas of the bladder, ureter, renal pelvis, and urethra, representing the fourth or fifth most common type of malignancy worldwide (1). Patients with advanced UC have extremely poor outcomes, with a median overall survival (OS) of <1 year (2). Platinum-based combination chemotherapy is widely performed as the gold standard first-line treatment for advanced UC (3). According to the findings of the phase III KEYNOTE-045 trial showing the significant survival benefit of pembrolizumab, an anti-programmed death (PD)-1 antibody, over chemotherapy as a second-line treatment for platinum-refractory advanced UC (4), standard sequential systemic therapy for treatment-naïve advanced UC involves the administration of platinum-based combination chemotherapy followed by pembrolizumab (5). However, according to the KEYNOTE-045 trial, the objective response rate (ORR) of pembrolizumab was only 21%.

There is increasing evidence to support the combination of chemotherapy with an immune checkpoint inhibitor (ICI); metastatic non-small-cell lung cancer is treated with platinum-based chemotherapy plus pembrolizumab as standard systemic therapy (6, 7). However, in the field of advanced UC, the KEYNOTE-361 trial, which compared chemotherapy alone versus chemotherapy plus pembrolizumab, did not show significant differences in progression-free survival (PFS) or OS (8). We herein focused on the immune microenvironment of UC, compared the therapeutic effects of different doses of cisplatin alone or cisplatin plus anti-mouse PD-1 antibody, and examined the infiltrating patterns of lymphocytes into tumor tissues after the completion of these treatments.

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Key Words: Urothelial carcinoma, cisplatin, anti-mouse PD-1 antibody immune checkpoint inhibitor.

Materials and Methods

Tumor cell line. MBT2 cells, established from FANFT (N-[4-(5-nitro-2-furyl)-2-thiazolyl] formamide)-induced murine bladder tumors arising in C3H mice, were purchased from the JCRB Cell Bank (Osaka, Japan), and were cultured as previously described (9).

Cell proliferation assay. The *in vitro* growth inhibitory effects of cisplatin and/or anti-mouse PD-1 antibody on MBT2 cells were assessed as previously described (9). Briefly, 5×10^3 cells were seeded in each well of a 96-well plate and allowed to attach overnight. The wells were treated with vehicle, cisplatin (Wako, Osaka, Japan), anti-mouse PD-1 antibody (Bio X Cell, Lebanon, NH, USA), or cisplatin plus anti-mouse PD-1 antibody. After an incubation for 48 h, the number of cells was counted by Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan).

Western blot analysis. A Western blot analysis was performed as previously described (9, 10).

Animal studies. Eight-week-old-female, pathogen-free, C3H/He mice were purchased from Japan SLC (Shizuoka, Japan) and housed as previously described (9). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Approximately 2×10^5 of MBT2 cells were injected subcutaneously with 100 μ l of Matrigel (CORNING, Corning, NY, USA). One week after the injection of MBT2 cells, the treatment of mice was initiated as described below. Each experimental group consisted of eight mice. The required sample size was selected based on the study by Charen *et al.* (11). Six groups of experiments were conducted and 48 mice were used.

In this experiment, the therapeutic effects of cisplatin, anti-mouse PD-1 antibody, and their combination were examined using mice receiving a subcutaneous injection of MBT2 cells. Mice were randomly divided into the following six groups: no treatment, low-dose cisplatin alone, high-dose cisplatin alone, anti-mouse PD-1 antibody alone, anti-mouse PD-1 antibody plus low-dose cisplatin, and anti-mouse PD-1 antibody plus high-dose cisplatin. After randomization, the low-dose cisplatin alone group and anti-mouse PD-1 antibody plus low-dose cisplatin group received intraperitoneal injections of cisplatin at a dose of 3 mg/kg, and the high-dose cisplatin alone group and anti-mouse PD-1 antibody plus high-dose cisplatin group received intraperitoneal injections of cisplatin at a dose of 6 mg/kg once per week for two weeks. The anti-mouse PD-1 antibody alone group, anti-mouse PD-1 antibody plus low-dose cisplatin group, and anti-mouse PD-1 antibody plus high-dose cisplatin group received intraperitoneal injections of 200 μ g of anti-mouse PD-1 antibody twice per week for two weeks.

After the subcutaneous injection of MBT2 cells mice were observed daily for 21 days after the initiation of treatment and were then sacrificed. The growth of subcutaneous tumors was measured and tumor volumes were calculated as previously described (9). Three weeks after the initiation of treatment, tumor tissues were harvested from mice. All animal experiments were approved by the Animal Care and Use Committee of Hamamatsu University School of Medicine (approved number: 2020082) and performed according to the guidelines of this committee and ARRIVE (12).

Immunohistochemical staining. Subcutaneous tumors were harvested from mice after the completion of treatment with cisplatin and/or anti-mouse PD-1 antibody as described above. The immunohistochemical

staining of tumor specimens was conducted as previously described (9, 13) using antibodies against mouse CD3, CD8 (Cell Signaling Technology, Danvers, MA, USA), CD11b, and Ki-67 (Abcam, Cambridge, UK). Quantitative assessments of the results of immunohistochemical staining were performed using Fiji software as previously described (9, 14).

Statistical analysis. All statistical analyses were conducted using EZR software (Saitama Medical Center, Jichi Medical University, ver. 1.40), and *p*-values < 0.05 were considered to be significant. All data are presented as the mean \pm standard deviation, and differences between the two groups were compared using the two-tailed Student's *t*-test.

Results

Effects of treatment with cisplatin and/or anti-mouse PD-1 antibody on the *in vitro* growth of MBT2. As shown in Figure 1A, cisplatin inhibited the *in vitro* growth of MBT2 cells in a dose-dependent manner; however, anti-mouse PD-1 antibody did not significantly affect the *in vitro* growth of MBT2 cells at any of the concentrations examined (Figure 1B). Furthermore, when combined with anti-mouse PD-1 antibody, the sensitivity of MBT2 cells to cisplatin was not significantly enhanced (Figure 1C).

Effects of treatment with cisplatin on PD-L1 and PD-L2 expression levels in MBT2 cells *in vitro*. Western blot analyses were used to examine whether the expression levels of PD-L1 and PD-L2 in MBT2 cells were affected by the treatment with cisplatin. As shown in Figure 2A, the treatment of MBT2 cells with cisplatin induced the time-dependent up-regulation of PD-L1, but not PD-L2. In addition, despite the lack of changes in PD-L2 after the cisplatin treatment, the treatment of MBT2 cells with 100 nM cisplatin induced the up-regulation of PD-L1, whereas that with 1 and 10 nM cisplatin did not (Figure 2B).

Therapeutic effects of treatment with cisplatin, anti-mouse PD-1 antibody, or their combination on mice receiving the subcutaneous injection of MBT2 cells. The treatment with cisplatin, anti-mouse PD-1 antibody, or their combination inhibited subcutaneous MBT2 tumor growth, and subcutaneous tumor volumes significantly differed between mice without any treatment and those receiving agents other than low-dose cisplatin alone. In addition, MBT2 tumor volumes were significantly larger in mice treated with low-dose cisplatin alone than in those treated with high-dose cisplatin alone. Combined treatment with cisplatin plus anti-mouse PD-1 antibody exerted significantly stronger growth inhibitory effects on MBT2 tumors than treatment with either agent alone, irrespective of cisplatin doses; however, no significant differences were noted in MBT2 tumor volumes between mice receiving anti-mouse PD-1 antibody plus high-dose cisplatin and those receiving anti-mouse PD-1 antibody plus low-dose cisplatin (Figure 3B).

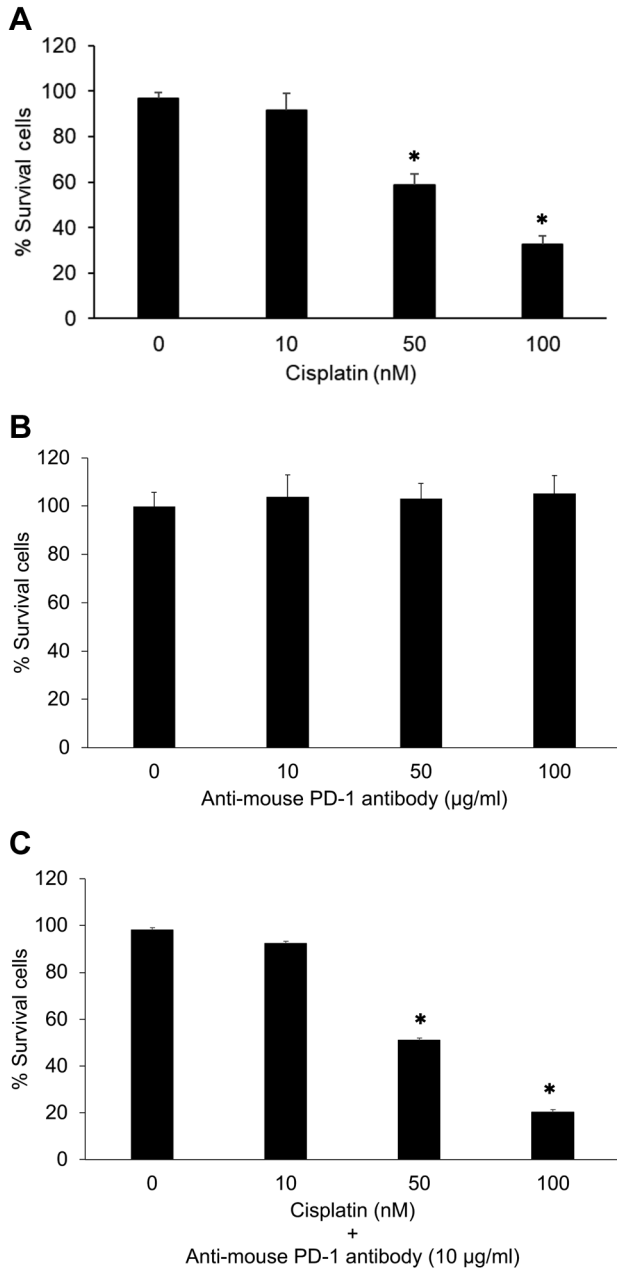


Figure 1. Effects of combination treatment with cisplatin and/or anti-mouse programmed death-1 (PD-1) antibody on MBT2 cell growth. MBT2 cells were treated with cisplatin (A), anti-mouse PD-1 antibody (B), or cisplatin plus 10 µg/ml anti-mouse PD-1 antibody (C). After incubation for 48 h, the number of viable cells was assessed using a cell proliferation assay. Each data point represents the mean of triplicate analyses; bars, standard deviations. *Differs from control ($p < 0.01$) by the Student's *t*-test.

Assessment of infiltrating patterns of T-lymphocytes into subcutaneous MBT2 tumors after treatment with cisplatin, anti-mouse PD-1 antibody, or their combination. The infiltration of CD3+, CD8+, and CD11b+ T-lymphocytes into

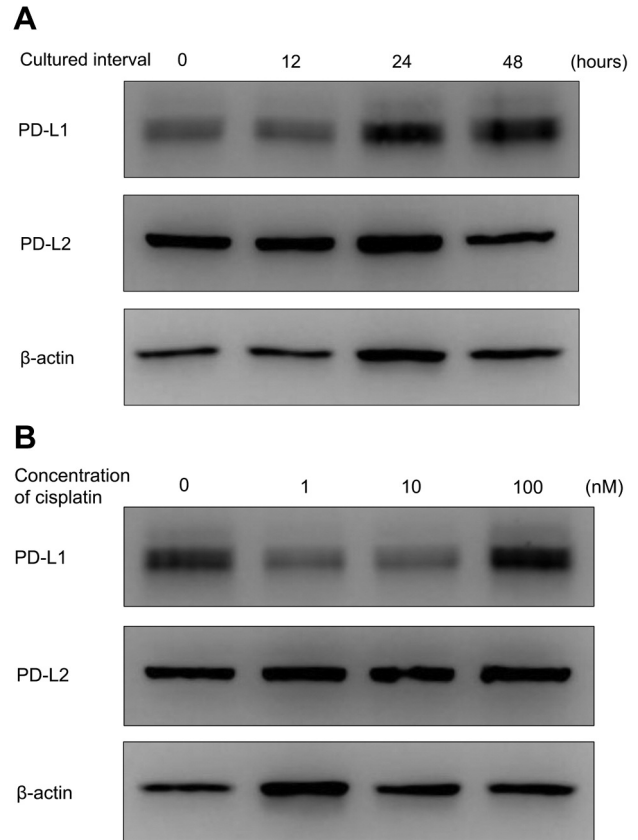


Figure 2. Effects of cisplatin treatment on programmed death-ligand 1 (PD-L1) and PD-L2 expression in MBT2 cells. Blots/gels in the figures have been cropped for clarity and conciseness. Figure 2A and B show different blots/gels. (A) MBT2 cells were treated with 100 nM cisplatin for several intervals, and protein was then extracted from cultured cells and analyzed for PD-L1, PD-L2, and β-actin levels using western blotting. These used the same membrane in all cases. (B) MBT2 cells were treated with several concentrations of cisplatin for 48 h, and protein was then extracted from cultured cells and analyzed for PD-L1, PD-L2 and β-actin levels using western blotting. These used the same membrane in all cases.

subcutaneous tumors after treatment with cisplatin, anti-mouse PD-1 antibody, or their combination was quantitatively evaluated using immunohistochemical staining. Figure 4A shows typical findings of the immunohistochemical staining of subcutaneous tumors. As shown in Figure 4B and C, CD8+ to CD3+ (CD8/CD3) and CD8+ to CD11b+ (CD8/CD11b) T-lymphocyte ratios in subcutaneous tumors were both significantly higher in the low-dose cisplatin alone group than in the high-dose cisplatin alone group. However, no significant differences were observed in the CD8/CD3 or CD8/CD11b ratio between MBT2 tumors treated with anti-mouse PD-1 antibody plus high-dose cisplatin and those treated with anti-mouse PD-1 antibody plus low-dose cisplatin.

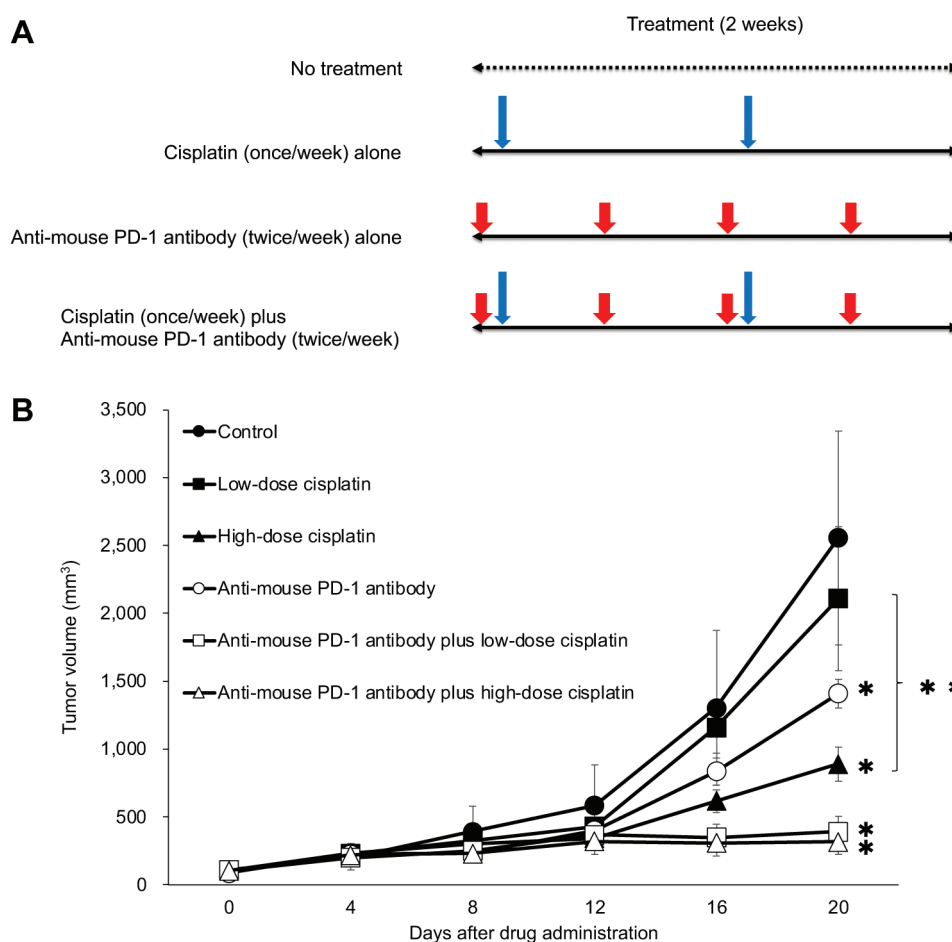


Figure 3. Therapeutic effects of combination treatment with cisplatin, anti-mouse programmed death-1 (PD-1) antibody, or their combination in the *in vivo* MBT2 model. (A) Schematic presentation of treatment schedules in this experiment. (B) One week after the subcutaneous injection of MBT2 cells, mice developed tumors with volumes of approximately 100 mm³ and were randomly divided into the following six groups: no treatment, low-dose cisplatin alone, high-dose cisplatin alone, anti-mouse programmed death-1 (PD-1) antibody alone, anti-mouse PD-1 antibody plus low-dose cisplatin, and anti-mouse PD-1 antibody plus high-dose cisplatin. Detailed treatment schedules using cisplatin and/or anti-mouse PD-1 antibody are described in the Materials and Methods section. Each data point represents the mean tumor volume in each experimental group (n=8 mice); bars, standard deviation. Mean tumor volumes 21 days after the initiation of treatment relative to that of no treatment group were as follows; -17.4%, low-dose cisplatin alone; -65.2%, high-dose cisplatin alone; -44.8%, anti-mouse PD-1 antibody alone; -84.7%, anti-mouse PD-1 antibody plus low-dose cisplatin; and -87.6%, anti-mouse PD-1 antibody plus high-dose cisplatin. *Differs from controls (p<0.05) according to the Student's t-test; **Differs from each group (p<0.05) according to the Student's t-test.

Discussion

Since the demonstration of OS benefits with pembrolizumab over chemotherapy in patients with platinum-refractory advanced UC (1), pembrolizumab has been widely accepted as a standard second-line agent against advanced UC (2). However, according to the KEYNOTE-045 trial, the ORR of pembrolizumab was only 21% (1). The efficacy of the combination of chemotherapy and ICI was recently investigated in the KEYNOTE-361 trial, and the findings obtained revealed that PFS and OS showed no advantage over

chemotherapy alone (8). Therefore, the present study focused on the tumor microenvironment and compared the therapeutic effects of different doses of cisplatin alone or cisplatin plus anti-mouse PD-1 antibody using the mouse bladder tumor model MBT2.

Since the main aim of the present study was to examine the *in vivo* effects of several treatments, including anti-mouse PD-1 antibody, and the mechanisms mediating their antitumor activities, human UC cell lines were not suitable; therefore, MBT2, the most frequently used mouse bladder tumor cell line, was selected for this study. We initially confirmed that the

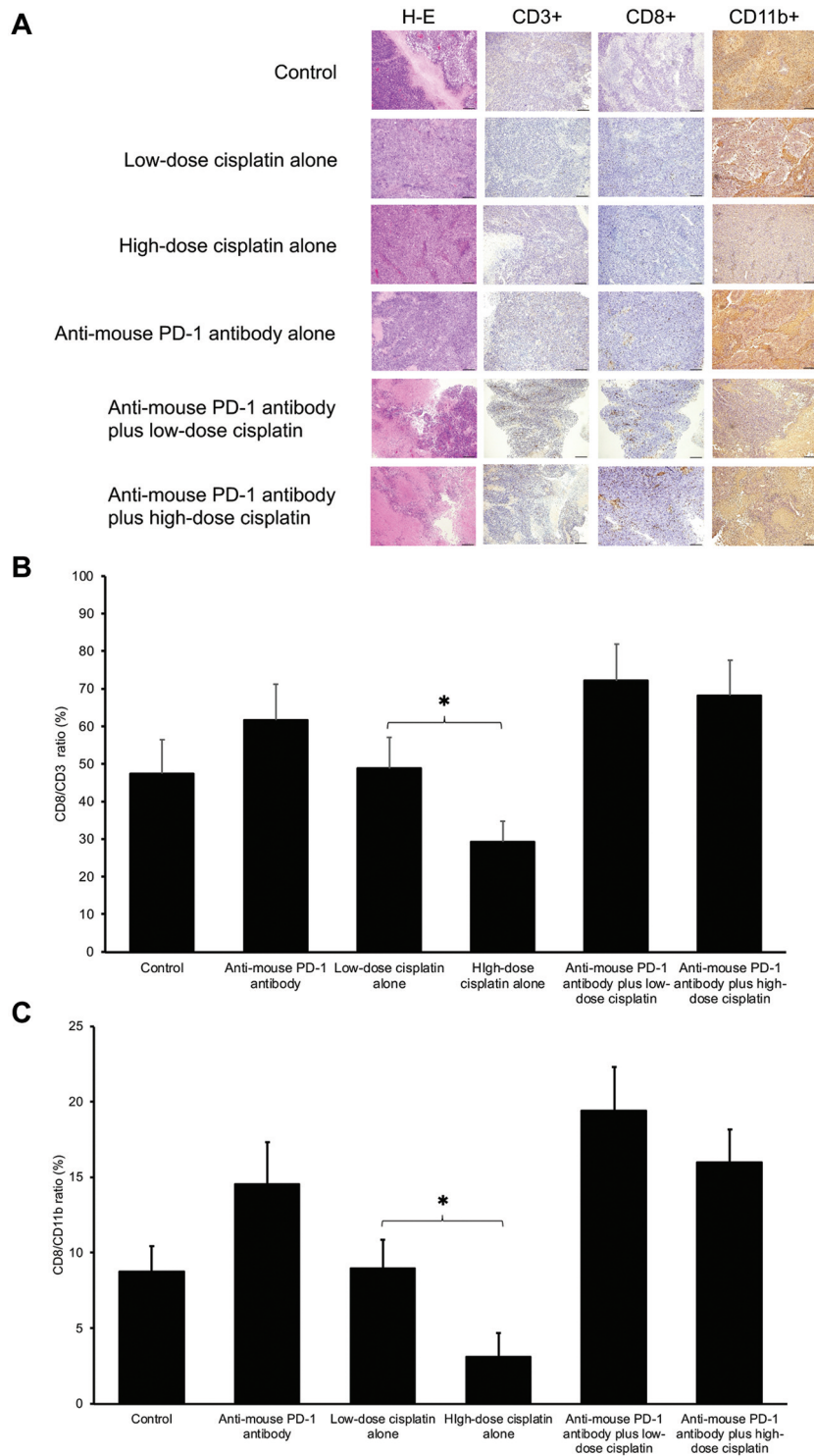


Figure 4. Histopathological study on MBT2 tumors after treatment with cisplatin and/or anti-mouse programmed death-1 (PD-1) antibody. *In vivo* subcutaneous tumors were harvested from nude mice after the completion of treatment according to the schedule shown in this figure. Sections from each tumor tissue were examined following hematoxylin-eosin (H-E) staining and immunohistochemical staining with antibodies for CD3, CD8, and CD11b. (A) Representative findings of the histopathological study are shown (scale bar=100 μ m). (B) Quantification of the CD8/CD3 ratio in MBT2 tumors based on the results of immunohistochemical staining in the following treatment groups: no treatment, low-dose cisplatin alone, high-dose cisplatin alone, anti-mouse PD-1 antibody alone, anti-mouse PD-1 antibody plus low-dose cisplatin, and anti-mouse PD-1 antibody plus high-dose cisplatin. Each data point represents the mean of eight tumors; bars, standard deviations (SDs); *Differs from each group ($p < 0.05$) according to the Student's *t*-test.

treatment with cisplatin dose-dependently inhibited the growth of MBT2 cells *in vitro*, whereas treatment with anti-mouse PD-1 antibody, either alone or in combination with cisplatin, did not. Furthermore, after the treatment of MBT2 cells with cisplatin *in vitro*, despite the lack of marked changes in the expression of PD-L2, that of PD-L1 was significantly up-regulated. In a previous study by Powles *et al.*, the up-regulation of an adaptive immunosuppressive pathway, including PD-L1, during platinum-based chemotherapy was demonstrated in several preclinical models (15). To date, several studies have indicated that an increase in PD-L1 expression in infiltrating immune cells is triggered by cytokines, such as interferon- γ , in the tumor microenvironment, which contributes to adaptive immune resistance (16, 17). Collectively, these findings provide a strong rationale for combining ICI with cisplatin as a systemic approach for advanced UC.

In the *in vivo* experiments performed in the present study, the therapeutic effects of two concentrations of cisplatin, anti-mouse PD-1 antibody, and their combination were compared in mice subcutaneously administered MBT2 cells. Increases in the cisplatin dose resulted in favorable tumor growth inhibition in monotherapy, but not in the combination therapy with anti-mouse PD-1 antibody. Further studies are needed to elucidate the mechanisms underlying these outcomes. Chemotherapeutic agents have been shown to induce immunogenic cell death under specific conditions, which triggers the production of immune-activating molecules and activates dendritic cells as well as specific T cell responses, resulting in the activation of acquired immunity against tumor cells (18). However, chemotherapy leads to resistance to immunotherapy due to immune cell exhaustion and the induction of PD-L1 expression in tumor or immune cells (19, 20). Based on these findings, balancing the positive and negative aspects of chemotherapy by adjusting chemotherapy doses may be important in the combination of chemotherapy and ICI.

The present results also showed changes in the patterns of tumor-infiltrating lymphocytes (TIL) after treatment with anti-mouse PD-1 antibody and different concentrations of cisplatin. Although the significance of TIL subsets after ICI-based systemic therapy remains unclear, previous studies examined several TILs in tumor tissues prior to systemic therapy, and the percentages of specific TILs were shown to be associated with prognosis (21, 22). Peng *et al.* demonstrated that high CD8/CD3 ratios in live oligometastases from colorectal cancer independently correlated with better recurrence-free survival and OS (21). In the present study, CD8/CD3 and CD8/CD11b ratios in subcutaneous tumors both closely reflected differences in tumor growth inhibition between cisplatin monotherapy and combination therapy with anti-mouse PD-1 antibody at different cisplatin concentrations. Accordingly, assessments of TIL subsets during ICI-based therapies may be useful as both predictive and prognostic biomarkers for advanced UC.

Study limitations. Since the mouse bladder tumor model MBT2 was used throughout this study, further research is needed to confirm whether the results obtained herein are reproducible when using other tumor-derived animal models. Furthermore, in the assessment of TIL subsets, the infiltration of CD11b+ T lymphocytes was quantified based on the results of immunohistochemical staining; however, it is preferable to evaluate myeloid-derived suppressor cells rather than CD11b+ T lymphocytes. Regarding the application of the present results to clinical practice, further studies that investigate the safety profiles of combinations of cisplatin and anti-mouse PD-1 antibody are needed.

Conclusion

The present results showed that at higher concentrations, cisplatin alone, but not in combination with anti-mouse PD-1 antibody, efficaciously inhibited tumor growth in mice after the subcutaneous injection of MBT2 cells, and these therapeutic effects were proportional to post-treatment CD8/CD3 and CD8/CD11b ratios in subcutaneous tumors. Collectively, these results suggest an optimal dose of cisplatin for combination therapy with anti-PD-1 antibody to prevent lymphocyte exhaustion while achieving good antitumor effects for advanced UC.

Conflicts of Interest

The present study was partially supported by Pfizer Japan Inc.

Authors' Contributions

Conceptualization: Ryo Sato and Hideaki Miyake. Data curation: Ryo Sato and Hideaki Miyake. Formal analysis: Ryo Sato and Hideaki Miyake. Funding acquisition: Hideaki Miyake. Investigation: Ryo Sato. Methodology: Ryo Sato and Hideaki Miyake. Project administration: Ryo Sato and Hideaki Miyake. Writing and making figures-original draft: Ryo Sato and Hideaki Miyake. Review and editing: All Authors.

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- 2 McGuire WL and Chamnes GC: Studies on the oestrogen receptor in breast cancer. In: *Receptors for Reproductive Hormones*. O' Malley BW, Chamnes GC (eds.). New York City, NY, USA, Plenum Publ Corp., pp 113-136, 1973.
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