Received: 4 August 2017 | Revised: 2 October 2017 | Accepted: 9 October 2017

DOI: 10.1111/cas.13424

ORIGINAL ARTICLE

WILEY Cancer Science

PD-L1 expression is mainly regulated by interferon gamma associated with JAK-STAT pathway in gastric cancer

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Despite multidisciplinary treatment for patients with advanced gastric cancer, their prognosis remains poor. Therefore, the development of novel therapeutic strategies is urgently needed, and immunotherapy utilizing anti-programmed death 1/-programmed death ligand-1 mAb is an attractive approach. However, as there is limited information on how programmed death ligand-1 is upregulated on tumor cells within the tumor microenvironment, we examined the mechanism of programmed death ligand-1 regulation with a particular focus on interferon gamma in an in vitro setting and in clinical samples. Our in vitro findings showed that interferon gamma upregulated programmed death ligand-1 expression on solid tumor cells through the JAK-signal transducer and activator of transcription pathway, and impaired the cytotoxicity of tumor antigen-specific CTL against tumor cells. Following treatment of cells with anti-programmed death ligand-1 mAb after interferon gamma-pre-treatment, the reduced anti-tumor CTL activity by interferon gamma reached a higher level than the non-treatment control targets. In contrast, programmed death ligand-1 expression on tumor cells also significantly correlated with epithelial-mesenchymal transition phenotype in a panel of solid tumor cells. In clinical gastric cancer samples, tumor membrane programmed death ligand-1 expression significantly positively correlated with the presence of CD8-positive T cells in the stroma and interferon gamma expression in the tumor. The results suggest that gastric cancer patients with high CD8-positive T-cell infiltration may be more responsive to anti-programmed death 1/-programmed death ligand-1 mAb therapy.

KEYWORDS
CD8, gastric cancer, IFN-γ, PD-L1, TILs

Kousaku Mimura and Jun Liang Teh contributed equally to this study.

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Funding information
National Research Foundation Singapore; Singapore Ministry of Education; Clinician Scientist-Individual Research Grant, Grant/Award Number: NMRC/CIRG/1364/2013; Clinician Scientist Award, Grant/Award Number: NMRC/CSA/0043/2012

1 | INTRODUCTION

Programmed death ligand-1 (PD-L1) is a B7 protein family member that interacts with programmed death 1 (PD-1) expressed on activated T cells, B cells and natural killer cells.[1,2] Activation of the PD-1/PD-L1 pathway leads T cells to apoptosis and anergy, resulting in downregulation of anti-tumor responses of T cells.[3,4] Marked expression of PD-L1 has been reported in various types of human cancer and tumor-infiltrating immune cells.[5-7] Indeed, we and others have recently demonstrated that PD-L1 expression on tumors inhibits the effector phase of CTL function, and blockade of PD-L1 can augment the tumor-specific CTL response in vitro.[3,4,8-10] Moreover, several clinical trials on the inhibition of PD-1/PD-L1 interaction with therapeutic mAb induced significant and durable responses in several types of refractory tumors,[11-15] suggesting that the PD-1 and PD-L1 interaction might have a pivotal role in anti-tumor immunity.

Programmed death ligand-1 on tumor cells is often upregulated within the tumor microenvironment.[16,17] Several mechanisms for the upregulation of PD-L1 on tumor cells, which include epigenetic factors, oncogenic signaling and acquired immune responses, have been proposed.[18] Constitutive oncogenic signaling has been reported to induce PD-L1 expression on tumor cells either through the phosphatidylinositol-3-kinase-protein kinase B (PI3K-AKT) pathway or signal transducer and activator of transcription (STAT) 3 signaling.[19,20] Acquired immune response is also considered to manifest through PD-L1 upregulation on tumor cells by endogenous anti-tumor immunity factors in the tumor microenvironment, such as interferon gamma (IFN-γ) produced by tumor infiltrating lymphocytes (TIL).[21] Furthermore, it has recently been shown that the microRNA-200/zinc-finger E-box-binding homeobox 1 (ZEB-1) axis, which is closely related to the epithelial-mesenchymal transition (EMT) conversion, could regulate PD-L1 expression.[22] However, there is still limited information on how PD-L1 on tumor cells is upregulated in the tumor microenvironment, especially in the human tumor system.

Gastric cancer (GC) is the fourth most common malignancy in the world and second leading cause of cancer death worldwide, accounting for 723 000 deaths annually.[23] Despite various treatments such as surgical resection combined with chemotherapy, the prognosis for patients with advanced GC remains poor. The 5-year overall survival rates of patients with pathological stage IV disease who underwent resection between 2001 and 2007 is 16.4% in Japan.[24] Previous studies have reported that PD-L1 is expressed in 42% of GC tissue, and is correlated with tumor size, invasion, lymph node status and patient survival.[25] In a clinical trial using the anti-PD-1 mAb, pembrolizumab, for GC patients who were refractory to standard chemotherapy, an overall response of 22% and 6-month progression free survival of 26% were reported by Muro et al.[26] Utilization of immunotherapy with anti-PD-1/-PD-L1 mAb is, therefore, an attractive approach for GC.

In this study, we examined the mechanisms of PD-L1 regulation with a particular focus on IFN-γ, and how PD-L1 is related to tumor-specific CTL function using a panel of human solid tumors. Furthermore, we evaluated in detail the relationship between PD-L1 expression on tumor cells and types of TIL by immunohistochemistry (IHC) in surgically resected specimens of GC.

2 | MATERIALS AND METHODS

2.1 | Tumor cell lines

The 30 cell lines used in this study are summarized in Table S1. All cell lines except for HEK293T were cultured in RPMI-1640 containing L-glutamine (Invitrogen, Carlsbad, CA, USA) with 5% FCS (Invitrogen) and 1% Penicillin-Streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The HEK293T cell line was cultured in DMEM (Invitrogen) with 10% FCS (Invitrogen) and 1% Penicillin-Streptomycin (Sigma-Aldrich). All cell lines were verified as authentic through short tandem repeat DNA profile analysis.

2.2 | Clinical samples

This study examined samples in tissue arrays of intestinal and diffuse GC tissues constructed and analyzed previously.[27] The tissue arrays spanned four tissue blocks with 1 mm in diameter cores of primary gastric tumors from 150 patients who underwent surgery for GC at the National University Hospital of Singapore between 2000 and 2008. All GC staging was performed in accordance with the latest American Joint Committee on Cancer (7th edition). This study was approved by the Domain Specific Review Board of the National Healthcare Group of Singapore (Reference 2015/00209).

2.3 | Cell treatment and reagents

The following (sources in parentheses) were used: IFN-γ (R&D Systems, Minneapolis, MN, USA), PD98059 (Cell Signaling Technology, Danvers, MA, USA), wortmannin (Cell Signaling Technology), lapatinib (GlaxoSmithKline, Brentford, UK) and MIH1 PD-L1 mAb (eBioscience, Santa Clara, CA, USA). DMSO (Sigma-Aldrich) was used as a vehicle and negative control for all treatments. Cells were cultured and treated in 12-well plates (Thermo Fisher Scientific, Wilmington, DE, USA).

2.4 | Flow cytometry

The cells were stained with antibodies as previously described.[28] The following antibodies were used for staining: Annexin V-FITC (Becton Dickinson, San Jose, CA, USA), 7-Aminoactinomycin D (Becton Dickinson) and anti-human CD274 (PD-L1, B7-H1) PE (clone: MIH1, eBioscience). An isotype-matched immunoglobulin served as a negative control, and staining was detected using an LSRII flow cytometer (Becton Dickinson). Dead and/or apoptotic cells were excluded using Annexin-V and 7-Aminoactinomycin D. The relative mean fluorescence intensity (rMFI) was calculated according to the formula: [(MFI with specific mAb − MFI with isotype mAb)/MFI with isotype mAb]/[(MFI with specific mAb of control treatment − MFI with isotype mAb of control treatment)/MFI with isotype mAb of control treatment].[8,28]

2.5 | Western blotting

The following were used as primary antibodies and purchased from Cell Signaling Technology: JAK2 mAb, phospho-JAK2 mAb, STAT1 mAb, phospho-STAT1 mAb, p44/42 MAPK (ERK1/2) mAb, phospho-p44/42 MAPK (ERK1/2) mAb and β-actin antibody. An HRP-linked anti-rabbit IgG (Cell Signaling Technology) was used as secondary antibody. All samples were prepared and stained with antibodies as previously described,[8] and blots were visualized by ECL Prime (Amersham Pharmacia, Uppsala, Sweden) and with a Konica SRX-101A film processor (JZ Imaging & Consulting, Willoughby, OH, USA).

2.6 | Gene expression array analysis

Total RNA was isolated using TRIZOL (Invitrogen) and assessed using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific) and Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Microarray gene expression analysis was performed using Illumina HT-12 v4 Expression BeadChips (Illumina, San Diego, CA, USA). The RNA was first biotinylated and amplified using the Illumina TotalPrep-96 RNA Amplification Kit, followed by cDNA synthesis, cDNA purification, cRNA synthesis and cRNA purification. The samples were then hybridized onto the arrays for 16 hour at 56°C. The arrays were washed and scanned using the Illumina BeadArray Reader. Data were then exported and analyzed using GenomeStudio v2011.11 software (Illumina).

An EMT score was calculated as the average expression level of mesenchymal genes minus the average expression level of epithelial genes from a 76 gene EMT signature.[22,29] The mRNA expression z-scores of the 76 genes analyzed on the Affymetrix HG-U133 Plus 2.0 platform were obtained from the Cancer Cell Line Encyclopedia database through cBioPortal (http://www.cbioportal.org/).[30,31]

2.7 | Immunohistochemistry stains

Immunohistochemistry was performed using the BOND-MAX autostainer (Leica, Wetzlar, Germany). Five-micron sections underwent automated de-paraffinization using the bake and de-wax protocol at 60°C for 30 minute followed by three rinses of the BondDewax solution at 72°C. Slides were incubated with ER1 solution for 20 minute. Slides were incubated with specific antibodies before the application of 3,3′-diaminobenzidine for 10 minute and then counterstaining for 10 minute with hematoxylin. For PD-L1, antibody incubation comprised blocking with protein for 30 minute, followed by incubation with PD-L1 antibody (clone 4059; ProSci, Loveland, CO, USA) at 1:200 dilution for 15 minute. IFN-γ antibody (ab9657; Abcam, Cambridge, UK) was applied at 1:400 dilution for 30 minute without protein blocking. IHC for CD3, CD4 and CD8 was performed using the Benchmark XT system (Ventana Medical Systems, Tucson, AZ, USA). Positive and negative controls consisted of placental and tonsil tissues stained with and without primary antibody, respectively.

2.8 | Assessment of immunohistochemistry stains

Assessment of IHC stains was performed using the Vectra slide imaging system (Perkin Elmer, Waltham, MA, USA) under the supervision of a pathologist. The system was programmed to segment tumor and stroma, as well as individual cells, including immune-shaped cells, in each compartment (Fig. S1a,b). The system was also programmed to identify tumor membrane, converting the image into a membrane score map to score the percentage of cells with tumor membrane staining at intensity levels of 0-3 (Fig. S1c). In this study, only cases that the Vectra slide imaging system could evaluate were used for analysis.

An H-score of membranous PD-L1 expression on the tumor cells was calculated by adding the products of proportions and intensity at each intensity levels of 0-3 to a maximum score of 300 (which corresponds to 100% of tumor cells positive for PD-L1 with a staining intensity score of 3).[32] Similarly, an IFN-γ H-score was calculated based on the amount of cells with cytoplasmic staining in the tumor. The CD3-, CD4- and CD8-positive cell density was defined as the percentage of immune-shaped cells staining for CD3, CD4 or CD8 of the total number of immune-shaped cells present within the tumor and stromal compartments, respectively.

2.9 | Generation of a human leukocyte antigen-A24 restricted, cancer-testis antigen-specific CTL clone

The human leukocyte antigen (HLA)-A24, LY6K (lymphocyte antigen-6 complex locus K)-peptide-specific CTL clone was established from HLA-A24-positive peripheral blood mononuclear cells from healthy donors as previously described.[8,33] Briefly, T cells were stimulated with LY6K-peptide-loaded, mitomycin C (Kyowa Hakko Kirin, Tokyo, Japan)-treated autologous mature dendritic cells every 7 days. One week after the third stimulation, the CTL lines were tested for their antigen specificity for the LY6K peptide using the ELISpot assay. A peptide-specific CTL clone was established from an HLA-A24-restricted, LY6K-peptide-specific CTL line using a limiting dilution method.

2.10 | Cytotoxicity assay

Cytotoxicity was measured using a calcein-release assay, as previously described.[8,28] Briefly, target cells were incubated with 5 μmol L^{-1} of calcein-AM (Dojindo Laboratories, Kumamoto, Japan) for 30 minute at 37°C and 5% CO_2. Stained targets (5×10^3/well) were co-cultured at various ratios of LY6K-peptide-specific CTL clones in 200 μL of culture medium for 4 hour. Assays were performed in triplicate in a 96-well U-bottomed plate. After a 4-hour incubation, 100 μL of the supernatant was transferred from the culture plate to a 96-well flat-bottomed plate and the fluorescence of each supernatant was measured at 485-nm excitation and 528-nm emission using an Infinite 200 plate reader (Tecan Group, Männedorf, Switzerland). Spontaneous release was obtained from target cells incubated without effector cells, and maximum release was obtained from detergent-released target cells. The percentage of specific lysis was calculated according to the formula: %specific lysis = 100 × (experimental release − spontaneous release)/(maximum release − spontaneous release).

2.11 | Statistics

Associations between protein expression levels was assessed using Spearman's rho test. Associations with survival of clinical and

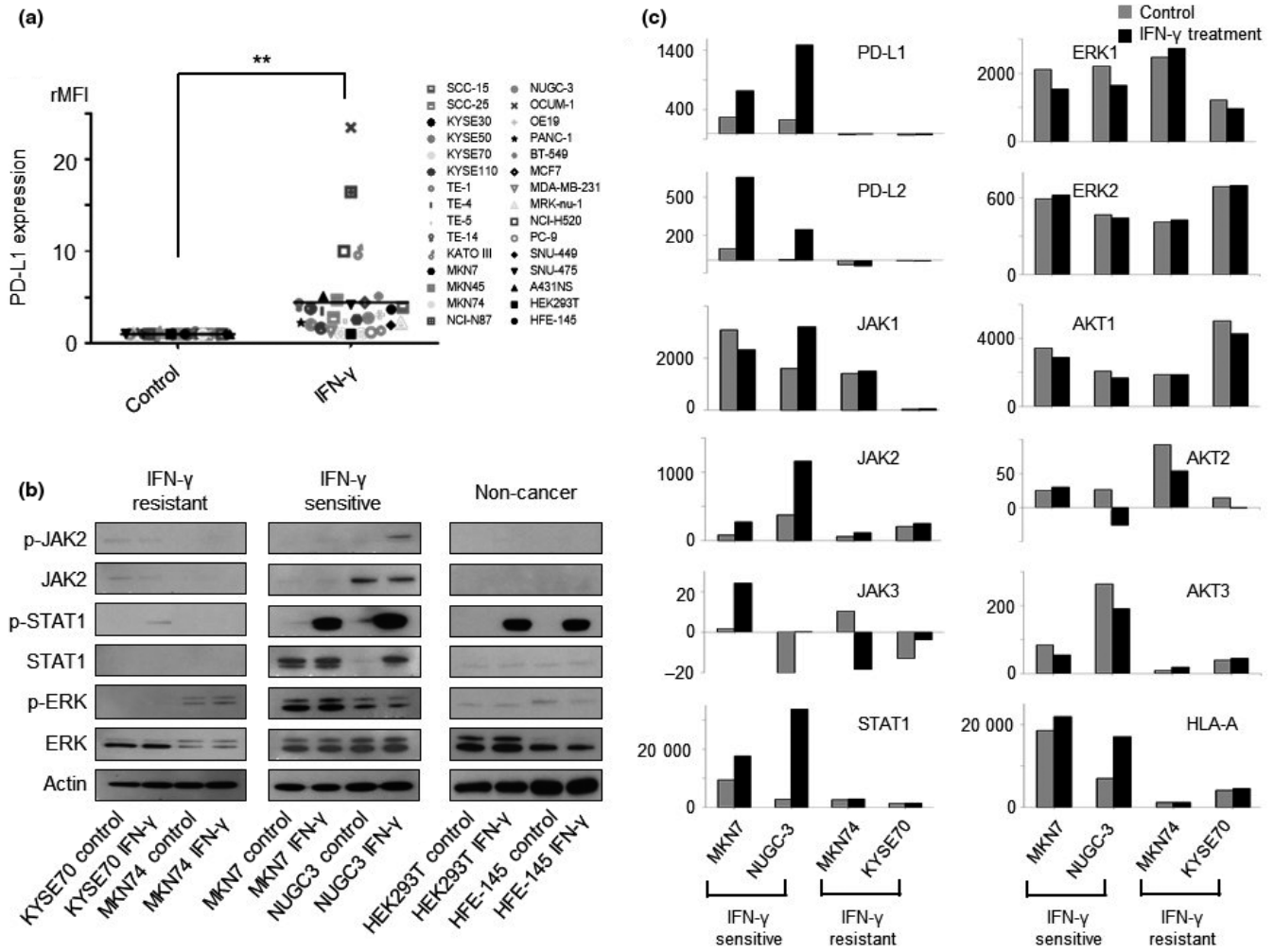


FIGURE 1 Effect of interferon gamma (IFN-γ) on programmed death ligand-1 (PD-L1) and signaling pathways. A, PD-L1 expression was measured by flow cytometry in cell lines 48 h after treatment without (control) and with 10 ng/mL IFN-γ. $^{**}P < .01$. B, Western blot analysis of relevant proteins in cell lines 1 h after treatment without (control) or with 10 ng/mL IFN-γ. Representative results out of three independent experiments are shown. C, Expression levels of relevant genes in cell lines treated without (control) or with 10 ng/mL IFN-γ. Gene expression levels are normalized to those of *ACTB*, *GAPDH*, *PPIA* and *HPRT*

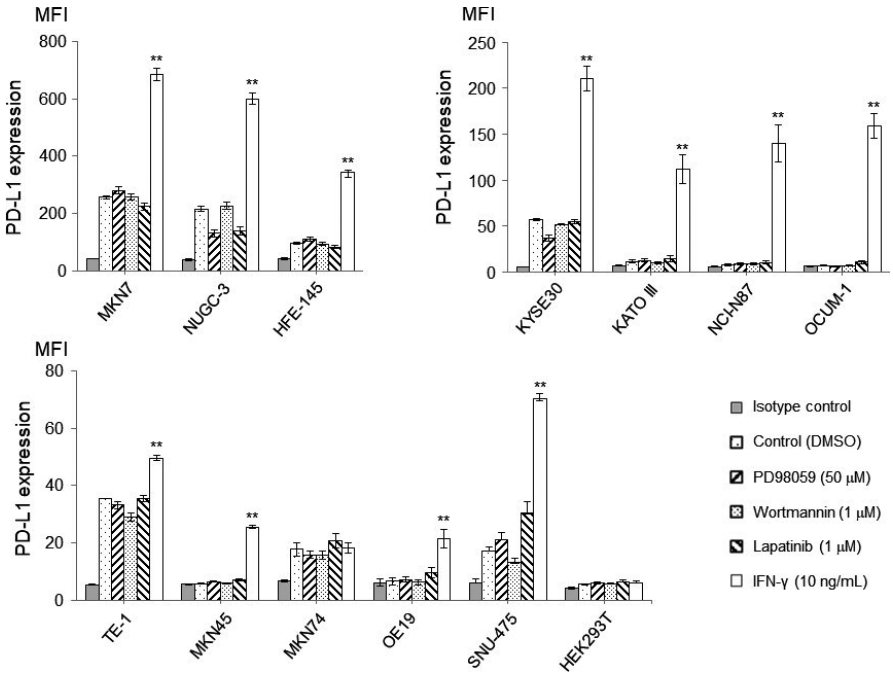


FIGURE 2 Effect of interferon gamma (IFN-γ) and kinase inhibitors on programmed death ligand-1 (PD-L1) expression. PD-L1 expression was measured by flow cytometry in cell lines 48 h after treatment with 10 ng/mL IFN-γ, 50 μmol L^{-1} PD98059 (MAPK inhibitor), 1 μmol L^{-1} wortmannin (PI3K-AKT inhibitor) and 1 μmol L^{-1} lapatinib (combined epidermal growth factor receptor/human epidermal growth factor receptor 2 tyrosine kinase inhibitor). DMSO was used as a vehicle and negative control. $^{**}P < .01$ between the treated and control cells

molecular features as continuous variables on a univariate basis, and multivariate basis using the backward conditional selection, were calculated using the Cox's proportional hazard model method. All patients included in the study were at least 5 years post-surgery with available follow-up data. Overall survival was analyzed by the Kaplan-Meier method for patients and statistical differences were analyzed by a log-rank test. To generate the survival curves for membranous PD-L1, and CD3/CD4/CD8 tumor and stroma, we used the median of the values; those lesser than the median was determined as low and those equal or above the median was determined as high. Only death from GC was considered as an event. The follow-up times of patients who were alive or who had died of other causes were censored at the date of last follow-up. Statistical significance was considered at $P < .05$. Analyses were performed using SPSS Statistics Package version 22 (IBM, Chicago, IL, USA).

3 | RESULTS

3.1 | Upregulation of programmed death ligand-1 by interferon gamma in cell lines

In this study, we used two non-cancer cell lines, HEK293 (human embryonic kidney cell line) and HFE-145 (human gastric epithelial cells), and 28 solid cancer cell lines, including 10 esophageal squamous cell carcinoma cell lines (SCC-15, SCC-25, KYSE30, KYSE50, KYSE70, KYSE110, TE-1, TE-4, TE-5 and TE-14), 8 GC cell lines (KATOIII, MKN7, MKN45, MKN74, NCI-N87, NUGC-3 and OCUM-1 OE19), 1 pancreatic cancer cell line (PANC-1), 4 breast cancer cell lines (BT-549, MCF7, MDA-MB-231 and MRK-nu-1), 2 lung cancer cell lines (NCI-H520 and PC-9), 2 hepatocellular carcinoma cell lines (SNU-449 and SNU-475) and an epidermoid carcinoma cell line (A432NS). Treatment with 10 ng/mL IFN-γ significantly increased PD-L1 expression to different degrees in 28 out of 30 cell lines at 48 hour as measured by flow cytometry (Figure 1A). PD-L1 expression was not increased in KYSE70 and MKN74 cell lines. The treatment conditions used were determined from initial titration experiments with NUGC-3 cells (Fig. S2).

3.2 | Upregulation of programmed death ligand-1 by interferon gamma is associated with the JAK-STAT but not the MAPK and PI3K-AKT pathway activation

It has been reported that IFN-γ can stimulate the MAPK pathway in addition to the JAK-STAT pathway, and the MAPK pathway was a major contributor to IFN-γ-induced overexpression of PD-L1 in malignant plasma cells and lymphoma.[34-36] Another study recently reported that oncogenic signaling induces PD-L1 expression on tumor cells through the PI3K-AKT pathway.[19,37] Therefore, we assessed the effect of IFN-γ on the JAK-STAT, MAPK and PI3K-AKT pathway using western blot and gene expression array analyses in two IFN-γ resistant (KYSE70 and MKN74) and two sensitive (MKN-7 and NUGC-3) GC cell lines, as well as two non-cancer (HEK293T and HFE-145) cell lines. Western blot analysis revealed that IFN-γ increased p-STAT1 in sensitive and non-cancer cell lines but not resistant cell lines (Figure 1B). p-JAK2 was also increased in NUGC3 IFN-γ sensitive cell lines. p-ERK levels were not altered by IFN-γ treatment in all cell lines. Gene expression array analysis showed PD-L1, PD-L2, HLA-A and the JAK-STAT pathway (JAK2 and STAT1) but not the MAPK pathway (ERK1 and ERK2) or the PI3K-AKT pathway (AKT1, AKT2, and AKT3) genes were increased by IFN-γ in the IFN-γ sensitive cell lines (Figure 1C). There was no significant change in the expression of these genes in IFN-γ resistant cell lines

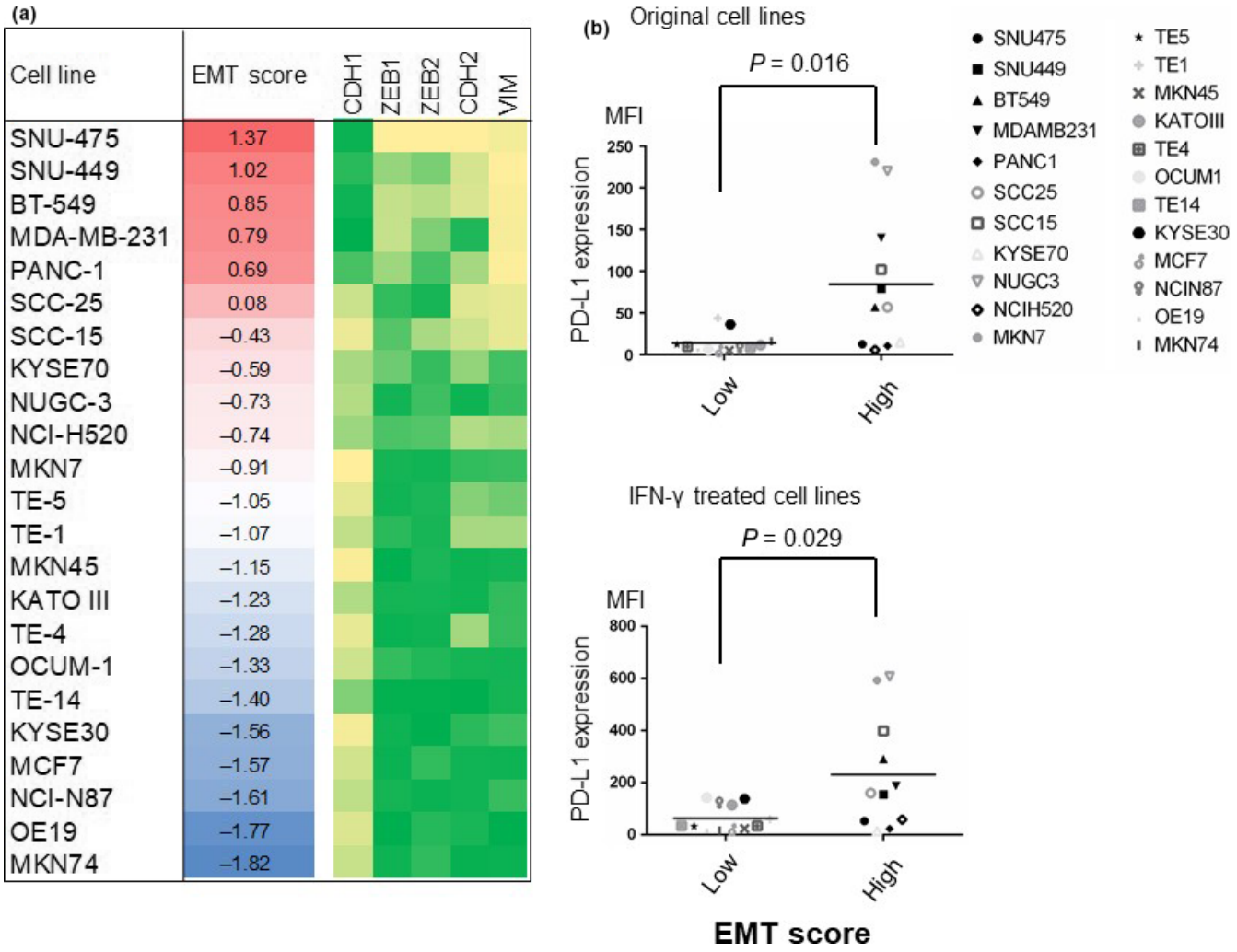


FIGURE 3 Association between epithelial-mesenchymal transition (EMT) score and PD-L1 expression in cells treated with interferon gamma (IFN-γ). A, EMT score and expression of component genes in cell lines. B, PD-L1 expression in cell lines with a high (>−1.0) and low (≤−1.0) epithelial-mesenchymal transition (EMT) score, treated without (original) and with 10 ng/mL IFN-γ

(Figure 1C). IFN-γ treatment also increased the expression of many HLA and antigen-processing machinery (APM) component genes in IFN-γ sensitive and not IFN-γ resistant cell lines (Table S2). Taken together, IFN-γ induces the upregulation of PD-L1 and PD-L2 mainly through the JAK-STAT pathway in the majority of the gastrointestinal tract cell lines.

3.3 | Upregulation of programmed death ligand-1 expression is induced by interferon gamma but not MAPK and PI3K-AKT inhibitors

To further analyze the mechanism of PD-L1 expression in solid cancer cells, we evaluated the expression of PD-L1 on cancer cells and non-cancer cells treated with IFN-γ (10 ng/mL) or MAPK inhibitor, PD98059 (50 μmol L^{-1}), or PI3K-AKT inhibitor, wortmannin (1 μmol L^{-1}), or the combined epidermal growth factor receptor/human epidermal growth factor receptor 2 tyrosine kinase inhibitor, lapatinib (1 μmol L^{-1}), by flow cytometry. The optimal conditions, including concentration and incubation time of these reagents, were already assessed in our previous study.[28] As shown in Figure 2, PD-L1 expression was consistently and significantly upregulated in all tested cell lines when treated with IFN-γ. In contrast, there was no significant alteration in PD-L1 expression when treated with PD98059 or wortmannin or lapatinib that could inhibit the MAPK and PI3K-AKT pathways (Figure 2).

3.4 | Programmed death ligand-1 expression correlates with the epithelial-mesenchymal transition phenotype

Chen et al[22] report that the microRNA-200/ZEB-1 axis can regulate PD-L1 expression. Because the microRNA-200/ZEB-1 axis has been implicated in EMT,[22] we evaluated the correlation between PD-L1 expression and an EMT score derived from the expression of 76 EMT-related genes. The EMT of the 30 cell lines in this study was calculated, and cell lines with EMT scores above and below −1.0

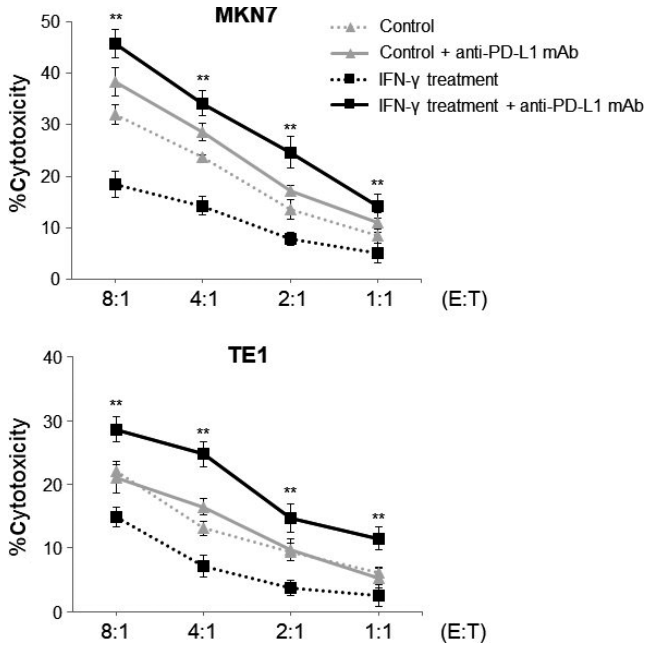


FIGURE 4 Effect of anti-programmed death ligand-1 (PD-L1) mAb treatment on CTL activity in cells treated with interferon gamma (IFN-γ). Cytotoxicity of CTL clones against MKN7 and TE1 cells treated with anti-PD-L1 mAb or isotype control for 1 h after pre-treatment with or without 10 ng/mL IFN-γ for 48 h. $^{**}P < .01$ between IFN-γ pre-treated cells treated with and without anti-PD-L1 mAb. E:T, effector:target

were classified as EMT high and low, respectively (Figure 3A). PD-L1 expression was significantly higher in EMT high compared to EMT low cell lines, in both original and IFN-γ treated cells (Figure 3B).

3.5 | Anti- programmed death ligand-1 antibody can enhance tumor-specific CTL activity

To evaluate the effect of the PD-1/PD-L1 interaction on anti-tumor CTL activity, HLA-A24-positive, LY6K-positive tumor cells, namely MKN7 and TE1,[8] were pre-treated with or without IFN-γ for 48 hour. The cells were then treated with anti-PD-L1 mAb or isotype control for 1 hour and subjected to cytotoxic assay. The specificity of the HLA-A24-restricted CTL clone to the LY6K-peptide, and treatment conditions were established previously.[8] IFN-γ-pre-treated cells showed lower susceptibility to the CTL clone as compared to the control target (Figure 4). Treatment with anti-PD-L1 mAb led to a higher cytotoxicity in cells with IFN-γ pre-treatment as compared to controls (Figure 4), consistent with a role for IFN-γ in PD-L1 and HLA-A upregulation.

3.6 | Programmed death ligand-1 expression on tumor cells significantly correlated with CD8-positive T cells in the stroma and interferon gamma positive cells in the tumor

We used IHC to evaluate the expressions of CD3, CD4, CD8, PD-L1 and IFN-γ in primary tumors from 150 patients with GC. Patients' demographics and clinical characteristics are summarized in Table 1. Mean age of the patients was 63.6 ± 11.8 years. The majority of patients (n = 128, 85%) were Chinese and 107 (71%) were male. Mean duration of follow-up was 46.2 ± 44.9 months and a total of 78 (52%) patients had died due to GC at the time of analysis. Representative patterns of expression of each molecule are shown in Figure 5A.

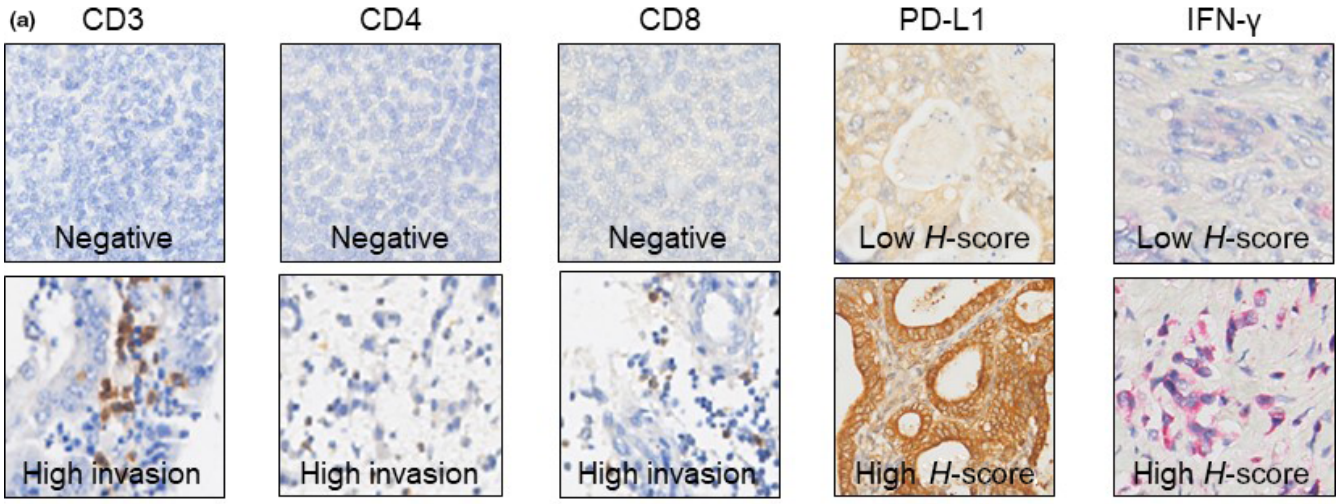
TABLE 1 Patient and tumor characteristics (n = 150)

Age (y)	
Mean	63.6 ± 11.8
Range	32-87
Gender	
Male	107
Female	43
Tumor[a]	
T1	10
T2	71
T3	57
T4	12
Lymph node metastasis[a]	
N0	48
N1	51
N2	30
N3	21
Lauren's classification	
Intestinal type	83
Diffuse type	38
Mixed type	15
Missing	14
Stage[a]	
I	39
II	28
III	47
IV	36

[a]Tumor, lymph node metastasis and stage are according to the TNM classification for gastric cancer (UICC, 7th edition).

Because there is no established H-score cutoff to define positive PD-L1, CD3, CD4 and CD8, these molecular features were analyzed as continuous variables. The H-score (mean ± SD) for PD-L1 (tumor membrane) was 53.5 ± 29.1, CD3 (tumor) was 5.8 ± 4.6, CD3 (stroma) was 22.6 ± 12.7, CD4 (tumor) was 0.9 ± 3.3, CD4 (stroma) was 1.1 ± 2.6, CD8 (tumor) was 35.3 ± 22.7 and CD8 (stroma) was 13.6 ± 12.7. PD-L1 levels significantly correlated with CD8 (stroma) levels ($P = .018$), but not with CD3 nor CD4 in tumor/stroma (Figure 5B). PD-L1 levels were also significantly positively correlated with tumor IFN-γ levels (Figure 5C). These results are in line with a recent report that upregulation of PD-L1 may result from increased IFN-γ production by CTL which migrate to the tumor during immune activation.[38]

In the univariate survival analysis, advanced disease stage, CD3 tumor, CD3 stroma and CD8 stroma were significantly associated



(b)

Correlation between membranous PD-L1 expression on tumor cells and each character of TILs		
	Correlation coefficient	*P*-value
CD3 tumor	0.046	0.611
CD3 stroma	–0.07	0.443
CD4 tumor	–0.086	0.362
CD4 stroma	–0.005	0.956
CD8 tumor	0.09	0.34
CD8 stroma	0.22	0.018

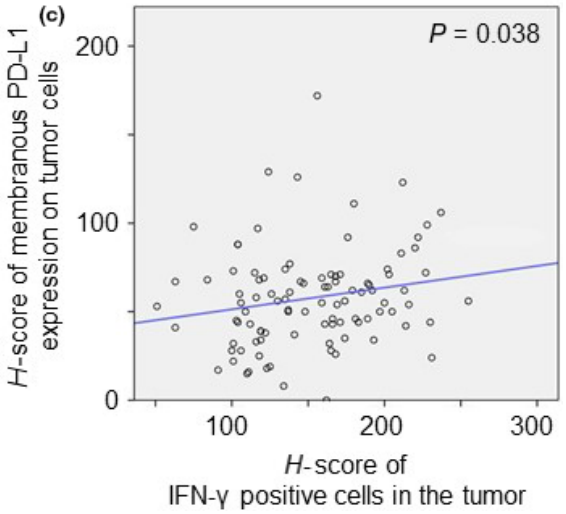


FIGURE 5 Correlation of CD3, CD4, CD8, PD-L1 and interferon gamma (IFN-γ) expression in tumor and stroma by immunohistochemistry. A, Representative immunohistochemistry stains. B, Correlation between tumor programmed death ligand-1 (PD-L1) and T-lymphocyte types in the tumor and stroma. C, Correlation between PD-L1 and IFN-γ expression in the tumor cells

with patient survival, a result similar to those in previously published studies on GC as well as other cancers,[39-41] but PD-L1 alone was not an independent predictor of patient survival (Table 2). In a multivariate analysis, considering factors significantly associated with survival in the univariate analysis confirmed that the factors independently associated with survival were advanced disease stage and CD3 stroma (Table 2).

In the analysis of overall survival, progress of disease stage was significantly inversely associated with survival. Furthermore, the survival numbers in the high infiltration group of CD8 stroma, CD3 stroma, CD4 stroma and CD3 tumor were superior to those of the low infiltration group (Figure 6).

4 | DISCUSSION

Interferon gamma is considered to have a double-edge sword effect on anti-tumor immunity, upregulating HLA class I expression and APM components on tumor cells, but also being one of the most potent inducers of PD-L1.[5,8,18] There are several reports suggesting that PD-L1 expression on tumor cells in the tumor microenvironment could be upregulated by genomic alteration, oncogenic signaling, EMT conversion and IFN-γ produced by TIL.[18-22] To date, there is still limited information on how PD-L1 on cancer cells is upregulated in the tumor microenvironment, especially in the human system. In this study, we showed that PD-L1 expression on solid tumor cells was upregulated by IFN-γ mainly through JAK-STAT pathway and membranous PD-L1 expression on tumor cells in the tumor microenvironment of GC was significantly positively correlated with tumor stroma CD8-positive T cells and tumor IFN-γ levels.

Programmed death ligand-1 was upregulated by IFN-γ treatment in 28 out of 30 cell lines (Figure 1), suggesting that the majority of solid tumors respond to IFN-γ and upregulate PD-L1 expression within the tumor microenvironment. Although IFN-γ is known to be the most potent inducer of PD-L1 through the JAK-STAT pathway, previous reports showed that the MAPK and PI3K-

TABLE 2 Univariate and multivariate analysis to correlate between survival and clinical features

	Univariate analysis			Multivariate analysis		
Variables	Hazard ratio	95% CI	*P*-value	Hazard ratio	95% CI	*P*-value
Male	1.165	0.699-1.941	.559	-	-	-
Age	1.007	0.981-1.034	1.007	-	-	-
Stage	1.84	1.472-2.300	<.01	1.57	1.224-2.017	<.001
Lauren's classification	1.263	0.813-1.963	.298	-	-	-
Grade	0.751	0.520-1.086	.128	-	-	-
CD3 tumor	0.9	0.838-0.967	.004	1.046	0.949-1.154	.367
CD3 stroma	0.955	0.935-0.974	<.01	0.951	0.929-0.975	<.001
CD4 tumor	0.937	0.801-1.098	.422	-	-	-
CD4 stroma	0.816	0.663-1.004	.054	-	-	-
CD8 tumor	1.001	0.991-1.012	.787	-	-	-
CD8 stroma	0.974	0.954-0.995	.015	1.004	0.979-1.030	.751
Membranous PD-L1	0.996	0.987-1.005	.348	-	-	-

PD-L1, programmed death ligand-1; -, not tested.

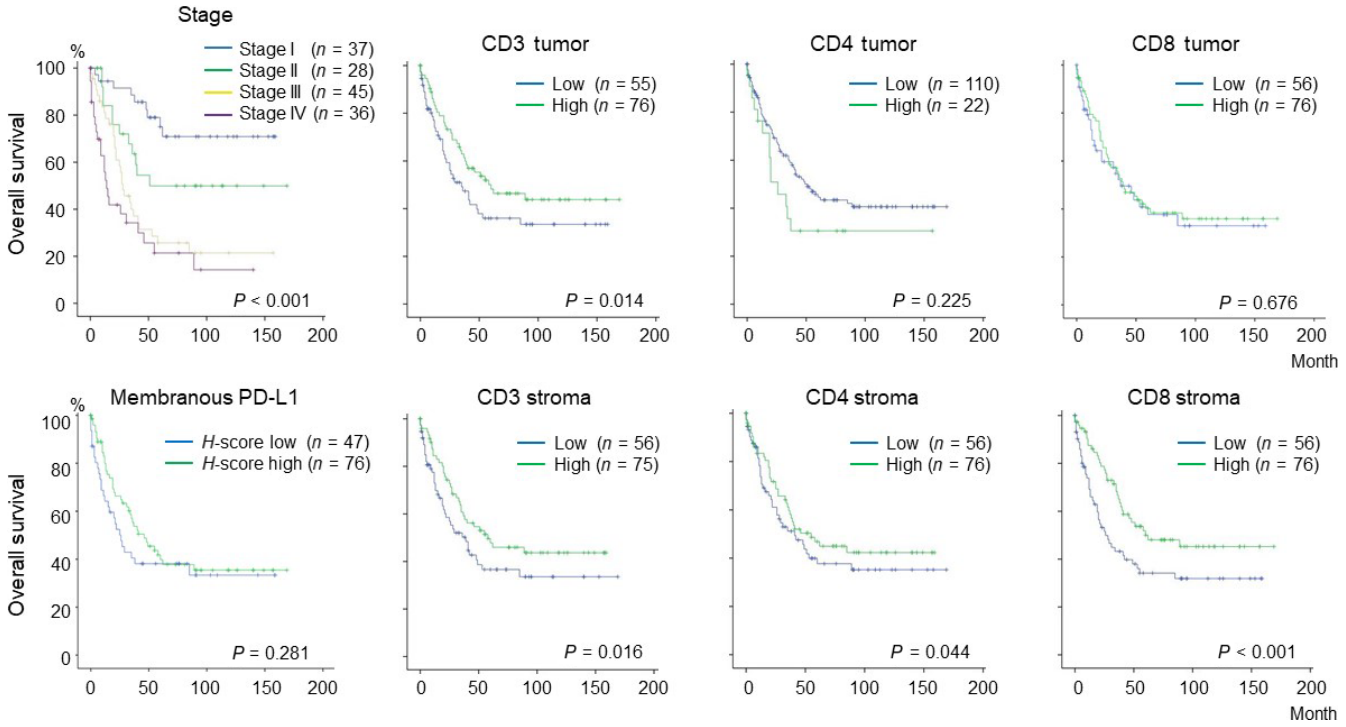


FIGURE 6 Overall survival of disease stage, tumor programmed death ligand-1 (PD-L1) and T-lymphocyte types in the tumor and stroma

AKT pathways are also involved in PD-L1 overexpression induced by IFN-γ in malignant plasma cells, lymphoma[35,36] and melanoma.[42] However, in the panel of human solid cancer cells in this study, upregulation of PD-L1 was induced by IFN-γ associated with the JAK-STAT but not the MAPK and PI3K-AKT pathways activation (Figure 1). These results suggest that PD-L1 regulation by IFN-γ may be mediated through different pathways depending on the cell types.

In order to invade, disseminate to distant tissues, and subsequently form metastatic colonies, cancer cells must shift into a more mesenchymal phenotype and this shift can be achieved by EMT.[43] Chen et al[22] report that ZEB1 induces both EMT conversion and PD-L1 expression on cancer cells through inhibition of microRNA-200, resulting in tumor invasion and immunosuppression in the tumor microenvironment. Similarly, we showed that PD-L1 expression significantly correlated with EMT phenotype in a panel of solid

cancer cells (Figure 3). There is, therefore, a possibility that EMT status also plays a role in regulating PD-L1 expression, as well as IFN-γ-related regulation, in the tumor microenvironment.

Because IFN-γ can upregulate both PD-L1 and HLA class I, including APM components on tumor cells simultaneously,[5,8,18] the antitumor CTL activity may depend on a balance between inhibitory (upregulated PD-L1) and stimulatory (upregulated HLA class I) effects of IFN-γ. In a cytotoxic assay with anti-PD-L1 mAb (Figure 4), we showed that IFN-γ treatment for target cells results in significant impairment of tumor-specific CTL activity, and the impairment was recovered by the addition of anti-PD-L1 mAb. Furthermore, CTL activity with anti-PD-L1 mAb treatment after IFN-γ exposure was significantly enhanced in comparison to those without IFN-γ exposure (Figure 4), consistent with our previous data.[8] Collectively, our findings highlight PD-L1 expression as potentially one of the key limiting factors for tumor-specific CTL activity in the tumor microenvironment.

According to our flow cytometry data (Figure 1A), only 2 out of 30 cell lines, MKN7 and NUGC3, originally expressed relatively high PD-L1 expression, while the other cell lines expressed faint PD-L1 expression but were inducible to express PD-L1 in response to IFN-γ, except for KYSE70 and MKN74. Our IHC results of GC showed that membranous PD-L1 expression on tumor cells was significantly correlated with the number of CD8-positive stromal T cells and IFN-γ positive cells in the tumor. Therefore, we proposed that IFN-γ produced by CD8-positive T cells may upregulate membranous PD-L1 expression on tumor cells in the tumor microenvironment of GC.

Molecular characterization of GC in The Cancer Genome Atlas elucidated four subtypes of GC: tumors positive for Epstein-Barr virus, micro satellite unstable tumors, tumors with chromosomal instability and genomically-stable tumors.[44] Recent evidence demonstrated that micro satellite unstable tumors are predictors of clinical response to pembrolizumab in colon cancers.[45] According to our concept described above, because tumors positive for Epstein-Barr virus display elevated neo-antigens, infiltration of TIL and amplification of PD-L1, and micro satellite unstable tumors show elevated neo-antigens,[44] these two GC subtypes may be good candidates for PD-1/PD-L1 antagonists.

In conclusion, PD-L1 expression is a key limiting factor for tumor-specific CTL activity, and is significantly upregulated by IFN-γ exposure. Furthermore, PD-L1 expression on GC cells is positively correlated with the presence of CD8-positive T cells in the stroma and IFN-γ expression in the tumor. Taken together, we suggest that PD-1/PD-L1 antagonists are more efficacious for patients with GC in whom there are CD8-positive T cells in the tumor microenvironment.

ACKNOWLEDGMENTS

This work was supported by a Clinician Scientist Award and Clinician Scientist-Individual Research Grant from the National Medical Research Council of Singapore. This research was also supported by the National Research Foundation Singapore and the Singapore Ministry of Education under its Research Centres of Excellence initiative.

We would like to thank Yuka Kimura (Department of Radiation Oncology, Gunma University Graduate School of Medicine) for her technical assistance.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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SUPPORTING INFORMATION

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How to cite this article: Mimura K, Teh JL, Okayama H, et al. PD-L1 expression is mainly regulated by interferon gamma associated with JAK–STAT pathway in gastric cancer. *Cancer Sci*. 2018;109:43–53. https://doi.org/10.1111/cas.13424