Immune Suppression by PD-L2 against Spontaneous and Treatment-Related Antitumor Immunity

Tokiyoshi Tanegashima1,2, Yosuke Togashi1, Koichi Azuma3, Akihiko Kawahara3, Ko Ideguchi4, Daisuke Sugiyama4, Fumio Kinoshita2,5, Jun Akiba6, Eiji Kashiwagi2, Ario Takeuchi2, Takuma Irie1, Katsunori Tatsugami2, Tomoaki Hoshino3, Masatoshi Eto2, and Hiroyoshi Nishikawa1,4

Abstract

**Purpose:** To evaluate the detailed immunosuppressive role(s) of PD-L2 given that its detailed role(s) remains unclear in PD-1 signal blockade therapy in animal models and humans.

**Experimental Design:** We generated mouse cell lines harboring various status of PD-L1/PD-L2 and evaluated the tumor growth and phenotypes of tumor-infiltrated lymphocytes using several PD-1 signal blockades in animal models. In humans, the correlation between immune-related gene expression and CD274 (encoding PD-L1) or PDCD1LG2 (encoding PD-L2) was investigated using The Cancer Genome Atlas (TCGA) datasets. In addition, PD-L1 or PD-L2 expression in tumor cells and CD8+ T-cell infiltration were assessed by IHC.

**Results:** In animal models, we showed that PD-L2 expression alone or simultaneously expressed with PD-L1 in tumor cells significantly suppressed antitumor immune responses, such as tumor antigen–specific CD8+ T cells, and was involved in the resistance to treatment with anti-PD-1 mAb alone. This resistance was overcome by anti-PD-1 mAb or combined treatment with anti-PD-L2 mAb. In clinical settings, antitumor immune responses were significantly correlated with PD-L2 expression in the tumor microenvironment in renal cell carcinoma (RCC) and lung squamous cell carcinoma (LUSC).

**Conclusions:** We propose that PD-L2 as well as PD-L1 play important roles in evading antitumor immunity, suggesting that PD-1/PD-L2 blockade must be considered for optimal immunotherapy in PD-L2–expressing cancers, such as RCC and LUSC.

Introduction

Immune checkpoint blockade (ICB), including PD-1 blockade, has been approved to treat various cancers, such as malignant melanoma, non–small cell lung cancer (NSCLC), and renal cell carcinoma (RCC), leading to a paradigm shift in cancer therapy (1–4). However, as the clinical efficacy is limited, more effective therapies and predictive biomarkers stratifying responders from nonresponders are urgently needed. Tumors employ the PD-1 pathway to evade antitumor immunity, particularly CD8+ T cells against tumor antigens (5, 6). PD-1 is mainly expressed by activated T cells and binds to its ligands, PD-L1 and PD-L2 (5, 6), resulting in immunosuppression. PD-L1 is expressed by both antigen-presenting cells (APC) and tumor cells (7, 8). PD-1 signal blockade unleashes antitumor T-cell responses by augmenting signals from the T-cell receptor and CD28 (9–11), an essential costimulatory molecule. Consistent with the importance of the PD-1/PD-L1 interaction, several studies revealed favorable clinical courses using treatment with anti-PD-1/PD-L1 mAbs in patients harboring PD-L1–expressing tumors (12, 13). However, some patients harboring PD-L1–expressing tumors do not respond to PD-1 signal blockade treatment. In addition, patients with PD-L1–negative tumors occasionally experience clinical efficacy (4, 12–14). In large phase III trials, the clinical benefit from anti-PD-1 mAbs appears to be independent of PD-L1 expression in some cancers, such as RCC and lung squamous cell carcinoma (LUSC; refs. 4, 14–16).

The expression of another PD-1 ligand, PD-L2, was initially thought to be restricted in APCs (6). Recently, several studies showed that PD-L2 is expressed by both various immune cells and tumor cells, depending on microenvironmental stimuli (17–19). PD-L2 expression in tumor cells is a predictive factor for the clinical efficacy of anti-PD-1 mAb in some studies (20). In this study, we evaluated the detailed immunosuppressive role(s) of PD-L2 in animal models and humans. The Cancer Genome Atlas
Translational Relevance

Although PD-L1, a ligand of PD-1, has mainly been studied as a therapeutic target and predictive biomarker in PD-1 blockade therapy, the detailed role(s) of PD-L2, another PD-L1 ligand, remains unclear. Using preclinical animal models, we showed that PD-L2 expression alone or simultaneously expressed with PD-L1 by tumor cells significantly suppressed antitumor immune responses, such as tumor antigen–specific CD8⁺ T cells, and was involved in the resistance to treatment with anti-PD-L1 mAb. This resistance was overcome by anti-PD-1 mAb or combined treatment with anti-PD-L2 mAb. In clinical settings, antitumor immune responses were significantly correlated with PD-L2 expression in the tumor microenvironment in renal cell carcinoma (RCC) and lung squamous cell carcinoma (LUSC). We propose that PD-L2 plays an important role in evading antitumor immunity as well as PD-L1, suggesting that PD-1/PD-L2 blockade must be considered for optimal immunotherapy in PD-L2–expressing cancers, such as RCC and LUSC.

Materials and Methods

Patients

Twenty-nine patients with clear-cell and non–clear-cell RCC who underwent surgical resection at Kyushu University Hospital (Fukuoka, Japan) from September 2017 to May 2018 were enrolled in this study. Twenty-seven patients with LUSC who underwent surgical resection at Kurume University Hospital from January 2008 to December 2012 were enrolled in this study. The patients’ clinical information was obtained from their medical records. All patients provided written informed consent before undergoing the study procedures. The clinical protocol for this study was approved by the appropriate institutional review boards and ethics committees at Kyushu University Hospital and Kurume University Hospital (Kurume, Japan). This study was conducted in accordance with the Declaration of Helsinki.

IHC

Sections were immunostained using the Ventana Benchmark XT Automated Staining System (Ventana Medical System) according to the manufacturer’s protocol. Tumor PD-L1 and PD-L2 membrane expression was assessed. ≥1% was defined as positive and <1% as negative (4, 12, 14). The intertumoral CD8⁺ T cells were counted. Specifically, five areas (0.5 × 0.5 mm (0.25 mm²) fields) with the most abundant distribution were selected and counted in each case.

Cell lines and reagents

B16-F10 (mouse melanoma), MC-38 (mouse colon carcinoma), CT26 (mouse colon carcinoma) expressing NY-ESO-1 (CT26-NY-ESO-1), Renca (mouse kidney adenocarcinoma), CMS5a (mouse fibrosarcoma), and A20 (mouse reticulum cell sarcoma) cell lines were maintained in RPMI medium (FUJI FLM Wako Pure Chemical Corporation) supplemented with 10% FCS (Bio sera). CT26-NY-ESO-1 is a cell line derived from CT26 stably transfected with NY-ESO-1 (21). CT26, Renca, B16-F10, and A20 cell lines were obtained from the ATCC. MC-38 cell line was obtained from Kerafast. CMS5a cell line was provided by the late Dr. Lloyd J. Old, Memorial Sloan Kettering Cancer Center (New York, NY; ref. 22). All cell lines were used after confirming Mycoplasma testing with a PCR Mycoplasma Detection Kit (Takara Bio) according to the manufacturer’s instructions. Murine IFNγ, IL2, IL4, TNFα, and granulocyte macrophage colony stimulating factor (GM-CSF) were obtained from PeproTech. Murine IFNγ, IFNβ, and tumor growth factor-β1 were obtained from R&D Systems, Inc. Rat anti-mouse PD-1 (RMP1-14) mAb, anti-PD-L1 (10F.9G2) mAb, anti-PD-L2 (TY25) mAbs, and control rat IgG2a (RTK2758) mAb used in the in vivo study were obtained from BioLegend. Anti-CD4 (GK1.5) mAb and anti-CD8β (Lyt 3.2) mAbs were obtained from BioXcell.

Constructs, viral production, and transfection

Mouse PDCD1LG2 cDNA was subcloned into pMXs-ires-GFP, which was transfected into the packaged cell line using Lipofectamine 3000 Reagent (Thermo Fisher Scientific). After 48 hours of retroviral production, the supernatant was concentrated and dissolved in water. Subsequently, the viral lysate was transfected with MC-38 and CT26-NY-ESO-1, and the PD-L2–expressing cell lines were named MC-38-L2 and CT26-NY-ESO-1-L2, respectively. GFP-expressing cell lines were also created for the controls and named MC-38-GFP and CT26-NY-ESO-1-GFP. To generate the PD-L1–knockout (KO) MC-38 cell line, we used a CRISPR/Cas9 system. Briefly, guide RNA (gRNA)-targeting mouse CD274 (5’-GCCCTGGTTAGTGGTGTACT-3’) was made using the GeneArt Precision gRNA Synthesis Kit (Thermo Fisher Scientific). The Cas9 protein (Thermo Fisher Scientific) and gRNA were electroporated into MC-38 cells using the Neon Transfection System (Thermo Fisher Scientific) per the manufacturer’s instructions. The knockout cell line was named MC-38-L1KO. Either GFP or PD-L2 was introduced into the MC-38-L1KO in the same manner, and these cell lines were named MC-38-L1KO-GFP and MC-38-L1KO-L2, respectively.

In vivo mouse studies

Female C57BL/6, BALB/c and BALB/c nude mice (6–8 week) were purchased from CLEA Japan and used at 7–9 weeks of age. Tumor cells (1 × 10⁵) were injected subcutaneously, and tumor size was monitored twice weekly. The mean of the long and short diameters was applied for the tumor growth curves. Mice were grouped when tumor volume reached approximately 100 mm³, and anti-PD-1/PD-1/L1/PD-1 mAbs were administered intraperitoneally three times every 3 days thereafter. For CD4⁺ T-cell or CD8⁺ T-cell depletion, anti-CD4 mAb (100 μg/mouse) or anti-CD8β mAb (100 μg/mouse) was administered intraperitoneally on days −1, 0, and every 7 days after tumor injection. Tumors were harvested 14 days after tumor injection, and tumor-infiltrating lymphocytes (TIL) were analyzed with flow cytometry. All mouse experiments were approved by the Animals Committee for Animal Experimentation of the National Cancer Center Japan. All experiments met the U.S. Public Health Service Policy on Humane Care and Use of Laboratory Animals.
Flow cytometry

Cells were washed using PBS with 2% FBS and stained with mAbs specific for CD4, CD8, CD44, CD62L, PD-1, PD-L1, and PD-L2 as well as for fixable viability dye (Thermo Fisher Scientific). To examine antigen-specific CD8⁺ T cells, T-Select H-2Kb MuLV p15E Tetramer-KSPWFTI APC (MBL) was used per the manufacturer’s instructions. After tetramer staining, cells were stained for cell surface markers (mAbs specific for CD4, CD8, and fixable viability dye). When intracellular cytokines were analyzed, cells were stimulated for 5 hours with phorbol 12-myristate 13-acetate (PMA; 100 ng/mL) and ionomycin (2 µg/mL; Sigma Aldrich). GolgiPlug Reagent (1.3 µL/mL; BD Biosciences) was added for the last 4 hours of the culture. These cells were stained for surface markers and then stained for intracellular cytokines (mAbs specific for TNFα and IFNγ). After washing, the cells were analyzed with an LSR Fortessa Instrument (BD Biosciences) and FlowJo Software (Tree Star).

Antibodies

Violet 500–conjugated anti-CD8 (53-6.7) mAb, Brilliant Violet 786 (BV786)–conjugated anti-CD4 (RM4-5) mAb, phycoerythrin (PE)- and Cy7–conjugated anti-CD44 (IM7) mAb, BV421-conjugated anti-PD-L2 (TV25) mAb, and BV605-conjugated anti-CD11c (HL3) were purchased from BD Biosciences. BV421-conjugated anti-PD-L1 (29F.1A12) mAb, peridinin chlorophyll protein complex, Cy5.5-conjugated anti-CD62L (ME14) mAb, BV241-conjugated anti-TNFα (MP6–X22) mAb, PE-conjugated anti-PD-L1 (10E.9G2) mAb, and AF488-conjugated anti-CD8 (53-6.7) mAb were purchased from eBioscience. FITC-conjugated anti-IFNγ (XMG1.2) mAb were obtained from eBioscience. PE-conjugated anti-CD11b (M1/70) mAb were purchased from Thermofisher Scientific. The antibodies used for IHC were PD-L1 (E1L3N, Cell Signaling Technology), PD-L2 (#176611, R&D Systems, Inc.), and CD8 (4B11, Leica Biosystems).

Real-time qRT-PCR

RNA was extracted using RNeasy Mini Kit (Qiagen). cDNA was generated using SuperScript VILO (Thermo Fisher Scientific), and real-time PCR reactions were performed with SYBR Green (Applied Biosystems) or Fast SYBR Green (Thermo Fisher Scientific). Gene expression changes relative to 18S ribosomal RNA as a housekeeping gene were calculated using the DDCT method. A forward primer (5’ [5AGCCGTCTGTCATCTGTC-3’) and reverse primer (5’ [5TCCGATCACCCATAGGCGG-3’) were used to detect PDCD11LG2. A forward primer (5’ [5TAGGTTGTT- CAAACGAGCCCGG-3’) and reverse primer (5’ [5CAACAAATA-GAACGCGCCG-3’) were used to detect 18S.

Statistical analysis

GraphPad Prism7 (GraphPad Software) was used for statistical analysis. The overall survival was analyzed with Kaplan-Meier method and compared with log-rank test. The relationships between the groups were compared using Fisher exact test or Welch t test. P < 0.05 was considered statistically significant.

Results

PD-L2 plays a limited role in immune suppression in murine tumor models

To elucidate the role of PD-L2 in tumor immunity, we first explored PD-L1 and PD-L2 expression in several murine tumor cell lines, including MC-38, CT26, B16-F10, Renca, CM55a, and A20. All tumor cell lines possessed variable PD-L1 expression that was enhanced by stimulants, especially IFNγ, β, and γ. In contrast, PD-L2 was minimally detected by any cell lines even after stimulation (Fig. 1A; Supplementary Figs. S1 and S2). While PD-L2 is reportedly expressed by APCs (6), low expression in APCs (CD45 MHCIIC/D11c/C111b cells) was observed in animal models (ref. 23; Supplementary Fig. S3). In contrast to PD-L2, APCs had considerable, although slightly lower than tumor cells, PD-L1 expression (Supplementary Fig. S3). We next examined in vivo antitumor effects of anti-PD-1/PD-L1/PD-L2 mAbs using MC-38 and CT26-NY-ESO-1 cells. Anti-PD-1 mAb and anti-PD-L1 mAb similarly inhibited MC-38 and CT26-NY-ESO-1 tumor growth, whereas anti-PD-L2 mAb was ineffective against both tumors (Fig. 1B). As neoantigen-specific T cells are reportedly essential for antitumor effects by ICB (24), MuLV p15E (a neoantigen of MC-38–specific CD8⁺ T cells) detected by MHC/peptide tetramers was examined in an MC-38 model. MuLV p15E tetramer CD8⁺ T cells were significantly primed/augmented by treatment with anti-PD-1 mAb or anti-PD-L1 mAb but not with anti-PD-L2 mAb alone (Fig. 1C). Activated CD8⁺ T cells, which were assessed by the proportion of CD44+CD62L⁻ effector/memory CD8⁺ T cells and the frequency of PD-1⁺ CD8⁺ T cells, were significantly higher in TILs in mice treated with anti-PD-1 mAb or anti-PD-L1 mAb compared with mice treated with isotype control or anti-PD-L2 mAb (Fig. 1D; Supplementary Fig. S4). In addition, TNFα IFNγ CD8⁺ T cells were significantly higher in mice treated with anti-PD-1 mAb or anti-PD-L1 mAb than in those treated with isotype control or anti-PD-L2 mAb (Fig. 1D; Supplementary Fig. S4). Therefore, PD-L1 plays a dominant role in immune suppression in murine tumor models.
PD-L2 Is Important for Evading Antitumor Immunity

Because PD-L2 was minimally expressed by the murine tumor cell lines that are frequently used in preclinical studies for PD-1 signal blockade although some human tumor cells highly express PD-L2 (20, 25), we hypothesized that the lesser appreciation of PD-L2 in PD-1 signal blockade treatment may solely reflect the lack of appropriate animal models using PD-L2–expressing tumor cell lines. The PD-L2–overexpressing cell lines MC-38 (MC-38-L2) and CT26-NY-ESO-1 (CT26-NY-ESO-1-L2) were thus developed to evaluate influence of PD-L2 on PD-1 signal blockade efficacies using anti-PD-1 mAb, anti-PD-L1 mAb, and anti-PD-L2 mAb. PD-L2 expression was confirmed at both mRNA and protein levels using real-time qRT-PCR (Fig. 2A) and flow cytometry (Fig. 2B), respectively. The level of PD-L2 expression was equivalent to that of endogenous PD-L1 expression after IFNγ treatment (Fig. 2A and B). PD-L2–expressing tumors grew rapidly compared with those in control mice injected with parental tumors or GFP-expressing tumors (Fig. 2C) although these tumors exhibited comparable tumor growth in immunocompromised mice (Supplementary Fig. S5A). Furthermore, to elucidate the role of CD4+ T cells and CD8+ T cells, the tumor growth of MC-38/GFP/L2 and CT26-NY-ESO-1/GFP/L2 was evaluated in CD4+ T-cell– or CD8+ T-cell–depleted mice. In CD8+ T-cell–depleted mice, the rapid tumor growth in PD-L2–expressing tumors was totally abrogated although PD-L2–expressing tumors grew rapidly compared with the parental tumors or GFP-expressing tumors in CD4+ T-cell–depleted mice (Supplementary Fig. S5B and 5C), indicating the importance of CD8+ T-cell suppression by PD-L2. Accordingly, in the MC-38-L2 tumors, MuLV p15E tetramer+ CD8+ T cells were reduced compared with those of the control mice (Fig. 2D). In addition, activated CD8+ T cells (CD44+CD62L− effector/memory CD8+ T cells and PD-1+ CD8+ T cells) and TNFα+ IFNγ+ CD8+ T cells in TILs were significantly lower in MC-38-L2 tumors than in the control parental tumors or GFP-expressing tumors (Fig. 2E, Supplementary Fig. S6). Taken together, PD-L2–expressed tumors by cell lines functions as an immunosuppressive molecule against antitumor immunity, particularly CD8+ T cells.

PD-L2 blockade is necessary for controlling PD-L2–expressing tumors

We next addressed how PD-L2 influenced the antitumor effects of anti-PD-1 mAb, anti-PD-L1 mAb, and anti-PD-L2 mAb. Anti-PD-1 mAb significantly inhibited PD-L2–expressing tumor growth compared with anti-PD-L1 mAb or anti-PD-L2 mAb alone. The combination of anti-PD-L1 mAb and anti-PD-L2 mAb showed a comparable tumor growth inhibition in PD-L2–expressing tumor growth as observed by anti-PD-1 mAb (Fig. 3A). In addition, anti-PD-1 mAb did not show any additional antitumor effects against PD-L2–expressing tumors when combined with anti-PD-L2 mAb, indicating that anti-PD-1 mAb alone can inhibit PD-1/PD-L2 interaction sufficiently to reinvigorate an effective antitumor immunity. (Supplementary Fig. S7). The tumor growth inhibition by these antibodies including anti-PD-L2 mAb was totally abrogated in immunocompromised mice or CD8+ T-cell–depleted mice although they exhibited antitumor effects against PD-L2–expressing tumors in CD4+ T-cell–depleted mice as well as in control mice (Supplementary Fig. S8A), indicating that the antitumor efficacy against PD-L2–expressing tumors was attributed for unleashing CD8+ T cells. In addition, anti-PD-L2 mAb did not exhibit antitumor immunity against parental tumors or GFP-expressing tumors in immunocompromised mice (Supplementary Fig. S8B). Thus, the antitumor efficacy of anti-PD-L2 mAb against PD-L2–expressing tumors was dependent on the reactivation of CD8+ T cells, but not caused by a direct effect of PD-L2 blockade against PD-L2–expressing tumors. Accordingly, MuLV p15E tetramer+ CD8+ T cells were significantly primed/augmented by anti-PD-1 mAb or the combination of anti-PD-L1 mAb and anti-PD-L2 mAb compared with anti-PD-L1 mAb or anti-PD-L2 mAb alone in MC-38-L2 tumor–bearing mice (Fig. 3B). Activated CD8+ T cells (CD44+CD62L− effector/memory CD8+ T cells and PD-1+ CD8+ T cells) and TNFα+ IFNγ+ CD8+ T cells in TILs were also significantly higher in mice treated with the anti-PD-1 mAb or the combination of anti-PD-L1 mAb and anti-PD-L2 mAb compared with untreated mice or mice treated either by anti-PD-L1 mAb or by anti-PD-L2 mAb alone (Supplementary Fig. S9). Therefore, PD-L2 endows resistance to anti-PD-1 mAb monotherapy, which can be overcome by combined treatment with anti-PD-1 mAb and anti-PD-L2 mAb.

To further confirm the role of PD-L2, a PD-L1–knockout MC-38 cell line (MC-38-L1KO) was generated using a CRISPR/Cas9 system, and the lack of PD-L1 protein expression was confirmed with flow cytometry (Supplementary Fig. S10). Either PD-L2 or GFP was then expressed in MC-38-L1KO cells (MC-38-L1KO-L2 and MC-38-L1KO-GFP, respectively; Supplementary Fig. S10). MC-38-L1KO tumors grew more slowly than the parental MC-38 tumors, whereas PD-L2 expression allowed MC-38-L1KO-L2 tumors to grow comparably with the parental MC-38 tumors (Fig. 3C). In contrast, these tumors grew similarly in immunocompromised mice (Supplementary Fig. S10). Anti-PD-1 mAb and anti-PD-L2 mAb significantly inhibited tumor growth, but anti-PD-L1 mAb did not (Fig. 3D). Thus, PD-L2 alone sufficiently hampers antitumor immunity, allowing rapid tumor growth.

PD-L2 expression is associated with antitumor immune responses in various cancers

Because PD-L2 significantly suppressed antitumor immunity in animal models, we investigated the correlation between immune-
related gene expression and CD274 (encoding PD-L1) or PDCD1LG2 (encoding PD-L2) in humans using TCGA datasets. The expression levels of 43 genes were defined for the immune cell types, CD8⁺ T cells, costimulatory APCs, costimulatory T cells, co-inhibitory APCs, co-inhibitory T cells, and cytolytic activity (ref. 26; Supplementary Table S1). We analyzed bladder cancer, gastric cancer, head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma, LIUSC, and RCC datasets because the efficacy of anti-PD-1 mAb or anti-PD-L1 mAb has been demonstrated in these cancers, and the published datasets were available (4, 12, 14–16, 27–30). The correlation coefficient between the expression level of each gene and that of CD274 or PDCD1LG2 was calculated (Fig. 4A; Supplementary Fig. S11). Especially in RCC, PDCD1LG2 expression rather than CD274 expression was significantly positively correlated with immune-related gene expression (Fig. 4A). We further evaluated the correlation between CD274, PDCD1LG2, or PDCD1 expression and the INTERFERON_GAMMA_RESPONSE gene signature (Fig. 4B; ref. 31). The heatmap highlights the quantitative differences in association between PD-1–related molecules and immune responses in the TME across tumor types. In RCC and LIUSC, PDCD1LG2 expression was strongly correlated with IFNγ-related gene expression, although CD274 expression was less significantly correlated. These data suggest that PD-L2 expression in RCC and LIUSC strongly controls antitumor immune responses in the TME.

We next examined the relationship between CD274 or PDCD1LG2 expression and survival from RCC and LIUSC TCGA datasets. In RCC, no correlation between CD274 or PDCD1LG2 expression and survival was observed. On the other hand, high expression of CD274 or PDCD1LG2 equally correlated with a better survival in LIUSC, indicating the possible correlation between antitumor immune responses and a favorable prognosis (Supplementary Fig. S12).

CD8⁺ T-cell infiltration is accompanied by PD-L2 expression in RCC and LIUSC

Because the correlation between immune-related genes and CD274 expression was relatively weak in RCC and LIUSC, the expression of PD-L1 or PD-L2 in tumor cells and the number of infiltrated CD8⁺ T cells were assessed by IHC in RCC (n = 29) and LIUSC (n = 27) cohorts to address a role of PD-L2 expression for the suppression against antitumor immunity, particularly CD8⁺ T cells. PD-L1- and PD-L2–expressing tumors (≥1% of tumor cells) were observed in 17.2% (5/29) and 72.4% (21/29) in RCC (Supplementary Table S2) and 55.6% (15/27) and 85.2% (23/27) in LIUSC (Supplementary Table S3), respectively. Correlations between PD-L1 or PD-L2 expression and clinicopathologic features of RCC and LIUSC are summarized in Supplementary Tables S2 and S3, respectively. In RCC, age, sex, Fuhrman nuclear grade, pathological stage (pStage), and histology did not correlate with PD-L1 or PD-L2 expression (Supplementary Table S2). In LIUSC, age, pStage, smoking status, and driver gene status did not correlate with PD-L1 or PD-L2 expression (Supplementary Table S3).

In RCC, CD8⁺ T cells significantly highly infiltrated into PD-L2–expressing tumors, such as RCC_12, compared with PD-L2–negative tumors, such as RCC_25. In LIUSC, CD8⁺ T cells showed higher infiltration into PD-L2–expressing tumors, such as LIUSC_21, than PD-L2–negative tumors, such as LIUSC_27. In contrast, CD8⁺ T-cell infiltration was unrelated to PD-L1 expression in both tumors (Fig. 5; Supplementary Tables S2 and S3). In accordance with our animal models, PD-L2 expression in immune cells was very low compared with tumor cells. Together with TCGA dataset analyses, PD-L2 in tumor cells appears to play an important role in evading antitumor immunity in several cancers, including RCC and LIUSC.

**Discussion**

Accumulating evidence shows that the important immunosuppressive role of PD-L1 in the TME, while that of PD-L2 has not been fully elucidated. Using animal models, we showed that PD-L2 played an important role in evading antitumor immunity. In addition, we revealed that PD-L2 was dominantly correlated with antitumor immune responses in the TME rather than PD-L1 in RCC and LIUSC using TCGA datasets and clinical samples. In preclinical animal models, anti-PD-1 mAb was effective against the PD-L2–expressing tumors, whereas PD-L2–expressing tumors were resistant to anti-PD-L1 mAb alone. This limitation of anti-PD-L1 mAb was overcome by combined treatment with anti-PD-L2 mAb. To our knowledge, this is the first report to directly show the importance of PD-L2 as a mechanism for evading antitumor immunity using preclinical animal models and clinical samples.

CD8⁺ T cells play major roles in antitumor immunity in the TME by directly killing tumor cells (32). PD-L1 expressed by tumor cells inhibits antitumor effects of CD8⁺ T cells (7). PD-L1 expression in tumor cells is induced by two mechanisms: intrinsic induction by genetic alterations (innate expression) and stimulation by IFNγ released from effector T cells, including CD8⁺ T cells (acquired expression; ref. 33). In many cancers, tumor cells express PD-L1 through the latter mechanism (acquired expression; ref. 34, 35); that is, PD-L1 expression seems to be a surrogate marker for the presence of antitumor immune responses, such as CD8⁺ T cell responses in the TME. PD-1 signal blockade unleashes suppressed antitumor T-cell immunity, resulting in tumor regression; thereby, PD-L1 expression becomes a predictive biomarker (12, 36). However, in some cancers, including RCC and LIUSC, PD-L1 expression was not accompanied by clinical effectivity of PD-1 signal blockade treatment (4, 13, 14). Consistent with this finding, we found that PDCD1LG2 expression was more strongly correlated with immune-related gene expression than CD274 expression, especially in RCC and LIUSC, using TCGA datasets. Our cohort also revealed that PD-L2 expression in tumor cells was correlated with CD8⁺ T-cell infiltration rather than PD-L1 expression in RCC and LIUSC, suggesting that PD-L2 expression can be a more dominant immunosuppressive mechanism than PD-L1 in these cancer types. In addition, our *in vivo* animal models showed that PD-L2 can be related to resistance to anti-PD-L1 mAb alone, which can be overcome by combined treatment with anti-PD-L2 mAb. Thus, the PD-1/PD-L2 interaction plays a more important role in evading antitumor immunity than the PD-1/PD-L1 interaction in these cancers.

Approximately 30% of patients with RCC possess locally advanced or metastatic RCC at diagnosis, and 40% of patients with localized RCC develop metastasis after primary surgical treatment (37). Therefore, developing effective systemic therapies is critical in RCC treatment. Immunotherapeutic agents, such as IL2 and IFNα, have been used to treat advanced RCC with limited success (a ~10%–22% response rate; refs. 38, 39). Along with understanding RCC biology, several molecularly targeted agents, including VEGF and mTOR, have been clinically introduced (40, 41). While VEGF and mTOR inhibitors have provided marked clinical benefits in advanced RCC, some patients are inherently
resistant to these therapies, and most patients acquire resistance to them (42). Lung cancers, in which approximately 80% are classified as NSCLC, are leading causes of cancer-related mortality worldwide (43). Among NSCLCs, several molecular-targeted therapies have provided significant clinical benefits in lung adenocarcinoma (44). However, systemic therapeutic options against LUSC remain limited, resulting in poor prognoses compared with lung adenocarcinoma (44). Thus, more effective systemic

Figure 3.
PD-L2 is Important for Evading Antitumor Immunity

**A**. In vivo efficacies of various ICBs including combinations against MC-38-L2 and CT26-NY-ESO-1-L2 tumors. MC-38-L2 cells (1.0 × 10^6) were injected subcutaneously on day 0, and ICB treatments as indicated were started on days 3, 6, and 9 (left). Tumor growth was monitored twice a week (n = 8 per group). CT26-NY-ESO-1-L2 cells (1.0 × 10^6) were injected subcutaneously on day 0, and ICB as indicated were administered on days 7, 10, and 13 (right). Tumor growth was monitored twice a week (n = 5 per group).

**B**. Tumor growth of MC-38-L1KO tumors treated with the indicated mAb on day 14, and tumor antigen-specific CD8+ T cells were detected by MuLV p15E/H-2Kb tetramers. Summary of the MuLV p15E/H-2Kb tetramer+ CD8+ T-cell frequencies (n = 8 per group; right).

**C**. In vivo tumor growth of MC-38-, MC-38-L1KO, MC-38-L1KO-GFP, or MC-38-L1KO-L2 tumors. Tumor cells (1.0 × 10^6) were injected subcutaneously, and tumor growth was monitored twice a week (n = 8 per group). Tumor growth was monitored twice a week (n = 5 per group).

**D**. In vivo efficacies of various ICBs against MC38-L1KO-L2 tumors. MC38-L1KO-L2 cells (1.0 × 10^6) were injected subcutaneously on day 0, and ICB treatments as indicated were started on days 3, 6, and 9. Tumor growth was monitored twice a week (n = 8 per group). N.S., not significant; *P < 0.05; **P < 0.01; ***P < 0.001. In vivo experiments were performed at least twice. Representative data are shown from two to three independent experiments.
Figure 4.

PDCD1LG2 (encoding PD-L2) expression rather than CD274 (encoding PD-L1) is correlated with immune-related gene expression in RCC and LUSC. A, The scattergram and real numbers of the Pearson correlations between expression levels (RPKM) of CD274 or PDCD1LG2 and immune-related genes in RCC from TCGA datasets. Critical immune-related gene expression, including CD8A, GZMA, PRF1, and PDCD1, are shown in red. B, Heatmaps of correlation coefficients between CD274, PDCD1LG2, or PDCD1 expression and the hallmark gene sets of the INTERFERON_GAMMA_RESPONSE signatures in bladder cancer (BLCA), gastric cancer (GC), HNSC, lung adenocarcinoma (LUAD), LUSC, and RCC.
therapies against these cancers are necessary, and PD-1 signal blockade treatment, which has been approved to treat these cancer types (4, 14), is thought to play a major role as a key therapeutic strategy. Yet, as the clinical efficacy is unsatisfactory, predictive biomarkers are urgently needed. PD-L1 expression evaluated by IHC, which is used as a biomarker of PD-1 signal blockade, is not a predictive biomarker of PD-1 signal blockade in RCC or LUSC (4, 14). Furthermore, in recent clinical trials, anti-PD-L1 mAb monotherapy was ineffective against RCC (45) and seemed less valuable against LUSC compared with lung adenocarcinoma (28). These findings are consistent with our current findings showing that not only PD-1/PD-L1 but also PD-1/PD-L2 interaction could be important in evading antitumor immunity in RCC and LUSC.

Figure 5.
PD-L2 expression is accompanied by CD8$^+$ T-cell infiltration in the TMEs of RCC and LUSC. PD-L1, PD-L2 expression, and CD8$^+$ T-cell infiltration in the TME of RCC (A) and LUSC (B) were examined by IHC. Representative staining of PD-L1, PD-L2, and CD8 (top). Correlation between the number of infiltrated CD8$^+$ T cells and PD-L1 expression (left) and PD-L2 expression (right; bottom). RCC_12 and LUSC_21, PD-L2-expressing tumor; RCC_25 and LUSC_27, PD-L2-negative tumor; Scale bar, 100 μm; N.S., not significant; ***, P < 0.001.
humans. We therefore generate genetically engineered PD-L2 models. Thus, little attention has been paid to PD-L2 expression. It has been shown that PD-L2 is expressed by tumor cells similar to our current human clinical sample study (20, 25), suggesting a discrepancy in PD-L2 expression between mouse models and humans. We therefore generate genetically engineered PD-L2 expressing murine cancer cell lines harboring the comparable level of PD-L2 expression with endogenous PD-L1. The PD-L2–overexpressing tumors grew faster than the controls regardless of PD-L1 expression, although this was not observed in immunocompromised mice, clearly indicating that PD-L2 expressed by tumor cells can be involved in evading antitumor immunity. Accordingly, PD-L2–expressing tumors were resistant to anti-PD-L1 mAb alone, which was overcome by combined treatment with anti-PD-L2 mAb, thus highlighting that more attention should be paid to PD-L2 expression in clinical settings. However, why some cancers, including RCC and LLISC, exhibit PD-L2 expression rather than PD-L1 expression is unclear. A recent study revealed a difference between the regulations of PD-L1 and PD-L2 expression (46). It was reported that the IFNγ–JAK1/JAK2–STAT1/STAT2/STAT3–IRF1 axis primarily regulates PD-L1 expression with IRF1 binding to its promoter. On the other hand, PD-L2 responded equally to IFNβ and γ and is regulated through both IRF1 and STAT3, which bind to the PD-L2 promoter (46). In addition, genetic and/or epigenetic abnormalities may inhibit PD-L1 expression, and PD-L2 compensates the immunosuppressive role of PD-L1, or genetic and/or epigenetic abnormalities in the PD-L2 gene induce a more dominant expression than those of PD-L1 (47, 48).

In conclusion, we demonstrated that PD-L2 expression in tumor cells appears to be strongly correlated with antitumor immune responses in the TME especially in RCC and LLISC. Although little attention has been paid to PD-L2 expression in animal models and clinical settings, mainly due to the lack of appropriate cell lines and examination methods for PD-L2 expression, respectively. PD-L2 clearly contributes to evading antitumor immunity, suggesting that the PD-1/PD-L2 interaction should be blocked when treating PD-L2–expressing tumors. Currently, both anti-PD-1 mAb and anti-PD-L1 mAb are clinically available, although the differences between these reagents are unclear. Anti-PD-1 mAb may be clinically effective against broader types of cancer expressing either PD-L1 or PD-L2. Yet, as it is still an open question that PD-L1 may have other receptors, it is difficult to conclude that anti-PD-1 mAb is more useful in any types of cancer (49). Furthermore, because anti-PD-1 mAb does not inhibit PD-L2, the frequencies of immune-related adverse events (irAE), such as endocrine system disorders and pneumonitis, are reported to be lower in patients treated with anti-PD-L1 mAb than in those treated with anti-PD-1 mAb (50). Thus, considering the proper use of PD-1 signal blockade treatment with reflective PD-L2 and PD-L1 expression in addition to minimizing irAEs is an important issue for optimal cancer immunotherapy and our findings help clinicians to select optimal immunotherapy.

Disclosure of Potential Conflicts of Interest
Y. Togashi reports receiving other commercial research support from Ono Pharmaceutical, Bristol-Myers Squibb, and AstraZeneca, and reports receiving speakers bureau honoraria from Ono Pharmaceutical and Chugai. K. Azuma reports receiving speakers bureau honoraria from Ono Pharmaceutical, Bristol-Myers Squibb, AstraZeneca, and Chugai. M. Eto reports receiving commercial research grants from Ono Pharmaceutical, Takeda, Pfizer, Astellas, and Kissei, and reports receiving speakers bureau honoraria from Ono Pharmaceutical, Bristol-Myers Squibb, Pfizer, Novartis, Bayer, and Takeda. H. Nishikawa reports receiving speakers bureau honoraria and other commercial research support from Ono Pharmaceutical, Bristol-Myers Squibb, and Chugai. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions
Conception and design: Y. Togashi, M. Eto, H. Nishikawa
Development of methodology: T. Tanegashima, Y. Togashi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): T. Tanegashima, K. Azuma, A. Kawahara, K. Ideguchi, D. Sugiyama, F. Kinoshita, J. Akiba, A. Takeuchi, K. Tatsugami, T. Hoshino, M. Eto
Writing, review, and/or revision of the manuscript: T. Tanegashima, Y. Togashi, H. Nishikawa
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Kashiwagi
Study supervision: H. Nishikawa

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References


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Tokiyoshi Tanegashima, Yosuke Togashi, Koichi Azuma, et al.


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