

PD-L1 on Tumor Cells Is Induced in Ascites and Promotes Peritoneal Dissemination of Ovarian Cancer through CTL Dysfunction

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Abstract

Purpose: Ovarian cancer often progresses by disseminating to the peritoneal cavity, but how the tumor cells evade host immunity during this process is poorly understood. Programmed cell death 1 ligand 1 (PD-L1) is known to suppress immune system and to be expressed in cancer cells. The purpose of this study is to elucidate the function of PD-L1 in peritoneal dissemination.

Experimental Design: Ovarian cancer cases were studied by microarray and immunohistochemistry. PD-L1 expression in mouse ovarian cancer cell line in various conditions was assessed by flow cytometry. PD-L1-overexpression cell line and PD-L1-depleted cell line were generated, and cytotoxicity by CTLs was analyzed, and alterations in CTLs were studied by means of timelapse and microarray. These cell lines were injected intraperitoneally to syngeneic immunocompetent mice.

Results: Microarray and immunohistochemistry in human ovarian cancer revealed significant correlation between PD-L1 expression and peritoneal positive cytology. PD-L1 expression in mouse ovarian cancer cells was induced upon encountering lymphocytes in the course of peritoneal spread *in vivo* and coculture with lymphocytes *in vitro*. Tumor cell lysis by CTLs was attenuated when PD-L1 was overexpressed and promoted when it was silenced. PD-L1 overexpression inhibited gathering and degranulation of CTLs. Gene expression profile of CTLs caused by PD-L1-overexpressing ovarian cancer was associated with CTLs exhaustion. In mouse models, PD-L1 depletion resulted in inhibited tumor growth in the peritoneal cavity and prolonged survival.

Conclusion: PD-L1 expression in tumor cell promotes peritoneal dissemination by repressing CTL function. PD-L1-targeted therapy is a promising strategy for preventing and treating peritoneal dissemination. *Clin Cancer Res*; 19(6); 1363–74. ©2012 AACR.

Introduction

Ovarian cancer is the most lethal disease among gynecologic malignancies. Unlike other epithelial tumors, peritoneal dissemination is the most common mechanism of disease progression in ovarian cancer, and up to 70% of cases present with massive malignant ascites and peritoneal implants (1). Control of dissemination seems to be the most important strategy in controlling ovarian cancer because the median overall survival and progression-free

survival are 81.1 and 35.0 months, respectively, if macroscopically complete surgical resection of the disseminated tumors is achieved in Federation Internationale des Gynaecologues et Obstetristes (FIGO) stage IIIC cancers, whereas these measures are only 34.2 and 14.5 months, respectively, if the disseminated tumor remains after the initial surgery (2). The peritoneal cavity is also the most frequent site of recurrence, and most patients who undergo intraperitoneal recurrence die from this disease (3).

At least 3 steps, cell detachment, immune evasion, and implantation, are required for dissemination. Various molecules expressed by cancer cells have been reported to be involved in these steps (4). In cell detachment, molecules that cause epithelial-to-mesenchymal transition, such as TGF- β or Snail, have an important role (5–7). In implantation, extracellular matrix proteins and VEGF are thought to be important (8). In addition, cancer cells potentially must escape from attack by the immune cells that they encounter in the peritoneal cavity. Immune evasion during peritoneal dissemination is the most enigmatic step. Lymphocytes isolated from malignant ascites have shown

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Translational Relevance

Immune evasion is one of the emerging hallmarks of cancer, though most of its mechanisms remain unveiled. Ovarian cancer often progresses by disseminating to the peritoneum, but how the tumor cells evade host immunity during this process is poorly understood. In this study, we have shown that ovarian cancer cells express programmed cell death 1 ligand 1 (PD-L1) upon encountering lymphocytes in the peritoneal cavity, and as a consequence, inhibit CTL function, escape from CTLs, and disseminate into the peritoneal cavity. Depleting PD-L1 expression in tumor cells resulted in inhibited tumor growth in the peritoneal cavity and prolonged survival of the mice. These data show for the first time that host-tumor immunity, especially tumor immune escape mechanisms, has a pivotal role in peritoneal dissemination. Our data suggest that restoring immune function by inhibiting immune-suppressive factors such as PD-L1 is a promising strategy for controlling the peritoneal dissemination of malignant tumors, including ovarian cancer.

tumoricidal activity (9), but the mechanisms by which the tumor cells evade these cells are not clearly understood. Secretion of Fas ligands by ovarian cancer cells (10), the recruitment of regulatory T cells (11), and the T-cell suppressor cytokine phenotype of monocytes and macrophages (12) have been reported to be included in this step, but the precise mechanism of tumor evasion from immune cells remains unclear.

Recent studies have added immune evasion as one of the important hallmarks of cancer (13). Restoring immune function in cancer microenvironment has immense potential for a new cancer therapy (14). We have attempted to elucidate the mechanism of immune escape in ovarian cancer and reported that in the ovarian cancer microenvironment, molecules such as ULBP2 (NKG2D ligand), COX-1, COX-2, and programmed cell death 1 ligand 1 (PD-L1) or the combined expression of these molecules are related to limited infiltration by lymphocytes and an unfavorable prognosis (15–18). PD-L1 (also known as B7-H1 or CD274) is a coregulatory molecule that is expressed on the surface of various types of cells, including immune cells and epithelial cells. By binding to its receptor PD-1 on lymphocytes, it generates an inhibitory signal toward the T-cell receptor (TCR)-mediated activation of lymphocytes (19, 20). We have reported that PD-L1 expression in tumor cells is an independent unfavorable prognostic factor in human ovarian cancer (15), and that PD-L1 expression showed the closest relation to unfavorable prognosis among other immunosuppressive molecules that we have tested (18). These data suggest that PD-L1 has a role in the clinical course of ovarian cancer by affecting the local immune microenvironment and that PD-L1/PD-1 signal could be a potential therapeutic target. Actually, a recent clinical trial

of systemic administration of anti-PD-1 or anti-PD-L1 antibody showed a promising clinical effect in several solid tumors (21–23). However, the role of PD-L1 or the precise mechanism of immune escape in the process of peritoneal dissemination is poorly understood.

The aim of this study was to investigate the mechanism by which PD-L1 on cancer cells in ascites enables immune evasion during peritoneal dissemination, by using both clinical samples and mouse models.

Materials and Methods

Survival analysis of ovarian cancer patients

A total of 65 patients with epithelial ovarian cancer (KOV-IH-65), who underwent primary operation at Kyoto University Hospital (Kyoto, Japan) between 1997 and 2002 and the outcome and peritoneal cytology was evaluable from the chart was included in the study under the approval of the Kyoto University Graduate School and Faculty of Medicine Ethics Committee. Ascites or the peritoneal wash fluid was collected at operation and served for pathologic diagnosis. Patient characteristics are listed in Supplementary Table S1.

Microarray profiling of ovarian cancer tissues

Ovarian cancer specimens were obtained from 64 patients (KOV-MA-64), who underwent primary surgery for epithelial ovarian cancer at Kyoto University Hospital between 1997 and 2011. Ten patients in KOV-IH-65 were included in KOV-MA-64. All tissue specimens were collected under written consent approved by the Facility Ethical Committee. Patient characteristics are listed in Supplementary Table S1. Samples were selected to have more than 70% tumor cell nuclei and less than 20% necrosis. Total RNA expression was analyzed on Human Genome U133 Plus 2.0 Array (Affymetrix). Robust multiarray average (RMA) normalization was conducted using R (R: a language and environment for statistical computing; <http://www.R-project.org>). Probes showing expression value more than 5.0 in at least one of the samples and SD more than 0.2 across all the samples were selected, and *t* test was conducted between cytology-positive and -negative groups. Enrichment for Gene Ontology terms was analyzed using GOEAST software (<http://omicslab.genetics.ac.cn/GOEAST/>; ref. 24) for the set of probes highly expressed in cytology-positive or -negative groups, respectively ($P < 0.05$). A publicly accessible gene set of IFN- γ -upregulated genes was downloaded (http://www.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=SANA_RESPONSE_TO_IFNG_UP; ref. 25). Gene set enrichment analysis (GSEA) for positive ascites cytology and negative cytology was conducted using GSEA software (<http://www.broadinstitute.org/gsea/downloads.jsp>).

Immunohistochemistry

Formalin-fixed, paraffin-embedded specimens of ovarian cancer were obtained from KOV-IH-65 patients under written consent as earlier. Immunohistochemical staining for PD-L1 was conducted using a PD-L1 antibody as previously described (15, 18). PD-L1 expression was analyzed by 2

independent gynecologic pathologists without any prior information about the clinical history of the patients, and the samples were categorized into a positive expression group (equal to or stronger than the positive control) and a negative expression group (weaker than the positive control) based on the intensity of the staining. Placenta was used as positive control. Samples with staining in less than 50% of tumor cells was considered negative.

Animals

Female C57BL/6 (B6) and B6C3F1 and C.B-17/lcr-scid/scidJcl [severe combined immunodeficient mice (SCID)] mice were purchased from CLEA Japan. 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, and animals were maintained under specific pathogen-free conditions. To evaluate the effect of PD-L1 on the survival and progression of peritoneal dissemination and ascites formation, HM-1 cells (1×10^6) or ID8 cells (5×10^6) were injected into the abdominal cavity. The body weight gain was calculated every other day. Mice were euthanized before reaching the moribund state.

Cell lines

The ID8 mouse ovarian cancer cell line (26, 27) was kindly provided by Dr. Margit Maria Janát-Amsbury (Department of Obstetrics and Gynecology, Division of Gynecologic Oncology, Baylor College of Medicine, Houston, TX; ref 27). The cells were maintained in RPMI-1640 medium (Nacalai Tesque) supplemented with 10% FBS (v/v; Biowest) and penicillin–streptomycin (Nacalai Tesque). The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from RIKEN BioResource Center and cultured as previously described (7). Human ovarian cancer cell lines were cultured as described previously (28). The ID8-GFP cells and HM1-GFP cells were generated by retroviral transfection as described previously (29).

The PD-L1–overexpressing cell lines, ID8-pdl1 and HM1-pdl1, were generated by lentiviral transfection of ViraPower pLenti6/V5-DEST Gateway Vector (Invitrogen) carrying mouse PD-L1 cDNA. Full-sequenced cDNA was purchased from OpenBiosystems and amplified by PCR using the following primers:

Forward; CACCAACATGAGGATATTTGCTGG
Reverse; TCAACACTGCTTACGTCTCC

Expression vector was generated using pENTR Directional TOPO Cloning Kit (Invitrogen).

The PD-L1–depleted cell lines, ID8-Mirpd11 and HM1-Mirpd11, were generated using the BLOCK-iT HiPerform Lentiviral Pol II miR RNAi Expression System with EmGFP (Invitrogen) according to the protocol provided by the manufacturer. Briefly, double-stranded oligos were generated from designed single-stranded DNA oligos listed later, and cloned into pcDNATM6.2-GW/EmGP-miR expression vector. Then, it was linearized and BP/LR Reaction was

conducted using pDONRTM221 vector and pLenti6.4/R4R2/V5-DEST and pENTRTM5' promoter clone to generate Lentiviral expression clone. The sequence of the miR DNA oligos used for PD-L1 depletion is as follows:

Top strand oligo;

TGCTGTTCACGCCACATTTCTCCACGTTTGGCCACT-GACTGACGTGGAGAAGTGGCGTTGAA

Bottom strand oligo;

CCTGTTCACGCCACTTCTCCACGTCAGTCAGTGGCCA-AAACGTGGAGAAATGTGGCGTTGAAC

Sequence control cell lines (ID8-control and HM1-control) were generated using a nonsilencing miR oligo provided by the manufacturer.

A concentration of 20 ng/mL recombinant human IFN- γ (R&D Systems) or recombinant mouse IFN- γ (PeproTech) was added to the culture medium for 24 hours before analysis for IFN- γ stimulation. For the other recombinant mouse cytokines, 200 ng/mL interleukin (IL)-2 (eBioscience) or 20 ng/mL IL-6 (R&D Systems), TGF- β (PeproTech), IL-10 (PeproTech), or TNF- α (PeproTech) was added to the culture medium for 24 hours before analysis.

Flow cytometry

Cultured cells were harvested and incubated with phycoerythrin (PE)-conjugated PD-L1 (mouse clone MIH5, human clone MIH1; BD Biosciences) or a matched isotype control (BD Biosciences) at 4°C for 30 minutes, washed twice, and analyzed using a FACSCalibur cytometer (Beckton Dickinson). The results were analyzed using CellQuest Pro software.

Analysis of PD-L1 expression on tumor cells in ascites

Mice were challenged with an intraperitoneal injection of the GFP-labeled cell lines. Mice with ascites formations were sacrificed and the ascites were collected. After briefly centrifuging, red blood cells were lysed, and the remaining cells were washed twice, incubated with antibodies, and analyzed by flow cytometry as mentioned previously. 7-AAD Staining Solution (BD Biosciences) was added 10 minutes before analysis to gate out nonviable cells. GFP-positive and 7-amino-actinomycin D–negative gated cells were analyzed as ascites tumor cells.

CD8⁺ T lymphocyte collection from ascites

Mouse ascites cells were collected and washed with PBS supplemented with 2% FBS. CD8⁺ T lymphocyte was collected by magnetic separation using mouse CD8a MicroBeads (Miltenyi Biotec).

Detection of intracellular IFN- γ in mouse lymphocytes

For intracellular IFN- γ staining, BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and PE-conjugated anti-mouse IFN- γ antibody (BD Biosciences) were used. A matched isotype control was used to determine IFN- γ –negative quadrant. PerCP-conjugated anti-mouse CD3e antibody (BD Biosciences), Alexa Fluor 647–

conjugated anti-mouse CD8a antibody (BD Biosciences), and fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4 antibody were used to gate lymphocytes and CD4⁺ or CD8⁺ cells.

Multiplexed bead assay for cytokines in ascites

BD CBA Mouse Th1/Th2/Th17 Kits (BD Biosciences) was used according to the manufacturer's protocol. Concentrations of each cytokines were calculated using BD Cytometric Bead Array Software version 1.4 (BD Biosciences).

Proliferation assay

A water soluble tetrazolium-8 assay using Cell Count Reagent SF (Nacalai Tesque) was conducted according to the manufacturer's protocol, and the proliferation rate for each cell line was calculated and plotted.

Activation of CTLs

B6 splenocytes underwent T-cell depletion using CD90.2 Microbeads (Miltenyi Biotec) and were incubated with 10 µg/mL OVA₂₅₇₋₂₆₄ peptide (SIINFEKL, Bachem Bioscience) at 37°C for 1 hour. Then they were coincubated with CD8⁺ cells that were isolated from female OT-1-GFP mice using CD8a⁺ T Cell Isolation Kit II (Miltenyi Biotec) for 4 to 6 days. Subsequently, the CTLs were collected by CD8a MicroBeads (Miltenyi Biotec) and were used for further experiments. RPMI-1640 medium supplemented with 10% FBS, 50 µmol/L 2-mercaptoethanol (Nacalai Tesque), 2 mmol/L L-glutamine (Invitrogen), and penicillin-streptomycin (Nacalai Tesque) was used for lymphocyte cultures.

Cytotoxicity assay

As target cells, ID8 cells were loaded with 10 µg/mL OVA₂₅₇₋₂₆₄ peptide (Bachem Bioscience) at 37°C for 1 hour. As effectors, activated OT-1 CD8⁺ CTLs were prepared as described earlier. The target cells were cocultured with the effector cells at various E/T (effector-to-target) ratios. After 5 hours of incubation, the levels of lactate dehydrogenase in the culture supernatant were determined using the cytotoxicity detection kit CytoTox96 (Promega). We used 0.9% Triton X to determine maximum target cell lysis. Percentage lysis was calculated according to a modified standard formula:

$$\frac{(\text{OD}_{\text{experimental}} - \text{OD}_{\text{spontaneous targets}} - \text{OD}_{\text{spontaneous effectors}})}{(\text{OD}_{\text{maximum}} - \text{OD}_{\text{spontaneous targets}})} \times 100.$$

CD107a expression assay

After 4 hours of coincubation of target cells and OT-1-GFP mouse CTLs at an E/T ratio of 30, the cells were incubated with an Alexa Fluor 647-conjugated anti-CD107a antibody (BioLegend) and were washed twice and analyzed by flow cytometry. GFP-positive cells were gated as OT-1-GFP mouse CTLs.

Time-lapse photography of CTLs attacking target cells

CTLs from OT-1-GFP mouse were activated as described earlier. A total of 3×10^6 /mL CTLs were mixed

with 1×10^5 /mL ID8-control or ID8-pdl1 cells loaded with OVA peptide and observed under a laser microscope (Olympus TH4-100) at magnification of $\times 200$. Images of GFP-positive cells were acquired every 2 minutes for total of 68 times (=136 minutes) using DP71-MetaMorph system. Time-lapse video was made from these images (10 frames/s) using MetaMorph software (Molecular Devices).

Microarray profiling of CTLs

OT-1-GFP-mouse CTLs were collected from 4 mice (mouse A to D) and activated as described earlier. CTLs from mouse A to D were divided into 2 groups and coincubated with ID8-pdl1 (PD-L1 group) or ID8-Mirpdl1 (Mir group) for 4 hours at an E/T ratio of 30. Then, the activated CTLs were collected by magnetic separation using CD8a Microbeads (Miltenyi Biotec). From these 8 samples of CTLs, whole RNA was extracted with RNeasy Kit (Qiagen) and hybridized to Affymetrix Mouse Genome430 2.0 Array as previously described (5). RMA normalization was conducted as described earlier. Gene sets for CTL_PD-L1_UP (high in PD-L1 group) and CTL_PD-L1_DN (high in Mir group) were generated using paired *t* test between the 2 groups ($P < 0.01$). GSE24026 dataset, which analyzed downstream of PD-1 signaling (30), was downloaded from Gene Expression Omnibus (GEO) DataSets (<http://www.ncbi.nlm.nih.gov/gds>) to analyze the association of PD-1 signaling with our experiments.

Statistics

For the analysis of immunohistochemistry, Fisher exact test and the χ^2 test were used to analyze the associations between PD-L1 expression and ascites cytology. Survival was analyzed using the Kaplan-Meier survival analysis with the log-rank test by GraphPad Prism 5 software. A *P* value less than 0.05 was considered to be significant.

Results

Positive cytology of peritoneal wash or ascites is related to poor overall and progression-free survival in ovarian cancer patients

Survivals of 65 patients with ovarian cancer (KOV-IH-65) were studied. A cytologic examination at the time of operation revealed viable malignant cells in the ascites of 42 patients ("cytology-positive" cases) in this group. Positive cytology was related to poor overall survival ($P < 0.001$; Supplementary Fig. S1A) and poor progression-free survival ($P < 0.001$; Supplementary Fig. S1B) indicating that positive cytology in ascites was a significant poor prognostic factor in ovarian cancer as previously reported (1, 4).

Genes in Gene Ontology term related to immunity are enriched in cytology-positive cases

Microarray analysis of ovarian cancer tissue from 64 patients (KOV-MA-64) was conducted. Thirty patients were cytology positive in this group. Among 1,692 probes that were highly expressed in ascites-cytology-positive cases,

genes belonging to Gene Ontology terms related to immunity, such as "regulation of immune system process," "positive regulation of immune effector process," or "regulation of IFN- γ production" were enriched. Significantly enriched Gene Ontology terms in cytology-positive cases are listed in Supplementary Table S2. PD-L1 (CD274) was included in Gene Ontology term "regulation of immune system process."

Genes upregulated by IFN- γ , including PD-L1, are enriched in cytology-positive cases

GSEA revealed that the genes upregulated in response to IFN- γ were significantly enriched in cytology-positive cases in KOV-MA-64 (Fig. 1A). FDR q value was 0.242. Genes upregulated in response to IFN- γ are shown in heatmap in Supplementary Fig. S2. Again, PD-L1 (CD274) was included in the enriched genes. These data indicate that ascites-cytology-positive cases in ovarian cancer are distinctly characterized by regulation of immune response, especially by IFN- γ -induced genes, including PD-L1.

PD-L1 protein expression in human ovarian cancer is related to positive peritoneal cytology and poor prognosis

To determine if PD-L1 protein expression also correlates to the positive peritoneal cytology, immunohistochemistry

for PD-L1 in the sampled tissue was conducted (Fig. 1B). Forty-four cases were positive for PD-L1. Positive cytology cases showed tendency to have positive PD-L1 expression in the tumor tissue ($P = 0.048$, χ^2 test; $P = 0.058$, Fisher exact test; Fig. 1C).

Overall survival of PD-L1-positive patients in KOV-IH-65 was significantly shorter ($P = 0.023$) as compared with PD-L1-negative patients (Fig. 1D).

Human and mouse ovarian cancer cell lines express various levels of PD-L1

We examined the PD-L1 expression on several human and mouse ovarian cancer cell lines by flow cytometry. Two of 6 tested human cell lines expressed high levels of PD-L1, whereas 4 cell lines expressed very low levels of or no PD-L1 (Fig. 2A). The mouse ovarian cancer cell line ID8 did not express PD-L1, whereas HM-1 expressed very low level of PD-L1 (Fig. 2B).

Next, we assessed whether IFN- γ alters PD-L1 expression on these cell lines because IFN- γ is reported to induce PD-L1 expression (31, 32). Human recombinant IFN- γ (20 ng/mL) for human cells or mouse recombinant IFN- γ (20 ng/mL) for mouse cells was added to the culture medium. IFN- γ induced PD-L1 expression in 3 human cell lines and in ID8 and HM-1, whereas OV90 did not express PD-L1 even after

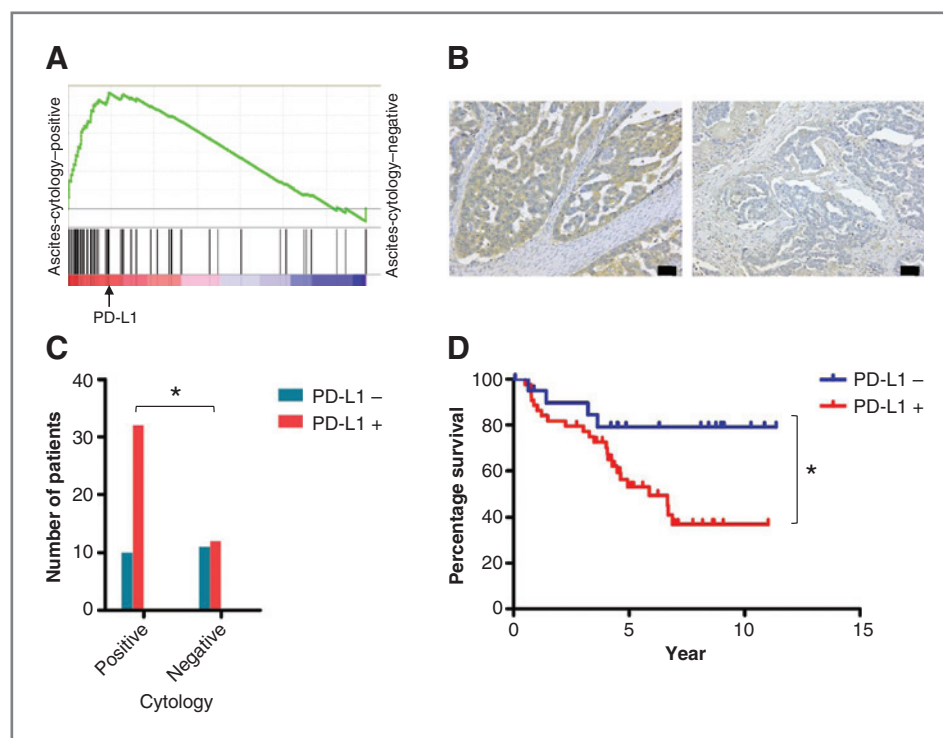


Figure 1. PD-L1 expression on human ovarian cancer cells is related to tumor survival in ascites. A, enrichment of the gene set described for response to IFN- γ in the ascites-cytology-positive cases, relative to the ascites-cytology-negative cases. Black vertical bars represent genes in this gene set. The position of the gene PD-L1 is shown by an arrow. Position to the left indicates enrichment in ascites-cytology-positive cases; a position to the right indicates enrichment in ascites-cytology-negative cases. B, PD-L1 expression in human ovarian cancer tissue. Representative samples with high expression (left) and low expression (right; magnification $\times 200$). Bars, 50 μ m. C, the result of immunohistochemistry of PD-L1 in KOV-IH-65. Positive cytology cases tend to have positive PD-L1 expression. *, $P = 0.048$, χ^2 test; $P = 0.058$, Fisher exact test. D, overall survival of KOV-IH-65. PD-L1 immunohistochemistry positive (red line) and negative (blue line). *, $P = 0.023$.

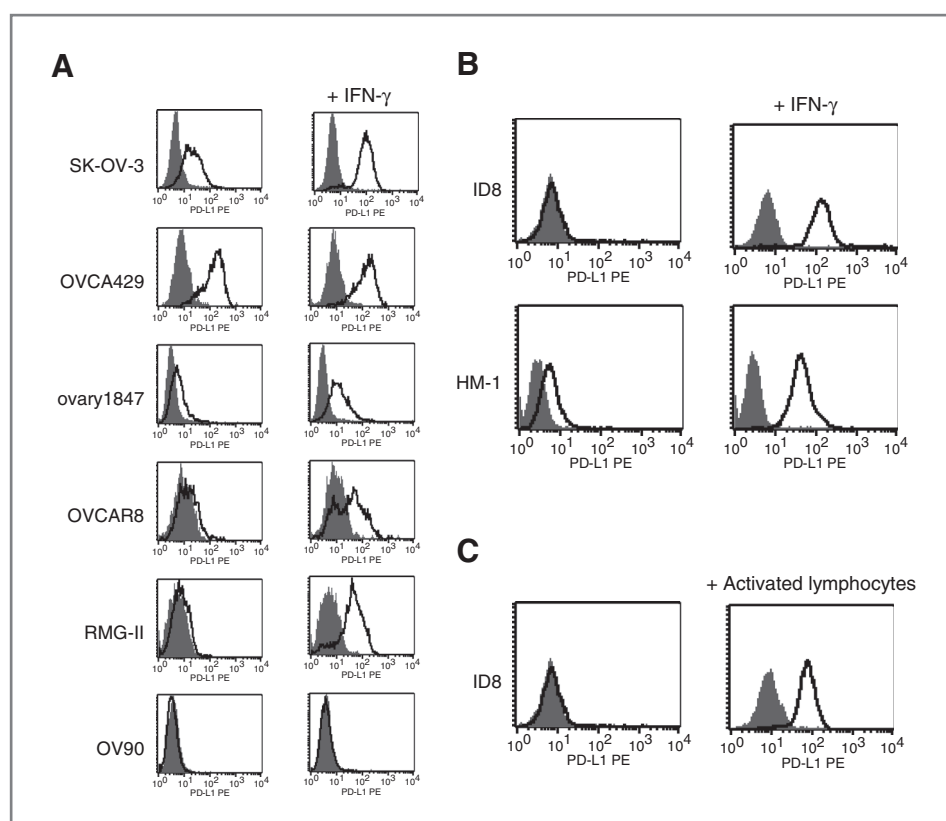


Figure 2. Human and mouse ovarian cancer cell lines express various levels of PD-L1. A, PD-L1 expression in 6 human ovarian cancer cell lines with (right) or without (left) IFN- γ exposure. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. B, PD-L1 expression in 2 mouse ovarian cancer cell lines with or without IFN- γ exposure. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. C, PD-L1 expression in ID8 cells coincubated with or without activated lymphocytes for 24 hours. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody.

IFN- γ exposure, indicating that this cell line has some functional loss in IFN- γ pathway (Fig. 2A and B).

Coculture with activated lymphocytes induces PD-L1 expression in mouse ovarian cancer cell lines

To determine whether activated lymphocytes, which are a possible source of IFN- γ *in vivo*, induce PD-L1 on ovarian cancer cells, we cocultured activated lymphocytes with ID8 cells. Lymphocytes from B6 mouse spleen were stimulated with 1 μ g/mL of anti-mouse CD3 antibody (BioLegend) and 2 μ g/mL of anti-mouse CD28 antibody (BioLegend) for 4 days before the experiment. After 24 hours of coculture, the ID8 cells were analyzed for PD-L1 expression by flow cytometry. PD-L1 expression was markedly increased after coculture with activated lymphocytes (Fig. 2C). Similarly, PD-L1 on HM-1 was also induced by coculture with syngeneic activated lymphocytes (data not shown). Thus, coculture with activated T lymphocytes induces PD-L1 in mouse ovarian cancer cells.

Ovarian cancer cells in mouse ascites express PD-L1 by encountering lymphocytes

As mouse models of ovarian cancer dissemination, ID8 and HM-1 formed cancerous ascites and massive peritoneal dissemination after intraperitoneal injection into syngeneic mice. ID8-GFP cells and HM-1-GFP cells in the ascites expressed PD-L1 (Fig. 3A), and as high as 19% of the CD8⁺ T lymphocytes in the ascites was positive for intracellular IFN- γ (Fig. 3B). In contrast, IFN- γ concentration in

ascites supernatant was very low, whereas IL-6, -10, and TNF- α were detected in higher concentrations (Fig. 3C). We tested IL-2, -6, TGF- β , TNF- α , and IL-10 to determine whether cytokines other than IFN- γ affect PD-L1 expression in the ascites, but none of the tested cytokines induced PD-L1 on HM-1 cells (Fig. 3D). As expected, adding the ascites supernatant to the culture medium did not affect PD-L1 expression on ID8 or HM-1 cells (Fig. 4A). Floating cultures in a nonadherent dish, a hypoxic culture in 1% oxygen, or both, which is a mimic of ascites condition, did not alter PD-L1 expression in HM-1 cells (Fig. 4B). However, coculture with mice ascites cells enhanced PD-L1 expression in HM-1 cells, and coculture with CD8⁺ cells isolated from mouse ascites induced even higher levels of PD-L1 in HM-1 cells (Fig. 4C).

Administration of HM-1-GFP to a SCID mouse also forms cancerous peritonitis. However, HM-1-GFP cells in SCID mouse ascites did not express PD-L1 (Fig. 4D). These data suggest that the tumor cells express PD-L1 in ascites as a consequence of their encounter with activated lymphocytes.

Generation of PD-L1-overexpressing and PD-L1-depleted cell lines

To examine the effects of PD-L1 expression on tumor cells, we established PD-L1-overexpressing cell lines (denoted ID8-pdl1 and HM1-pdl1) and PD-L1-depleted cell lines (denoted ID8-Mirpd1 and HM1-Mirpd1) from the ID8 and HM-1. PD-L1 expression is shown in Supplementary Fig. S1C. To confirm that PD-L1 depletion was successfully

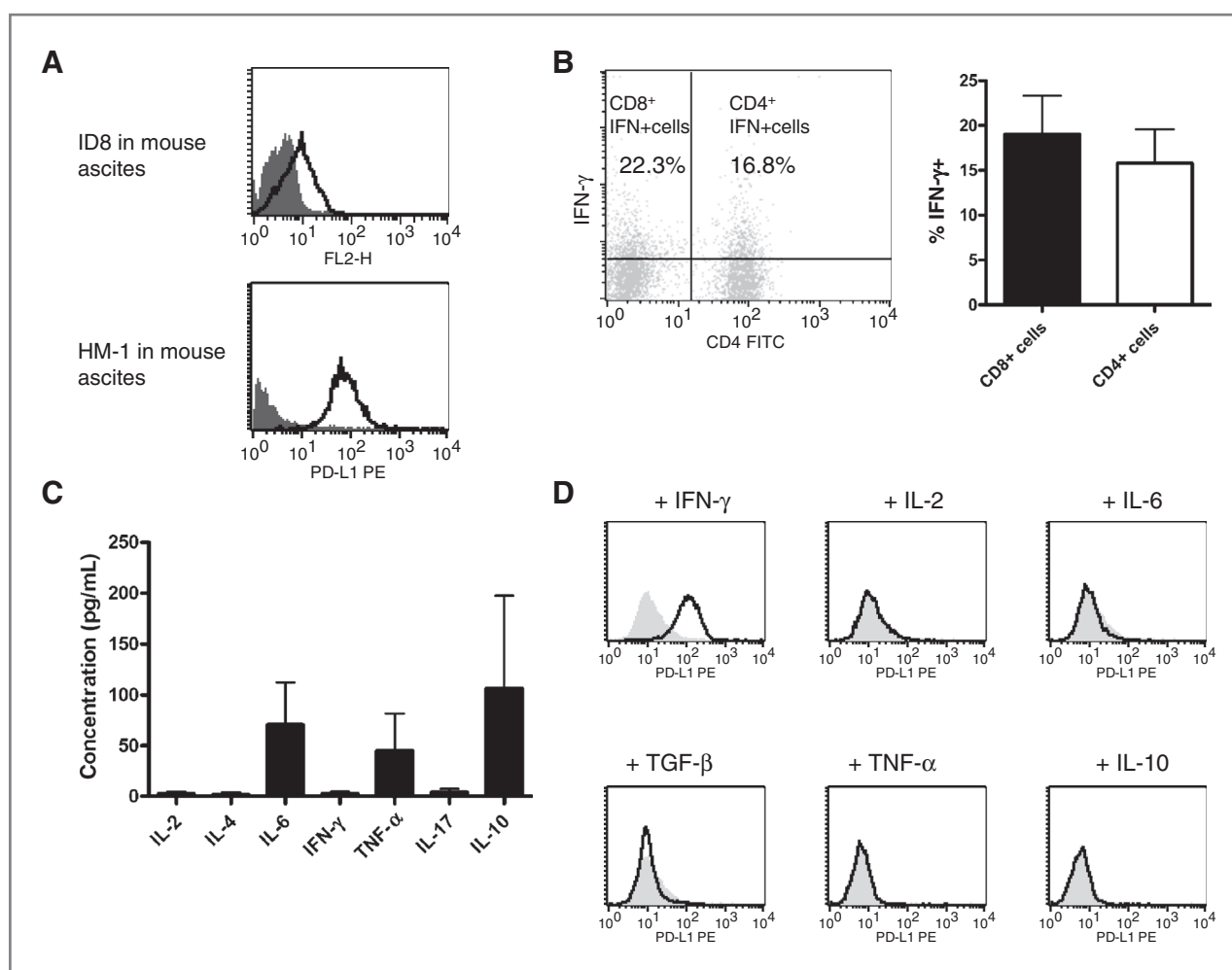


Figure 3. Ovarian cancer cells in mouse ascites express PD-L1. **A**, ovarian cancer cells in the ascites of the mouse ovarian cancer models express PD-L1. Flow cytometry histograms of ascites cells from a mouse inoculated with ID8-GFP (top) and HM-1-GFP (bottom) are shown. GFP-positive and 7-amino-actinomycin D-negative cells are gated as tumor cells. Shaded histogram, isotype control; open histogram, anti-PD-L1-antibody. Representative of 3 experiments with similar results. **B**, lymphocytes in the ascites of mouse ovarian cancer model are positive for intracellular IFN- γ . A representative result of 3 experiments (left) and percentage of intracellular IFN- γ -positive cells in mouse ascites T lymphocytes (right). Mean \pm SD ($n = 3$). CD3-positive cells are gated. **C**, cytokine concentration in ID8-bearing mouse ascites supernatant. Mean \pm SD ($n = 3$). **D**, PD-L1 expression after exposure to various cytokines. None of the tested cytokines other than IFN- γ induced PD-L1 on HM-1. Shaded histogram, PD-L1 expression without cytokine; open histogram, PD-L1 expression with cytokine added to the medium 24 hours before the assessment.

achieved, PD-L1-depleted cell line or control cell line was coincubated with ascites cells or ascites CD8⁺ cells, and PD-L1 expression in the depleted cell line was lower than in the control cell lines (Supplementary Fig. S1D).

In vitro cell proliferation is not affected by PD-L1 expression

A cell proliferation assay revealed that the proliferation curves of the PD-L1-manipulated cell lines were similar to those of the control cell lines (Fig. 5A), indicating that PD-L1 expression does not affect cell proliferation *in vitro*.

PD-L1 protects ovarian cancer cells from antigen-specific cytotoxicity by CTLs

We next conducted a cytotoxicity assay to examine antigen-specific cytotoxicity by CD8⁺ CTLs. The cytotoxicity curves

were significantly different between the cell lines. High levels of target cell lysis were observed in ID8-Mirpd1 cells, and low levels of target cell lysis were observed in ID8-pd1 cells (Fig. 5B), indicating that antigen-specific cytotoxicity by CTLs is inhibited by PD-L1 and can be promoted by PD-L1 depletion.

CTL function is inhibited by tumor-associated PD-L1

Alterations in CTLs following their encounter with tumor-associated PD-L1 were assessed. CTLs lyse target cells by secreting perforin and granzymes, and CD107a is a surface marker for the degranulation of activated CTLs. CD107a expression in the CTLs cocultured with ID8-pd1 was weaker than control, indicating that T-cell degranulation following antigen stimulation has been inhibited by tumor-associated PD-L1 (Fig. 5C). Under microscopic

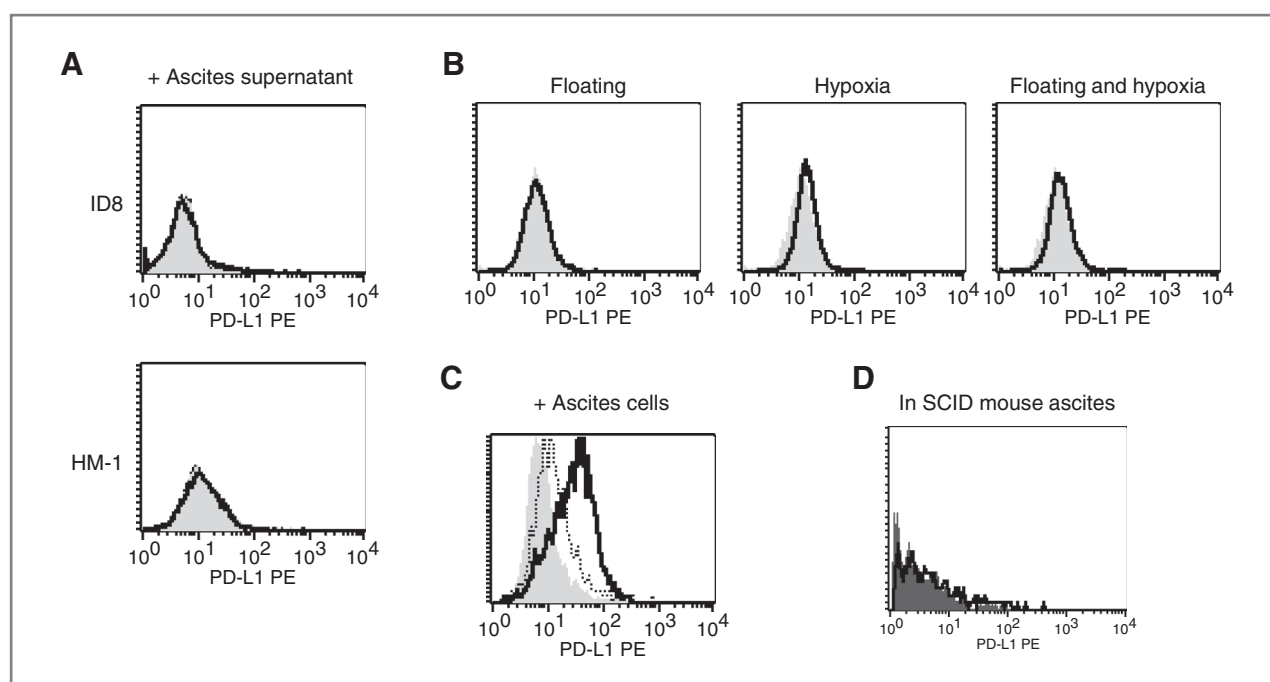


Figure 4. Lymphocytes in ascites induce PD-L1 on mouse ovarian cancer cells. A, ascites supernatant did not induce PD-L1 in ID8 or HM-1 cells. Shaded histogram, isotype control; open histogram, anti-PD-L1 antibody. Representative of 3 repeated independent experiments with similar results. B, PD-L1 expression under various culture conditions. Floating culture in nonadherent dish, culture under hypoxic condition (1% O₂), or both did not affect PD-L1 expression. Shaded histogram, PD-L1 expression in normal culture condition; open histogram, PD-L1 expression under floating, hypoxic, or both floating and hypoxic conditions. Representative of 3 repeated independent experiments with similar results. C, CD8⁺ T cells from mouse ascites induce PD-L1 expression on HM-1. Shaded histogram, cultured without any ascites cells; dotted line histogram, ascites cells added to the culture; solid line histogram, ascites CD8⁺ cells added to the culture. Representative of 3 repeated independent experiments with similar results. D, mouse ovarian cancer cells in SCID mouse ascites do not express PD-L1. Representative of 3 mice with similar results.

observation while coculturing with these target cells, CTLs gathering to the tumor cells were markedly inhibited in ID8-pdl1 (Fig. 5D and Supplementary Videos S1 and S2). These results indicate that PD-L1 on tumor cells inhibit CTL function.

Gene expression profile of mouse CTLs affected by PD-L1 shows correlations to PD-1 signal genes in human

PD-L1 is reported to transmit an inhibitory signal through its receptor, PD-1, in lymphocytes. To examine the alteration in gene expression profiles in mouse CTLs associated with PD-L1, microarray analysis for CTLs cocultured with ID8-pdl1 or ID8-Mirpd11 was conducted, and the gene expression profile was compared by GSEA with a publicly accessible gene set of human functionally impaired CD8⁺ T cells by positive PD-1 signal (30). Up- and down-regulated genes in mouse CTLs are shown in Supplementary Table S3. Interestingly, the genes upregulated in PD-L1-affected mouse CTLs were significantly enriched in upregulated genes in PD-1 downstream genes in human CTLs. Furthermore, the genes downregulated in PD-L1-affected mouse CTLs were also significantly downregulated in PD-1 signal-transmitted human CTLs (Supplementary Table S4). This result is consistent with the fact that PD-L1 on tumor cells transfers inhibitory signal through PD-1 on CTLs and also validate the similar mechanism of PD-L1/PD-1 effect in human and mouse CTLs.

PD-L1 promotes tumor progression in mouse ovarian cancer dissemination models

HM-1-pdl1, HM-1-Mirpd11, or HM1-control cells were injected intraperitoneally to syngeneic mice. After 7 days, the body weight of the mice, a reliable marker of tumor growth, in all 3 groups increased (Fig. 6A). However, in the HM1-Mirpd11 group, the body weight decreased after 10 days (Fig. 6A, right). Therefore, PD-L1 depletion decreased the tumor that once grew in the peritoneal cavity.

The survival of the mice is shown in Fig. 6B–D. The HM-1-pdl1 group lived shorter ($P = 0.039$) than the control group, and the HM-1-Mirpd11 group lived longer ($P = 0.0029$; Fig. 6B). In ID8-injected mice, the survival of the ID8-pdl1 group and the ID8-control group were similar (Fig. 6C), indicating that differences in PD-L1 expression upon injection is eventually abrogated in slow-progressing tumors because PD-L1 is induced in the peritoneal cavity. However, the mice in the ID8-Mirpd11 group had significantly longer survival times than the control group ($P < 0.001$; Fig. 6C). PD-L1 expression in tumor cells did not affect the survival of SCID mice following intraperitoneal injection (Fig. 6D).

Discussion

Although various molecules expressed by cancer cells have been implicated in the process of peritoneal dissemination, the influence of immunologic factors is poorly understood. In this study, we first focused on the state of

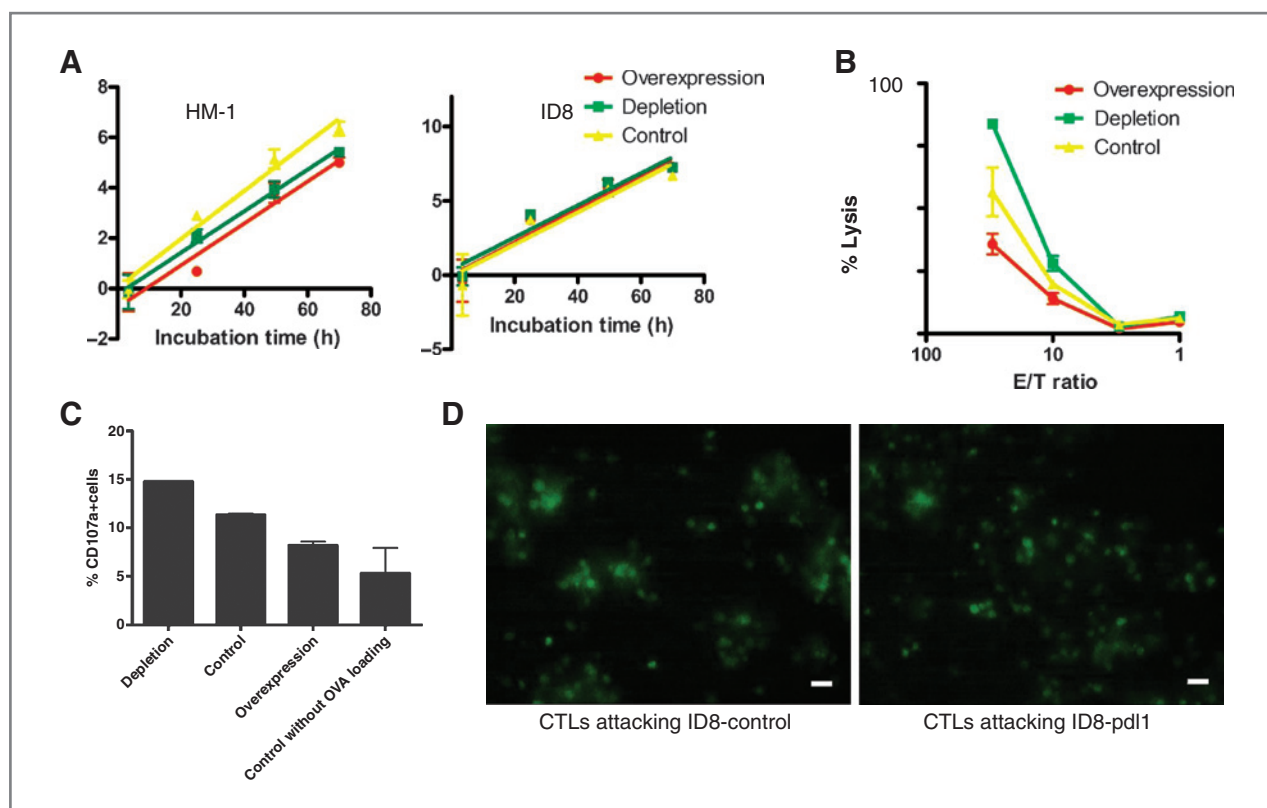


Figure 5. PD-L1 protects tumor cells from CTLs. **A**, cell proliferation assay of the PD-L1-manipulated HM-1 cell lines (left) and ID8 cell lines (right). Y-axis, relative number of cells in log₂ scale. X-axis, incubation time (h). Mean \pm SD ($n = 6$) from 1 representative experiment of 2 repeated experiments with similar results. Relative number of cells is calculated in the following formula: (Relative number of cells) = (number of cells estimated by water soluble tetrazolium-8 assay)/(seeded cells). **B**, cytotoxicity assay of the PD-L1-manipulated ID8 cell lines. Mean \pm SD ($n = 4$) from 1 representative experiment of 3 repeated experiments with similar results. **C**, CD107a⁺ CTLs following coincubation with OVA-loaded ID8-Mirpd1, OVA-loaded ID8-control, OVA-loaded ID8-pd1, or ID8-control without OVA loading. Mean \pm SD ($n = 3$) from 1 of 3 repeated experiments with similar results. **D**, microscopic image of activated GFP⁺ CTLs, after 136 minutes of coincubation with ID8-control (left) or ID8-pd1 (right). Bars, 50 μ m. Time-lapse video available in Supplementary Videos S1 and S2. One of 3 repeated experiments with similar results.

"positive peritoneal cytology," which represents the status that the tumor cells are surviving in peritoneal cavity without being destroyed by host immunity. We confirmed that positive cytology adversely affects the overall and progression-free survival of the patients. Then, we analyzed PD-L1 expression in the primary tumor, both in mRNA and protein levels, and found for the first time that it significantly correlates to positive peritoneal cytology. Furthermore, in microarray analyses, gene profile associated with positive peritoneal cytology was significantly enriched of immune-related genes, including PD-L1, assessed by a Gene Ontology analysis. An IFN- γ -induced gene signature, which also includes PD-L1, was also significantly associated with positive peritoneal cytology by GSEA. Together, these data imply that peritoneal spread of ovarian cancer accompanies with local immune modification, and that PD-L1 functions as a key molecule in this process. These data prompted us to further investigate the function of PD-L1 in ovarian cancer cells, especially as related to the peritoneal dissemination.

The mechanism by which PD-L1 expression is regulated is quite ambiguous, especially in cancer cells. In an early

report, PD-L1 was reported to be expressed only in immune cells under natural circumstances and to be highly expressed in some tumor cells (31). Subsequent reports have shown that PD-L1 is expressed constitutively in some normal tissues including eyes and placenta (33, 34), and that PD-L1 can be induced in cancer cells and noncancer cells by IFN- γ (35, 36). However, the precise mechanism of PD-L1 induction, especially *in vivo*, is still unclear. Therefore, we initially examined PD-L1 expression under natural culture conditions as well as upon various cytokine stimulations, including IFN- γ , in 6 human and 2 mouse ovarian cancer cell lines. The results suggest that there are 3 types of cells with regards to PD-L1 expression: type A cells (e.g., SK-OV-3) always express PD-L1; type C cells (e.g., OV90) never express PD-L1; and type B cells (e.g., OVARY1847) do not express PD-L1 at baseline but express PD-L1 when exposed to IFN- γ . Type B was most frequent in the tested human ovarian cancer cell lines. It is assumed that PD-L1 expression is not constitutive in these cells but is induced by the influence of other factors. In a mouse experiment, we used 2 type B mouse ovarian cancer cells, ID8 and HM-1. Both cell lines expressed PD-L1 when administered into the

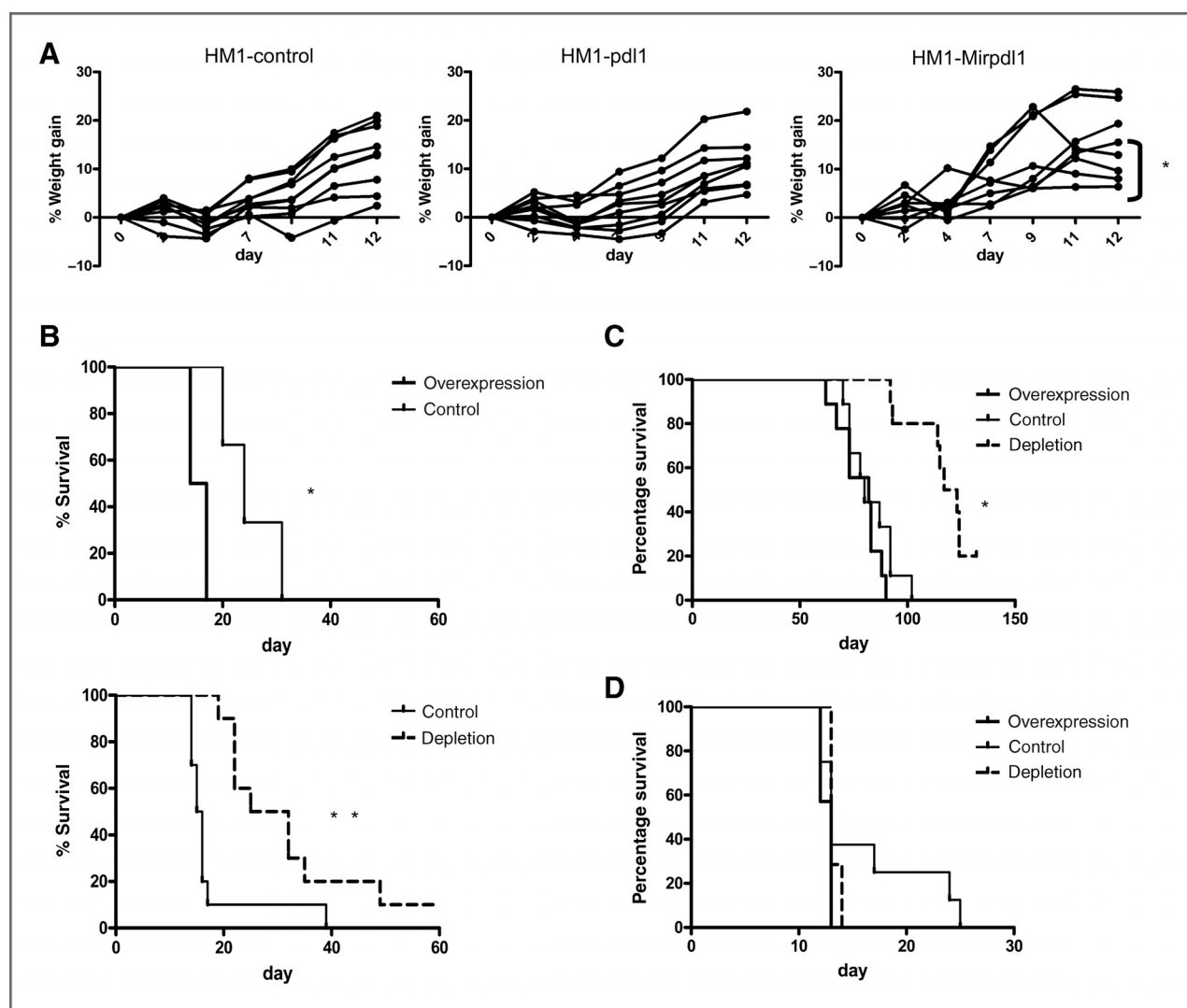


Figure 6. PD-L1 depletion prevents tumor progression and prolongs mouse survival. **A**, mouse body weight gain is plotted after intraperitoneal injection of HM1-control (left), HM1-pd11 (middle), or HM1-Mirpd11 (right). Weight is a reliable marker of tumor growth. Body weight decreased in 4 of 8 mice in HM1-Mirpd11 group (*). **B**, survival of HM1-pd11-injected mice (thick line) and HM1-control-injected mice (thin line); *, $P = 0.039$ ($n = 5$; top), and survival of HM1-control-injected mice (thin line) and HM1-Mirpd11-injected mice (dotted line); **, $P = 0.0029$ ($n = 10$; bottom). **C**, survival of ID8-pd11-injected mice (thick line), ID8-control-injected mice (thin line), and ID8-Mirpd11-injected mice (dotted line). ID8-control versus ID8-Mirpd11; *, $P < 0.001$ ($n = 10$). **D**, survival of SCID mice intraperitoneally injected with HM1-pd11 (thick line), HM1-control (thin line), and HM1-Mirpd11 (dotted line). Differences between the groups are not significant ($n = 10$).

mouse peritoneal cavity, whereas IFN- γ concentration in ascites supernatant was too low to induce PD-L1 expression. However, flow cytometric analysis of ascites cells indicated that there are numerous T lymphocytes positive for intracellular IFN- γ , and cocubation with ascites cells, ascites CD8⁺ lymphocytes, or *in vitro* activated spleen-derived lymphocytes induced PD-L1 on ovarian cancer cells, whereas hypoxic condition or floating culture did not. Notably, HM-1 cells did not express PD-L1 in SCID mouse ascites, suggesting that the copresence of lymphocytes is required for the induction of PD-L1. Taken together, our study indicates that type B cancer cells begin to express PD-L1 when they encounter activated lymphocytes in ascites. Although precise mechanism to explain the difference in

PD-L1 expression is not fully understood, there are several reports showing that PD-L1 is overexpressed under influence of oncogenic mutation such as PTEN loss (37) or NPM/ALK (38), which might be the case in type A tumors. On the other hand, type C tumors, which do not respond to IFN- γ , may have some impairment in IFN-receptors or its downstream signals. Namely, tumor cells express PD-L1 depending on both the cell nature (types A, B, or C) and its immune microenvironment. Therefore, in selecting the patients for PD-L1-targeted therapy in ovarian cancer, it might be necessary to assess not only the PD-L1 status of the primary tumor but also the PD-L1 and immune status in the ascites, to predict whether the case will be sensitive to the therapy or not.

Next, we generated PD-L1–overexpressing and PD-L1–depleted cell lines, which are representative of types A and C tumor cells, respectively. PD-L1 manipulation did not affect cell proliferation *in vitro*. In contrast, the *in vivo* proliferation of both the rapid- and slow-growing mouse ovarian cancer cell lines, HM-1 and ID8, was markedly affected, suggesting that PD-L1 has an important role in cancer spreading into the peritoneal cavity. There are several reports about immune responses in ascites and peritoneal dissemination (4, 39). In malignant ascites, abundant activated lymphocytes are found. These lymphocytes can easily attack tumor cells, so surviving in ascites should be difficult for tumor cells (9). In our mouse model, there were numerous IFN- γ –producing activated lymphocytes in the ascites. Nonetheless, the PD-L1–expressing tumor cells progressed. In contrast, the progression of PD-L1–depleted tumor cells was inhibited in this environment. The difference between the 2 groups was observed 10 days after inoculating with tumor cells, indicating that the difference is not due to tumor proliferation itself or an innate immune response but rather is due to an adapted immune response. Survival of SCID mouse was not affected by tumor PD-L1, indicating that the difference is due to interaction between PD-L1 and lymphocytes.

There is some controversy about how and in which phase PD-L1 works in tumor immunity. Dong and colleagues reported that tumor-associated PD-L1 promotes T-cell apoptosis but does not alter CTL cytotoxicity (31). Hirano and colleagues reported that PD-L1 on tumor cells forms a molecular shield to prevent destruction by CTLs without impairing CTL function (40). In contrast, Blank and colleagues reported that PD-L1 inhibits the effector phase of tumor rejection and alters target cell lysis by CD8⁺ T cells (41). To further elucidate these possibilities, we conducted several *in vitro* assays to evaluate CTL activity against ovarian cancer cells with varying PD-L1 status. A cytotoxicity assay revealed that PD-L1 expression on ID8 cells inhibits the antigen-specific cytotoxicity by CTLs. The assessment of CD107a expression on CTL surface indicated that CTL degranulation following encounter with PD-L1–overexpressing ID8 cells is significantly suppressed. These data clearly suggest that PD-L1 attenuate CTL activity in effector phase. A time-lapse analysis revealed that gathering of the CTLs to the target tumor cells was markedly inhibited and

CTLs behaved as if they ignored tumor cells when the tumor cells overexpressed PD-L1. We also conducted microarray analysis to elucidate the influence of PD-L1 stimuli on gene expression of CTLs. Altered gene profiles of mouse CTLs caused by PD-L1–expressing ovarian cancer cells was significantly coincident with a gene signature associated with human CTL exhaustion (30). These data collectively indicate that, in both human and mouse peritoneal dissemination, PD-L1 induces peripheral tolerance in CTLs and enables tumor cells to evade from the immune system in the peritoneal cavity.

In summary, our study showed for the first time the close relationships between PD-L1 and peritoneal dissemination of cancer cells. PD-L1 expression and peritoneal positive cytology showed a significant correlation in patients with ovarian cancer, and silencing PD-L1 suppressed tumor progression in the mouse peritoneal cavity and prolonged mouse survival. Our data indicate that restoring immune function by inhibiting PD-L1/PD-1 pathway may serve as a promising strategy for controlling the peritoneal dissemination of malignant tumors, including ovarian cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: K. Abiko, M. Mandai, J. Hamanishi

Development of methodology: K. Abiko, M. Mandai, J. Hamanishi

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): K. Abiko, M. Mandai, R. Murakami, A. Yamamoto
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): K. Abiko, N. Matsumura, T. Baba, K. Yamaguchi, R. Murakami

Writing, review, and/or revision of the manuscript: K. Abiko, M. Mandai, N. Matsumura, K. Kosaka

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K. Abiko, J. Hamanishi, Y. Yoshioka, T. Baba, B. Kharma

Study supervision: T. Baba, I. Konishi

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Clinical Cancer Research

PD-L1 on Tumor Cells Is Induced in Ascites and Promotes Peritoneal Dissemination of Ovarian Cancer through CTL Dysfunction

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