PD-L1 on tumor cells is induced in ascites and promotes peritoneal dissemination of ovarian cancer through CTL dysfunction

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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 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 on 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.

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 on 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 cytolysis 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 on
mouse ovarian cancer cells was induced upon encountering lymphocytes in the course of peritoneal
spread in vivo and co-culture 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 on tumor cell promotes peritoneal dissemination by repressing
CTL function. PD-L1-targeted therapy is a promising strategy for preventing and treating peritoneal
dissemination.
Introduction

Ovarian cancer is the most lethal disease among gynecological 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 appears to be the most important strategy in controlling ovarian cancer because the median overall survival and progression-free survival are 81.1 months and 35.0 months, respectively, if macroscopically complete surgical resection of the disseminated tumors is achieved in FIGO (International Federation of Gynecology and Obstetrics) stage IIIc cancers, while these measures are only 34.2 months 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 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 PD-L1 (programmed cell death 1 ligand 1) 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 co-regulatory 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 TCR-mediated activation of lymphocytes (19, 20). We have reported that PD-L1 expression by 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 epithelial ovarian cancer patients (KOV-IH-65) who underwent primary operation at Kyoto University Hospital 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 pathological 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 >70% tumor cell nuclei and <20% necrosis. Total RNA expression was analyzed on Human Genome U133 Plus 2.0 Array (Affymetrix). Robust Multi-Array Average (RMA) normalization was performed using R (R: A language and environment for statistical computing. http://www.R-project.org.). Probes showing expression value>5.0 in at least one of the samples and standard deviation>0.2 across all the samples were selected, and t test was performed between cytology-positive and -negative groups. Enrichment for Gene Ontology (GO) terms was analyzed using GOEAST software (http://omicslab.genetics.ac.cn/GOEAST/) (24) for the set of probes highly expressed in cytology-positive or -negative groups, respectively (p<0.05). A publicly accessible gene set of interferon (IFN)-γ-up-regulated genes was downloaded (http://www.broadinstitute.org/gsea/msigdb/geneset_page.jsp?geneSetName=SANA_RESPONSE_TO_IFNG_UP) (25). Gene set enrichment analysis (GSEA) for positive ascites cytology and
negative cytology was performed 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 above. Immunohistochemical staining for PD-L1 was performed using a PD-L1 antibody as previously described (15, 18). PD-L1 expression was analyzed by two independent gynecologic pathologists without any prior information regarding 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 <50% of tumor cells was considered negative.

**Animals**

Female C57BL/6 (B6) and B6C3F1 and C.B-17/ICR-scid/scidJcl (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 (1x10^6) or ID8 cells (5x10^6) 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, Texas. ref 27). The cells were maintained in RPMI1640 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-Mirpdl1 and HM1-Mirpdl1, 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 below, and cloned into pcDNA™6.2-GW/EmGP-miR expression vector. Then it was linearized and BP/LR Reaction was performed using pDONR™221 vector and pLenti6.4/R4R2/V5-DEST and pENTR™5’ 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;
TGCTGTCCAACGCCACATTTCTCCACGTTTTGGCCACTGACTGACGTGGAGAAGTGG
CGTTGAA

Bottom strand oligo;

CCTGTTCCAACGCCACTTCTCCACGTCAGTCAGTGGCCAAAACGTGGAGAAATGTGG
CGTTGAAC

Sequence control cell lines (ID8-control and HM1-control) were generated using a non-silencing 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 prior to analysis for IFN-γ stimulation. For the other recombinant mouse cytokines, 200 ng/ml 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 prior to 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 was 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-AAD-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 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 WST-8 assay using Cell Count Reagent SF (Nacalai Tesque) was performed 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 10µg/ml OVA<sub>257-264</sub> peptide (SIINFEKL, Bachem Bioscience) at 37°C for 1 hour. Then they were co-incubated with CD8<sup>+</sup> cells that were isolated from female OT-1-GFP mice using CD8a<sup>+</sup> 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.

RPMI1640 medium supplemented with 10% FBS, 50 µM 2-mercaptoethanol (Nacalai Tesque), 2 mM 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 OVA<sub>257-264</sub> peptide (Bachem Bioscience) at 37°C for 1 hour. As effectors, activated OT-1 CD8<sup>+</sup> CTLs were prepared as described above. The target cells were co-cultured 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:

\[
\text{Percentage lysis} = \left( \frac{OD_{\text{experimental}} - OD_{\text{spontaneous targets}} - OD_{\text{spontaneous effectors}}}{OD_{\text{maximum}} - OD_{\text{spontaneous targets}}} \right) \times 100.
\]
CD107a expression assay

After 4 hours of co-incubation 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 above. 3×10⁶/ml CTLs were mixed with 1×10⁵/ml ID8-control or ID8-pdl1 cells loaded with OVA peptide and observed under a laser microscope (Olympus TH4-100) at magnification of x200. 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/second) 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 above. CTLs from mouse A to D were divided into two groups and co-incubated 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 RNAeasy Kit (Qiagen), and hybridized to Affymetrix Mouse Genome430 2.0 Array as previously described (5). RMA normalization was performed as described above. Gene sets for CTL_PDL1_UP (high in PD-L1 group) and CTL_PDL1_DN (high in Mir group) were generated using paired t test between the two groups (p<0.01). GSE24026 dataset, which analyzed downstream of PD-1 signaling (30), was downloaded.
from 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's exact test and the χ² 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 ovarian cancer patients (KOV-IH-65) were studied. A cytological 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 performed. Thirty patients were cytology positive in this group. Among 1692 probes that were highly expressed
in ascites-cytology-positive cases, genes belonging to GO 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 GO terms in
cytology-positive cases are listed in Supplementary Table S2. PD-L1 (CD274) was included in GO
term “regulation of immune system process”.

**Genes up-regulated by IFN-γ, including PD-L1, are enriched in cytology-positive cases**

GSEA revealed that the genes up-regulated 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 up-regulated in
response to IFN-γ are shown in heat map in Supplementary Figure 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 performed (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 χ² test, P=0.058 Fisher's exact test) (Figure 1C).
Overall survival of PD-L1-positive patients in KOV-IH-65 was significantly shorter (P=0.023)
compared to PD-L1-negative patients (Figure 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 out of 6 tested human cell lines expressed high levels of PD-L1, while 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, while 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, while OV90 did not express PD-L1 even after IFN-γ exposure, indicating that this cell line has some functional loss in IFN-γ pathway (Fig. 2A and B).

Co-culture 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 co-cultured activated lymphocytes with ID8 cells. Lymphocytes from B6 mouse spleen were stimulated with 1µg/ml of anti-mouse CD3 antibody (BioLegend) and 2µg/ml of anti-mouse CD28 antibody (BioLegend) for 4 days prior to the experiment. After 24 hours of co-culture, the ID8 cells were analyzed for PD-L1 expression by flow cytometry. PD-L1 expression was markedly increased after co-culture with activated lymphocytes (Fig. 2C). Similarly, PD-L1 on HM-1 was also induced by co-culture with syngeneic activated lymphocytes (data not shown). Thus, co-culture 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, while IL-6, IL-10, TNF-α were detected in higher concentrations (Fig. 3C). We tested IL-2, IL-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 non-adherent 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, co-culture with mice ascites cells enhanced PD-L1 expression in HM-1 cells, and co-culture 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-Mirpdl1 and HM1-Mirpdl1) from the ID8 and HM-1. PD-L1 expression is shown in Supplementary Fig. S1C. To confirm that PD-L1 depletion was successfully achieved,
PD-L1-depleted cell line or control cell line was co-incubated 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 cytolysis by CTLs**

We next performed a cytotoxicity assay to examine antigen-specific cytolysis by CD8+ CTLs. The cytotoxicity curves were significantly different between the cell lines. High levels of target cell lysis were observed in ID8-Mirpdl1 cells, and low levels of target cell lysis were observed in ID8-pdl1 cells (Fig. 5B), indicating that antigen-specific cytolysis 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 co-cultured with ID8-pdl1 was weaker than control, indicating that T-cell degranulation following antigen stimulation has been inhibited by tumor-associated PD-L1 (Fig. 5C). Under microscopic observation while co-culturing with these target cells, CTLs gathering to the tumor cells were markedly inhibited in ID8-pdl1 (Fig.
5D and Supplementary Video S1, 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, on lymphocytes. To examine the alteration in gene expression profiles in mouse CTLs associated with PD-L1, microarray analysis for CTLs co-incubated with ID8-pdl1 or ID8-Mirpdl1 was performed, 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 up-regulated in PD-L1-affected mouse CTLs were significantly enriched in up-regulated genes in PD-1 downstream genes in human CTLs. Furthermore, the genes down-regulated in PD-L1-affected mouse CTLs were also significantly down-regulated 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-Mirpdl1, 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 three groups increased (Fig. 6A). However, in the HM1-Mirpdl1 group, the body weight decreased after 10 days (Fig. 6A, right panel). Therefore, PD-L1 depletion decreased the tumor that once grew in the peritoneal cavity.
The survival of the mice is shown in Fig. 6 B-D. The HM-1-pdl1 group lived shorter (P=0.039) than the control group, and the HM-1-Mirpdl1 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-Mirpdl1 group had significantly longer survival times than the control group (P<0.001) (Fig. 6C). PD-L1 expression on 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 immunological factors is poorly understood. In this study, we first focused on the state of “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 on cancer cells and non-cancer 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 two Type B mouse ovarian cancer cells, ID8 and HM-1. Both cell lines expressed PD-L1 when administered into the mouse peritoneal cavity, while 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 coincubation with ascites cells, ascites CD8+ lymphocytes, or in vitro activated spleen-derived lymphocytes induced PD-L1 on ovarian cancer cells, while hypoxic condition or floating culture did not. Notably, HM-1 cells did not express PD-L1 in SCID mouse ascites, suggesting that the co-presence 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 (Type 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, in order 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 Type A and Type 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 regarding 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 two 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 concerning how and in which phase PD-L1 works in tumor immunity. Dong et al. reported that tumor-associated PD-L1 promotes T-cell apoptosis but does not alter CTL cytolysis (31). Hirano et al. reported that PD-L1 on tumor cells forms a molecular shield
to prevent destruction by CTLs without impairing CTL function (40). In contrast, Blank et al. 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 cytolysis 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 performed 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 demonstrated 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 ovarian cancer patients, 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.

Acknowledgements

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References


Figure legends

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 panel) and low expression (right panel) (magnification x200). Bars, 50 µm.

C. The result of immunohistochemistry of PD-L1 in KOV-IH-65. Positive cytology cases tends to have positive PD-L1 expression. * P=0.048 χ² test, P=0.058 Fisher’s 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 co-incubated with or without activated lymphocytes for 24 hours. Shaded histogram: isotype control, open histogram: anti-PD-L1-antibody.
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 (upper panel) and HM-1-GFP (lower panel) are shown. GFP-positive and 7-AAD-negative cells are gated as tumor cells. Shaded histogram: isotype control, open histogram: anti-PD-L1-antibody. Representative of three 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 panel) and percentage of intracellular IFN-γ-positive cells in mouse ascites T lymphocytes (right panel). Mean±SD (n=3). CD3-positive cells are gated.

C. Cytokine concentration in ID8-bearing mouse ascites supernatant. Mean±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 prior to the assessment.

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 three repeated independent experiments with similar results.

B. PD-L1 expression under various culture conditions. Floating culture in non-adherent 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 three 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 three repeated
independent experiments with similar results.

D. Mouse ovarian cancer cells in SCID mouse ascites do not express PD-L1. Representative of
three mice with similar results.

Figure 5. PD-L1 protects tumor cells from CTLs.

A. Cell proliferation assay of the PD-L1-manipulated HM-1 cell lines (left panel) and ID8 cell
lines (right panel). Y axis: relative number of cells in Log₂ scale. X axis: incubation time (hours).
Mean±SD (n=6) from one representative experiment of two repeated experiments with similar
results. Relative number of cells is calculated in the following formula.

(Relative number of cells) = (number of cells estimated by WST-8 assay) / (seeded cells).

B. Cytotoxicity assay of the PD-L1-manipulated ID8 cell lines. Mean±SD (n=4) from one
representative experiment of three repeated experiments with similar results.

C. CD107a⁺ CTLs following co-incubation with OVA-loaded ID8-Mirpdl1, OVA-loaded
ID8-control, OVA-loaded ID8-pdl1, or ID8-control without OVA loading. Mean±SD (n=3) from
one of three repeated experiments with similar results.

D. Microscopic image of activated GFP+ CTLs, after 136 minutes of co-incubation with
ID8-control (left panel) or ID8-pdl1 (right panel). Bars, 50 µm. Time-lapse video available in
supplementary video S1 and video S2. One of three repeated experiments with similar results.
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 panel) or HM1-pdl1 (middle panel) or HM1-Mirpdl1 (right panel). Weight is a reliable marker of tumor growth. Body weight decreased in 4/8 mice in HM1-Mirpdl1 group(*).

B. Survival of HM1-pdl1-injected mice (thick line) and HM1-control-injected mice (thin line), * P=0.039 (n=5) (upper panel), and survival of HM1-control-injected mice (thin line) and HM1-Mirpdl1-injected mice (dotted line), ** P=0.0029 (n=10) (lower panel).

C. Survival of ID8-pdl1-injected mice (thick line), ID8-control-injected mice (thin line), and ID8-Mirpdl1-injected mice (dotted line). ID8-control vs ID8-Mirpdl1, * P<0.001 (n=10).

D. Survival of SCID mice intraperitoneally injected with HM1-pdl1 (thick line), HM1-control (thin line), and HM1-Mirpdl1 (dotted line). Differences between the groups are not significant (n=10).
Figure 1  Abiko et al.

A

Ascites cytology positive

Ascites cytology negative

PD-L1

B

C

Number of patients

PD-L1 -

PD-L1 +

Cytology

positive

negative

D

Percent survival

PD-L1 -

PD-L1 +

year

0 5 10 15
Figure 2 Abiko et al.

A

<table>
<thead>
<tr>
<th>Cell Line</th>
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<td></td>
<td></td>
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<tr>
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B

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<td></td>
</tr>
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<td>HM-1</td>
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C

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<tr>
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<tbody>
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</table>
Figure 3 Abiko et al.

**A**

ID8 in mouse ascites

HM-1 in mouse ascites

**B**

<table>
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<tr>
<th>CD8+ IFN+ cells</th>
<th>CD4+ IFN+ cells</th>
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<td>22.3%</td>
<td>16.8%</td>
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**C**

![Graph showing concentration of cytokines](image1)

**D**

- + IFN-γ
- + IL-2
- + IL-6
- + TGF-β
- + TNF-α
- + IL-10
Figure 4  Abiko et al.

A + ascites supernatant

ID8

HM-1

B floating

hypoxia

floating and hypoxia

C + ascites cells

D in SCID mouse ascites
Figure 5  Abiko et al.

A

HM-1

ID8

% lysis

B

E/T ratio

C

%CD107a+cells

depletion control overexpression control without OVA loading

D

CTLs attacking ID8-control

CTLs attacking ID8-pdl1
Figure 6  Abiko et al.

A

HM1-control  HM1-pdl1  HM1-Mirpdl1

B

C

D

% survival

% survival

% survival

% survival

% weight gain

% weight gain

% weight gain

overexpression  control  overexpression  control  overexpression  control  overexpression  control

depletion  depletion

depletion

depletion

day  day  day  day

0 20 40 60

0 100

0 20 40 60

0 20 40 60

0 20 40 60

0 20 40 60

0 100

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PD-L1 on tumor cells is induced in ascites and promotes peritoneal dissemination of ovarian cancer through CTL dysfunction

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