

## Clinical Significance and Therapeutic Potential of the Programmed Death-1 Ligand/Programmed Death-1 Pathway in Human Pancreatic Cancer

Takeo Nomi,<sup>1</sup> Masayuki Sho,<sup>1</sup> Takahiro Akahori,<sup>1</sup> Kaoru Hamada,<sup>2</sup> Atsushi Kubo,<sup>3</sup> Hiromichi Kanehiro,<sup>1</sup> Shinji Nakamura,<sup>1</sup> Koji Enomoto,<sup>1</sup> Hideo Yagita,<sup>4</sup> Miyuki Azuma,<sup>5</sup> and Yoshiyuki Nakajima<sup>1</sup>

**Abstract Purpose:** The programmed death-1 ligand/programmed death-1 (PD-L/PD-1) pathway has been recently suggested to play a pivotal role in the immune evasion of tumors from host immune system. In this study, we tried to reveal the clinical importance and therapeutic potential of the PD-L/PD-1 pathway in pancreatic cancer, which is one of the most aggressive and intractable malignant tumors.

**Experimental Design:** We used immunohistochemistry to investigate PD-L expression in 51 patients with pancreatic cancer who underwent surgery and explored the therapeutic efficacy of blocking the PD-L1/PD-1 pathway in murine pancreatic cancer *in vivo*.

**Results:** PD-L1 – positive patients had a significantly poorer prognosis than the PD-L1 – negative patients, whereas there was no significant correlation of tumor PD-L2 expression with patient survival. PD-L1 expression was inversely correlated with tumor-infiltrating T lymphocytes, particularly CD8<sup>+</sup> T cells. These clinical data have suggested that the PD-L1/PD-1 pathway may be a critical regulator in human pancreatic cancer. Monoclonal antibodies against PD-L1 or PD-1 induced a substantial antitumor effect on murine pancreatic cancer *in vivo*. PD-L1 blockade promoted CD8<sup>+</sup> T-cell infiltration into the tumor and induced local immune activation. Furthermore, the combination of anti – PD-L1 monoclonal antibody and gemcitabine exhibited a significant synergistic effect on murine pancreatic cancer and resulted in complete response without overt toxicity.

**Conclusion:** Our data suggest for the first time that PD-L1 status may be a new predictor of prognosis for patients with pancreatic cancer and provide the rationale for developing a novel therapy of targeting the PD-L/PD-1 pathway against this fatal disease.

Pancreatic cancer is still one of the most aggressive and intractable human malignant tumors and a leading cause of cancer-related deaths worldwide (1, 2). Due to its extremely high malignant potential, it is usually diagnosed at an advanced stage and often recurs even after curative surgical excision (3). Despite significant advances in cancer therapy, including

surgery, radiation, chemotherapy, or combinations of these, the overall pancreatic cancer mortality rate has not been dramatically changed (1, 2). Therefore, novel approaches against pancreatic cancer need to be developed and established to improve patient prognosis.

Programmed death-1 (PD-1) is an immunoglobulin superfamily member related to CD28 and CTLA-4 (4–6). PD-1 is induced on T cells, B cells, and monocytes on activation. Accumulating evidence indicates that PD-1 plays a crucial role in regulating peripheral tolerance and autoimmunity (4, 7). PD-1 has two ligands: PD-1 ligand 1 (PD-L1; B7-H1) and PD-1 ligand 2 (PD-L2; B7-dendritic cells; refs. 8–12). PD-L1 and PD-L2 are involved in the negative regulation of cellular and humoral immune responses by engaging PD-1 receptor (4). PD-L1 is expressed on resting T cells, B cells, dendritic cells, and macrophages. PD-L1 is also expressed on parenchymal cells, including vascular endothelial cells and pancreatic islet cells (7). In contrast, PD-L2 is inducibly expressed only on dendritic cells and macrophages (4, 8). The distinct expression patterns of PD-L1 and PD-L2 suggest that their relative functions may depend on the tissue microenvironment (4, 7, 10). Recent studies have also shown that the PD-L/PD-1 pathway might play critical roles in tumor immunity (4, 13, 14). PD-L1 on tumors or antigen-presenting cells in tumor environment has been proposed to promote tumor growth and induce apoptosis

**Authors' Affiliations:** <sup>1</sup>Department of Surgery, <sup>2</sup>Second Department of Internal Medicine, <sup>3</sup>First Department of Internal Medicine, Nara Medical University, Nara, Japan; <sup>4</sup>Department of Immunology, Juntendo University School of Medicine; and <sup>5</sup>Department of Molecular Immunology, Graduate School, Tokyo Medical and Dental University, Tokyo, Japan

Received 11/17/06; revised 12/17/06; accepted 12/28/06.

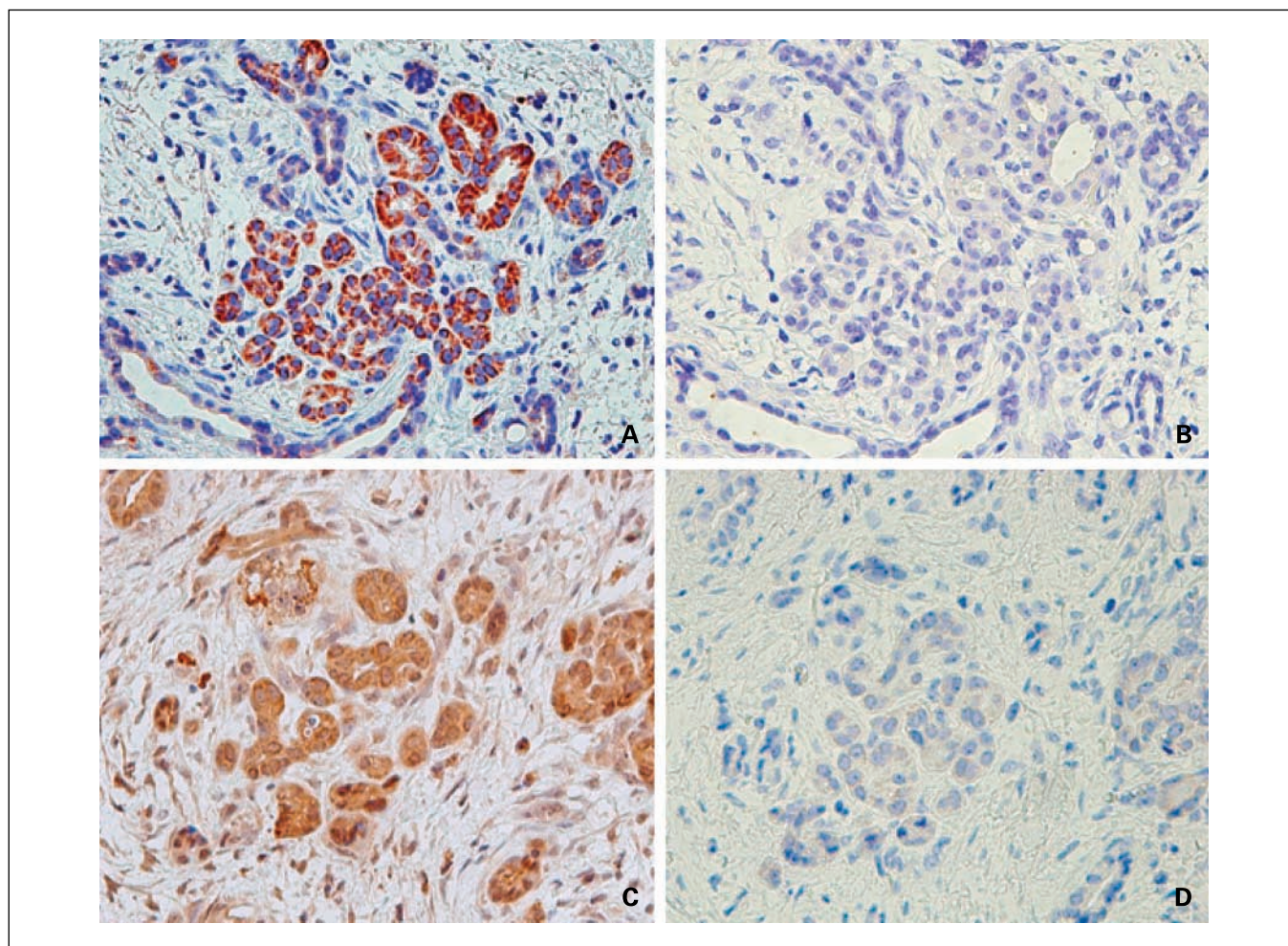
**Grant support:** Grants-in-Aid 16591343 for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan; Research Grant from Pancreas Research Foundation of Japan; Research Grant from Foundation for Promotion of Cancer Research in Japan; and Research Grant from Daiwa Securities Health Foundation (M. Sho).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

**Requests for reprints:** Masayuki Sho, Department of Surgery, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8522, Japan. Phone: 81-744-29-8863; Fax: 81-744-24-6866; E-mail: m-sho@narmed-u.ac.jp.

© 2007 American Association for Cancer Research.

doi:10.1158/1078-0432.CCR-06-2746



**Fig. 1.** Immunohistochemical staining of human pancreatic cancer tissue for PD-L1 and PD-L2. Representative case of PD-L1 – positive (A) and PD-L2 – positive (C) tumor. B and D, negative controls stained with mouse immunoglobulin G. Original magnification,  $\times 400$ .

of tumor-reactive T cells expressing PD-1 (13, 14). More recently, we and others have also shown the potential negative regulatory role for PD-L1 in clinical cancers (15–18). Thus, PD-L1 may play an important role in the immune evasion from host immune system in cancer patients. On the other hand, some studies have suggested that, in contrast to PD-L1, PD-L2 might serve as a costimulator in tumors (19, 20). They have shown that PD-L2 expression led to rapid tumor rejection by enhancing tumor-reactive T-cell priming and effector function (19). PD-L2 also augmented T helper 1 and CTL response *in vivo* (21). However, analysis of the clinical data on tumor PD-L2 expression has been limited at present (17).

In this study, we investigated the clinical significance of PD-L1 and PD-L2 expression in human pancreatic cancer and the therapeutic efficacy of targeting the PD-L/PD-1 pathway toward future clinical application for the treatment of pancreatic cancer.

## Materials and Methods

**Patients.** We examined 51 patients with pancreatic cancer who underwent surgery at Department of Surgery, Nara Medical University,

between 1996 and 2004. The median age of the patients was 63 years, with a range of 46 to 73 years. Tissues were obtained from the resected specimens and then were rapidly frozen at  $-80^{\circ}\text{C}$  for storage until use. For immunohistochemistry, a part of fresh tumor tissue specimen was immediately embedded in optimum cutting temperature compound (Miles, Kankakee, IL), and frozen sections were then cut on the cryostat to thickness of 5 mm. A serial section from each specimen was stained with H&E for histologic evaluation. The median follow-up for all patients was 22 months, with a range of 3 to 91 months. Most of the patients received systemic chemotherapy after surgery. Positive surgical margin was identified in 24 (47.1%) patients and negative in 27 (52.9%).

**Animal and cell line.** Female C57BL/6 mice (8–12 weeks old) were obtained from CLEA Japan (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at Nara Medical University. All experiments were conducted under a protocol approved by our institutional review board. A murine pancreatic adenocarcinoma, PAN 02, was obtained from the DCTD Tumor Repository, National Cancer Institute (Frederick, MD). Cells were grown in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum. To evaluate the inducible expression of PD-L1 on PAN 02, cells were treated for 72 h with recombinant murine IFN- $\gamma$  (10 ng/mL; PharMingen, San Diego, CA). Then, PD-L1 expression was measured by fluorescence-activated cell sorting analysis.

**Antibodies.** Monoclonal antibodies (mAb) against human PD-L1 (MIH1, mouse immunoglobulin G1) and PD-L2 (MIH18, mouse

immunoglobulin G1) were previously described (17). RMP1-14 and MIH6 against mouse PD-1 and PD-L1 (rat immunoglobulin G) were generated as previously described (10). Antihuman CD4 and CD8 T-cell mAbs were purchased from Dako Japan (Kyoto, Japan). Antimouse CD4 and CD8 T-cell mAbs and isotype immunoglobulin were purchased from PharMingen. Gemcitabine was a generous gift of Eli Lilly Japan (Kobe, Japan).

**Immunohistochemistry.** Immunohistochemical staining was done using Dako Envision system (Dako Japan) as previously described (17). After neutralization of endogenous peroxidase, cryostat sections on glass slides were preincubated with blocking serum and then were incubated overnight with each mAb. After three washes in PBS, the sections were incubated for 1 h with biotinylated antimouse immunoglobulin G, washed thrice with PBS, incubated with avidin-biotin-peroxidase complex for 1 h, and again washed for 10 min with PBS. Reaction products were visualized with 3,3'-diaminobenzidine tetrahydrochloride and the slides were counterstained with hematoxylin.

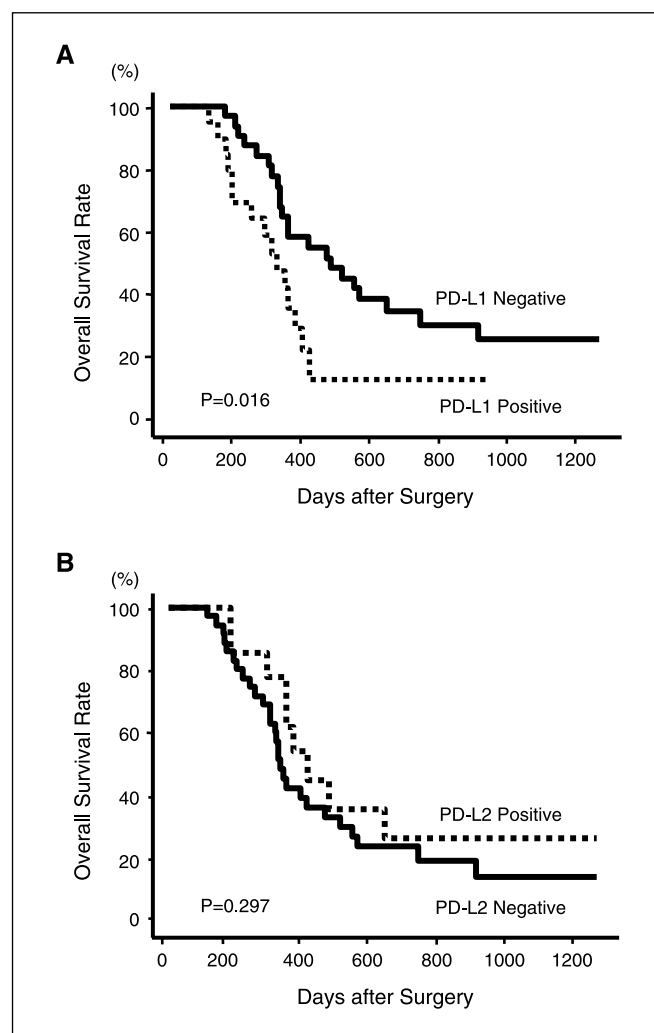
**Evaluation of immunostaining.** Immunohistochemistry for PD-L was evaluated as previously described (17). All of the immunostained sections were examined under low power (4× objective) to identify regions containing low-staining tumor cells. In case of multiple areas with low intensity, five randomly selected areas were scored, and in sections where all of the staining appeared intense, one field was selected at random. The proportion of tumor cells showing high and low staining in each selected field was determined by counting individual tumor cells at high magnification. At least 200 tumor cells were scored per ×400 field. Because most prognostic factors are usually considered as dichotomized, discontinuous variables, a cutoff point was selected to give the optimal separation between low risk and high risk of the overall survival as previously described by others and us (17, 22, 23). Then, we selected 10% as cutoff point based on preliminary analysis and recent report on PD-L1 expression in human cancer (16). Specimens with a ≥10% PD-L-positive tumor cells were classified as positive. Immunohistochemistry for CD4<sup>+</sup> and CD8<sup>+</sup> T cells was evaluated. An average number of >50 accumulating CD4<sup>+</sup> and CD8<sup>+</sup> tumor-infiltrating T lymphocytes (TIL) per field at ×200 magnification were scored. We used the mean number of infiltrating cells as a cutoff point to divide all tumors into groups as having either positive or negative infiltration by TILs in tumor tissue. Fifty-one cases were classified as having positive (≥150) levels of T-cell infiltration in tumor tissue and the remainder as having negative (<150) infiltration. Tumor samples were examined and classified by two observers who had no knowledge of the patients' clinical status and outcome.

**Extraction of total RNAs and real-time reverse-transcriptase PCR analysis.** Total RNA was isolated using the guanidine isothiocyanate method (RNeasy Protect Mini Kit, Qiagen, Tokyo, Japan) and was transcribed to cDNA using cDNA synthesis kit (Pharmacia, Piscataway, NJ) according to the manufacturer's protocol. A human tonsil tissue was used as the positive control for PD-L. Real-time quantitative PCR analysis was done by using ABI Prism 7700 sequence detector system (PE Applied Biosystems, Foster City, CA). All primer/probe sets were purchased from PE Applied Biosystems. PCR was carried out with the TaqMan Universal PCR Master Mix (PE Applied Biosystems) using 1 μL of cDNA in a 20-μL final reaction volume. The PCR thermal cycle conditions were as follows: initial step at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The expression level of the housekeeping gene *β<sub>2</sub>-microglobulin* was measured as an internal reference with a standard curve to determine the integrity of template RNA for all of the specimens. The ratio of mRNA level of each gene was calculated as follows: (absolute copy number of each gene) / (absolute copy number of *β<sub>2</sub>-microglobulin*).

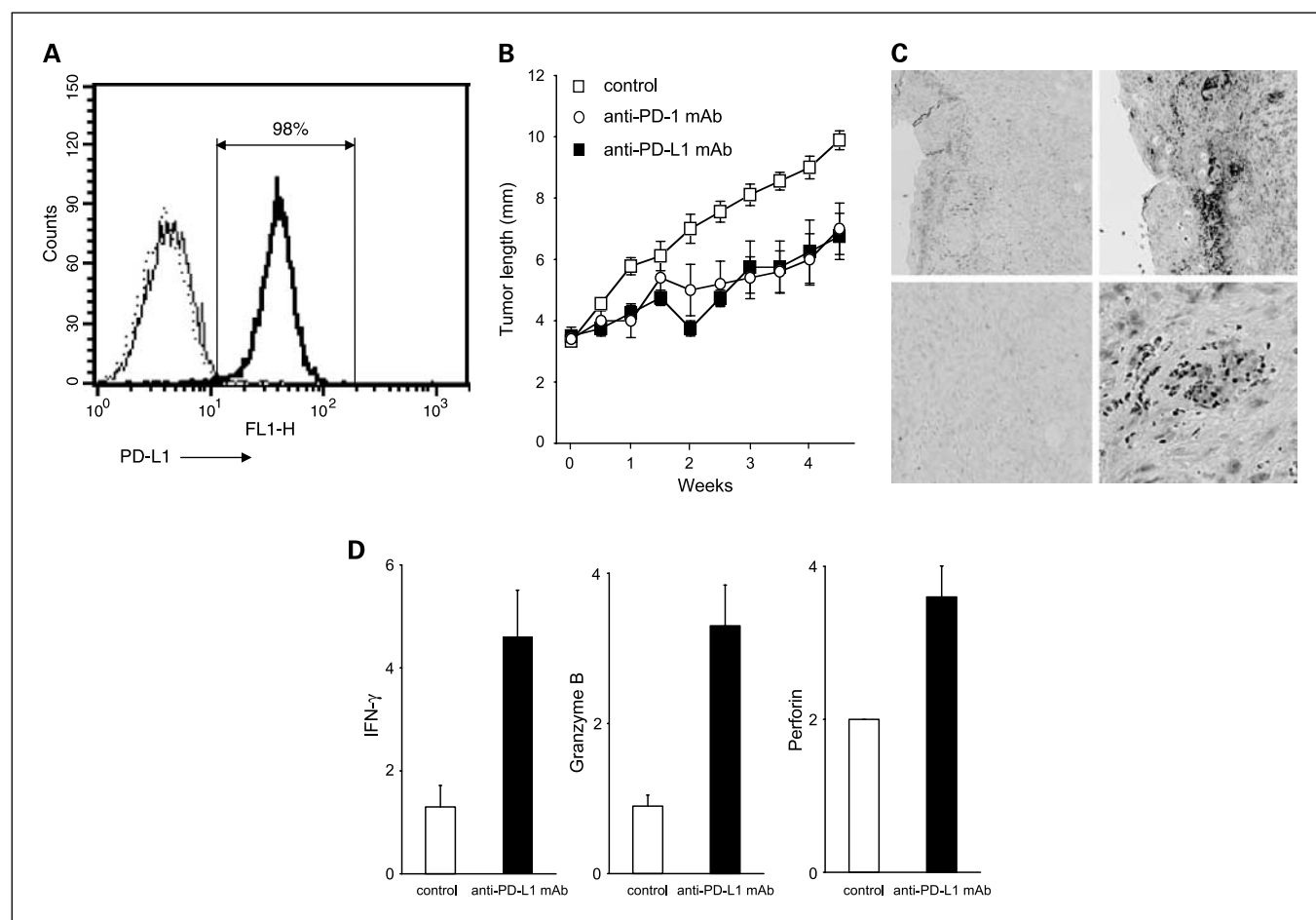
**Animal experimental protocol.** Cells (1 × 10<sup>6</sup> PAN 02) were s.c. inoculated on one side of the ventral surface in the lower flank region of syngeneic C57BL/6 mice. Treatment was started ~10 days later when a small palpable lump was evident, ranging from 3 to 4 mm in diameter.

Some mice were injected i.p. with 0.3 mg of each mAb thrice per week. Control mice received control immunoglobulin G. Gemcitabine was given i.p. at a dose of 60 μg/g every third day. The dose was elected based on our preliminary experiments as well as previous reports in murine tumor models. Tumor size was determined by caliper measurements. At 4 weeks after tumor establishment, mice were sacrificed and tumors were taken for further analysis.

**Statistical analysis.** The overall cancer-specific survival time was calculated from the date of surgery to the date of death from pancreatic cancer. The significance of the difference between PD-L expression and several clinical and pathologic variables was assessed by the  $\chi^2$  test or Mann-Whitney *U* test. The Kaplan-Meier method was used to estimate the probability of survival, and significance was assessed by the log-rank test. Multivariate analysis was done using the Cox regression model to study four factors (PD-L1 status, tumor status, nodal status, and metastatic status). We use the term of tumor status as T factor, nodal status as N factor, and metastatic status as M factor in tumor-node-metastasis classification, respectively. Some data in murine model were analyzed using Student's *t* test to determine significant differences between two different groups. *P* < 0.05 was considered as statistically significant.



**Fig. 2.** A, overall survival of 51 patients with pancreatic cancer in relation to tumor PD-L1 status. PD-L1-positive patients had a poorer prognosis than the PD-L1-negative patients (*P* = 0.016). B, overall survival of 51 patients with pancreatic cancer in relation to tumor PD-L2 status. There was no significant difference in prognosis between PD-L2-positive and PD-L2-negative patients (*P* = 0.297).



**Fig. 3.** Therapeutic efficacy of PD-L1/PD-1 blockade in murine pancreatic cancer. *A*, expression of PD-L1 on murine pancreatic cancer cells, PAN 02. IFN- $\gamma$  stimulation for 3 d *in vitro* induced its high expression (>95%) as estimated by flow cytometric analysis. Dashed line, negative control; thin line, naïve PAN 02; bold line, PAN 02 stimulated with IFN- $\gamma$ . *B*, PD-L1 or PD-1 blockade induced significant antitumor effect and inhibited tumor growth. Mice were treated with anti-PD-L1 or anti-PD-1 mAb at a dose of 0.3 mg thrice per week for 4 wk. Tumor size at 4 wk after tumor establishment: anti-PD-L1 mAb,  $6.3 \pm 1.0$  mm,  $n = 5$ ; anti-PD-1 mAb,  $6.0 \pm 0.8$  mm,  $n = 5$ ;  $P = 0.0087$  and  $P = 0.0025$ , respectively, compared with controls ( $9.0 \pm 0.4$  mm,  $n = 10$ ). Points, mean maximal tumor size; bars, SE. *C*, immunohistochemical staining of CD8<sup>+</sup> T cells in tumor tissue at 4 wk after tumor establishment. Representative pictures of three mice for each treatment. PD-L1 blockade induced massive CD8<sup>+</sup> T-cell infiltration (magnification: *top right*,  $\times 100$ ; *bottom right*,  $\times 400$ ) compared with controls (*top left*,  $\times 100$ ; *bottom left*,  $\times 400$ ). *D*, induction of local immune activation by PD-L1 blockade. The expressions of IFN- $\gamma$ , granzyme B, and perforin were significantly higher in tumors treated with anti-PD-L1 mAb than in controls ( $P = 0.030$ ,  $P = 0.015$ , and  $P = 0.012$ , respectively). Points, mean of real-time PCR data from three individual mice for each group; bars, SE.

## Results

**PD-L expression in human pancreatic cancer.** Of the 51 tumors evaluated in this study, 20 (39.2%) were positive for PD-L1 expression at protein level and 31 (60.8%) were negative, whereas PD-L2 was positive in 14 (27.5%) and negative in 37 (72.5%). PD-L1 and PD-L2 were expressed mainly in the plasma membrane and cytoplasm of cancer cells (Fig. 1). In addition, PD-L1 and PD-L2 expression was also found in some infiltrating lymphocytes as well as stromal cells in pancreatic cancer tissue.

**Correlation between PD-L expression and postoperative prognosis.** Among the 51 patients, PD-L1-positive patients had significantly poorer survival than the PD-L1-negative patients ( $P = 0.016$ ; Fig. 2A). One-year postoperative survival rate of PD-L1-positive and PD-L1-negative patients was 33.5% and 60.3%, respectively. On the other hand, there were no significant differences in postoperative prognosis between PD-L2-positive and PD-L2-negative patients (Fig. 2B). There was no significant correlation between tumor PD-L1 status

and clinical indicators including tumor status, nodal status, metastatic status, and pathologic stage. However, subgroup analysis has indicated that significant differences were noted in 1-year survival rate after surgery between PD-L1-positive and PD-L1-negative patients when categorized by the following variables: T<sub>3</sub> status, N<sub>0</sub> status, M<sub>0</sub> status, and pathologic stage IIa (40.8% versus 62.9%,  $P = 0.019$ ; 20.0% versus 87.5%,  $P = 0.019$ ; 33.3% versus 63.2%,  $P = 0.035$ ; and 20.0% versus 100%,  $P = 0.006$ , respectively; Table 1). Surgical margin status had no significant effect on the prognosis of either PD-L1-positive or PD-L1-negative patients. Furthermore, the multivariate analysis using Cox regression model has shown that tumor PD-L1 status was defined to be a significant independent prognostic factor ( $\chi^2 = 5.26$ , RR = 2.3,  $P = 0.022$ ). Although there was a trend showing that advanced-stage tumors such as T<sub>4</sub>, N<sub>1</sub>, and M<sub>1</sub> had poorer survival, T, N, M status did not reach statistical significance.

**Inverse correlation between tumor PD-L1 and TILs.** We then analyzed the correlation between tumor PD-L1 expression and TILs. There was a significant inverse correlation between PD-L1

expression and TILs, particularly CD8<sup>+</sup> T cells (CD4<sup>+</sup> T cells,  $P = 0.019$ ; CD8<sup>+</sup> T cells,  $P < 0.0001$ ; Table 2).

**Therapeutic efficacy of PD-L1/PD-1 blockade in pancreatic cancer in vivo.** For clinical application, we explored the therapeutic efficacy of blocking the PD-L1/PD-1 pathway in pancreatic cancer *in vivo*. To this end, we used a murine pancreatic adenocarcinoma, PAN 02. Although constitutive expression of PD-L1 was minimal in PAN 02, IFN- $\gamma$  stimulation induced its high expression (>95%) as estimated by flow cytometric analysis (Fig. 3A). *In vivo* treatment with either anti-PD-L1 or anti-PD-1 blockade on PAN 02 induced a substantial antitumor effect *in vivo* and significantly inhibited tumor growth (Fig. 3B). There was no significant difference in tumor growth between PD-L1 and PD-1 blockade. To define the underlying mechanisms in tumor growth inhibition by PD-L1 blockade, we first assessed the intratumoral T cells in this model. Marked CD8<sup>+</sup>, but minimal CD4<sup>+</sup>, T-cell infiltration in implanted tumor tissues was identified by immunohistochemistry (Fig. 3C). Quantification by real-time PCR further confirmed the effect of PD-L1 blockade on T cells [CD8<sup>+</sup> T cells,  $P = 0.0004$ ; CD4<sup>+</sup> T cells, not significant ( $P > 0.05$ ), compared with controls]. Then, we analyzed local immune status in tumors. The expressions of IFN- $\gamma$ , granzyme B, and perforin were significantly higher in tumors treated with anti-PD-L1 mAb than in controls ( $P = 0.030$ ,  $P = 0.012$ , and  $P = 0.015$ , respectively; Fig. 3D).

**Synergy between PD-L1 blockade and chemotherapy.** Finally, we evaluated the combination of chemotherapy with PD-L1 blockade in pancreatic cancer. We used gemcitabine that is currently the standard chemotherapeutic agent for pancreatic cancer. The delayed 2-week treatment of anti-PD-L1 mAb starting on day 15 after tumor establishment had minimal inhibitory effect on tumor progression (Fig. 4A). The treatment of gemcitabine alone resulted in significant inhibition of tumor growth (Fig. 4A). This antitumor effect of gemcitabine was further enhanced by the combination with the delayed PD-L1 blockade (Fig. 4A). Furthermore, the combined treatment of gemcitabine

and 4-week treatment of PD-L1 blockade displayed a substantial synergistic antitumor effect on pancreatic cancer, thereby resulting in complete response in treated mice (Fig. 4B). There were no overt toxicity and death in mice during and after treatment.

## Discussion

Pancreatic cancer is among the most lethal cancers due, in part, to a lack of effective therapies (1, 2). To improve the patient survival, a variety of both basic and clinical researches have been conducted. However, despite substantial efforts to develop novel therapy, there has been little change in the overall pancreatic cancer mortality rate (1). Among the proposed novel therapies, immunotherapy may be a potentially potent strategy (2). Several strategies such as vaccine to stimulate patient's own immune system and achieve an antitumor response have been clinically attempted. Unfortunately, the therapeutic efficacy was only limited thus far, partly because tumor may evade host immune response through various mechanisms. Recent studies have suggested a novel mechanism of tumor escape through negative regulation of PD-L and PD-1 interaction (13, 14). The expression of PD-L on the cell surface of tumor itself or antigen-presenting cells in tumor environments might induce apoptosis on tumor-reactive T cells through engagement of PD-1 and promote tumor growth (13, 14). Several clinical studies conducted by us and others further showed that tumor PD-L1 expression has significant clinical implications (15–18).

In this study, we first investigated the tumor PD-L expression in human pancreatic cancer. Then we confirmed both PD-L1 and PD-L2 expression in several pancreatic cancer tissues and found that tumor PD-L1 but not PD-L2 expression significantly correlated with postoperative prognosis. We also found that PD-L1 expression was inversely correlated with TILs, particularly CD8<sup>+</sup> T cells. Furthermore, the subgroup analysis has indicated the significance of PD-L1 in early stage including

**Table 1.** One-year survival rate of 51 patients with pancreatic cancer according to clinicopathologic characteristics and tumor PD-L1 status

Variable	n (%)	PD-L1 positive, n (%)	PD-L1 negative, n (%)	P
Tumor status				
T <sub>1</sub>	1 (100)	1 (ND)	0 (—)	ND
T <sub>2</sub>	7 (42.9)	3 (0)	4 (75.0)	0.093
T <sub>3</sub>	40 (54.7)	15 (40.8)	25 (62.9)	0.019
T <sub>4</sub>	3 (54.7)	1 (ND)	2 (ND)	ND
Nodal status				
N <sub>0</sub>	15 (61.9)	6 (20.0)	9 (87.5)	0.019
N <sub>1</sub>	36 (46.2)	14 (40.8)	22 (50.0)	0.252
Metastatic status				
M <sub>0</sub>	45 (52.8)	17 (33.0)	28 (63.2)	0.035
M <sub>1</sub>	6 (33.3)	3 (33.3)	3 (33.3)	0.486
Pathologic stage				
Ia, Ib	2	50.0 (0)	— (2)	ND (ND)
IIa	13 (64.2)	6 (20.0)	7 (100)	0.006
IIb	29 (50.6)	11 (42.4)	18 (55.6)	0.426
III	1 (0)	0 (—)	1 (ND)	ND
IV	6 (33.3)	3 (33.3)	3 (33.3)	0.486
Total	51 (50.4)	20 (33.5)	31 (60.3)	0.016

Abbreviation: ND, not determined.

**Table 2.** Inverse correlation between tumor PD-L1 status and TILs

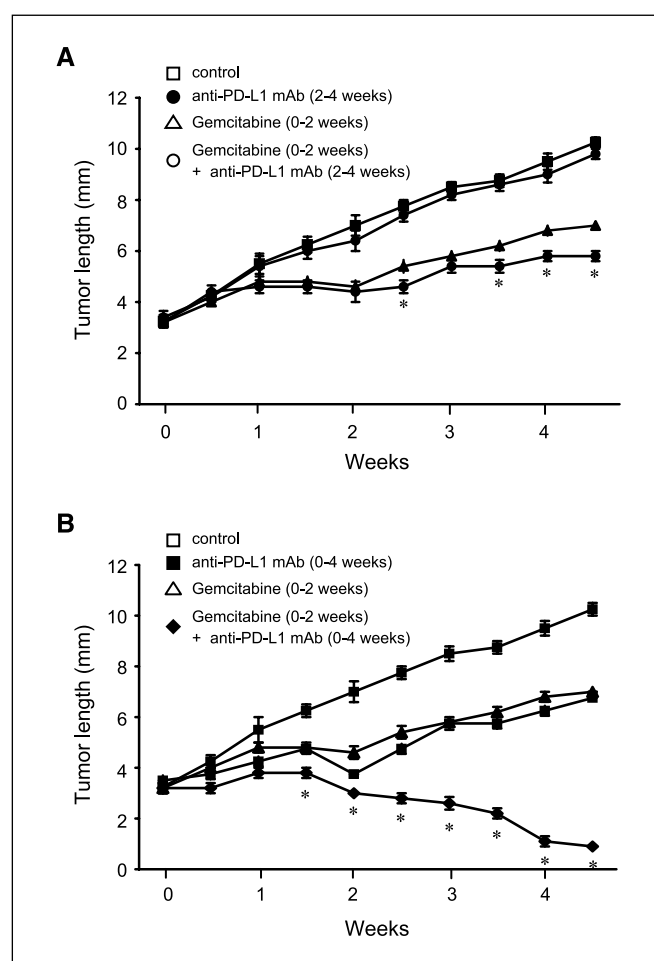
PD-L1	CD4		CD8	
	Positive	Negative	Positive	Negative
Positive	9	11	5	15
Negative	24	7	25	6

$N_0$  and  $M_0$  status, suggesting that PD-L1 might play more important role in tumor progression rather than in tumor metastasis of pancreatic cancer. Our *in vivo* study in murine pancreatic cancer model may further support that PD-L1 is a critical regulator in tumor growth of pancreatic cancer. In our previous study, in contrast to pancreatic cancer, both PD-L1 and PD-L2 status had a significant effect in advanced stage of esophageal cancer with positive lymph node and distant metastasis (17). Such differences between pancreatic and esophageal cancer may depend on tumor biology and malignancy of each cancer. Taken together with other recent studies, this study might further corroborate that tumor-associated PD-L expression plays a critical role in human cancers (15–18).

Next, using a murine pancreatic cancer model, we investigated the therapeutic efficacy of blocking the PD-L1/PD-1 pathway in pancreatic cancer toward future clinical application. Then, we found several important data in this model. First, both anti-PD-1 and anti-PD-L1 mAbs had a significant antitumor effect on the inhibition of tumor growth, suggesting that the PD-L1/PD-1 pathway is critical for growth of pancreatic cancer. Our data corroborate several previous studies and strengthen the therapeutic potential of targeting the PD-L1/PD-1 pathway for the treatment of cancer patients (13, 24–27). Second, we confirmed that PD-L1 blockade promoted tumor-reactive CD8<sup>+</sup> T-cell infiltration into the established tumor, thereby resulting in local immune activation as evidenced by the up-regulation of several potent effectors including IFN- $\gamma$ , granzyme B, and perforin. However, further studies may be required to clarify the therapeutic effect of PD-L1/PD-1 blockade on the metastasis of pancreatic cancer using other cancer metastasis models because metastasis and metastatic relapse are the most frequent causes of cancer-related death in pancreatic cancer (1, 3, 28). Third, more importantly, data indicated that the combination of gemcitabine with PD-L1 blockade exerted a synergistic antitumor effect on pancreatic cancer. Chemotherapy and immunotherapy have usually been regarded as unrelated or potentially antagonistic forms of therapy (29). This is because most chemotherapies kill target cells by apoptosis and similarly induce cell death of activated T cells by recognizing tumor antigen. In addition, lymphopenia is a common side effect of many anticancer drugs, and this has been assumed to be detrimental to sufficient antitumor immune response. Our data showed that the delayed PD-L1 blockade following preceding gemcitabine treatment significantly augmented antitumor efficacy. Furthermore, the combination of gemcitabine with simultaneous blockade of PD-L1 has exerted a significant synergistic effect on pancreatic cancer and induced complete response without overt toxicity. Gemcitabine is currently the standard chemotherapeutic agent for pancreatic cancer because several clinical trials have proved the some favorable effect on patient survival (1, 30). However, the effect of

gemcitabine alone is limited and most patients develop resistance to the therapy. Therefore, gemcitabine in combination with other approaches is currently under investigation (26, 30). Thus, our data may be clinically important and support future application of PD-L1/PD-1 blockade for the treatment of pancreatic cancer.

In conclusion, we have shown for the first time that PD-L1 is a novel prognostic marker for human pancreatic cancer. Furthermore, our data have indicated that targeting PD-L1/PD-1, especially in combination with standard chemotherapy, exhibited significant therapeutic efficacy. Little change in pancreatic cancer mortality for decades has created an urgent demand for the development of new strategies directed toward novel targets. Our data may provide the rationale of developing a novel immunotherapy targeting the PD-L1/PD-1 pathway for this fatal malignant disease.



**Fig. 4.** Combination of PD-L1 blockade and gemcitabine. *A*, gemcitabine and delayed PD-L1 blockade significantly inhibited tumor growth of pancreatic cancer. Gemcitabine was given *i.p.* on days 1, 4, 7, 10, and 13 at a dose of 60  $\mu$ g/g. Anti-PD-L1 mAb was given at a dose of 0.3 mg on days 15, 17, 19, 22, 24, and 26. Tumor size at 4 wk after tumor establishment: gemcitabine + anti-PD-L1 mAb, 5.8  $\pm$  0.2 mm;  $P = 0.0077$ , versus gemcitabine alone, 6.8  $\pm$  0.2 mm;  $P < 0.0001$ , versus anti-PD-L1 mAb alone, 9.0  $\pm$  0.3 mm. *B*, gemcitabine and simultaneous PD-L1 blockade had a synergistic antitumor effect and resulted in complete response in treated mice. Anti-PD-L1 mAb was given at a dose of 0.3 mg for three per week for 4 wk. Tumor size at 4 wk after tumor establishment: gemcitabine + anti-PD-L1 mAb, 1.2  $\pm$  0.2 mm;  $P < 0.0001$ , versus gemcitabine alone, 6.8  $\pm$  0.2 mm;  $P = 0.001$ , versus anti-PD-L1 mAb alone, 6.2  $\pm$  1.0 mm. Points, mean tumor size of five mice; bars, SE.

## References

- Li D, Xie K, Wolff R, Abbruzzese JL. Pancreatic cancer. *Lancet* 2004;363:1049–57.
- Laheru D, Jaffee EM. Immunotherapy for pancreatic cancer—science driving clinical progress. *Nat Rev Cancer* 2005;5:459–67.
- Traverso LW. Pancreatic cancer: surgery alone is not sufficient. *Surg Endosc* 2006;20 Suppl 2:S446–9.
- Greenwald RJ, Freeman GJ, Sharpe AH. The B7 family revisited. *Annu Rev Immunol* 2005;23:515–48.
- Nishimura H, Honjo T. PD-1: an inhibitory immunoreceptor involved in peripheral tolerance. *Trends Immunol* 2001;22:265–8.
- Ishida Y, Agata Y, Shibahara K, Honjo T. Induced expression of PD-1, a novel member of the immunoglobulin gene superfamily, upon programmed cell death. *EMBO J* 1992;11:3887–95.
- Keir ME, Liang SC, Guleria I, et al. Tissue expression of PD-L1 mediates peripheral T cell tolerance. *J Exp Med* 2006;203:883–95.
- Latchman Y, Wood CR, Chernova T, et al. PD-L2 is a second ligand for PD-1 and inhibits T cell activation. *Nat Immunol* 2001;2:261–8.
- Freeman GJ, Long AJ, Iwai Y, et al. Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation. *J Exp Med* 2000;192:1027–34.
- Youngnag P, Kozono Y, Kozono H, et al. Differential binding properties of B7-1 and B7-DC to programmed death-1. *Biochem Biophys Res Commun* 2003;307:672–7.
- Tseng SY, Otsuji M, Gorski K, et al. B7-DC, a new dendritic cell molecule with potent costimulatory properties for T cells. *J Exp Med* 2001;193:839–46.
- Dong H, Zhu G, Tamada K, Chen L. B7-1, a third member of the B7 family, co-stimulates T-cell proliferation and interleukin-10 secretion. *Nat Med* 1999;5:1365–9.
- Curiel TJ, Wei S, Dong H, et al. Blockade of B7-1 improves myeloid dendritic cell-mediated antitumor immunity. *Nat Med* 2003;9:562–7.
- Dong H, Strome SE, Salomao DR, et al. Tumor-associated B7-1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793–800.
- Ghebeh H, Mohammed S, Al-Omair A, et al. The B7-1 (PD-L1) T lymphocyte-inhibitory molecule is expressed in breast cancer patients with infiltrating ductal carcinoma: correlation with important high-risk prognostic factors. *Neoplasia* 2006;8:190–8.
- Thompson RH, Kuntz SM, Leibovich BC, et al. Tumor B7-1 is associated with poor prognosis in renal cell carcinoma patients with long-term follow-up. *Cancer Res* 2006;66:3381–5.
- Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. *Clin Cancer Res* 2005;11:2947–53.
- Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res* 2004;10:5094–100.
- Liu X, Gao JX, Wen J, et al. B7DC/PDL2 promotes tumor immunity by a PD-1-independent mechanism. *J Exp Med* 2003;197:1721–30.
- Shin T, Yoshimura K, Shin T, et al. *In vivo* costimulatory role of B7-DC in tuning T helper cell 1 and cytotoxic T lymphocyte responses. *J Exp Med* 2005;201:1531–41.
- Matsumoto K, Inoue H, Nakano T, et al. B7-DC regulates asthmatic response by an IFN- $\gamma$ -dependent mechanism. *J Immunol* 2004;172:2530–41.
- Tandon AK, Clark GM, Chamness GC, Chirgwin JM, McGuire WL. Cathepsin D and prognosis in breast cancer. *N Engl J Med* 1990;322:297–302.
- Ikeda N, Nakajima Y, Tokuhara T, et al. Clinical significance of aminopeptidase N/CD13 expression in human pancreatic carcinoma. *Clin Cancer Res* 2003;9:1503–8.
- Iwai Y, Ishida M, Tanaka Y, Okazaki T, Honjo T, Minato N. Involvement of PD-L1 on tumor cells in the escape from host immune system and tumor immunotherapy by PD-L1 blockade. *Proc Natl Acad Sci U S A* 2002;99:12293–7.
- Hirano F, Kaneko K, Tamura H, et al. Blockade of B7-1 and PD-1 by monoclonal antibodies potentiates cancer therapeutic immunity. *Cancer Res* 2005;65:1089–96.
- Strome SE, Dong H, Tamura H, et al. B7-1 blockade augments adoptive T-cell immunotherapy for squamous cell carcinoma. *Cancer Res* 2003;63:6501–5.
- Blank C, Kuball J, Voelkl S, et al. Blockade of PD-L1 (B7-1) augments human tumor-specific T cell responses *in vitro*. *Int J Cancer* 2006;119:317–27.
- Iwai Y, Terawaki S, Honjo T. PD-1 blockade inhibits hematogenous spread of poorly immunogenic tumor cells by enhanced recruitment of effector T cells. *Int Immunol* 2005;17:133–44.
- Lake RA, Robinson BW. Immunotherapy and chemotherapy—a practical partnership. *Nat Rev Cancer* 2005;5:397–405.
- Hochster HS, Haller DG, de Gramont A, et al. Consensus report of the international society of gastrointestinal oncology on therapeutic progress in advanced pancreatic cancer. *Cancer* 2006;107:676–85.