# IFN-γ from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer

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**Background:** PD-L1 (programmed cell death 1 ligand 1) on tumour cells suppresses host immunity through binding to its receptor PD-1 on lymphocytes, and promotes peritoneal dissemination in mouse models of ovarian cancer. However, how PD-L1 expression is regulated in ovarian cancer microenvironment remains unclear.

**Methods:** The number of CD8-positive lymphocytes and PD-L1 expression in tumour cells was assessed in ovarian cancer clinical samples. PD-L1 expression and tumour progression in mouse models under conditions of altering IFN-γ signals was assessed.

**Results:** The number of CD8-positive cells in cancer stroma was very high in peritoneally disseminated tumours, and was strongly correlated to PD-L1 expression on the tumour cells ($P<0.001$). In mouse models, depleting IFNGR1 (interferon-γ receptor 1) resulted in lower level of PD-L1 expression in tumour cells, increased the number of tumour-infiltrating CD8-positive lymphocytes, inhibition of peritoneal disseminated tumour growth and longer survival ($P=0.02$). The injection of IFN-γ into subcutaneous tumours induced PD-L1 expression and promoted tumour growth, and PD-L1 depletion completely abrogated tumour growth caused by IFN-γ injection ($P=0.01$).

**Conclusions:** Interferon-γ secreted by CD8-positive lymphocytes upregulates PD-L1 on ovarian cancer cells and promotes tumour growth. The lymphocyte infiltration and the IFN-γ status may be the key to effective anti-PD-1 or anti-PD-L1 therapy in ovarian cancer.

Ovarian cancer is the most lethal disease among the gynaecological malignancies. Most cases present massive peritoneal dissemination, and, although many of the cases respond to initial chemotherapy following tumour reductive surgery, the majority of cases recur and eventually become resistant to chemotherapy (Coleman *et al*, 2013). Therefore, a new strategy for peritoneal dissemination other than conventional chemotherapy is urgently needed.

Immune evasion is an important hallmark of cancer, and a better understanding of this mechanism is essential to develop a strong strategy against cancers (Hanahan and Weinberg, 2011). In ovarian cancer, multiple inhibitory mechanisms are present, including regulatory T cells (Curiel *et al*, 2004; Barnett *et al*, 2010), tumour-associated macrophages (Zhang *et al*, 2012), myeloid-derived suppressor cells (MDSCs) (Godoy *et al*, 2013) and inhibitory molecules expressed on tumour cells (Kandalaft *et al*, 2010; Thibodeaux and Curiel, 2011). These players work as strong brakes for antitumour immunity and obstruct the elimination of cancer by the host immune system (Junttila and de Sauvage, 2013). We have attempted to elucidate the inhibitory immune molecules in ovarian cancer, such as ULBP2 (NKG2D ligand), COX-1, COX-2 and PD-L1 (programmed cell death 1 ligand 1, CD274) (Hamanishi *et al*, 2007; Li *et al*, 2009; Liu *et al*, 2009). Among

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them, PD-L1, a coinhibitory factor that is expressed on many types of cancer cells, showed the closest relation to unfavourable prognosis in ovarian cancer (Hamanishi *et al*, 2011). By binding to its receptor, PD-1 on lymphocytes, PD-L1 transmits a signal that inhibits lymphocyte activation (Freeman *et al*, 2000). Efficacy of anti-PD-1 antibodies in multiple cancer types is reported (Topalian *et al*, 2012), and clinical trials in ovarian cancer cases are in process.

Interferon-γ is a cytokine that is critical for innate and adaptive immunity. Once antigen-specific immunity develops, IFN-γ is secreted by activated effector T cells (Dunn *et al*, 2006). Interferon-γ upregulates MHC class I and class II molecules and promotes antigen presentation on tumour cells (Freedman *et al*, 2000). By these functions, IFN-γ was expected to work as an antitumour agent. Nevertheless, in a clinical trial, tumour progression was promoted upon administration of IFN-γ to ovarian cancer patients (Alberts *et al*, 2008). The mechanisms of tumour promotion by IFN-γ remain unknown.

Interferon-γ is also known to upregulate PD-L1 expression on tumour cells (Blank *et al*, 2004). In mouse melanoma models, IFN-γ secreted from CD8-positive T cells was reported to upregulate PD-L1 (Spranger *et al*, 2013). Similarly, in mouse ovarian cancer peritoneal dissemination models, we have shown that IFN-γ secreted from lymphocytes induces PD-L1 on tumour cells (Abiko *et al*, 2013). However, in clinical samples of ovarian cancer, especially in peritoneal dissemination, the association between PD-L1 expression and IFN-γ has not been clarified so far.

In the present study, using clinical samples of ovarian cancer including peritoneal dissemination, we analyse the correlation between CD8-positive lymphocytes and PD-L1 expression on ovarian cancer cells. Furthermore, the relationship between IFN-γ status and number of infiltrating lymphocytes is assessed. Then, using ovarian cancer mouse models, we investigate the role of IFN-γ in the microenvironment of peritoneal dissemination. Our findings shed light on the relationship between PD-L1 expression and tumour microenvironment, and may provoke a discussion on biomarkers for anti-PD-1 therapy.

## MATERIALS AND METHODS

**Microarray data sets of cell lines.** We downloaded a cancer cell line expression data set from the Cancer Cell Line Encyclopedia (CCLE; http://www.broadinstitute.org/ccle) (Barretina *et al*, 2012). It consists of 990 cell lines of 24 tissue origins and includes 51 cell lines of ovarian origin. Robust multiarray average normalisation was performed using R (R: A language and environment for statistical computing; http://www.R-project.org). The cutoff line for positive expression was determined as reported previously (Yoshihara *et al*, 2013).

**Cell lines.** The OV2944-HM-1 (HM-1) mouse ovarian cancer cell line was purchased from RIKEN BioResource Center and cultured as described previously (Yamamura *et al*, 2012). The ID8 mouse ovarian cancer cell line was kindly provided by Janat-Amsbury *et al* (2006), and were maintained as described previously (Abiko *et al*, 2013). The immortalised human ovarian surface epithelial cell line, HOSE1-E7/hTERT (HOSE), was kindly provided by Dr Hironori Tashiro and was maintained as described (Maeda *et al*, 2005).

The IFNGR1 (interferon-γ receptor 1)-depleted cell lines, HM1-shIFNGR1 and ID8-shIFNGR1, were generated by lentiviral transfection of short hairpin RNAs (shRNAs) targeting IFNGR1 using Mouse GIPZ Lentiviral shRNAmir individual clone viral particles (Thermo Scientific, Waltham, MA, USA; clone ID V2LMM_76619, gene target sequence 5′-CTAATACTAACCACA-TAGA-3′). Control cell lines, HM1-control and ID8-control, were

generated by transfecting a non-silencing, control shRNA (Thermo Scientific; clone ID V12070603). The PD-L1-overexpressing cell lines, HM1-pdl1, PD-L1-depleted cell line, HM1-Mirpdl1, and the control cell line, HM1-Mircontrol, were generated and cultured as described previously (Abiko *et al*, 2013).

**Flow cytometric analysis of PD-L1 expression on tumour cells in patient ascites.** Under the approval of the Institutional Ethical Committee and with written informed consent from each patient, ascites from ovarian cancer patients were collected during their initial operations. After brief centrifugation, red blood cells were lysed, and the remaining cells were washed two times and incubated in RPMI 1640 medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% FBS ($v\,v^{-1}$; Biowest, Nuaille, France) with or without adding $20\,ng\,ml^{-1}$ recombinant human IFN-γ (R&D Systems, Minneapolis, MN, USA) to the culture medium for 24 h before analysis. At analysis, ascite cells were incubated with fluorescein isothiocyanate (FITC)-conjugated anti-EpCAM (epithelial cell adhesion molecule; CD326) antibody (clone 9C4; BioLegend, San Diego, CA, USA) and phycoerythrin-conjugated anti-PD-L1 antibody (cloneMIH1; BD Biosciences, San Jose, CA, USA) or a matched isotype control (BD Biosciences) at 4 °C for 30 min, washed two times and analysed using a FACSCalibur cytometer (Beckton Dickinson, San Jose, CA, USA) and CellQuest Pro software (Beckton Dickinson). 7-Amino-actinomycin D (AAD) Staining Solution (BD Biosciences) was added 10 min before analysis to gate out nonviable cells. EpCAM-positive and 7-AAD-negative gated cells were analysed as tumour cells, as reported previously (Kitayama *et al*, 2014).

**Immunohistochemistry for ovarian cancer cases.** Under the approval of the Institutional Ethical Committee, written informed consent was obtained from the patients, and surgical specimens from 27 ovarian cancer cases who underwent an initial operation in Kyoto University Hospital between 1996 and 2008 were obtained for immunohistochemical analysis. The clinical characteristics of the 27 cases are shown in Supplementary Table S1. All 27 cases were advanced ovarian cancer cases accompanied by peritoneal dissemination to the omentum or pelvic peritoneum. Formalin-fixed, paraffin-embedded blocks from primary ovarian tumours and peritoneal disseminated sites were sliced into 4-μm-thick sections and used for immunohistochemistry.

Immunohistochemical staining for human PD-L1 was performed as described previously (Hamanishi *et al*, 2007). Two independent gynaecologic pathologists, who had no prior information regarding the clinical history of the patients, analysed the PD-L1 expression. The samples were evaluated according to the intensity of the staining and scored as follows: 0, negative; 1, very weak expression; 2, moderate expression but weaker than the positive control; and 3, equivalent to or stronger than the positive control. Placenta was used as a positive control. Samples with heterogeneous staining were scored by the intensity of the staining in the largest area. Samples with staining in < 50% of tumour cells was considered negative.

Immunohistochemical staining for CD8 (clone C8/144B; Nichirei, Tokyo, Japan) and CD4 (clone 1F6; Nichirei) was performed (Hamanishi *et al*, 2007, 2011) and evaluated as described previously (Barnett *et al*, 2010).

**Microarray profiling of ovarian cancer tissues.** Ovarian cancer specimens were newly obtained from 12 patients, who underwent primary surgery for ovarian cancer at Kyoto University Hospital between 2010 and 2013 and served for microarray analysis. These microarray data (deposited in NCBI's Gene Expression Omnibus, accession number GSE55512, http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55512) was added to 63 patients' data from our previously reported data set of 64 ovarian cancer patients, KOV-MA-64 (GEO accession GSE39204) (Abiko *et al*, 2013), and

termed KOV-75. One case in KOV-MA-64 was excluded because we could not obtain slides for immunohistochemistry. The patient characteristics of KOV-75 are listed in Supplementary Table S2.

HOSE cells were incubated in eight separate culture dishes, four dishes with and four dishes without $500\,IU\,ml^{-1}$ recombinant human IFN-γ (R&D Systems) in the culture medium for 6 h before the analysis. The cells were then harvested, and total RNA was extracted using the RNAeasy Kit (Qiagen, Hilden, Germany) and analysed using the Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA). The results were deposited in Gene Expression Omnibus, and are accessible through GEO series accession number GSE55510 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE55510). A Student's $t$-test was performed between the IFN-γ-added and IFN-γ-free groups, and probes showing an expression value $> 5.0$ in at least one of the samples, a fold change of over 2 and a $P$-value $< 0.001$ were selected as 'IFN-γ signature genes (295 probes)'. A publicly accessible microarray data set of IFN-treated cells (GEO accession GSE3920) was downloaded (Indraccolo *et al*, 2007). Single sample gene set enrichment analysis (ssGSEA) (Barbie *et al*, 2009) for 'IFN-γ signature' in GSE3920 and KOV-75 was performed using R, as reported previously (Kawasaki *et al*, 2014). Briefly, R code for ssGSEA was downloaded from Genepattern (http://www.broadinstitute.org/cancer/software/genepattern/), and gmt file for IFN-γ signature gene symbols and gct file for KOV75 were used as input files and 'ssGSEA-Projection.Library.R' and 'common R' were calculated using R.

**Animals.** Female B6C3F1 and C57BL/6 mice were purchased from CLEA Japan (Tokyo, Japan). All 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 IFNGR1 on the survival and progression of peritoneal dissemination, HM-1 cells $(1 \times 10^6)$ were injected into the abdominal cavities of syngeneic mice. Mice were killed before reaching the moribund state. To evaluate the effect of IFN-γ on tumour growth, HM-1 $(1 \times 10^6)$ or ID8 cells $(5 \times 10^6)$ were injected subcutaneously into the right flanks of syngeneic mice, tumour size was calculated every other day and the volume of the tumour was estimated using the following formula:

$$\text{Estimated tumour volume} = (r1 \times r2 \times r3)/2$$

**Immunohistochemistry for mouse tumours.** Mouse tumour cryosections (10-μm-thick) were fixed on a glass slide with acetone and stained with an anti-CD4 (clone H129.19; BD Biosciences) or anti-CD8 antibody (clone KT15; SeroTec, Kidlington, UK), as described previously (Hamanishi *et al*, 2010). Anti-mouse-PD-L1 antibody (R&D Systems) was used according to the same protocol.

**Western blotting analysis of mouse tumours.** Tumour tissue was lysed in RIPA buffer with a protease inhibitor cocktail (Merck Millipore, Darmstadt, Germany) and a phosphatase inhibitor cocktail (Nacalai Tesque). Protein was separated by SDS–PAGE gel and immunoblotted with anti-mouse-PD-L1 antibody (R&D Systems) or anti-GAPDH antibody (Abcam, Cambridge, UK). Specific proteins were visualised using ECL Plus Western Blotting Reagent (GE Healthcare Life Sciences, Piscataway, NJ, USA).

**Flow cytometric analysis for mouse cell line.** Cultured cells were harvested and incubated with an FITC-conjugated antibody
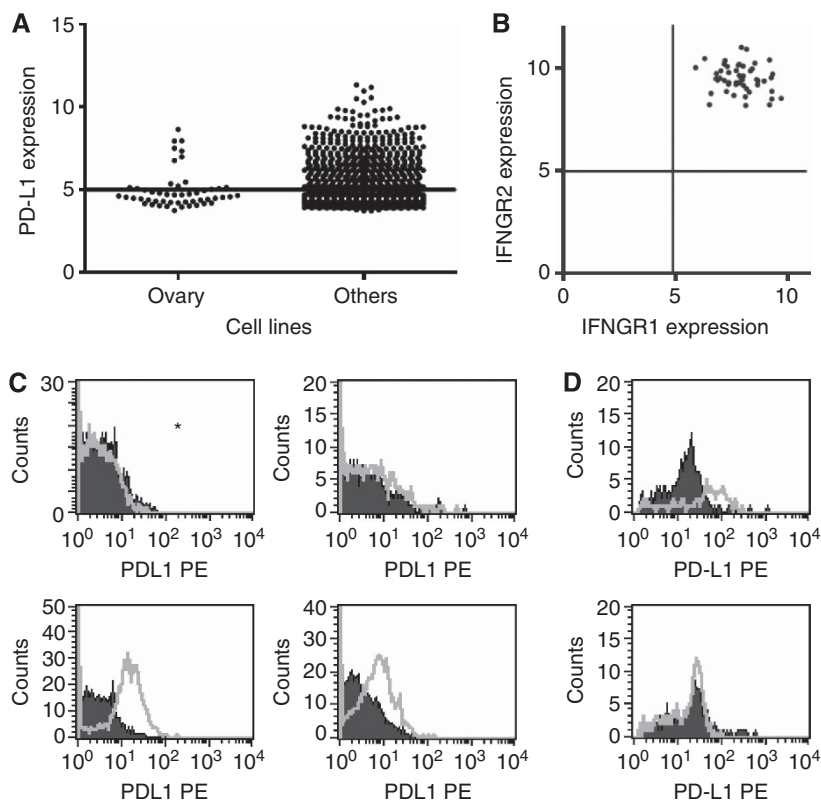


Figure 1. PD-L1 expression on ovarian cancer cells is induced by IFN-γ. (A) PD-L1 expression in cell lines from the CCLE data set. The horizontal line at value 4.950718 represents the determined cutoff value, as mentioned in the Materials and Methods section. (B) Expression of *IFNGR1* and *IFNGR2* in cell lines of ovarian origin from the CCLE data set. The horizontal and vertical lines represent the determined cutoff values. (C) PD-L1 expression in ascites tumour cells from four cases of ovarian cancer. CD326-positive and 7-AAD-negative cells were gated as viable tumour cells. Open histogram with grey line: anti-PD-L1 antibody; filled histogram: isotype control. PD-L1-positive cases (lower panels) and -negative cases (upper panels) are shown. (D) Ascites cells designated by * in (C) were incubated with (upper panel) or without (lower panel) IFN-γ for 24 h, and PD-L1 expression is shown. Open histogram with grey line: anti-PD-L1 antibody; filled histogram: isotype control.

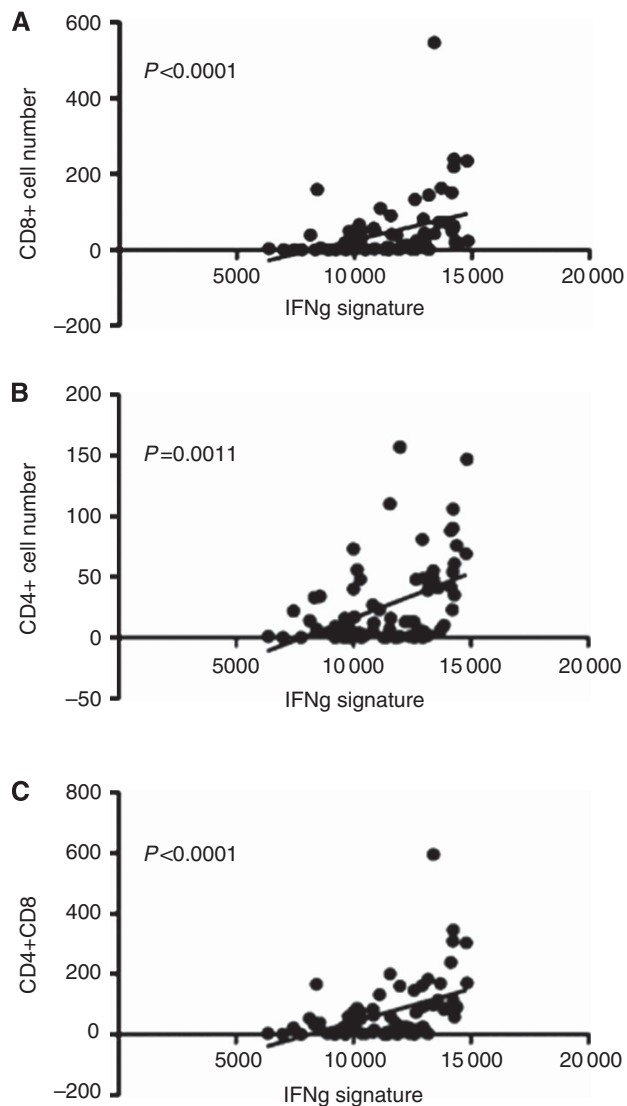**Figure 2. Interferon-γ signature score is correlated to lymphocyte infiltration in ovarian cancer clinical samples.** The IFN-γ signature score (x axis) and the number of CD8-positive (**A**), CD4-positive (**B**) or both (**C**) lymphocytes (y axis) showed a significant, positive correlation in KOV75 cases.

against mouse IFNGR1 (clone 2E2; eBioscience, San Diego, CA, USA) or a matched isotype control (eBioscience) at 4 °C for 30 min, washed two times and analysed as described above.

**Flow cytometric analysis of tumour-infiltrating lymphocytes in mice.** Mice with tumour formations were killed, and the tumours were collected. After straining through a 70-µm cell strainer (BD Biosciences), the tumour cells were washed two times and incubated with a PerCP-conjugated anti-mouse CD3e antibody (BD Biosciences), FITC-conjugated anti-mouse CD4 antibody (BD Biosciences), Alexa Fluor 647-conjugated anti-mouse CD8a antibody (BD Biosciences) or matched isotype controls. For analysis of MDSCs, cells were stained with PerCP-conjugated anti-mouse Gr-1 antibody (BD Biosciences) and APC-conjugated anti-mouse CD11b antibody (BD Biosciences) or matched isotype controls. Flow cytometric analysis was performed as described above.

**Statistics.** A paired *t*-test and Student's *t*-test were used for the immunohistochemistry analysis of human samples and mice tumours. Survival was analysed using the Kaplan–Meier survival analysis with the log-rank test using the GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). The one-way ANOVA test was used to analyse tumour volume in mouse subcutaneous tumours. A *P*-value < 0.05 was considered significant.

## RESULTS

**The majority of unstimulated ovarian cancer cell lines does not express PD-L1, but does express IFN-γ receptors.** The expression of *PD-L1* mRNA in the CCLE data set of 990 cell lines was evaluated. As shown in Figure 1A, the average PD-L1 expression levels were relatively low in 51 ovarian cancer cell lines when compared with the cell lines of other origins. Only 16 of 51 ovarian cancer cell lines (31%) were PD-L1-positive, whereas 411 of 939 cell lines of other origins (44%) were PD-L1 positive.

However, we have reported that PD-L1 protein expression in ovarian cancer is positive in 68–69% of the cases using immunohistochemistry (Hamanishi *et al*, 2007; Abiko *et al*, 2013). As IFN-γ induced PD-L1 in many types of ovarian cancer cell lines (Abiko *et al*, 2013), we hypothesised that PD-L1 in ovarian cancer cells in patients is not expressed constitutively, but expressed transiently in response to IFN-γ in the tumour microenvironment. The IFN-γ receptor is a heterodimer that consists of two chains: IFNGR1 and IFNGR2. As it is reported that
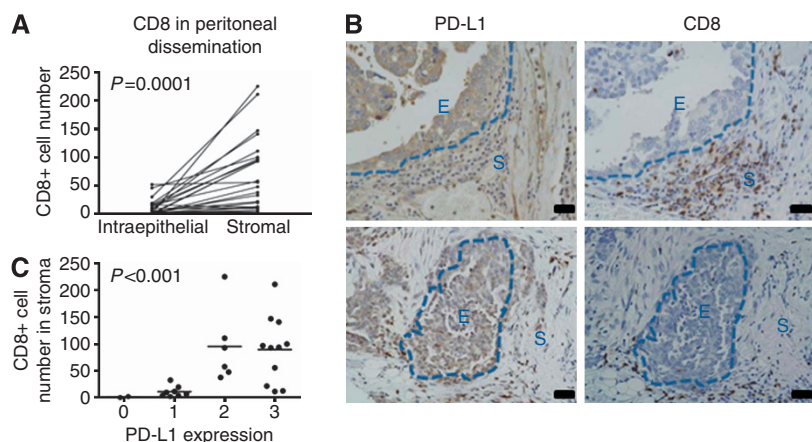


**Figure 3. Stromal CD8-positive cells are prominent in peritoneal dissemination and are related to PD-L1 expression on tumour cells in ovarian cancer clinical samples.** (**A**) Number of intraepithelial and stromal CD8-positive cells in peritoneal dissemination. (**B**) Immunohistochemistry for PD-L1 and CD8. Representative cases are shown. The area marked 'E' represents tumour epithelium, and the area marked 'S' represents tumour stroma. Note the abundant stromal infiltration of CD8-positive cells, and dense PD-L1 staining on both cancer cells and immune cells. Bars = 50 µm. (**C**) PD-L1 expression level and stromal CD8-positive cell number in a peritoneal dissemination sample. PD-L1 staining score 0–1 *vs* 2–3; *P* < 0.001.

some ovarian cancer cells lack protein expression of IFNGR1 (Duncan *et al*, 2007), we then evaluated the expression of *IFNGR1* and *IFNGR2* mRNA in the same data set. As shown in Figure 1B, all 51 ovarian cancer cell lines expressed *IFNGR1* and *IFNGR2*. Thus, the majority of ovarian cancer cell lines does not express *PD-L1* without induction, but does express two subunits of the IFN-γ receptor.

**IFN-γ induces PD-L1 on ovarian cancer cells.** It is known that IFN-γ induces PD-L1 on many cell types *in vitro* (Muhlbauer *et al*, 2006; Waeckerle-Men *et al*, 2007) including ovarian cancer cell lines (Abiko *et al*, 2013). To show that IFN-γ can also induce PD-L1 on tumour cells in ovarian cancer patients, we performed flow cytometric assays on ovarian cancer patient ascites cells. Two of four analysed cases were positive for PD-L1 expression on tumour cells (Figure 1C). When cells from the PD-L1-negative case (*) were incubated with IFN-γ, the PD-L1 expression on tumour cells was markedly induced (Figure 1D).

**The IFN-γ signature score is high in samples with high CD4- and CD8-positive lymphocyte infiltration.** To analyse the IFN-γ status in ovarian cancer clinical samples and because immuno-histochemistry for IFN-γ is technically difficult, we generated an IFN-γ signature using microarray data to evaluate the amount of IFN-γ in each case. A set of 295 probes targeting 194 known gene and 17 unknown gene sites were significantly upregulated by IFN-γ and was named the 'IFN-γ signature' (Supplementary Table S3). To validate the IFN-γ signature, we applied this signature using ssGSEA to a publicly accessible gene data set, GSE3920, and found that IFN-γ signature score was high in IFN-treated cells (Supplementary Figure S1).

We then applied IFN-γ signature to KOV-75 microarray data, which included 75 ovarian cancer clinical samples, by means of ssGSEA and obtained IFNγ signature score for each sample. Immunohistochemistry for CD4 and CD8 was performed, and the CD4- and CD8-positive cell number was counted (Supplementary Figure S2A and Supplementary Table S4). Strikingly, the IFN-γ signature score was strongly correlated to the number of infiltrating CD8- or CD4-positive lymphocytes, or the sum of them ($P < 0.0001$, $= 0.0011$ and $< 0.0001$, respectively; Figures 2A–C). These data suggest that the IFN-γ in the ovarian cancer microenvironment is derived from lymphocytes, and an IFN-γ-rich microenvironment is strongly correlated to a lymphocyte-rich microenvironment.

**The stromal CD8-positive cell number is high in peritoneally disseminated ovarian cancer and is correlated with PD-L1 expression.** Immunohistochemical analysis for CD8 was performed in paired surgical specimens of primary ovarian tumour and peritoneal dissemination from 27 ovarian cancer cases. In both primary ovarian and peritoneally disseminated tumours, CD8-positive cell numbers tended to be higher in the cancer stroma than in the tumour epithelium ($P = 0.09$ and 0.0001, respectively; Supplementary Figure S2B and Figure 3A). Furthermore, the number of CD8-positive cells in the cancer stroma was significantly high in the peritoneal dissemination samples compared with the primary ovarian tumour samples ($P = 0.004$; Supplementary Figure S2C), whereas the CD8-positive cell number in the cancer epithelium was not significantly different between the peritoneal dissemination and primary ovarian tumour samples (Supplementary Figure S2D).
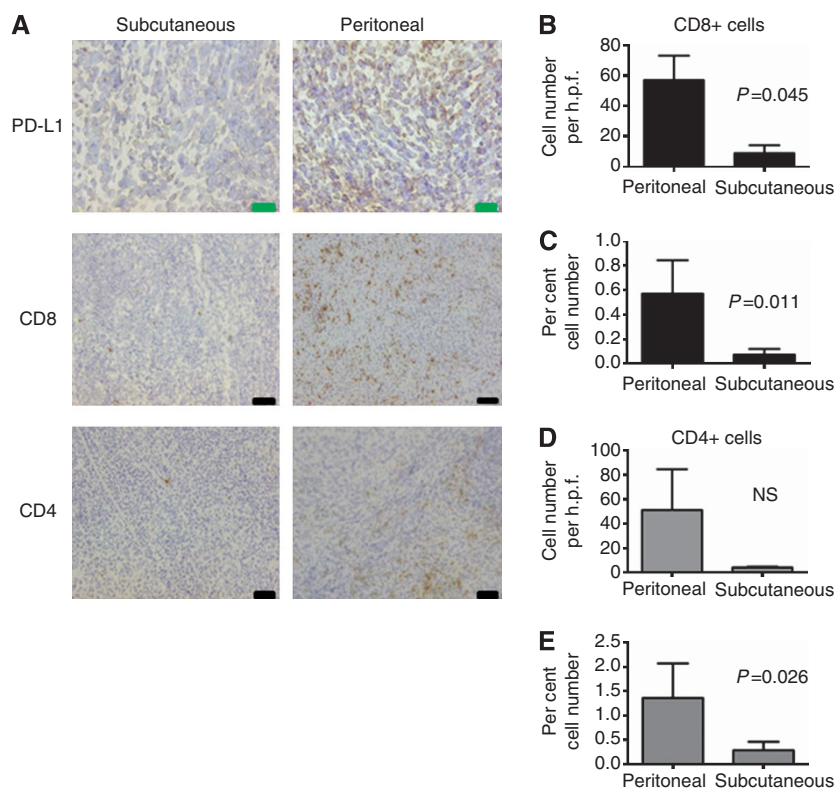


Figure 4. PD-L1 expression and lymphocyte infiltration vary between mouse subcutaneous and peritoneal tumours. (**A**) Representative images of immunohistochemistry for PD-L1 and CD8 for subcutaneous and peritoneal disseminated tumours of HM-1. Green bars: 25 μm; black bars: 50 μm. (**B**) CD8-positive cell number per high-power field (HPF) using immunohistochemistry (*n* = 3). (**C**) The percentage of CD8-positive cells in the total cells of tumour tissue analysed by flow cytometry (*n* = 4). (**D**) CD4-positive cell number per high-power field using immunohistochemistry (*n* = 3). (**E**) The percentage of CD4-positive cells in the total cells in tumour tissue analysed using flow cytometry (*n* = 4). NS = not significant.

When serial sections from peritoneal disseminations were stained with the anti-PD-L1 antibodies (Figure 3B), the CD8-positive cell number in the stroma were strongly correlated to PD-L1 expression (PD-L1 staining score 0–1 vs 2–3, $P < 0.001$; Figure 3C and Supplementary Figure S2E).

**Subcutaneous or peritoneal mouse tumours show different levels of PD-L1 expression and lymphocyte infiltration.** As CD8-positive lymphocytes are reported to be the source of IFN-γ in cancer microenvironment (Kooi et al, 1993; Taube et al, 2012), we next attempted to investigate the relationship between PD-L1 expression on tumour cells and the tumour microenvironment by using two types of mouse models, focusing on IFN-γ in the tumour microenvironment. The mouse ovarian cancer cell line, HM-1, forms subcutaneous tumours or peritoneally disseminated tumours after injection into syngeneic immune-competent B6C3F1 mice. Peritoneally disseminated tumour cells strongly expressed PD-L1, whereas subcutaneous tumours showed low PD-L1 expression (Figure 4A). Tumour-infiltrating CD8-positive cells were scarce in subcutaneous tumours, whereas they were prominent in peritone-ally disseminated tumours ($P = 0.045$; Figure 4B). Strong infiltra-tion of CD8-positive cells in peritoneally disseminated tumours was also confirmed using flow cytometry ($P = 0.011$; Figure 4C). Although the difference in CD4-positive cell count between subcutaneous and peritoneally disseminated tumours did not reach statistical significance using immunohistochemistry (Figure 4D), flow cytometric analysis showed significant difference between the two groups ($P = 0.026$; Figure 4E).

**IFN-γ signal blockade in tumour cells resulted in lower PD-L1 expression and longer mouse survival in a mouse peritoneal dissemination model.** To examine the effects of IFN-γ on tumour cells, we established an IFNGR1 knockdown cell line, HM1-shIFNGR1, using shRNA. Stable depletion of the IFNGR1 protein was confirmed using flow cytometry (Figure 5A). After injection into the mouse peritoneal cavity, both HM1-shIFNGR1 and HM1-control formed peritoneally disseminated tumours, but the expression of PD-L1 was weaker in HM1-shIFNGR1 cells (Figures 5B and C). The number of tumour-infiltrating CD8-positive lymphocytes was significantly larger in HM1-shIFNGR1 group (Figure 5D). Dissemi-nation occurred earlier and was prominent in the HM1-control group on day 19, whereas it was macroscopically invisible in the HM1-shIFNGR1 group (Figure 6A). Mouse survival was significantly longer in the HM1-shIFNGR1 group ($P = 0.021$; Figure 6B), which indicated that the IFN-γ signal associated with PD-L1 upregulation accelerated tumour progression of ovarian cancer cells in the peritoneal cavity.

**IFN-γ induces PD-L1 expression on tumour cells and facilitates tumour growth in a mouse subcutaneous model.** To further examine the effects of IFN-γ on tumour cells, we administered IFN-γ to subcutaneous tumours in the mice using two previously established cell lines, HM1-Mirpdl1 (PD-L1-depleted HM-1) or HM1-Mircontrol. The protocol scheme is shown in Supplementary Figure S3A. PD-L1 expression was induced by IFN-γ (Supplementary Figure S3B) and tumour growth was significantly accelerated by IFN-γ in HM1-Mircontrol subcutaneous tumours ($P < 0.05$; Supplementary Figure S3C). On the other hand, HM1-Mirpdl1 IFN-γ-injected tumours showed growth that was similar to that of
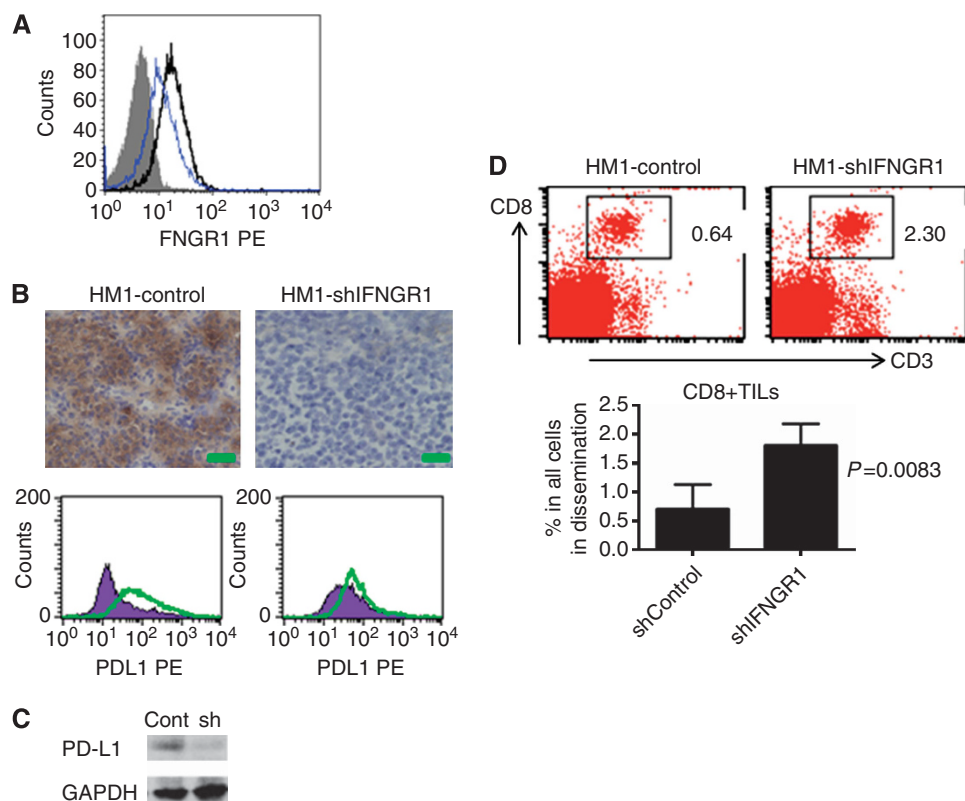


Figure 5. Blocking the IFN-γ signal in the mouse peritoneal dissemination model resulted in lower PD-L1 expression and more TILs. (A) IFNGR1 expression on HM-1 cells was successfully depleted by shRNA. HM1-control (black line histogram), HM1-shIFNGR1 (blue line histogram) and matched isotype control (shaded histogram). (B) Upper panels: Immunohistochemistry for PD-L1 in mouse peritoneal disseminated tumours. Green bars: 25 µm. Lower panels: Expression of PD-L1 on tumour cells. Green line histogram: anti-PD-L1 antibody; filled histogram: isotype control. Representative data from four mice per group with similar results. (C) Western blotting analysis for mouse peritoneally disseminated tumours. Cont = HM1-control; sh = HM1-shIFNGR1. (D) CD8-positive cells in mouse peritoneally disseminated tumour. Upper panel: representative dot plots. The percentage of CD3-positive, CD8-positive cells in total tumour cells are shown in the box. Lower panel: bar graph ($n = 4$). GAPDH = glyceraldehyde 3-phosphate dehydrogenase.

PBS-injected tumours, indicating that tumour growth caused by IFN-γ was completely abrogated by knockdown of PD-L1 (Supplementary Figure S3C). We repeated this procedure in another mouse cell line, ID8. Although the ID8 subcutaneous tumours were smaller and the difference between groups did not reach statistical significance, the results were comparable, with the ID8-control with IFN-γ administration presenting the largest tumour size (Supplementary Figure S3D).

**PD-L1 attenuates CD8-positive lymphocytes infiltration into the tumour.** To investigate the effect of PD-L1 on host immunity, we compared the tumour-infiltrating immune cells in peritoneal disseminated tumour of HM1-pdl1 (PD-L1-overexpressing HM-1) and in that of HM1-Mirpdl1. As shown in Supplementary Figure S4A, the number of CD8-positive tumour-infiltrating lymphocytes (TILs) was significantly larger in HM1-Mirpdl1, indicating that PD-L1 reduces the absolute number of infiltration of the CD8-positive TILs. On the other hand, the number of CD4-positive TILs and MDSCs was similar in both groups (Supplementary Figures S4B and C).

## DISCUSSION

We previously reported that PD-L1 expression is positive in nearly 70% of ovarian cancer cases (Hamanishi *et al*, 2007), and is significantly related to positive malignant cells in ascites and unfavourable prognosis (Abiko *et al*, 2013). We also reported that PD-L1 can be induced by IFN-γ secreted from CTLs in mouse models (Abiko *et al*, 2013). Although PD-L1 was not expressed in most of the ovarian cancer cell lines, ovarian cancer cells in patient ascites expressed PD-L1 in response to IFN-γ, and the IFN-γ signature score was closely related to lymphocyte infiltration into the tumour in clinical samples (Figure 1). These results are compatible with the report in melanoma (Taube *et al*, 2012; Spranger *et al*, 2013), which indicates that IFN-γ derived from CD8-positive T lymphocytes induces PD-L1 *in vivo*.

It was reported that a high number of intraepithelial CTLs in ovarian cancer is associated with favourable prognosis (Hwang *et al*, 2012), but few reports concerning the stromal CTL number have been published. Here we reported for the first time that the number of CTLs in cancer stroma was very high in peritoneal dissemination, and was strongly correlated to PD-L1 expression in peritoneally disseminated tumour cells (Figure 2). Abundant lymphocytes are originally present in the omentum, and they may eventually distribute into the stroma of disseminated tumours. Interestingly, intraepithelial CTL number was low in both primary tumour and peritoneal dissemination (Supplementary Figure S2E). As PD-L1 on tumour cells decreases CD8 + lymphocyte infiltration (Supplementary Figure S4A), PD-L1 on tumour cells, induced by IFN-γ from CTLs, may expel CTLs from the tumour epithelium to the stroma, especially in peritoneal dissemination. Although PD-L1 is known to suppress cytotoxic activity of CTLs (Iwai *et al*, 2002; Hirano *et al*, 2005; Abiko *et al*, 2013), function of TILs was similar regardless of different expression status of PD-L1 on tumour cells (data not shown), probably because TIL function is inevitably suppressed by various mechanisms other than PD-L1 expression (Walunas *et al*, 1996; Curiel *et al*, 2004; Gabrilovich and Nagaraj, 2009; Lob *et al*, 2009).

The biological function of IFN-γ includes increasing antigen presentation and promoting Th1 differentiation, leading to cellular immunity (Dunn *et al*, 2006). Therefore, IFN-γ has been expected to possess antitumour effects in cancer patients. Nevertheless, upon administration of IFN-γ to ovarian cancer patients, the opposite results were observed in two phase III clinical trials (Windbichler *et al*, 2000; Alberts *et al*, 2008); tumour progression was promoted when IFN-γ was combined with standard chemotherapy (Alberts
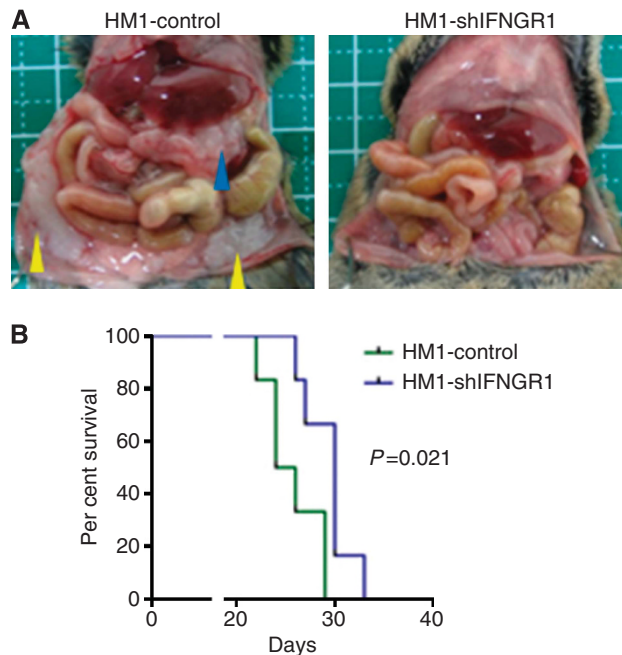


Figure 6. Blocking the IFN-γ signal in the mouse peritoneal dissemination model resulted in slower tumour progression and longer survival. (**A**) Photograph of mouse peritoneum from HM1-control- or HM1-shIFNGR1-injected group on day 19. Yellow arrowheads: peritoneally disseminated tumours on peritoneum; blue arrowheads: disseminated tumours on omentum. (**B**) Survival of HM1-shIFNGR1-injected mice (blue line) and HM1-control-injected mice (green line) (n = 6).

*et al*, 2008). Recent studies have shown that IFN-γ seems to have two faces, one good and one bad: immunoactivating/immuno-regulatory and antitumour/tumour-promoting (Zaidi and Merlino, 2011). One indirect tumour-promoting mechanism of IFN-γ was described as the tumour-immunoediting theory (Dunn *et al*, 2002, 2006), in which IFN-γ works through selective pressure and forces the evolution of tumour cells to IFN-γ-resistant phenotypes. However, no direct mechanism to explain the tumour-promoting effect of IFN-γ was identified. To explain the tumour-promoting effect of IFN-γ, we hypothesised that induction of PD-L1 by IFN-γ overwhelms the antitumour effects of IFN-γ. To prove our hypothesis, we conducted animal experiments to describe tumours grown in different microenvironment. The subcutaneous model refers to a tumour grown in a microenvironment with a smaller number of CTL infiltration, and the peritoneal dissemination model refers to a microenvironment with a larger amount of CTL infiltration, as is the case in many cases of human ovarian cancer peritoneal dissemination. IFN-γ signal inhibition resulted in lower PD-L1 expression in the tumour cells and prolonged survival in the peritoneal dissemination model (Figures 5 and 6). IFN-γ administration resulted in higher PD-L1 expression in the tumour cells and the acceleration of tumour progression in the subcutaneous model (Supplementary Figure S3). PD-L1 knockdown in tumour cells abrogated the tumour-promoting effect of IFN-γ (Supplementary Figure S3C). These data indicate that IFN-γ promotes tumour growth by inducing PD-L1 in the tumour.

The anti-PD-1 antibody, Nivolumab, shows striking effects in melanoma, lung and renal cancers (Topalian *et al*, 2012). In our ongoing clinical trial for ovarian cancer, we have also found that some patients strikingly respond to Nivolumab but others do not (Hamanishi *et al*, unpublished data). In 2012, a report suggesting that the predictive biomarker for anti-PD-1 therapy was PD-L1 expression in the tumour won attention (Topalian *et al*, 2012).

However, upon further investigation, conflicting reports followed, and now the issue is controversial (Wolchok *et al*, 2013). Our data in this study imply that PD-L1 expression on ovarian cancer cells can be altered by its microenvironment. Therefore, verifying PD-L1 expression on a single slide from one single biopsy site may not represent the immune microenvironment of the entire tumour and, thus, may not be sufficient for predicting the efficacy of Nivolumab in the entire patient. Instead, indicators such as IFN-γ signature or lymphocyte infiltration, which reflect the IFN-γ status in the tumour microenvironment may serve as potential biomarkers. Further investigation regarding these markers and response to anti-PD-1 therapy in clinical trial is required.

In conclusion, stromal CTLs in ovarian cancer peritoneal dissemination are strongly affecting antitumour immunity through PD-L1 expression. IFN-γ from stromal CTLs induces PD-L1 and promotes tumour progression in ovarian cancer microenvironment. Our results indicate that PD-L1 expression on ovarian cancer cells varies according to the microenvironment. Lymphocyte infiltration and the IFN-γ status may be the keys for effective immunotherapy, including anti-PD-1 therapy.

## ACKNOWLEDGEMENTS

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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