

# Lentivirus-mediated RNA interference targeting programmed death receptor ligand 1 increases the immunologic anti-tumor effect of dendritic cell vaccination against pancreatic cancer in SCID-hu mice

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**Abstract.** Programmed death receptor ligand 1 (PD-L1), which belongs to the B7 family, is overexpressed in a variety of human cancer types and serves a crucial role in immune escape by malignant cells. Programmed death receptor 1 (PD-1) is a specific PD-L1 receptor. PD-1/PD-L1 signaling inhibits the antitumor effects of dendritic cell (DC) immunization for tumor treatment. The aim of the present study was to determine whether inhibiting PD-L1 may increase the immunologic anti-tumor effect of dendritic cells against pancreatic cancer. In the present study, PD-L1 levels in non-cancerous and malignant tissue samples were compared, and the impact of PD-L1 downregulation on human pancreatic cancer PaTu8988 cells was determined by lentivirus-based RNA interference and DC immunotherapy. PD-L1 expression in pancreatic specimens was assessed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and immunohistochemistry. PaTu8988 cells expressing reduced levels of PD-L1 were generated by lentivirus-based knockdown to assess the mechanism by which the inhibition of PD-L1 signaling in DC immunization affects therapeutic outcomes in pancreatic cancer-bearing SCID-hu mice. PD-L1 levels were markedly elevated in pancreatic adenocarcinoma samples compared with in non-cancerous tissue. PD-L1 silencing in pancreatic adenocarcinoma cells resulted in improved treatment

outcomes of DC immunization *in vitro* and *in vivo* compared with traditional DC immunization. PD-L1 silencing enhances the antitumor response of cytotoxic T cells by increasing interferon  $\gamma$  production *in vitro*. *In vivo*, this method prevented tumor growth and lung metastasis, and prolonged survival in the SCID-hu model. In conclusion, the results of the present study suggested that suppressing PD-L1 in malignant cells during DC immunization may be a useful tool for immunotherapy in pancreatic adenocarcinoma.

## Introduction

Pancreatic adenocarcinoma is a common and highly aggressive malignancy that affects the digestive system (1). Occult infiltration and early rapid dissemination make pancreatic adenocarcinoma the fourth deadliest cancer worldwide, with the mortality rate being ~7% in both sexes (2,3). Surgery is the exclusive method for radical treatment of pancreatic cancer, with adjuvant chemotherapeutics such as gemcitabine and S-1 often administered post-surgery (4). Despite recent therapeutic advances, the incidence of pancreatic adenocarcinoma is steadily increasing with the rate of new cases being ~3% in females, and the 5-year survival rate in definite cases is only ~8%, largely due to the low rate of early diagnosis and the limited resection rate of advanced tumors (3,5). It is estimated that by 2030, pancreatic cancer will overtake breast cancer to become the second deadliest malignant tumor in the USA (6). In addition, there are no effective therapeutic options currently available to eradicate metastasis (7).

Immunotherapy induces cancer-specific immune reactions in the host, with remarkable therapeutic effects, which are enhanced by cytokines and interleukins (8). Successful tumor immunotherapy should counteract immune evasion and tolerance in cancer cells. Immunotherapy aims to inhibit cancer cells by specifically activating antitumor immune reactions (9), which are controlled, in particular, by cytotoxic (CD8<sup>+</sup>) T cells. Their precursors, dendritic cells (DCs), are the most powerful antigen-presenting cells that stimulate naive

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T cells with high efficiency to produce CD8<sup>+</sup> T cells (10). Immature DCs populate peripheral tissues to capture antigens, followed by maturation and migration through afferent lymphatic vessels into the draining lymph nodes (11). DCs are crucial in inducing tumor-specific immune responses, particularly through presentation of major histocompatibility complex-class I antigens and cytotoxic T cell production (12). DC-based tumor immunization represents a novel experimental immunotherapeutic approach in (13).

Programmed death receptor ligand 1 (PD-L1), which belongs to the B7 family of membrane proteins, was the first co-inhibitory protein the levels of which were reported to strongly correlate with cancer-specific survival (14). A number of studies have revealed the mechanisms by which tumors evade destruction by immune reactions associated with PD-L1 expression in cells (15-17). Therefore, the present study assessed whether lentivirus-mediated RNA interference (RNAi) targeting PD-L1 combined with DC vaccination may enhance T cell activity in pancreatic cancer. In addition, the effects of PD-L1 knockdown in cancer cells combined with DC vaccination on the inhibition of metastasis and survival were evaluated in a SCID-hu pancreatic tumor mouse model.

## Materials and methods

**Pancreatic cancer and clinical specimens.** The present study was approved by the Ethics Committee of The First Affiliated Hospital of Soochow University (Suzhou, China). A total of 46 patients with pancreatic adenocarcinoma surgically treated between January and December 2010 in the Department of General Surgery, The First Affiliated Hospital of Soochow University (Suzhou, China) were enrolled in the study. In the present study, the pancreatic cancer cases included 27 men and 19 women, with an average age of 61.5 years (range, 42-71 years). There were 26 patients with stage I-II tumors and 20 patients with stage III-IV tumors according to the 8th edition American Joint Committee on Cancer pancreatic cancer staging criteria (18). The criteria for including patients within the study included, patients who were diagnosed with pancreatic cancer prior to surgery and a diagnosis was confirmed via postoperative pathology. Exclusion criteria were: i) Treatment with chemotherapy or radiotherapy; ii) other pancreatic surgery prior to the surgical treatment of pancreatic adenocarcinoma. Pancreatic adenocarcinoma tissues were collected following signed written informed consent from the patients. Non-cancerous pancreatic tissue specimens were used as control samples. Hematoxylin and eosin (H&E) staining was performed to confirm diagnosis. During preparation and analysis, the tissue samples were divided in two by dissection under aseptic conditions; one part of the tissue specimens was submitted to fixation with 10% buffered methanol at room temperature for 24 h and used for PD-L1 detection by immunohistochemistry (IHC); the remaining part was frozen in liquid nitrogen for RNA extraction followed by reverse transcription-quantitative PCR (RT-qPCR) for PD-L1 mRNA level assessment. Peripheral blood samples were collected from healthy volunteers, which were prepared in order to extract peripheral blood mononuclear cells (PBMCs).

**Immunohistochemical staining.** Following overnight fixation with formalin at room temperature, the samples were embedded in paraffin and sectioned to 4- $\mu$ m-thick slices using a microtome. The activity of endogenous peroxidase was inactivated by 3% H<sub>2</sub>O<sub>2</sub> for 15 min, and 3% BSA was used to block non-specific binding at 37°C for 30 min. The sections were incubated overnight with rabbit anti-PD-L1 (dilution, 1:100; cat. no. ab205921; Abcam) primary antibody at 4°C, and further incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:1000; cat. no. ab6721; Abcam) at 37°C for 30 min. The tissue sections were counterstained with hematoxylin, dehydrated in gradient concentrations of ethanol, and mounted in neutral resin. The presence of brown diaminobenzidine precipitates, which represent positive reactivity, was evaluated under a light microscope (magnification, x200 and x400). Brown cytoplasmic signals were considered to indicate PD-L1 expression.

**RNA extraction and RT-qPCR.** Total RNA extraction from 46 cases of resected pancreatic adenocarcinoma and non-cancerous pancreatic specimens was performed using the RNeasy<sup>®</sup> Mini kit (Qiagen GmbH) following the manufacturer's protocol. RNA amounts were assessed by UV spectrophotometry, and cDNA was obtained using the QuantiTect<sup>®</sup> Reverse Transcription kit (Qiagen GmbH) according to the manufacturer's protocol. qPCR was performed using SYBR Green real-time PCR Master Mix (Takara Bio, Inc.) on 7500 Fast Real-Time PCR system (Bio-Rad Laboratories, Inc.), and the experimental results were analyzed using the 2<sup>- $\Delta\Delta$ C<sub>q</sub></sup> method to determine relative expression levels (19). GAPDH was used as a reference gene for normalization. The thermocycling conditions were as follows: Initial denaturation at 95°C for 15 sec, followed by 45 cycles of 95°C for 5 sec and 60°C for 30 sec. The primers used were: PD-L1 forward, 5'-ACTGGCATT TGCTGAACG-3' and reverse, 5'-TCCTCCATTTCCCAA TAGAC-3' (148 bp); GAPDH forward, 5'-TGACTTCAACAG CGACACCCA-3' and reverse, 5'-CACCTGTTGCTGTA GCCAAA-3' (121 bp).

**Cells and culture.** Pancreatic adenocarcinoma PaTu8988 cells were provided by the Institute of Biochemistry and Cell Biology (Chinese Academy of Science). The media contained 10% fetal bovine serum (Atlanta Biologicals, Inc.) and 1% penicillin-streptomycin (Thermo Fisher Scientific, Inc.), and cells were cultured at 37°C in a humid atmosphere containing 5% CO<sub>2</sub>. Cells were harvested at 80-90% confluency using 0.25% trypsin. Small hairpin RNA (shRNA) of human PD-L1 (GenBank ID, NM\_014143) harboring a lentivirus gene transfer vector that encodes green fluorescent protein (GFP) was obtained from Shanghai GeneChem Co., Ltd. The targeting sequence of PD-L1 was confirmed by sequencing to be 5'-GACCTATATGTGGTAGAGTAT-3'. The recombinant lentiviral vector expressing shRNA targeting PD-L1 (RNAi) and the non-targeted control mock lentivirus (NC) were adjusted to 5x10<sup>9</sup> transfection units/ml. PaTu8988 cells were seeded in 6-well plates (5x10<sup>4</sup> cells/well) overnight prior to transfection with lentiviral particles (multiplicity of infection, 100). The transfection was implemented at 37°C for 12 h, then culture medium was replaced by the normal culture medium, followed by incubation in a medium containing puromycin

(5  $\mu\text{g}/\text{ml}$ ) for selecting stably-transfected cells. Fluorescent microscopy was used to evaluate GFP expression and assess infection efficiency 3 days after transfection. Non-transfected PaTu8988 cells were used as controls. PD-L1 mRNA knock-down in transfected cells was confirmed by RT-qPCR and immunoblotting.

**Flow cytometric analysis.** PaTu8988 cells that had not been subjected to any other treatments were digested with EDTA-pancreatin, collected and washed with PBS. Cells ( $1 \times 10^6$ ) were incubated with a rabbit phycoerythrin-conjugated anti-PD-L1 antibody (1:100; cat. no. ab228415; Abcam) for 30 min at 4°C, followed by incubation with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. ab150077; Abcam) for 30 min at 4°C in the dark. The collected cells were resuspended in 0.5 ml PBS and analyzed using Cytomics FC 500 flow cytometer (Beckman Coulter, Inc.). Data analysis was performed using FlowJo v10.0 software (FlowJo LLC).

**Western blot analysis.** A total of  $1 \times 10^7$  cells were collected and washed twice prior to lysis on ice and then centrifuged for supernatant collection. The amounts of protein were assessed using Bio-Rad DC Protein assay (Bio-Rad Laboratories, Inc.). Proteins (50  $\mu\text{g}$  per lane) were separated via SDS-PAGE (10% gel) and transferred onto polyvinylidene difluoride (PVDF) membranes. Following blocking with 5% milk dissolved in TBS containing 0.05% Tween-20 (TBST), at room temperature for 1 h, the membranes were incubated overnight with rabbit anti-PD-L1 (1:1,000; cat. no. ab228415; Abcam) and rabbit anti-GAPDH (1:1,000; cat. no. ab22555; Abcam) primary antibodies at 4°C. A horseradish peroxidase-conjugated secondary goat anti-rabbit antibody (1:2,000; cat. no. ab6721; Abcam) was added for 1 h under shaker conditions at room temperature. Protein expression was determined using Enhanced Chemiluminescence Western Blot Detection Reagents (GE Healthcare).

**CD8<sup>+</sup> T cell antitumor response in vitro.** PBMCs were obtained by Ficoll-Hypaque gradient centrifugation. By using a bead-labeled anti-CD8 monoclonal antibody CD8-PE (1:50; cat. no. 130113720; Miltenyi Biotec GmbH) and a MACS separation column (Miltenyi Biotec GmbH) following the manufacturer's protocol, CD8<sup>+</sup> T lymphocytes were purified from PBMCs by immuno-magnetic reaction. The purity of CD8<sup>+</sup> T cells was >95%, and they were used for T cell response experiments.

CD14<sup>+</sup> monocytes (>95% purity) were obtained by magnetic cell separation (Miltenyi Biotec GmbH) and evaluated by flow cytometric analysis. The cells ( $3 \times 10^5$  cells/ml) were generated by following the classical technique for mature DC generation (20), and cultured in medium containing granulocyte macrophage colony-stimulating factor (GM-CSF; 25 ng/ml) and interleukin (IL)-4 (500 U/ml). Tumor necrosis factor (TNF)- $\alpha$  was supplemented at 5 days. Following two additional days of culture, the cells were prepared for T cell response experiments.

The control, NC and RNAi pancreatic cancer cell groups were washed with PBS three times following pre-treatment with mitomycin C (50 mg/ml; Sigma-Aldrich; Merck KGaA)

at 37°C for 30 min to inhibit proliferation. Each group of pancreatic cancer cells ( $5 \times 10^4$  cells/group) was co-cultured with human CD8<sup>+</sup> T lymphocytes ( $4 \times 10^5$  lymphocytes/well) and stimulated with CD14<sup>+</sup> monocytes ( $8 \times 10^4$  monocytes/well). Cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS at 200  $\mu\text{l}/\text{well}$  at 37°C in a humid environment containing 5% CO<sub>2</sub> for 72 h. Co-cultures were performed three times in 96-well plates. At 72 h of co-culture, supernatants from the three groups were collected separately for ELISA of soluble interferon (IFN)- $\gamma$  produced by CD8<sup>+</sup> T lymphocytes using an ELISA kit (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Pancreatic cancer cells were also co-cultured with human CD8<sup>+</sup> T lymphocytes without DCs as described above.

**Preparation of the dendritic cell vaccine.** Pancreatic cancer cells were thawed and cultured in RPMI-1640 containing 10% fetal bovine serum (FBS) (Sijiqing Biological Manufacturer Co., Ltd.) overnight at 37°C with 5% CO<sub>2</sub>. Monocyte culture was performed in presence of GM-CSF + IL-4 + isotype immunoglobulin G (IgG) for five days, and cells were treated with tumor lysates (cancer cell/DC ratio, 1:3) for 16 h. The cells were washed with PBS, and DC maturation was stimulated by adding TNF- $\alpha$  + isotype IgG to the culture medium for two additional days.

**Establishment and confirmation of the SCID-hu mouse model.** All experiments involving animals were approved by the Animal Care and Use Committee of Soochow University (Suzhou, China) and complied with current guidelines for animal welfare of the China National Committee for Animal Experiments. A total of 36, 6-week-old female SCID mice were supplied by Shanghai SLAC Laboratory Animal Co., Ltd., and kept under specific pathogen-free conditions in an environment with controlled humidity at 25-28°C in the dark. The mice had constant access to food and water. The SCID-hu model of pancreatic cancer was established as previously described (21). In brief, hemocytopenia in SCID mice was inhibited by administration of cerebrotendinous xanthomatosis at 40 mg/kg/d. Following intraperitoneal injections for four successive days, PBMCs ( $10^7$  cells/animal) were administered by injection to the abdominal cavity of each SCID mouse in order to generate the SCID-hu model. Human T and B cells and human IgG levels in peripheral blood were detected by fluorescence-assisted cell sorting and ELISA. Pancreatic adenocarcinoma model establishment was performed in SCID-hu mice by subcutaneous injection of 100  $\mu\text{l}$  pancreatic cancer cell suspension from each group (control, NC and RNAi;  $2 \times 10^4$  cells/mouse) for 4 weeks following engraftment. There were 12 mice per group. Tumor growth was monitored every two days. The animal studies were performed at the Laboratory Animal Center of Soochow University (Suzhou, China) according to a protocol approved by the Animal Care and Use Committee of Soochow University.

**Treatment of tumor-bearing mice.** Tumor diameter was ~1 cm four weeks post-cancer cell administration. Each group of tumor-bearing mice was randomly divided into two sub-groups (six mice per sub-group). In each group, one sub-group received subcutaneous injections of the DC vaccine

( $1 \times 10^6$  cells/mouse), while the other did not, which served as the control. Therapeutic DC vaccines were administered weekly for four consecutive weeks. Tumor sizes were measured by Vernier calipers twice weekly, and volumes were calculated as  $V = L \times W^2 \times 0.5$ . Mice were sacrificed by cervical dislocation 5 weeks later, and tumors were harvested five weeks following the completion of the DC vaccine treatment. Additionally, the thoracic cavity of each mouse was carefully opened to expose the trachea; India ink was intratracheally administered for lung staining, and the lung was rinsed with Fekete's solution. White nodules in the lung indicated metastatic pancreatic cancer cells. The number of metastatic tumor nodules on the lung surface was determined for each mouse. PD-L1 expression levels in the tumors and lung surface metastatic tumor nodules were determined by RT-qPCR as described above.

**Assessment of mouse survival following DC vaccine treatment.** The CD8<sup>+</sup> T cell-DC vaccine-SCID-hu mice were used to establish the pancreatic cancer model by subcutaneous injection of 100  $\mu$ l pancreatic cancer cell suspension (control, NC and RNAi;  $2 \times 10^4$  cells/mouse) four weeks following engraftment. There were 16 mice per group. Each group of tumor-bearing mice was randomly divided into two sub-groups (8 mice per sub-group) four weeks following tumor cell inoculation. In each group, one subgroup received the DC vaccine treatment as described above, whereas the other did not, which served as the control. The DC vaccines were administered weekly in all the treatment sub-groups for four consecutive weeks. Following the completion of treatment, the survival rates of mice were monitored for 10 weeks. The survival time for each mouse was recorded and the survival curve was drawn.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. The *in vitro* experiments were repeated three times. SPSS software (version 10.0; SPSS, Inc.) was used for the statistical analysis. Differences in values and percentages among groups were compared using a paired t-test,  $\chi^2$  test, Fisher's exact test or one-way analysis of variance followed by Dunnett's multiple comparison test, as appropriate. Survival curves and univariate analysis were estimated by the Kaplan-Meier method and the log-rank test using GraphPad Prism software (version 7.0; GraphPad Inc.).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**PD-L1 is found in most pancreatic adenocarcinoma tissues and absent in normal pancreatic tissues.** Relative expression levels of PD-L1 mRNA in pancreatic adenocarcinoma specimens were significantly increased compared with non-cancerous pancreatic tissue samples ( $P < 0.001$ ; Fig. 1). Immunohistochemical staining demonstrated significant over-expression of PD-L1 in pancreatic adenocarcinoma tissues compared with non-cancerous tissues ( $\chi^2 = 12.541$ ;  $P < 0.001$ ; Fig. 2). PD-L1 was identified in  $>70\%$  of cells in 34 out of 46 pancreatic cancer specimens, but not detected in normal pancreatic specimens (Fig. 2).

**Successful PD-L1 downregulation by RNA interference in PaTu8988 cells.** Flow cytometry was used to assess PD-L1

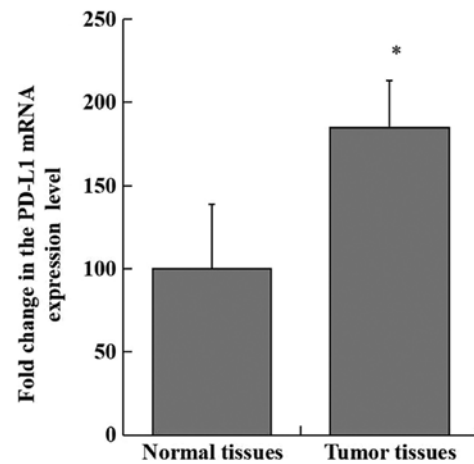


Figure 1. PD-L1 mRNA expression is increased in pancreatic adenocarcinoma compared with non-cancerous pancreatic tissues. Tumor tissues, pancreatic adenocarcinoma tissues obtained from patients with pancreatic adenocarcinoma. Normal tissues, paired precancerous tissue from patients with pancreatic adenocarcinoma. \* $P < 0.01$  vs. non-cancerous tissue.

expression levels in PaTu8988 cell lines prior to lentiviral transfection (Fig. 3A). The results revealed that PD-L1 was highly expressed in PaTu8988 cell lines. Fluorescence microscopy was used to demonstrate that the lentiviral infection of PaTu8988 cells with RNAi and NC was successful and highly efficient (Fig. 3B). In addition, PD-L1 mRNA and protein expression levels were assessed; PD-L1 mRNA expression levels were significantly reduced in the RNAi group compared with the NC and control groups ( $25 \pm 7\%$ ;  $P < 0.01$ ; Fig. 4A and B);  $\sim 75\%$  of gene expression was suppressed. The NC and control groups exhibited similar PD-L1 mRNA expression. PD-L1 protein expression levels detected by western blotting exhibited a similar pattern to mRNA expression (Fig. 4C). The present results indicated that RNAi downregulation of PD-L1 was specific and efficient.

**PD-L1 knockdown in PaTu8988 cells increases IFN- $\gamma$  production by CD8<sup>+</sup> T cells in co-culture with pancreatic adenocarcinoma cells and DCs.** CD8<sup>+</sup> T cells were simultaneously cultured with DCs and pancreatic adenocarcinoma cells to assess the role of PD-L1 expressed by pancreatic cancer cells in suppressing the effects of T-cells. Knockdown of PD-L1 significantly enhanced IFN- $\gamma$  biosynthesis by CD8<sup>+</sup> T lymphocytes ( $1,200 \pm 89$  ng/ml) compared with the NC ( $345 \pm 54$  ng/ml) and control ( $330 \pm 45$  ng/ml) groups ( $P < 0.01$ ; Fig. 5). No statistically significant differences in IFN- $\gamma$  levels were observed between the NC and control groups. Low levels of IFN- $\gamma$  were detected in the three groups without co-culture with DCs.

**PD-L1 silencing enhances the efficacy of DC vaccination in a subcutaneous tumor model.** The effects of PD-L1 on the tumor immune environment have been demonstrated by *in vitro* experiments assessing pancreatic cancer cells (22). Thus, the present study assessed whether such effects are present *in vivo*. DC vaccines were administered in tumor-bearing mice weekly for four consecutive weeks. In each pancreatic cancer model mouse, DC vaccination treatment inhibited

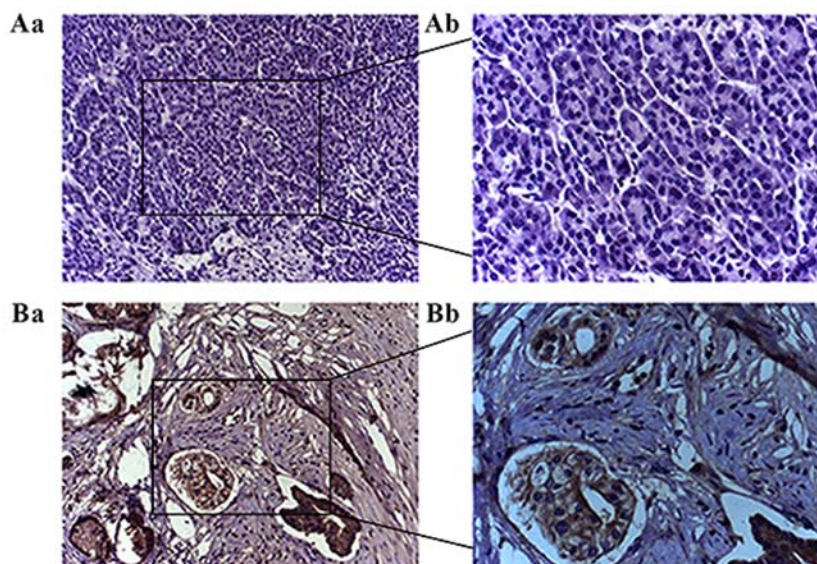


Figure 2. PD-L1 protein expression in clinical samples detected by immunohistochemistry. (A) No PD-L1 was observed in the non-cancerous pancreatic tissue. (B) PD-L1 was overexpressed in pancreatic adenocarcinoma. Magnification, A-a and B-a, x100; A-b and B-b, x200. PD-L1, programmed death receptor ligand.

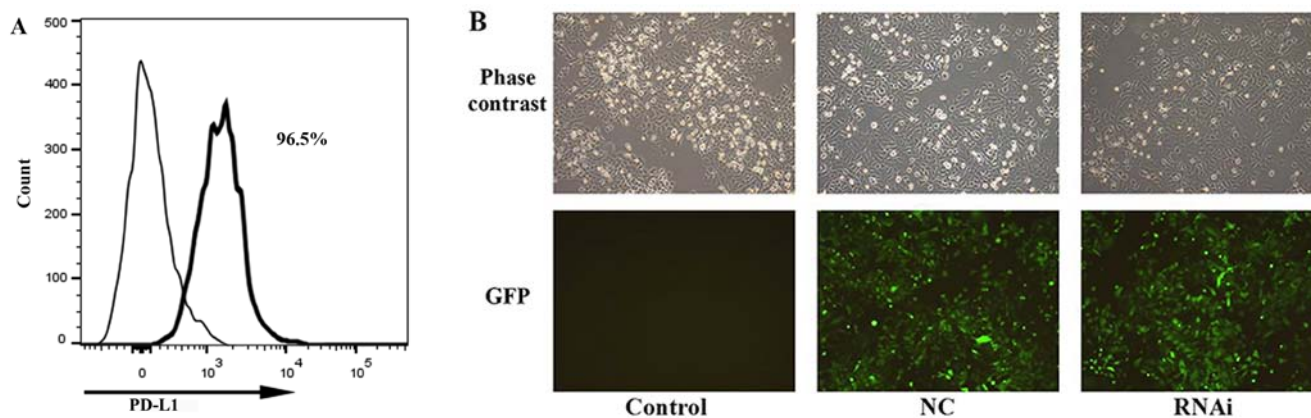


Figure 3. Efficiency of PD-L1 RNAi silencing. (A) PD-L1 expression levels in PaTu8988 cell lines prior to lentiviral infection evaluated by flow cytometry. (B) PaTu8988 cell infection with NC and RNAi lentiviruses. GFP expression was evaluated by fluorescence microscopy. Magnification, x100. B7-H3, cluster of differentiation 276; GFP, green fluorescent protein; NC, negative control; PD-L1, programmed death receptor ligand 1; RNAi, cells transfected with short hairpin RNA targeting PD-L1.

tumor growth compared with the untreated group (Fig. 6A). In addition, knockdown of PD-L1 combined with DC vaccination induced a more potent anti-tumor immunity compared with other groups: significant growth inhibition was observed in the RNAi + DC vaccine group five weeks following treatment ( $130 \pm 30 \text{ mm}^3$ ) compared with the NC + DC vaccine ( $254 \pm 66 \text{ mm}^3$ ) and control + DC vaccine ( $278 \pm 45 \text{ mm}^3$ ) groups ( $P < 0.01$ ; Fig. 6A). The mRNA expression levels of PD-L1 were significantly downregulated in the RNAi + DC vaccine group compared with the control + DC group ( $P < 0.01$ ; Fig. 6B).

Compared with DC vaccination only treatment, PD-L1 knockdown combined with DC vaccination demonstrated improved therapeutic effects on lung metastasis. Lung metastasis was significantly reduced in the RNAi + DC vaccine group five weeks following treatment ( $4 \pm 3$ ) compared with the NC + DC vaccine ( $23 \pm 9$ ) and control + DC vaccine ( $25 \pm 7$ ) groups ( $P < 0.01$ ; Fig. 7A and B). The non-treated (NT) RNAi group ( $33 \pm 6$ ) also exhibited significantly reduced

lung metastasis compared with the NC + NT ( $40 \pm 9$ ) and control + NT ( $45 \pm 8$ ) groups ( $P < 0.05$ ; Fig. 7B). In addition, PD-L1 mRNA expression levels were significantly downregulated in engrafted tumors and lung surface metastatic tumor nodules following treatment with RNAi + DC vaccine compared with the NC + DC vaccine and control + DC vaccine groups (Figs. 6B and 7C). These data indicated that inhibition of PD-L1 expression combined with DC treatment may decrease pancreatic adenocarcinoma metastasis *in vivo*. This result supported the *in vitro* observations that PD-L1 may be critical in inhibiting CD8<sup>+</sup> T cell antitumor immunological responses.

In mouse survival experiments, PD-L1 knockdown combined with DC vaccination prolonged the survival period of tumor-bearing mice (Fig. 8). Although DC vaccination exhibited certain antitumor effects compared with untreated groups, PD-L1 knockdown had a synergistic effect in prolonging the survival period of the animals ( $P < 0.01$ ; Fig. 8).

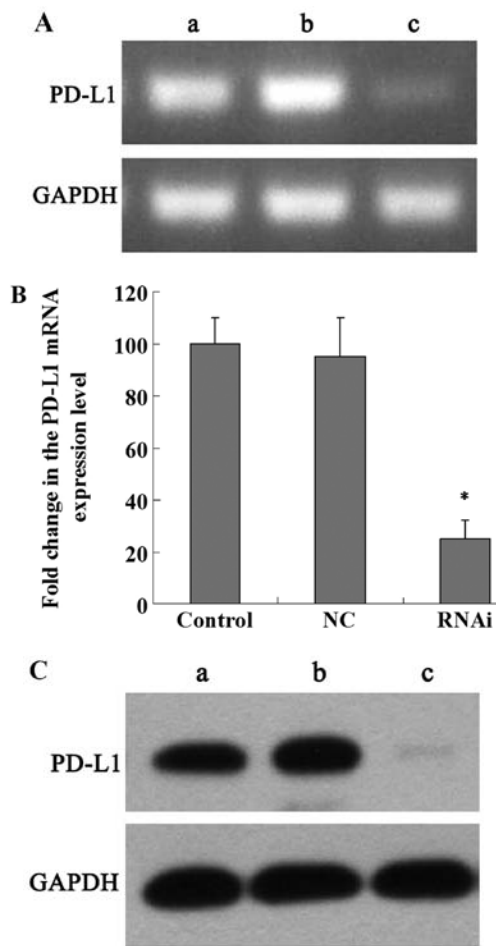


Figure 4. PD-L1 mRNA and protein expression levels are decreased following RNAi silencing. (A) Electrophoresis results of RT-qPCR products in the three groups of PaTu8988 cells (a, control; b, NC; c, RNAi). PD-L1 gene expression was reduced in the RNAi group compared with the control groups. (B) Quantification of PD-L1 mRNA levels by RT-qPCR following silencing with RNAi. PD-L1 mRNA levels were significantly decreased in the RNAi group. (C) PD-L1 protein expression levels detected by western blotting (a, control; b, NC; c, RNAi). PD-L1 protein expression in the RNAi group was reduced compared with that in the control and negative control groups. \*P<0.01 vs. control. NC, negative control; PD-L1, programmed death receptor ligand 1; RNAi, cells transfected with short hairpin RNA targeting PD-L1; RT-qPCR, reverse transcription-quantitative PCR.

**Discussion**

Antitumor immunity is mostly regulated by immune cells, particularly cancer-specific T cells (23). Although cancer-specific T lymphocytes exist in cancer patients and are activated by immunization, cancer cells are able to suppress T cell cytotoxicity by selecting certain immune checkpoints as the main immune resistance pathway (24). Therefore, blocking immune checkpoints is one of the most promising methods of inducing antitumor immune reactions (25). Among numerous immune checkpoints, PD-1 and its associated ligand PD-L1 have emerged as a promising target pair (26). PD-1/PD-L1 inhibition with monoclonal antibodies resulted in a breakthrough in cancer treatment, as it was revealed that antibody-mediated blockade of PD-L1 induced durable tumor regression and prolonged stabilization of disease in those patients with advanced disease stages (27,28).

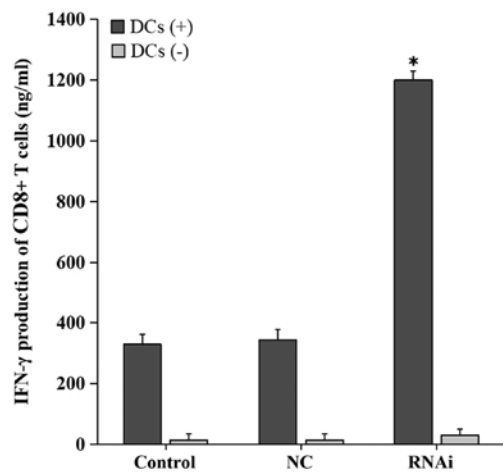


Figure 5. PD-L1 knockdown in cancer cells enhances T cell-associated IFN-γ secretion *in vitro*. CD8<sup>+</sup> T lymphocytes were cultured in presence of tumor cells with or without mature DCs (DC<sup>+</sup>). IFN-γ levels in the supernatants were evaluated by ELISA. IFN-γ secretion by CD8<sup>+</sup> T cells co-cultured with tumor cells of the RNAi group was significantly increased compared with that in the control groups. Data are from three independent experiments. \*P<0.01 vs. control. DC, dendritic cells; IFN-γ, interferon γ; NC, negative control; RNAi, cells transfected with short hairpin RNA targeting programmed death receptor ligand 1.

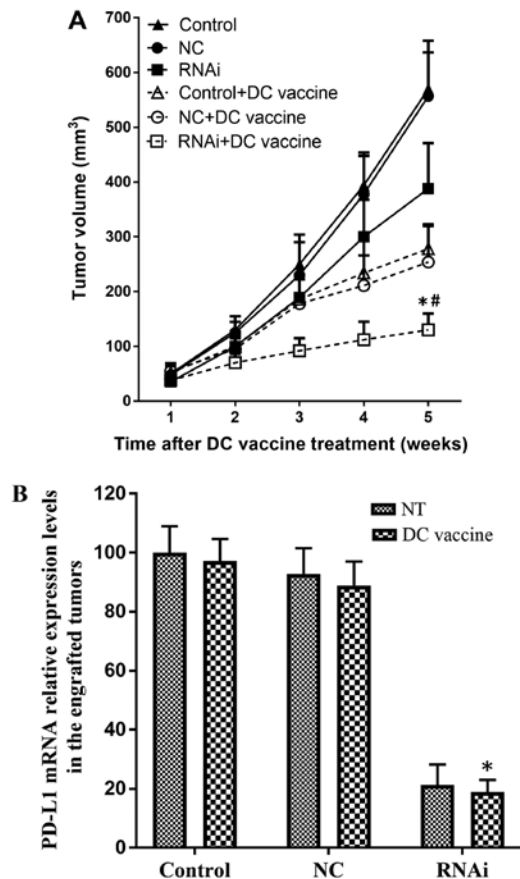


Figure 6. Subcutaneous tumor growth in the hu-SCID mouse model. (A) Growth curves of subcutaneously implanted pancreatic adenocarcinoma xenografts following various treatments (n=6 mice/group). The RNAi + DC vaccine group exhibited reduced tumor volumes compared with all other groups. (B) PD-L1 mRNA expression levels were downregulated in the RNAi + DC vaccine group compared with the control + DC group. \*P<0.01 vs. RNAi + NT. #P<0.01 vs. control + DC vaccine. DC, dendritic cells; NC, negative control; NT, no treatment; PD-L1, programmed death receptor ligand 1; RNAi, cells transfected with short hairpin RNA targeting PD-L1.



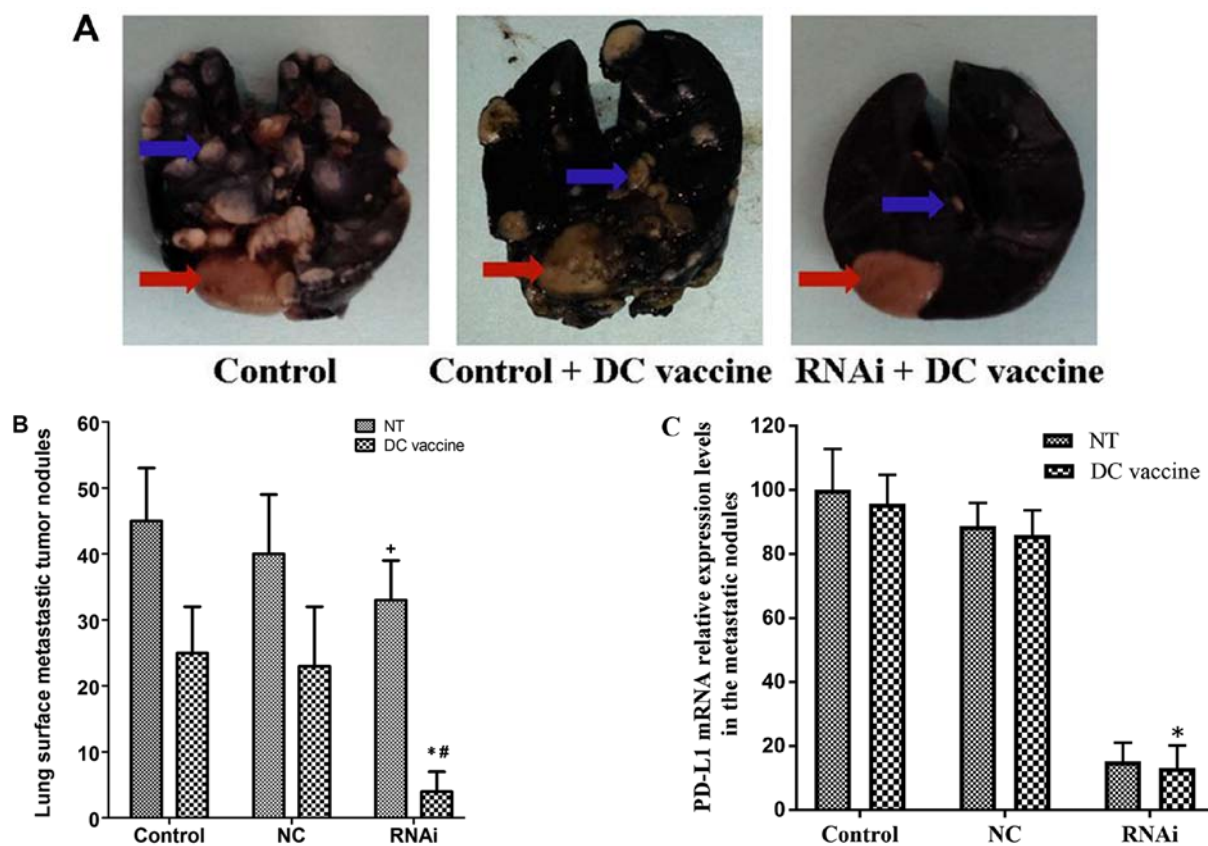


Figure 7. Lung surface metastatic nodules in the subcutaneous tumor hu-SCID mouse model. (A) Representative images of lung surface metastatic nodules. (B) The number of lung surface metastatic nodules in the RNAi + DC vaccine group was significantly decreased compared with that in the control + DC vaccine and the RNAi + NT groups. (C) PD-L1 mRNA expression in the RNAi + DC vaccine group was significantly reduced compared with that in the control + DC vaccine group. \*P<0.01 vs. control + DC vaccine; #P<0.01 vs. RNAi + NT; \*P<0.05 vs. control + NT. DC, dendritic cells; NC, negative control; NT, no treatment; PD-L1, programmed death receptor ligand 1; RNAi, cells transfected with short hairpin RNA targeting PD-L1.

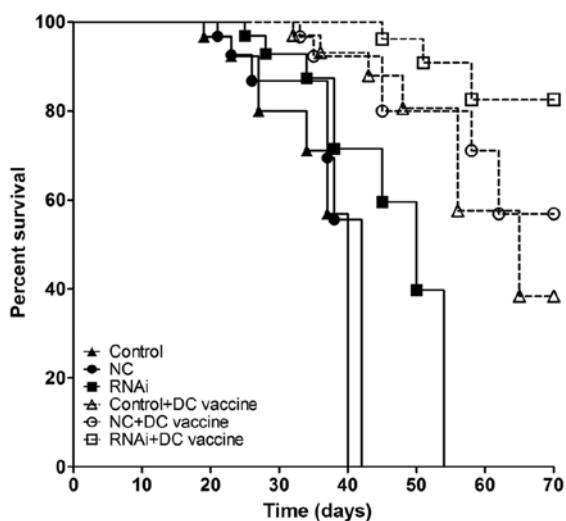


Figure 8. Knockdown of PD-L1 combined with DC vaccine increases hu-SCID mouse survival time. In the pancreatic adenocarcinoma mouse model, DC vaccine increased survival time compared with that in non-treated tumor-bearing animals. Compared with the RNAi alone and control + DC vaccination groups, the RNAi +DC vaccination group exhibited longer survival times.

As an immune-modulatory cell surface protein, PD-L1 binds PD-1 to negatively regulate cell-dependent immunity (29,30). Overexpression of PD-L1 has been described in various

human malignancies, including melanoma and glioblastoma, lung, breast, colon, ovary and renal cell cancers, impairing the antitumor effects of T cells (14,16,31-34). Aberrant PD-L1 expression is related to cancer aggressiveness and poor prognosis, which suggests that PD-L1 may serve an important role in tumor progression. The results of the immunohistochemical staining in the present study revealed that PD-L1 was overexpressed in pancreatic adenocarcinoma compared with normal pancreatic tissues. RT-qPCR was also used to verify the results of the immunohistochemical staining; the results coincided with those in a study by Loos *et al* (35), which revealed that PD-L1 may regulate Tregs and IFN- $\gamma$  to inhibit antitumor responses in pancreatic cancer, the mRNA expression levels of PD-L1 were upregulated in pancreatic adenocarcinoma.

The PD-L1/PD-1 interaction controlling cancer cell immune escape may be increased in pancreatic adenocarcinoma considering the high and ubiquitous expression of PD-L1 on cancer cells. The negative modulatory function of PD-L1 in cancer cells for tumor-specific T lymphocytes has been previously reported with transfected human cancer cells and animal experiments, which demonstrated altered tumor growth and metastasis control by immune cells (15-17,36). PD-L1 negatively regulates T-cell functions by binding PD-1; additionally, PD-1 is upregulated on activated T and B cells (37-39). Co-ligation of PD-1 reduces cytokine amounts and T cell proliferation (20,40-42). To determine the role of PD-L1 in antitumor immune evasion by pancreatic adenocarcinoma, a

stable PD-L1-silenced pancreatic adenocarcinoma cell line was established in the present study by lentivirus-mediated RNA interference. In an *in vitro* experiment, CD8<sup>+</sup> T lymphocytes were cultured in presence of mature DCs and pancreatic cancer cells, and the levels of secreted IFN- $\gamma$  were detected. The results demonstrated that PD-L1 knockdown in pancreatic adenocarcinoma cells induced significant upregulation of IFN- $\gamma$ , which is one of the most effective antitumor cytokines (43).

The *in vitro* results of the present study were further confirmed *in vivo*. In a SCID-hu subcutaneous tumor mouse model, PD-L1 RNAi knockdown combined with DC treatment decreased tumor growth and lung metastasis, and PD-L1 RNAi knockdown without DC treatment was also demonstrated to inhibit lung metastasis. Compared with the control and control + DC vaccine groups, the RNAi + DC vaccine group exhibited significantly reduced tumor volume and lung metastasis. Furthermore, knockdown of PD-L1 expression combined with DC treatment significantly prolonged the survival period of hu-SCID xenograft mice compared with the control and control + DC groups.

Previous findings indicate that PD-L1 pathway inhibition improves DC-mediated antitumor immunity. Curiel *et al* (31) demonstrated that PD-L1 pathway blockade enhances myeloid dendritic cell (MDC)-associated T cell activation, downregulates IL-10 as well as IL-2 and upregulates IFN- $\gamma$ . The authors concluded that PD-L1 pathway inhibition in MDCs in the tumor microenvironment constitutes a potential tool for tumor immunotherapy. Ge *et al* (44) revealed that blocking PD-L1 signaling enhances DC maturation and proliferation as well as secreted IL-12 levels, induces DC-primed T cell responses and reverses cancer cell-associated T cell impairment. In the present study, PD-L1 silencing during DC immunization resulted in improved treatment outcomes compared with routine DC immunization, reducing cancer cell proliferation and increasing survival time in a pancreatic cancer-bearing SCID-hu model. These findings suggested that PD-L1 knockdown combined with DC immunization may reverse the immune suppression effects exerted by pancreatic cancer cells and enhance T cell function. Following PD-L1 knockdown combined with DC immunization, IFN- $\gamma$  secretion was increased along with the proliferation of CD8<sup>+</sup> T cells, which may subsequently result in antitumor immune effects in the tumor microenvironment. However, PD-L1 knockdown was only performed in pancreatic cancer cells using lentivirus-mediated RNAi, whereas the PD-L1 pathway in DCs was not blocked, e.g. with PD-L1-specific antibodies. Nevertheless, a significant promotion of antitumor immunity was achieved by PD-L1 silencing combined with DC vaccine treatment, resulting in decreased cancer cell proliferation and metastasis, and enhanced survival in model animals. The present study provided novel insights into DC vaccine treatment, which may aid in the development of new immunotherapies for cancer.

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#### Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

#### Authors' contributions

XZhao designed the study, proposed the research project and is the guarantor. JW performed the experiments and drafted the manuscript. MS performed the experiments and analyzed the data. XZhu, HZ, DM and ZZ assisted in preparing and taking part in the experiments. All authors were involved in study design and data interpretation, and have reviewed the final version of the manuscript.

#### Ethics approval and consent to participate

All patients were informed about the study and provided written informed consent. The present study was approved by the Ethics Committee of First Affiliated Hospital of Soochow University (Suzhou, China). All experiments were performed in accordance with relevant guidelines and regulations.

#### Patient consent for publication

Not applicable.

#### Competing interests

The authors declare that they have no competing interests.

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