

Chemotherapy Induces Programmed Cell Death-Ligand 1 Overexpression via the Nuclear Factor- κ B to Foster an Immunosuppressive Tumor Microenvironment in Ovarian Cancer

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Abstract

Emerging evidence has highlighted the host immune system in modulating the patient response to chemotherapy, but the mechanism of this modulation remains unclear. The aim of this study was to analyze the effect of chemotherapy on antitumor immunity in the tumor microenvironment of ovarian cancer. Treatment of ovarian cancer cell lines with various chemotherapeutic agents resulted in upregulated expression of MHC class I and programmed cell death 1 ligand 1 (PD-L1) in a NF- κ B-dependent manner and suppression of antigen-specific T-cell function *in vitro*. In a mouse model of ovarian cancer, treatment with paclitaxel increased CD8⁺ T-cell infiltration into

the tumor site, upregulated PD-L1 expression, and activated NF- κ B signaling. In particular, tumor-bearing mice treated with a combination of paclitaxel and a PD-L1/PD-1 signal blockade survived longer than mice treated with paclitaxel alone. In summary, we found that chemotherapy induces local immune suppression in ovarian cancer through NF- κ B-mediated PD-L1 upregulation. Thus, a combination of chemotherapy and immunotherapy targeting the PD-L1/PD-1 signaling axis may improve the antitumor response and offers a promising new treatment modality against ovarian cancer. *Cancer Res*; 75(23); 5034–45. ©2015 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy worldwide and the fourth most common cause of cancer-related death in women, because more than 75% of patients are diagnosed at an advanced tumor stage (stage III or IV; refs. 1, 2). Although taxane- and platinum-based chemotherapies are effective in the treatment of the majority of ovarian cancer cases, more than 70% of patients suffer from recurrence and eventually develop chemoresistance (3). Considering the high mortality rate of ovarian cancer due to the absence of curative treatment for advanced stage or recurrent disease, new therapeutic modalities, including immunotherapy to complement chemotherapy, are urgently needed (4).

Anticancer chemotherapeutic agents have long been known to induce systemic immunosuppressive effects due to bone marrow

toxicity (5). However, recent reports have shown that several chemotherapeutic drugs altered the local immune state, affecting the response of the tumor to treatment (6–9). Several commonly used chemotherapeutic agents, including those commonly used for ovarian cancer such as paclitaxel and gemcitabine, induce immunoreactive effects such as promotion of tumor antigen presentation through upregulating the expression of tumor antigens or MHC class I molecules (10, 11). Other agents decrease the number of immunosuppressive cells, such as regulatory T cells or myeloid derived suppressor cells (MDSC), thereby increasing helper T-cell accumulation at the tumor site (7, 8). On the other hand, some chemotherapeutic agents lead to local immunosuppression via induction of a specific inflammasome, promoting tumor growth (12). Thus, it is unclear whether or not chemotherapeutic agents exert an immunoreactive effect on the tumor microenvironment.

Recent studies have revealed that tumor cells acquire several ways to escape the host immune attack in the tumor microenvironment; these constitute the cancer immune escape system (13–15). One of the most important components of this system is an immune checkpoint signal, the programmed death 1 (PD-1)/PD-1 ligand 1 (PD-L1) axis (16). PD-1 is mainly expressed by activated T cells, whereas PD-L1 is frequently expressed on tumor cells (17–19). Inhibition of PD-1/PD-L1 interaction enhances the T-cell response and mediates dramatic preclinical antitumor activity in patients with melanoma, renal cell cancer, and non-small cell lung cancer (17, 20–22). We previously reported that PD-L1 expression is associated with poorer prognosis in ovarian cancer patients

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(23) and promotes peritoneal dissemination of ovarian cancer via host immunosuppression (24). However, it is not clear how chemotherapeutic agents affect local immune status, including PD-1/PD-L1 signaling.

Herein, we investigate the molecular mechanism of chemotherapy-induced PD-L1 expression in human ovarian cancer cells and determine whether inhibition of the PD-1/PD-L1 signal increases the efficacy of chemotherapy in a syngenic mouse ovarian cancer model.

Materials and Methods

Microarray analysis

We analyzed four publicly available gene expression microarray datasets: GSE15622 (25), from clinical ovarian cancer samples collected before and after chemotherapy (paclitaxel, $n = 20$; carboplatin, CBDCA, $n = 14$); GSE18728 (26), from clinical breast cancer samples pre- and post-docetaxel and capecitabine ($n = 14$); GSE13525 (27), from human ovarian cancer cell line 36M2 treated with CBDCA and Cancer Cell Line Encyclopedia (CCLE) for 1,037 human tumor cell lines (28). Pathway analysis of these microarray data before and after chemotherapy was performed using Gene Annotation Tool to Help Explain Relationships (GATHER; ref. 29).

The NF- κ B gene expression signature that includes eight genes (*CCRN4L*, *CREB3L2*, *GAA*, *HIP1*, *HSPA5*, *KLF6*, *NFKBIZ*, and *SURF4*) has been implemented in a previous study (30). An NF- κ B signature score was calculated for each sample before and after chemotherapy using single sample Gene Set Enrichment Analysis (ssGSEA; ref. 31).

Immunohistochemistry

Immunohistochemical staining and counting of immune cells were performed as previously described (23, 32). Briefly, for human CD8 staining, paraffin-embedded sections were first stained with mouse anti-human CD8 monoclonal antibody. For mouse immunohistochemistry, peritoneal tumor cryosections were stained with rat anti-mouse CD4, rat anti-mouse CD8, goat anti-mouse PD-L1, anti-NF- κ B p65, or anti-mouse Gr-1. The numbers of CD4⁺, CD8⁺, and Gr-1⁺ cells at the tumor site were counted in a microscopic field at $\times 200$ magnification. Detailed information is provided in Supplementary Data.

Evaluation of PD-L1 expression on human ovarian cancer cells from ascites

Ascites fluid from an ovarian cancer patient was collected before and after chemotherapy (paclitaxel and CBDCA; days 4 and 11). These cells were harvested and incubated with phycoerythrin (PE)-conjugated anti-human PD-L1 antibody. EpCAM-positive (FITC-labeled anti-human CD326 clone 9C4; Biolegend) and 7-AAD-negative gated cells were scored as ovarian ascites tumor cells by flow cytometry.

Cell lines

The ID8 mouse ovarian cancer cell line was kindly provided by Dr. Margit Maria Janát-Amsbury from the Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Baylor College of Medicine (Houston, TX; ref. 24). The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from RIKEN BioResource Center (Ibaraki, Japan; ref. 32). Human ovarian cancer cell lines (ovary1847, SK-OV-3 and OVCAR8) were

provided by Dr. Susan K. Murphy from the Department of Obstetrics and Gynecology, Duke University (Durham, NC; ref. 33). RMGII were gifted by Dr. Daisuke Aoki from the Department of Obstetrics and Gynecology in Keio University (Tokyo, Japan; ref. 33). And all these cells were authenticated and were passaged fewer than 6 months after purchase or giving for all the experiments as previously described (33).

Chemotherapeutic agents

Gemcitabine (Eli Lilly Japan K.K.; 24 μ mol/L, 120 μ mol/L, 600 μ mol/L), paclitaxel (Bristol-Myers K.K.; 2, 10, 20 μ mol/L), CBDCA (Bristol-Myers K.K.; 20, 100, 500 μ mol/L), CPA (Shionogi & Co., Ltd.; 0.7, 7, 70 μ mol/L), or PBS were added to mouse cell lines ID8 and HM-1 for 24 hours and further monitored for an additional 9, 12, 24, 48 hours. Human cell lines ovary1847 and SK-OV-3 were treated with gemcitabine (24, 120, 600 μ mol/L), paclitaxel (2, 10, 20 μ mol/L) for 24 hours. RMGII and OVCAR8 were also treated with gemcitabine (120 μ mol/L), paclitaxel (10 μ mol/L) for 24 hours.

Flow cytometry

We used phycoerythrin (PE)-conjugated anti-PD-L1 antibody, PE-conjugated anti-mouse MHC class I antibody, PE-conjugated anti-mouse IFN γ receptor 1 (IFNGR1) antibody, PE-conjugated anti-mouse PD-1 antibody, FITC-conjugated anti-mouse CD4 antibody or AlexaFluor647-conjugated anti-mouse CD8 antibody. Detailed information is provided in Supplementary Data.

Biochemical and molecular biologic techniques

Western blotting, RT-PCR, knockdown of NF- κ B p65 (Rela) or Ifng1 expression in cells, generation of ID8OVA cells, cytotoxicity assays for ID8OVA cells and CD107a⁺ expression assays are described in Supplementary Data.

Mice

Female C57BL/6 (B6) and B6C3F1 mice were purchased from CLEA Japan. OVA-specific T-cell receptor transgenic mice (OT-1 mice) and CAG-GFP mice were purchased from The Jackson Laboratory and were interbred to generate OT-1-GFP mice. Animal experiments were approved by the Kyoto University Animal Research Committee.

Evaluation of PD-L1 depletion in tumor cells during chemotherapy in a mouse ovarian cancer model

A Pdl1-overexpressing cell line (ID8-pdl1), Pdl1-depleted cell line (ID8Mirpdl1; ID8-pdl1 KO), and sequence control cell line (ID8MirControl; ID8-control) were generated as previously described (24). Following intraperitoneal injection of ID8-control, ID8-pdl1 KO, or ID8-pdl1 (5×10^6 cells each) into syngenic mice, 8 mg/kg paclitaxel or saline was injected into the peritoneal cavities on days 14, 21, 28, and 35 after tumor implantation (each group, $n = 12$). Mice were euthanized before reaching the moribund state. This study was repeated using four 150 μ g/kg gemcitabine injections in place of paclitaxel.

Combination treatment with anti-PD-1 or anti-PD-L1 antibody and chemotherapy in a mouse ovarian cancer model

Mice (C57BL/6) were injected intraperitoneally with 5×10^6 ID8OVA cells. Paclitaxel (8 mg/kg) or saline was administered intraperitoneally on days 14, 21, 28, and 35 after tumor

implantation. Mice were treated intraperitoneally with 10 mg/kg of anti-mouse PD-1 monoclonal antibody (kindly provided by Ono Pharmaceutical Co., Ltd., Osaka, Japan,) or mouse IgG (clone MOPC 21; BioXcell) weekly on days 16, 23, 30, and 37. This study was repeated using six injections of 150 µg/kg gemcitabine (on days 14, 21, 28, 35, 42, and 49 after tumor implantation) in place of paclitaxel and six injections of 10 mg/kg of anti-mouse PD-1 monoclonal antibody (on days 16, 23, 30, 37, 44, and 51 after tumor implantation). In place of anti-PD-1 antibody, we also treated mice with 200 µg of rat anti-PD-L1 antibody (α PD-L1, clone 10F.9G2; BioLegend) alone, the combination of paclitaxel and α PD-L1, or saline and IgG2b κ (clone RTK4530; BioLegend) by intraperitoneal injection (control; each group, $n = 8$) on the same schedule as the anti-PD-1 antibody therapy. Survival analysis was performed by the Kaplan–Meier curves and log-rank test.

Cytotoxicity assay

As effector cells, activated OVA-specific CD8⁺ T cells were prepared as previously described (24). As target cells, Pdl1-depleted cells treated with paclitaxel (10 µmol/L) for 24 hours (paclitaxel-pretreated ID8-pdl1 KO), control cells treated with paclitaxel (10 µmol/L) for 24 hours (paclitaxel-pretreated ID8-control), control cells without paclitaxel treatment (ID8-control). Then, cells were loaded with 10 mg/mL OVA257–264 peptide (Bachem Bioscience) at 37°C for 1 hour, without OVA loading as a negative control (ID8-control without OVA loading). ID8-control, paclitaxel-pretreated ID8-control, paclitaxel-pretreated ID8-pdl1KO, or ID8-control without OVA loading were cocultured with activated OVA-specific CD8⁺ T cells at several effector-to-target (E:T) ratios. After 4 hours of incubation, the LDH in the coculture supernatant was detected with a nonradioactive cytotoxicity kit, CytoTox96 (Promega). Cytotoxicity for each E:T cell ratio was calculated as previously described (24).

Statistical analysis

The Student *t* test, paired *t* test or ANOVA was performed for the analysis of immunohistochemistry, Western blotting, and microarray data. The Kaplan–Meier curves and the log rank test were generated using GraphPad Prism 4 software. All *P* values < 0.05 were considered statistically significant.

Results

Chemotherapeutic agents alter the immunologic microenvironment in ovarian cancer

To evaluate the chemotherapeutic effect on the ovarian cancer microenvironment, we analyzed a microarray dataset, GSE15622, and found that in the top 100 pathways, 11 of apoptosis/cell death-related pathways, 9 of immune-related pathways, and 3 of NF- κ B-related pathways were upregulated following administration of chemotherapy (paclitaxel or CBDCA; Supplementary Table S1). Therefore, we suggest that chemotherapy (paclitaxel or CBDCA) leads not only to apoptotic cell death but also to an immune reaction with NF- κ B signal activation in the ovarian cancer microenvironment.

We also found that expression of *CD8* and *HLA-B* was upregulated not only in paclitaxel-treated samples but also in CBDCA-treated samples in the same GSE15622 microarray dataset (Fig. 1A and B), whereas *CD4* was upregulated only in paclitaxel-treated samples in this dataset (Fig. 1C). To

confirm NF- κ B signal activation in this dataset, we used ssGSEA to apply a previously established NF- κ B signature (23, 24) to the paclitaxel- or CBDCA-treated data in GSE15622; NF- κ B signaling was indeed significantly upregulated (Fig. 1D).

To confirm that the number of CD8⁺ TILs increases after chemotherapy in ovarian cancer patients we performed immunohistochemistry using an anti-CD8 antibody. We determined that the number of CD8⁺ TILs in ovarian cancer tissues from 21 patients was significantly increased after chemotherapy (Fig. 1E; Supplementary Table S2; CD8⁺ TILs count, 12.6 ± 19.7 – 20.6 ± 23.5 ; $P = 0.039$). Clinicopathologic features are shown in Supplementary Table S2.

Next, we evaluated whether overexpression of PD-L1 on ovarian cancer cells after chemotherapy in ovarian cancer patients was evident, because we previously reported that the expansion of CD8⁺ TILs was closely related to the amount of PD-L1 signaling by ovarian cancer cells (23). We collected cancer cells from massive ascites of a stage IIIc ovarian cancer patient both before and after (days 4 and 11) paclitaxel plus CBDCA chemotherapy at Kyoto University Hospital. PD-L1 expression was about 5-fold higher on day 4 than before chemotherapy. On day 11, however, PD-L1 expression had decreased to levels that were similar to those before chemotherapy (Fig. 1F). Therefore, the induction of PD-L1 expression by chemotherapy was reversible with or without continuous treatment with chemotherapeutic agents.

Chemotherapeutic agents induce NF- κ B p65 and MHC class I expression

To confirm the microarray results for dataset GSE15622 showing overexpression of NF- κ B p65 and MHC class I expression in clinical samples after chemotherapy, we incubated mouse ovarian cancer cell lines ID8 and HM-1 for 24 hours at several doses of gemcitabine, paclitaxel, CBDCA, or CPA. We found that NF- κ B p65 protein expression in ID8 and HM-1 was significantly induced with treatment in a dose-dependent manner (Fig. 2A and Supplementary Figs. S1 and S2). Treatment of human ovarian cancer cell lines (ovary1847, SK-OV-3, RMGII, and OVCAR8) with gemcitabine or paclitaxel also significantly induced total cellular NF- κ B p65 protein (Fig. 2B and Supplementary Fig. S3).

MHC class I expression was induced approximately 2-fold in mouse ovarian cancer cell lines ID8 or HM-1 after incubation with chemotherapeutic agents (gemcitabine, paclitaxel, or CBDCA; Fig. 2C and Supplementary Fig. S4A). Similarly, increased MHC class I protein was evident in human ovarian cancer cell lines ovary1847 and SK-OV3 treated with gemcitabine or paclitaxel in a dose-dependent manner (Fig. 2D and Supplementary Figs. S3 and S4B). MHC class I protein was also increased in human ovarian cancer cell lines RMGII and OVCAR8 treated with gemcitabine or paclitaxel (Supplementary Fig. S4C).

PD-L1 expression is modulated by chemotherapeutic agents

To determine whether PD-L1 expression is modulated by chemotherapeutic agents, we analyzed publicly available microarray dataset GSE13525 and found that PD-L1 mRNA expression is significantly induced in human ovarian cancer cell line 36M2 when incubated with CBDCA for 36 hours (Fig. 3A). In addition, analysis of a second dataset, GSE18728, showed that PD-L1 mRNA was elevated in breast cancer tissue from patients who

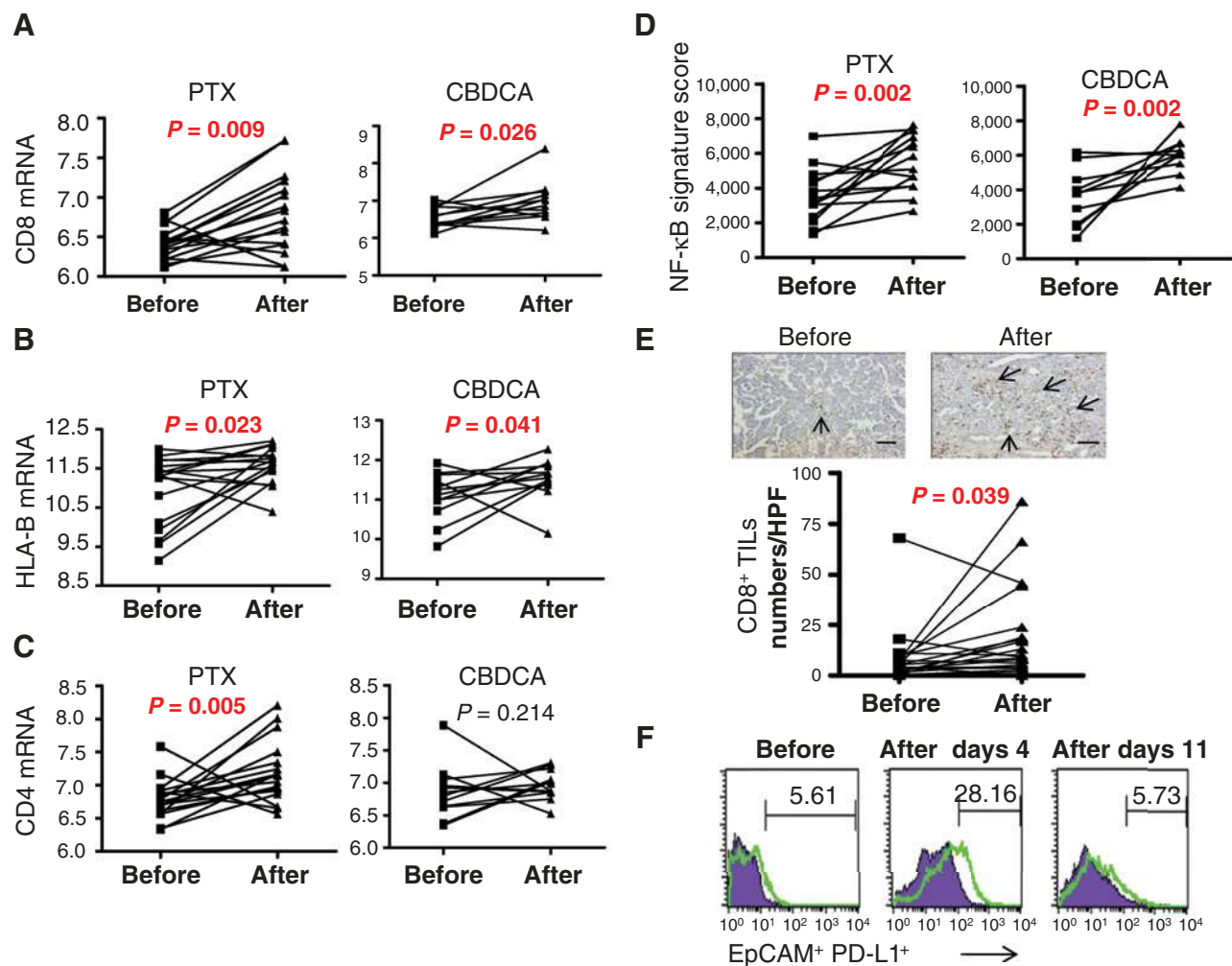


Figure 1. Chemotherapeutic agents alter the immunologic microenvironment in ovarian cancer. A–D, in ovarian cancer microarray dataset GSE15622, comparisons of CD8 mRNA (A), HLA-B mRNA (B), CD4 mRNA (C) expression, and the NF- κ B gene signature scores (D) before and after paclitaxel (PTX) or CBDCA treatment. E, a representative clinical sample of CD8⁺ TILs in ovarian cancer before and after paclitaxel plus CBDCA therapy (top). The number of CD8⁺ TILs from 21 ovarian cancer patients before and after chemotherapy (bottom). Bar, 100 μ m; arrow, CD8⁺ cells; HPF, high power field. F, human PD-L1 expression on cancer cells in ascitic fluid from an ovarian cancer patient before and after paclitaxel plus CBDCA chemotherapy at day 4 and day 11 (shaded histogram, isotype; green histogram, anti-PD-L1 antibody).

received docetaxel and capecitabine combination chemotherapy (Supplementary Fig. S5; $P = 0.002$).

To confirm PD-L1 induction by chemotherapeutic agents in cancer cells, we treated ID8 or HM-1 with gemcitabine, paclitaxel, or CBDCA for 0, 9, 24, and 48 hours. PD-L1 mRNA expression was significantly elevated in ID8 following incubation with gemcitabine in as little as 9 hours. There was a 3- to 4-fold induction of PD-L1 mRNAs in ID8 and HM-1 cells after 24 hours incubation with each chemotherapeutic agent (Fig. 3B). Treatment of ID8 or HM-1 cell lines with gemcitabine, paclitaxel, and CBDCA resulted in a 6- to 10-fold induction of cell surface PD-L1 protein expression (Fig. 3C and Supplementary Fig. S6A). Total PD-L1 protein expression in ID8 and HM-1 was induced in a dose-dependent manner when these cells were incubated with gemcitabine, paclitaxel, CBDCA, capecitabine, or IFN γ (Fig. 3D and Supplementary Figs. S2 and S6B–S6D). Similarly, a 1.5- to 5-fold increase in surface PD-L1 protein was

observed in human ovarian cancer cell lines ovary1847, SK-OV-3, RMGII, and OVCAR8 treated with gemcitabine or paclitaxel (Fig. 3E and Supplementary Fig. S7A and S7B). A significant increased total cellular PD-L1 is evident in RMGII and OVCAR8 treated with gemcitabine or paclitaxel (Supplementary Fig. S7C). An escalation of total cellular PD-L1 levels in ovary1847 or SK-OV-3 that was related with the treatment dose was evident (Fig. 3F and Supplementary Figs. S3 and S7D).

To evaluate the effect of chemotherapeutic treatment duration on PD-L1 induction, ID8 cells were treated with gemcitabine, paclitaxel or IFN γ for 24 hours, then incubated with drug-free medium for an additional 1, 2, 5, or 15 days. PD-L1 mRNA was induced after 1 day incubation with drug-free medium for gemcitabine -, paclitaxel -, and IFN γ (as a positive control; ref. 24) pretreated ID8 cells. However, after 2 days, PD-L1 mRNA expression was not detected in IFN γ -pretreated ID8 cells. PD-L1 was observed in paclitaxel or gemcitabine-pretreated ID8 cells until

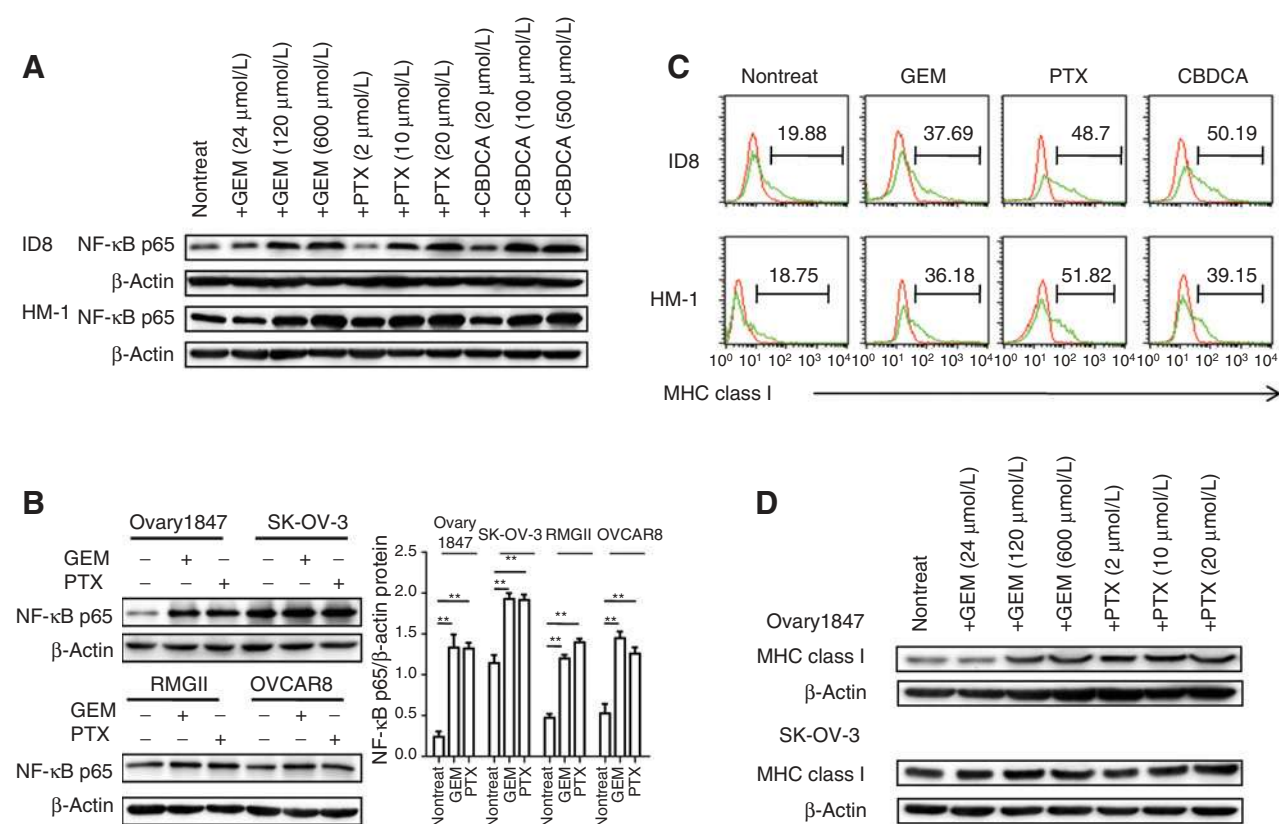


Figure 2. Upregulation of NF-κB p65 and MHC class I in ovarian cancer cell lines following treatment with chemotherapeutic agents. A, NF-κB p65 protein expression in mouse ovarian cancer cell lines (ID8 and HM-1) after gemcitabine (GEM; 24, 120, and 600 μmol/L), paclitaxel (PTX; 2, 10, and 20 μmol/L), or CBDCA (20, 100, and 500 μmol/L) treatment for 24 hours, analyzed by Western blotting. B, NF-κB p65 protein expression in human ovarian cancer cell lines (ovary1847, SK-OV-3, RMGII, and OVCAR8) after gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) treatment for 24 hours, analyzed by Western blotting. Densitometric analysis of NF-κB p65 was based on three independent experiments. **, $P < 0.01$. C, MHC class I expression in mouse ovarian cancer cell lines (ID8 and HM-1) after gemcitabine (120 μmol/L), paclitaxel (10 μmol/L), or CBDCA (100 μmol/L) treatment for 24 hours, analyzed by flow cytometry (red, isotype; green, anti-MHC class I antibody). D, MHC class I expression in human ovarian cancer cell lines (ovary1847, SK-OV-3) after gemcitabine (24, 120, and 600 μmol/L) or paclitaxel (2, 10, and 20 μmol/L) treatment for 24 hours, analyzed by Western blotting.

day 5, but not at day 15 (Fig. 3G). Therefore, the mechanism by which PD-L1 expression is induced by chemotherapeutic agents in ovarian cancer cells might differ from the way it is induced by IFN γ .

Chemotherapeutic agents upregulate PD-L1 and MHC class I via NF-κB signaling

To evaluate the relationship between gene expression of PD-L1 and MHC class I and NF-κB signaling, we first analyzed microarray data from 1,037 human malignant cell lines from the CCLE and found that the NF-κB gene signature score was significantly positively correlated with mRNA expression levels of HLA-A, HLA-B, HLA-C, and PD-L1 (Fig. 4A). To confirm these relationships, we analyzed ID8 or HM-1 cells transduced with Rela shRNA or control shRNA (Supplementary Fig. S8) and found that MHC class I expression in ID8shNF-κB (Rela69 knockdown) cell lines was not increased by administration of gemcitabine or paclitaxel (Fig. 4B and Supplementary Fig. S9). In human ovarian cancer cells (ovary1847, SK-OV-3, and OVCAR8), we found that MHC class I was similarly not elevated by gemcitabine or paclitaxel treatment when NF-κB p65 knockdown was achieved using NF-

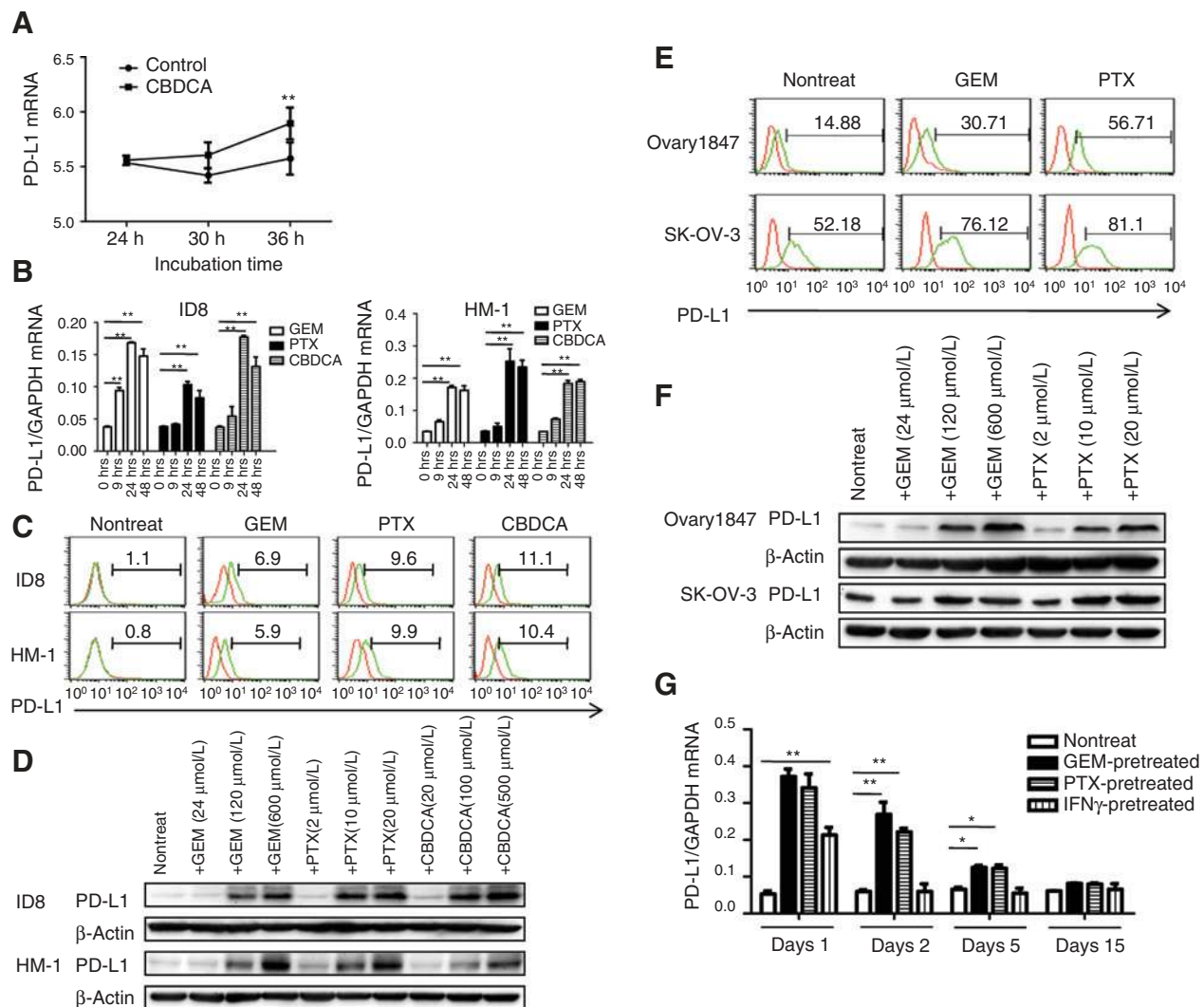
κB p65 siRNA, whereas it was elevated in control siRNA cell lines (Fig. 4C and Supplementary Figs. S10A–S10C and S11).

We then generated ID8 and HM-1 cells transfected with control siRNA or NF-κB p65 siRNA targeting the Rela gene. We found that knockdown of NF-κB p65 in ID8 and HM-1 cell lines impaired PD-L1 induction by gemcitabine or paclitaxel (Fig. 4D and Supplementary Figs. S12A–S12B and S13). As expected, PD-L1 expression in the Rela knockdown cell lines (ID8shRela69 or 70, HM1shRela69 or 70) was not elevated after incubation with gemcitabine or paclitaxel (Fig. 4E and Supplementary Figs. S12C and S14), whereas it was elevated in control shRNA cell lines (ID8 shControl or HM-1shControl).

We confirmed that PD-L1 protein was not elevated by either gemcitabine or paclitaxel treatment in NF-κB p65 knockdown human cell lines, ovary1847, SK-OV-3, and OVCAR8 (Fig. 4F and Supplementary Figs. S10A, S10B, S10D, and S11).

PD-L1 and MHC class I expression is induced by chemotherapeutic agents in the absence of the IFN γ signaling pathway

To determine whether the IFN γ signaling pathway is involved in the upregulation of PD-L1 by chemotherapeutic

**Figure 3.**

Upregulation of PD-L1 in ovarian cancer cell lines following chemotherapeutic treatment *in vitro*. A, PD-L1 mRNA expression in epithelial ovarian cancer cell line 36M2 (from microarray dataset GSE13525) incubated with CBDCA (100 μmol/L) for 24, 30, or 36 hours. **, $P < 0.01$. B, mouse ovarian cancer cell lines (ID8 and HM-1) were treated with gemcitabine (GEM; 120 μmol/L), paclitaxel (PTX; 10 μmol/L), or CBDCA (100 μmol/L) for 0, 9, 24, or 48 hours; PD-L1 mRNA was measured by qRT-PCR. Results are the average of triplicate determinations. **, $P < 0.01$. C, ID8 and HM-1 cells were incubated with gemcitabine (120 μmol/L), paclitaxel (10 μmol/L), or CBDCA (100 μmol/L) for 24 hours. Cell surface PD-L1 was detected by flow cytometry (red, isotype; green, anti-PD-L1 antibody). D, ID8 and HM-1 cells were incubated with gemcitabine (24, 120, and 600 μmol/L), paclitaxel (2, 10, and 20 μmol/L), or CBDCA (20, 100, and 500 μmol/L) for 24 hours. Total cellular PD-L1 protein was measured by Western blotting. E, human ovarian cancer cell lines (ovary1847, SK-OV-3) were cultured with gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) for 24 hours. Cell surface PD-L1 was detected by flow cytometry (red, isotype; green, anti-PD-L1 antibody). F, human ovarian cancer cell lines (SK-OV-3, ovary1847) were cultured with gemcitabine (24, 120, and 600 μmol/L) or paclitaxel (2, 10, and 20 μmol/L) for 24 hours. Total cellular PD-L1 was measured by Western blotting. G, The ID8 cell line was incubated with gemcitabine (120 μmol/L), paclitaxel (10 μmol/L), or IFNγ (20 ng/mL) for 24 hours, then washed with PBS and incubated with drug-free medium for 1, 2, 5, and 15 days. PD-L1 mRNA expression was analyzed by RT-PCR from triplicate experiments. *, $P < 0.05$; **, $P < 0.01$.

agents, ID8 cells transfected with control shRNA or IFNγ receptor 1 (IFNGR1) shRNA (ID8shControl or ID8shIfgr1) were treated with gemcitabine or paclitaxel (Supplementary Fig. S15). We found that PD-L1 and NF-κB p65 were upregulated by chemotherapeutic agent treatment, even in the IFNGR1 knockdown cell line (ID8shIfgr1; Fig. 5A and B and Supplementary Fig. S16). IFNγ significantly induced PD-L1, but this effect was partially abrogated in the IFNGR1 knockdown cell line (ID8shIfgr1; Fig. 5A and B and Supplementary Fig. S16). MHC class I expression in ID8shIfgr1 was also elevated after

chemotherapeutic agent treatment (Fig. 5C and D). Therefore, we clearly demonstrated that the induction of both PD-L1 and MHC class I following treatment with chemotherapeutic agents is affected by the NF-κB signaling pathway, even in the absence of the IFNγ signaling pathway.

Chemotherapy induces PD-L1 overexpression, NF-κB signal activation, and elevates TIL numbers

We next examined whether PD-L1/NF-κB signaling and local immunologic status was altered *in vivo* following

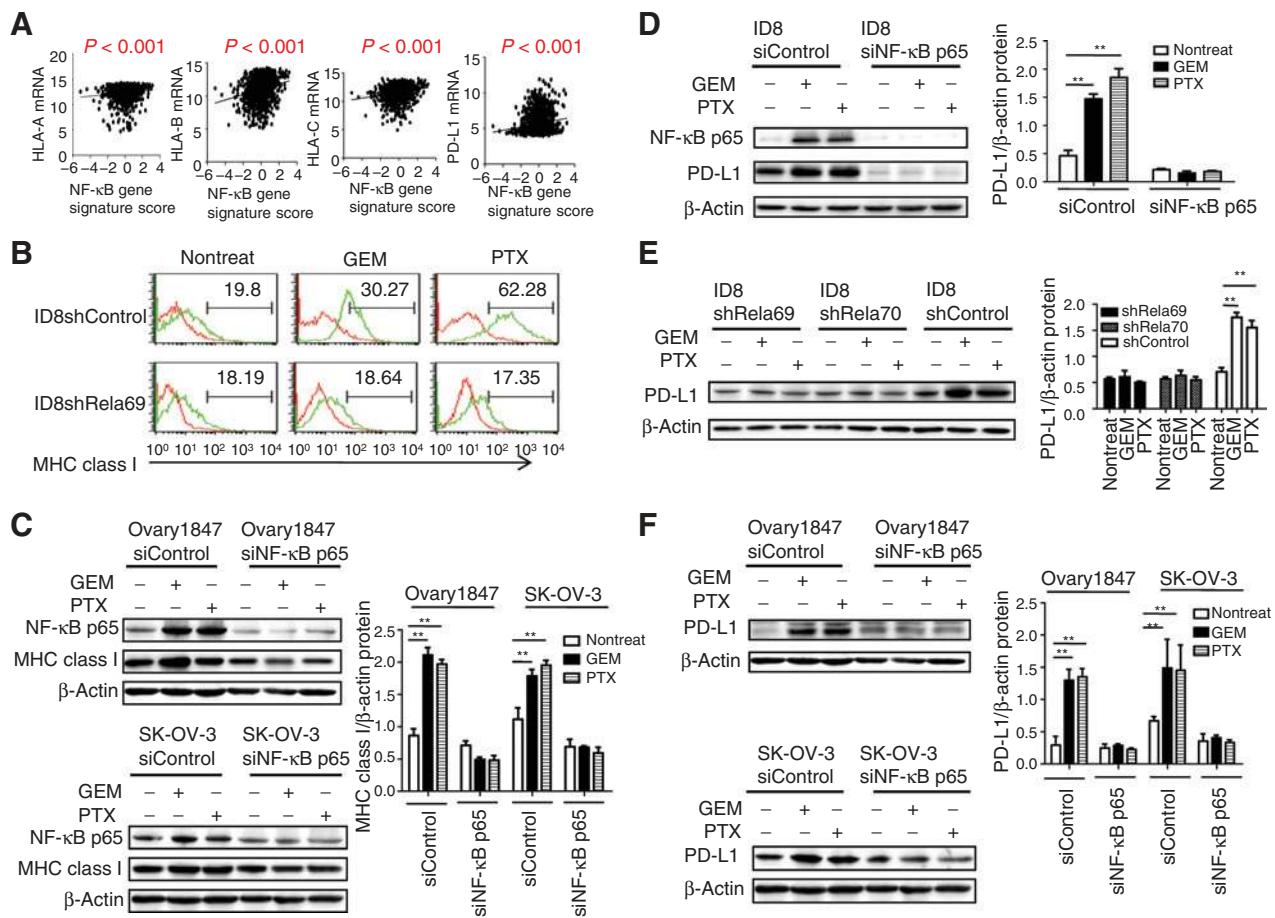


Figure 4. Chemotherapeutic agents upregulate MHC class I and PD-L1 via NF-κB in ovarian cancer cell lines *in vitro*. A, positive correlations are shown between the NF-κB gene signature score and HLA-A, HLA-B, HLA-C, or PD-L1 mRNA expression in 1,037 human malignant cell lines from the CCLE. B, mouse MHC class I expression in ID8 cells transfected with shNF-κB p65 (Rela69) or shControl after culturing with gemcitabine (GEM; 120 μmol/L) or paclitaxel (PTX; 10 μmol/L) for 24 hours, analyzed by flow cytometry (red, isotype; green, anti-MHC class I antibody). C, NF-κB p65 and MHC class I in human ovarian cancer cell lines ovary1847 or SK-OV-3 transfected with siNF-κB p65 or siControl after culturing with gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) for 24 hours, analyzed by Western blotting. Densitometric analysis of MHC class I was based on three independent experiments. **, $P < 0.01$. D, mouse NF-κB p65 and PD-L1 expression in ID8 cells transfected with siNF-κB p65 or siControl after culturing with gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) for 24 hours, analyzed by Western blotting. Densitometric analysis of PD-L1 was based on three independent experiments. **, $P < 0.01$. E, mouse PD-L1 expression in ID8 cells transfected with shNF-κB p65 (Rela69, 70) or shControl after culturing with gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) for 24 hours, analyzed by Western blotting. Densitometric analysis of PD-L1 was based on three independent experiments. **, $P < 0.01$. F, human PD-L1 expression in ovary1847 or SK-OV-3 cell lines transfected with siNF-κB p65 or siControl after culturing with gemcitabine (120 μmol/L) or paclitaxel (10 μmol/L) for 24 hours, analyzed by Western blotting. Densitometric analysis of PD-L1 was based on three independent experiments. **, $P < 0.01$.

paclitaxel or gemcitabine therapy. Mice (C57BL/6) were injected intraperitoneally with 5×10^6 ID8 ovarian cancer cells to establish a peritoneal disseminated tumor model. Paclitaxel was administered to a subset of mice by intraperitoneal injection. Both PD-L1 and NF-κB p65 were overexpressed in these tumor cells (Fig. 6A; Supplementary Table S3). Paclitaxel treatment significantly increased the number of CD8⁺ and CD4⁺ T cells in the ID8 peritoneal tumors (Fig. 6A; Supplementary Table S4). Administration of gemcitabine also increased PD-L1 and NF-κB p65 expression, as well as CD8⁺ and CD4⁺ TIL numbers in ID8 tumor-bearing mice (Supplementary Fig. S17; Supplementary Tables S3 and S4). These data clearly demonstrate that both paclitaxel and gemcitabine induce an immunosuppressive state *in vivo*, while they simul-

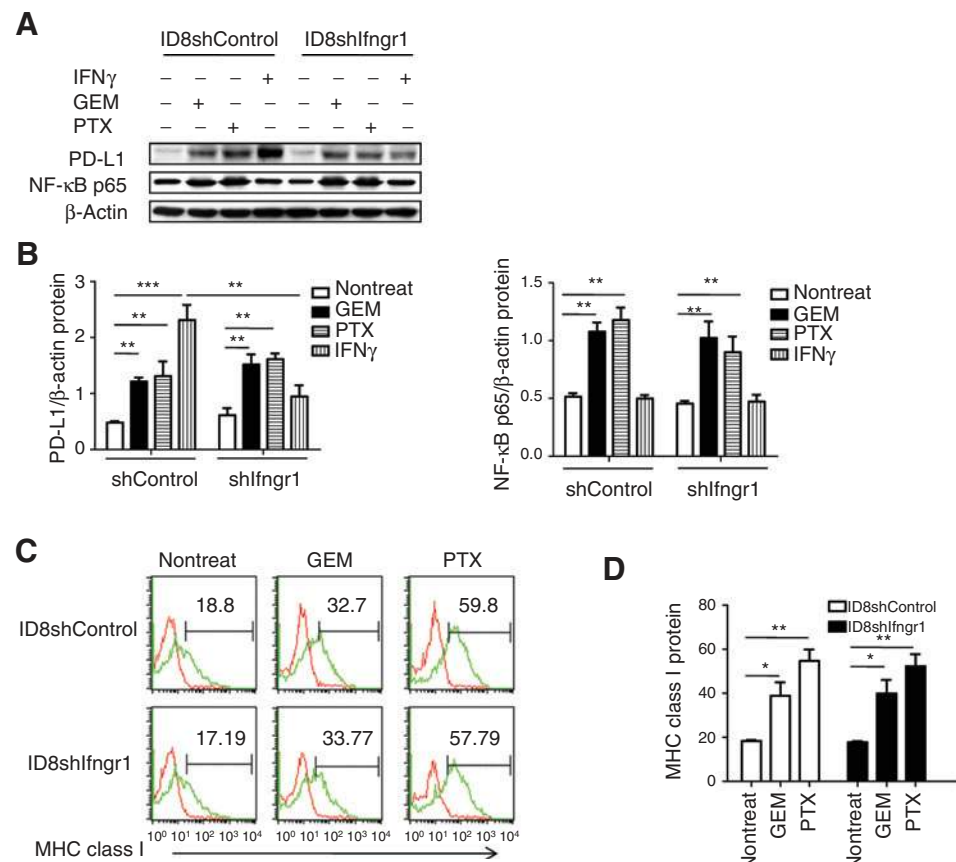
aneously induce immunoreactivation through accumulation of CD8⁺ T cells in the tumor site.

Paclitaxel combined with PD-L1 depletion leads to immune activation

We next assessed alterations in CD8⁺ TILs (CTL) following their encounter with paclitaxel-induced PD-L1 using cytotoxicity assay and CD107a degranulation assay. Following coculture with CD8⁺ T cells, paclitaxel-pretreated ID8-control cells show a lower lysis percentage than nonpretreated ID8-control cells, indicating that T-cell activation may be inhibited by paclitaxel (Fig. 6B; $P = 0.0001$). Next, paclitaxel-pretreated ID8-pd1KO, cocultured with CD8⁺ T cells show the highest lysis percentage, indicating that T cells are activated by paclitaxel following antigen

Figure 5.

PD-L1 or MHC class I expression is upregulated by cancer chemotherapeutic drugs in the absence of the IFN γ signaling pathway. A, ID8 cells were transfected with control shRNA or IFNGR1 shRNA, then cultured with gemcitabine (GEM; 120 μ mol/L), paclitaxel (PTX; 10 μ mol/L), or IFN γ (1 ng/mL) for 24 hours. Total cellular PD-L1 and NF- κ B p65 were measured by Western blotting. B, densitometric analysis of PD-L1 and NF- κ B p65 from Western blotting was based on three independent experiments. **, $P < 0.01$; ***, $P < 0.001$. C, ID8 cells were transfected with control shRNA or IFNGR1 shRNA, then cultured with gemcitabine (120 μ mol/L) or paclitaxel (10 μ mol/L) for 24 hours. Cell surface MHC class I was detected by flow cytometry (red, isotype; green, anti-MHC class I antibody). D, statistical analysis of MHC class I by flow cytometry was based on three independent experiments. *, $P < 0.05$; **, $P < 0.01$.



stimulation when PD-L1 is knocked down (Fig. 6B; $P = 0.0001$). Similarly, there were fewer CD107a⁺ CD8⁺ T cells following coculture with paclitaxel-pretreated ID8-control than with nontreated ID8-control, suggesting that T-cell activation may be inhibited by paclitaxel (Supplementary Fig. S17B; $P = 0.011$). Next, the number of CD107a⁺ CD8⁺ T cells significantly increased when they were cocultured with paclitaxel-pretreated ID8-pdl1KO instead of paclitaxel-pretreated ID8-control (Supplementary Fig. S17B; $P = 0.004$). This suggests that depletion of PD-L1 in ovarian cancer cells induces activation of CD8⁺ T cells. Finally, the number of CD107a⁺ CD8⁺ T cells following coculture with paclitaxel-pretreated ID8-pdl1KO decreased when they cocultured with nontreated ID8-control, indicating that PD-L1 knockout facilitate T cells activated by paclitaxel (Supplementary Fig. S17B; $P = 0.046$). Collectively, paclitaxel-induced overexpression of PD-L1 in tumor cells could be a mechanism of immune evasion from CD8⁺ T cells in ovarian cancer.

Paclitaxel combined with PD-L1 depletion prolongs mouse survival

In addition, we evaluated whether blocking PD-L1 in tumor cells reversed the decline in T-cell immunity and promoted tumor rejection after chemotherapy in a mouse ovarian cancer model. We examined survival in syngeneic mice with or without intraperitoneal injection of paclitaxel following establishment of intraperitoneal tumors using ID8-control, ID8-pdl1KO, or ID8-pdl1. ID8-pdl1 tumor-bearing mice without paclitaxel treatment had the worst prognosis, whereas pacli-

taxel administration significantly improved prognosis of the other groups (Fig. 6C; $P < 0.05$). ID8-control tumor-bearing mice treated with paclitaxel and ID8-pdl1KO tumor-bearing mice who did not receive paclitaxel had moderate survival, whereas ID8-pdl1KO tumor-bearing mice receiving paclitaxel had the best survival among the six groups (Fig. 6C; $P < 0.05$). *In vitro* data showed that expression of both PD-L1 and NF- κ B p65 was upregulated in ID8-control or ID8-pdl1 incubated with paclitaxel or gemcitabine. In contrast, PD-L1 mRNA and protein were not upregulated in the pdl1-depleted cell line after incubation with paclitaxel or gemcitabine, even though NF- κ B p65 was elevated (Supplementary Fig. S18). Thus, PD-L1 depletion effectively eliminates PD-L1 overexpression induced by paclitaxel. However, in contrast to paclitaxel, gemcitabine treatment combined with PD-L1 depletion did not improve prognosis in the mouse model as compared with gemcitabine treatment alone (Supplementary Fig. S19A; $P = 0.087$).

Combination therapy with paclitaxel and anti-PD-1 antibody enhances tumor regression via immune activation

To examine the effects of antigen-specific cytotoxicity by CD8⁺ T cells, we injected ID8OVA cells (Supplementary Fig. S20) intraperitoneally into syngeneic mice following treatment with paclitaxel alone, anti-PD-1 antibody (α PD-1) alone, the combination of paclitaxel and α PD-1, or saline and IgG intraperitoneal injection (control). Combined treatment of paclitaxel with α PD-1 resulted in the best survival among the four groups (Fig. 6D; $P < 0.0001$). ID8 tumor-bearing mice that received the

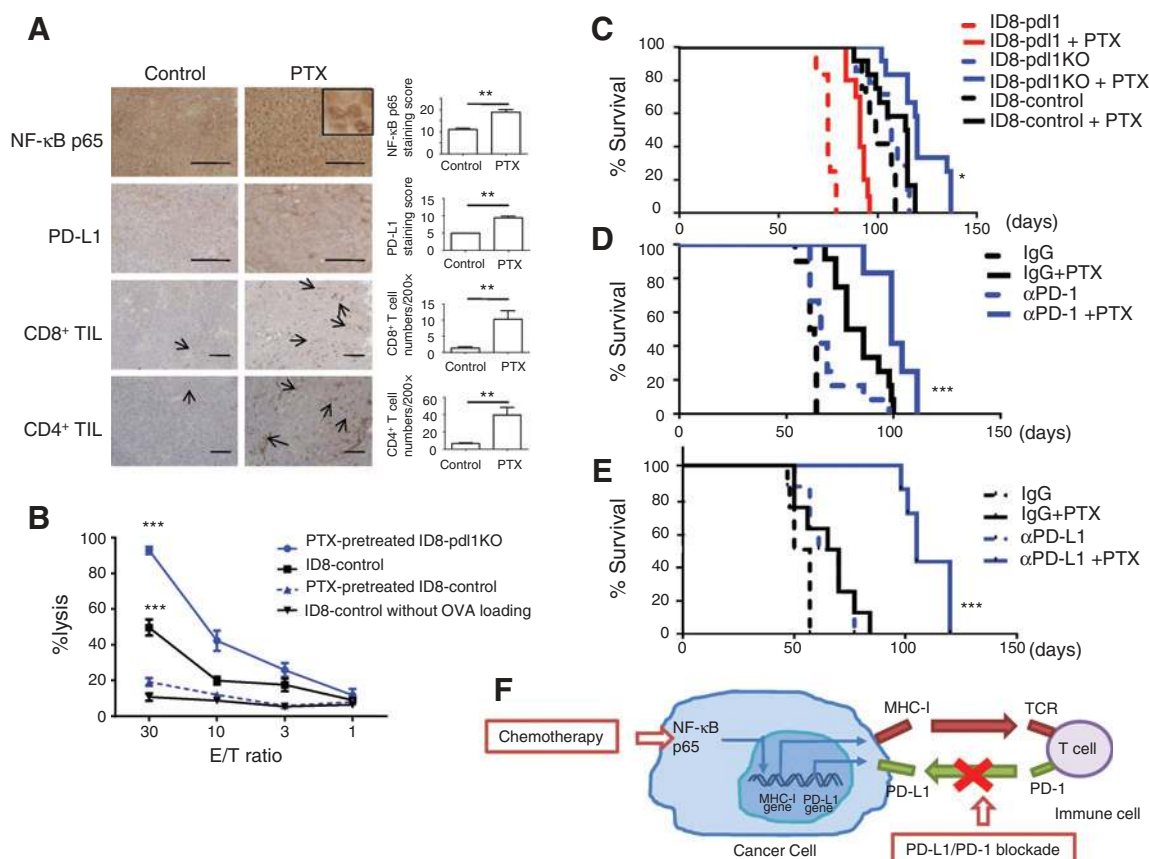


Figure 6. Combination therapy with paclitaxel (PTX) and PD-1/PD-L1 signal blockade enhances tumor regression via immune activation. A, frozen-section mouse tumor tissue analyzed by immunohistochemistry using anti-NF-κB p65 or anti-PD-L1 antibodies (original magnification, $\times 400$; scale bar, 100 $\mu\text{mol/L}$; left); anti-CD8 or anti-CD4 antibodies (original magnification, $\times 200$; scale bar, 100 $\mu\text{mol/L}$) from the ID8 tumor-bearing mice treated with vehicle (control) or paclitaxel. The staining score for NF-κB p65, PD-L1 and the number of CD8⁺ TILs, CD4⁺ TILs from the ID8 tumor-bearing mice treated with vehicle (control) or paclitaxel ($n = 5$ in each group; right). **, $P < 0.01$. B, cytotoxicity assays of the ovalbumin peptide (OVA)-manipulated cells. Ovalbumin-loaded paclitaxel-pretreated ID8-pd1KO (24 hours; 10 $\mu\text{mol/L}$), ovalbumin-loaded paclitaxel-pretreated ID8-control (24 hours; 10 $\mu\text{mol/L}$), ovalbumin-loaded ID8-control or non-ovalbumin-loaded ID8-control (negative control). Means \pm SD ($n = 6$). ***, $P < 0.001$. C, the Kaplan-Meier curves and log-rank test of overall survival analysis of ID8-pd1, ID8-control, or ID8-pd1KO tumor-bearing syngenic mice treated with or without paclitaxel (each group, $n = 12$). *, $P < 0.05$. D, the Kaplan-Meier curves and log-rank test of overall survival of ID8OVA tumor-bearing syngenic mice treated with paclitaxel alone, α PD-1 alone, the combination of paclitaxel and α PD-1, or saline and control IgG intraperitoneal injection (each group, $n = 12$). ***, $P < 0.0001$. E, the Kaplan-Meier curves and log-rank test of overall survival analysis of ID8OVA tumor-bearing syngenic mice treated with paclitaxel alone, anti-mouse PD-1 monoclonal antibody (α PD-L1) alone, the combination of paclitaxel and α PD-L1, or saline and IgG intraperitoneal injection (control; each group, $n = 8$). ***, $P < 0.0001$. F, schematic of immunologic changes in ovarian cancer cells following chemotherapy. Cancer chemotherapy upregulates PD-L1 and MHC class I via a NF-κB p65-dependent mechanism. Chemotherapy combined with a blockade of the PD-L1/PD-1 signal may be a promising approach to immune activation in ovarian cancer treatment.

combined treatment of paclitaxel with α PD-1 showed significantly better survival than those receiving monotherapy with paclitaxel ($P = 0.004$) or α PD-1 ($P < 0.0001$). In place of anti-PD-1 antibody, we also treated mice with α PD-L1 and found that the combined treatment of paclitaxel with α PD-L1 also resulted in the best survival (Fig. 6E; $P < 0.0001$). ID8 tumor-bearing mice that received the combined treatment of paclitaxel with α PD-L1 also showed significantly better survival than those receiving monotherapy with paclitaxel or α PD-L1 ($P < 0.0001$, each).

Consistent with our previous *in vivo* experiment (Fig. 6A), paclitaxel treatment again increased CD8⁺ and CD4⁺ TIL cell percentage at the tumor site in this *in vivo* experiment (Supplementary Fig. S21A and S21B). Moreover, combined therapy enhanced paclitaxel-induced immune activation not only by

increasing the number of CD8⁺ and CD4⁺ TILs, but also by decreasing the PD-1⁺ TIL percentage (Supplementary Fig. S21). This suggests that combined therapy with anti-PD-1 or anti-PD-L1 antibody and paclitaxel might induce tumor regression by attenuating the immunosuppressive PD-1/PD-L1 signal to achieve maximal immune activation. Therefore, we propose a model for immunologic changes in ovarian cancer cells with chemotherapy, upregulating PD-L1 and MHC class I via a mechanism dependent on NF-κB p65 (Fig. 6F). However, similar to the results from our *in vivo* study (Supplementary Fig. S19A), gemcitabine plus α PD-1 does not improve survival as compared with gemcitabine treatment alone (Supplementary Fig. S19B). Moreover, gemcitabine but not paclitaxel decreases the number of MDSCs in tumor site (Supplementary Fig. S22; Supplementary Table S4).

Discussion

In this study, chemotherapeutic agents commonly used for ovarian cancer enhanced both immunogenicity of tumor cells and immunosuppressive changes at the tumor site. First, we found that paclitaxel and CBDCA led to overexpression of MHC class I on tumor cells and increased CD8⁺ T-cell infiltration at the tumor site in the clinical setting (Fig. 1). These data were confirmed in a mouse intraperitoneal ovarian cancer model when tumor-bearing mice were treated with paclitaxel or gemcitabine (Fig. 6; Supplementary Fig. S21). These findings are consistent with previous reports (34–36). Second, we demonstrated that the immunosuppressive cosignaling molecule PD-L1 was overexpressed on human and mouse ovarian cancer cells following treatment with several chemotherapeutic agents in a dose-dependent manner (Figs 1 and 3) and in a mouse ovarian cancer model (Fig. 6 and Supplementary Fig. S19). These findings suggest a complicated immunologic status at the tumor site following chemotherapy and are consistent with previous studies (34–36). Third, a cytotoxicity assay and CD107a CTL assays revealed that paclitaxel inhibits the function of CD8⁺ T cells despite leading to increased numbers of CD8⁺ TILs in the *in vivo* mouse model (Fig. 6A and B and Supplementary Fig. S17B) and in the clinical setting (Fig. 1E), whereas paclitaxel activates CD8⁺ T-cell function when PD-L1 is depleted. Finally, we determined that these contradictory phenomena, including immunoreactivation and immunosuppression in ovarian cancer after chemotherapy, were connected through NF- κ B signaling induced by chemotherapy.

NF- κ B functions in cancer cells as a transcription factor and is related to tumor progression via its influence on prevention of apoptosis and enhancement of cell proliferation (37, 38). In prior reports, NF- κ B signal activation led to chemotherapeutic resistance in gastric, pancreatic, and lung cancer cells via the AKT-I κ B pathway (39–42). Consistent with these reports, we confirmed that several chemotherapeutic drugs also induce NF- κ B signaling in ovarian cancer cells in a dose-dependent manner *in vitro* and *in vivo* (Fig. 2A and B). As a relationship between NF- κ B signaling and MHC class I expression was demonstrated in neuroblastoma (43), we also found that NF- κ B signaling induced overexpression of MHC class I on ovarian cancer cells in the absence of IFN γ signaling caused by chemotherapy (Fig. 5). We are the first to report that chemotherapy upregulates MHC class I via NF- κ B signaling as well as the molecular mechanism.

In addition, we demonstrated that NF- κ B signaling induces overexpression of PD-L1 in ovarian cancer cells and this lead to immunosuppressive changes at the tumor site following chemotherapy (Fig. 4). Two prior studies identified a relationship between NF- κ B and PD-L1. NF- κ B signaling was shown to induce PD-L1 expression through binding of a PD-L1 promoter motif during the active phase of monocytes (44, 45). Other studies showed that PD-L1 induction resulted from PTEN loss or MEK activation in glioma or myeloma (46, 47), and from IFN γ in melanoma and plasma cell of multiple myeloma (47, 48). We previously showed that PD-L1 overexpression is induced by IFN γ produced by activated T cells in tumor ascites and that this promotes peritoneal dissemination in ovarian cancer (24). Therefore, to determine the function of the NF- κ B or IFN γ signal during chemotherapy, we generated the NF- κ B p65 or IFN γ receptor knockout cells in this study, which revealed that chemotherapy for

ovarian cancer leads to overexpression of PD-L1 in tumor cells in the absence of IFN γ signaling (Fig. 5). This is the first report to show that chemotherapeutic agents upregulate PD-L1 via NF- κ B signaling.

We selected paclitaxel and gemcitabine for our *in vivo* experiments because they are commonly used to treat ovarian cancer as a part of first-line (paclitaxel) and second-line (gemcitabine) conventional therapies. They have also been reported as immunogenic drugs that promote antigen presentation, increase CD8⁺ T-cell infiltration into the tumor and inhibit immunosuppressive T cells (32–34). Thus, on the basis of several prior reports, paclitaxel and gemcitabine were anticipated to be good candidates for combining with immunotherapy (49, 34). In fact, we found that these chemotherapeutic agents induced overexpression of PD-L1 and were the first to show that paclitaxel combined with PD-1/PD-L1 blockade treatment enhances antitumor effects relative to monotherapy in both the *in vitro* and *in vivo* systems (Fig. 6). And CBDCA, another part of first-line therapy with paclitaxel, also enhanced expression of PD-L1 on ovarian cancer cells so that anti-PD-1 antibody may be a good enhancer of conventional first line chemotherapy for ovarian cancer. Since 2011, we have opened a medical investigator-initiated phase II clinical trial with fully humanized anti-PD-1 antibody (Nivolumab) for platinum-resistant ovarian cancers (UMIN Clinical Trials Registry: UMIN000005714) and we have demonstrated safety and shown efficacy of the anti-PD-1 antibody in patients (50). From our experience, combined chemoimmune therapy with paclitaxel and anti-PD-1 antibody may offer promise as a new treatment strategy for ovarian cancers (Fig. 6E).

On the other hand, gemcitabine did not augment the antitumor effect of anti-PD-1 antibody treatment *in vivo* (Supplementary Fig. S19). Both paclitaxel and gemcitabine together induced CD8⁺ T-cell infiltration at the tumor site and upregulated PD-L1 expression *in vitro*. The reason for the difference in antitumor response between paclitaxel and gemcitabine seems partially due to the different immunogenic changes caused by each agent. Gemcitabine is known to decrease immunosuppressive cells such as MDSCs (34). Consistent with this report, we confirmed that gemcitabine decreased the number of MDSCs at the tumor site in the mouse ovarian cancer model, while paclitaxel did not (Supplementary Fig. S22). However, other unidentified mechanisms may also contribute to the lack of additional antitumor effects with combined gemcitabine and PD-1 signal blocking.

In addition, we were the first to reveal that PD-L1 expression induced via NF- κ B or IFN γ pathway signaling is not persistent in tumor cells but rather is transient. PD-L1 induction lasted less than 2 days via the IFN γ pathway, while it was extended to more than 5 days via NF- κ B treatment was stopped *in vitro* (Fig. 3G). *In vivo*, PD-L1⁺ cancer cells were detected and increased in ascites from an advanced ovarian cancer patient treated with paclitaxel and carboplatin chemotherapy after 4 days, but were decreased after 11 days. Therefore, the maximum antitumor effect of combined therapy with paclitaxel and the anti-PD-1 or anti-PD-L1 antibody may be dependent on specific timing of treatments.

In conclusion, we found that chemotherapy induced several immunologic changes in ovarian cancers, including MHC class I and PD-L1 upregulation via NF- κ B signaling. Thus, chemoimmunotherapy with paclitaxel and anti-PD-1 or anti-PD-L1 antibody may offer a novel and effective approach for future treatment strategies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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