Original Paper

Knockdown of PD-L1 in Human Gastric Cancer Cells Inhibits Tumor Progression and Improves the Cytotoxic Sensitivity to CIK Therapy

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Key Words

PD-L1 • Gastric cancer • RNAi • Immunohistochemistry • Cancer progression

Abstract

Background/Abstract: PD-L1 has been an important target of cancer immunotherapy. We have showed that in human gastric cancer tissues, over-expression of PD-L1 was significantly associated with cancer progression and patients' postoperative prognoses. However, as of now, how PD-L1 regulates the biological function of gastric cancer cells still remains elusive. **Methods:** We constructed the stable PD-L1 knockdown expression gastric cancer cell lines by using RNAi method, and further investigated the changes of biological functions including cell viability, migration, invasion, cell cycle, apoptosis, tumorigenicity *in vivo*, and the cytotoxic sensitivity to CIK therapy, in contrast to the control cells. **Results:** In the current study, we demonstrated that the knockdown of PD-L1 expression in human gastric cancer cells could significantly suppress the cell proliferation, migration, invasion, apoptosis, cell cycle, tumorigenicity *in vivo* and the cytotoxic sensitivity to CIK therapy. **Conclusion:** Our results indicate that PD-L1 contributes towards transformation and progression of human gastric cancer cells, and its intervention could prove to be an important therapeutic strategy against gastric cancer.

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Introduction

It has been suggested that numerous mediators in the tumor microenvironment can contribute to the escape of tumor cells from tumor suppressive immune response. These include dysfunctional antigen-presenting cells, regulatory T cells (Tregs), and over-

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expression of B7-family co-inhibitory molecules on immune cells or tumor cells [1, 2]. PD-L1 (also known as B7-H1 or CD274), an important member of the B7-family molecules, can interact with the PD-1 receptor to inhibit the T-cell activation, induce apoptosis of effector T cells, and finally impair the anti-tumor immunity [3, 4]. Numerous studies have reported that PD-L1 over-expression in many human cancers is significantly associated with tumor progression and patients' prognosis, and thus could be used as an important tumor marker and an immunotherapeutic target [5-9].

Recent clinical trial data have established that the antibody-mediated blockade of PD-L1 induced durable tumor regression and prolonged the stabilization of disease in patients with advanced cancers, including non-small-cell lung cancer, melanoma, and renal cell cancer, suggesting that anti-PD-L1 therapy has broad prospects in clinical application [10, 11]. Moreover, the PD-L1 expression status has equally been used as an important marker to evaluate the effect of anti-PD-1 targeted therapy against certain advanced human cancers [12-14]. In addition, PD-L1 expression in tumor cells not only dampens the T-cell mediated anti-tumor response, but also regulates the biological behaviors of the tumor cells, such as proliferation, apoptosis, migration, invasion, along with resistance to radiotherapy and chemotherapy [15-19]. PD-L1 over-expression in tumor cells has also been observed to be associated with induction of epithelial-mesenchymal transition in skin cancer, breast cancer, head and neck squamous cell carcinoma, and lung adenocarcinoma [16, 17, 20]. Therefore, it's necessary for us to systematically investigate the diverse role of PD-L1 in regulation of the biological functions of gastric cancer cells, which may uncover the mechanism of how PD-L1 could directly contribute to gastric cancer development.

In the present study, we focused on analyzing the contribution of PD-L1 in human gastric cancer cells to cancer progression, and its regulation of the cytotoxic sensitivity of cancer cells to CIK (cytokine induced killer cells) therapy. We successfully established the knockdown PD-L1 expression in SGC-7901 as well as AGS gastric cancer cell lines, using RNAi method, and investigated its role in the regulation of cellular proliferation, migration, invasion, apoptosis, cell cycle, tumorigenicity *in vivo*, and the cytotoxic sensitivity to CIK therapy. Our data demonstrated that knockdown of PD-L1 expression in human gastric cancer cells significantly inhibited tumor growth and improved the cytotoxic sensitivity to CIK therapy.

Materials and Methods

Antibodies and major reagents

APC-conjugated mouse anti-human PD-L1 monoclonal antibody and APC-conjugated mouse IgG1 isotype control were purchased from eBioscience (San Diego, CA, USA). Rabbit anti-human PD-L1 monoclonal antibody (NBP1-03220) was purchased from Novus Biologicals (Littleton, CO, USA). Mouse anti-human E-cadherin monoclonal antibody (MAB-0589) and mouse anti-human Vimentin monoclonal antibody (MAB-0178) were purchased from Maixin Biotechnology (Fuzhou, China). The HRP-labeled goat anti mouse/rabbit secondary antibodies (K500711) was purchased from Dako (Glostrup, Denmark). An RNeasy Mini Kit was purchased from Qiagen (Valencia, CA, USA), and SYBR Green Master Mix kits were purchased from TaKaRa (Dalian, China). RPMI-1640 media and fetal bovine serum (FBS) were purchased from Gibco (Cambrex, MD, USA).

Cell lines and cell culture

The human gastric cancer cell lines SGC-7901 and AGS were obtained from Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences (Shanghai, China). The cell lines were cultured in RPMI1640 media, supplemented with 10% FBS in the presence of benzylpenicillin (100 U/mL), streptomycin (100 µg/mL) and 2 mM L-glutamine, and were incubated at standard culture conditions (5% CO₂, 37°C).

PD-L1 RNAi lentivirus generation, infection and cell sorting

The human gastric cancer cell lines SGC-7901 and AGS were used in the PD-L1 RNAi study. The small hairpin RNA (shRNA) against human PD-L1 (NM_014143.2; GenBank) cloned into lentiviral gene transfer vector encoding the green fluorescent protein (GFP) was obtained from Shanghai GeneChem Co.

Ltd (Shanghai, China). The targeting sequence of PD-L1 shRNA was 5'-GACCTATATGTGGTAGAGTAT-3'. The recombinant PD-L1 targeting lentivirus (LV-PD-L1-siRNA virus) and the non-targeted control mock lentivirus (LV-NC virus) were prepared and transfected to SGC-7901 and AGS cell lines, according to the manufacturer's instructions. The infected cells were named LV-PD-L1-siRNA group and LV-NC group, respectively. Finally, the infected cells were analyzed by flow cytometry (Canto II, BD, USA) and the positive cell sorting was done using GFP tag with FL1 channel in a flow sorter (Aria II, BD, USA).

Real-time reverse transcriptase-polymerase chain reaction (RT-PCR)

The mRNA expression of PD-L1 in SGC-7901 and AGS cells after siRNA transfection was quantified by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from both cell lines were extracted by using TRIzol (Invitrogen) method, and PCR reaction was performed using ABI 7600 system (Applied Biosystems, USA) according to the manufacturer's instructions. Primer sequences for detection of the reference gene GAPDH and the target gene PD-L1 were synthesized as follows: human GAPDH forward primer: 5'-TGACTTCAACAGCGACACCA-3', human GAPDH reverse primer: 5'-CACCCTGTTGCTGTAGCAAA-3', human PD-L1 forward primer: 5'-ACTGGCATTGCTGAACG-3', and PD-L1 reverse primer: 5'-TCCTCCATTTCCTCAATAGAC-3'. Data were analyzed by comparing Ct ($2^{-\Delta\Delta Ct}$) method and expressed as fold change compared to GAPDH expression. The RT-PCR products for GAPDH and PD-L1 were also confirmed by electrophoresis on 1.8% agarose gel containing 0.1% ethidium bromide.

Western blot analysis

The protein expression of PD-L1 and GAPDH was determined by western blot analysis. Briefly, the whole cell extracts were prepared from 1×10^6 cells using RIPA lysis buffer (50 mM Tris/HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.25% Na-deoxycholate, 1 mM EDTA and protease inhibitor cocktail). Cells were lysed on ice for 30 min, and the lysates were collected in microtubes and centrifuged for 15 min at 12000 rpm/min at 4°C. After centrifugation, supernatants were collected and the protein concentrations were measured by using BCA Protein Assay Kit (Beyotime, Jiangsu, China). Equal amounts of denatured protein were separated by SDS-PAGE and transferred to a PVDF membrane (Millipore). The membranes were blocked by using 5% non-fat dry milk in TBS-T (20 mM Tris, pH 7.4, 137 mM NaCl, 0.05% Tween-20) for 3 hours at room temperature, and then were incubated with primary antibodies overnight at 4°C. Next day, blots were washed and then incubated for 1 hour with anti-rabbit secondary antibody and washed three times with PBST. Finally, the immuno-reactive protein bands were detected by using an Odyssey Scanning system (Li-Cor, Lincoln, NE, USA).

Cell viability assay

The cell proliferation was assessed by using Cell Counting Kit-8 (CCK-8, Beyotime, Shanghai, China), according to the manufacturer's instructions. Briefly, the LV-PD-L1-siRNA group and the LV-NC group cancer cells (2×10^3) were seeded into 96-well plates and incubated for 24, 48, 72 and 96 hours, respectively. CCK-8 reagent was added to each well at 3 hours before the endpoint of incubation, and absorbance at 450 nm was determined for each well by a microplate reader. An increase or decrease in absorbance values at 450 nm in the experimental wells relative to the initial value indicated cell growth or death, respectively. Experiments were repeated at least three times each time in triplicate.

Wound healing assay

Cell migration was evaluated by wound scrape assay to determine the role of PD-L1 in the regulation of gastric cancer cells migration. Briefly, the SGC-7901 and AGS cells from LV-PD-L1-siRNA and LV-NC groups were incubated in 6-well plates. A small wound area was created in the 90% confluent monolayer using a 200 μ l pipette tip in a lengthwise stripe. Cells were then washed twice with PBS and incubated in serum-free RPMI-1640 media at 37°C in a 5% CO₂ incubator for 24 hours. Photographs were taken at 2 time points for 0 and 24 hours, and wound width was measured using BX50 microscope (Olympus®) with a calibrated eyepiece grid. Data were averaged and expressed as a percent of the original width. These experiments were performed in triplicate.

Transwell assay

The co-culture system was used to evaluate the invasive ability of knockdown PD-L1 expression in SGC-7901 and AGS cells as described in our previous study [21]. Invasion assay was performed by using the Matrigel-coated invasion chambers (Corning, NY, USA). The cells from LV-PD-L1-siRNA and LV-NC groups (5

$\times 10^4$ cells/well) were serum starved for 24 hours and placed in the upper chamber in a serum-free medium. Medium containing 10% FBS was placed in the lower chamber as a chemo attractant. After 48 hours of incubation, the migrated cells in the lower chamber were collected and resuspended. Non-invading cells were removed with a cotton-tipped swab from the top of Matrigel and invading cells were fixed and stained with 0.1% crystal violet. The invaded cells were photographed under a microscope (Olympus, Tokyo, Japan) and counted from five random fields.

Cell apoptosis assay

The cell apoptosis assay was used to explore the effect of knockdown PD-L1 expression on the apoptosis of gastric cancer cells. The cells from LV-PD-L1-siRNA and LV-NC groups were serum starved for 24 hours, seeded into 6-well plates at the density of 1×10^6 cells/well. Later the cells were harvested and apoptosis was analyzed by flow cytometer using Annexin V-APC/PI according to the manufacturer's instructions. CellQuest software was used for data analysis.

Cell cycle assay

The SGC-7901 and AGS cells from LV-PD-L1-siRNA and LV-NC groups were inoculated in 6-well plates and cultured for 48 hours. Next the cells were washed with ice-cold PBS and fixed in a 70% (v/v) ice-cold ethanol solution overnight at 4°C. The following day, these cells were analyzed by flow cytometry according to the instruction of cell cycle analysis kit (Sigma, MO, USA). The cell cycle information was analyzed using ModFit LT 4.0 software.

CIK cells preparation

The protocol for the CIK cell preparation was performed according to our previous studies [22, 23]. In brief, 50ml peripheral blood was drawn from patients using heparin as an anticoagulant. The PBMCs were isolated by Ficoll-Conray density gradient centrifugation, and the viability of the PBMCs was assessed by trypan blue exclusion. PBMCs (2.0×10^6 /mL) were plated onto 6-well dishes (Nunc, Denmark) and cultured with the RPMI 1640 in the presence of human interferon-gamma (IFN- γ , 1.0×10^6 U/L, Shanghai Fosun Pharma Co., China), recombinant human interleukin 2 (IL2, 5.0×10^5 U/L, Shangdong Quangang Pharmaceutical Co., China), 10% inactivated human serum, 25 mM HEPES, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 μ g/ml). The cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C. After 24 h, monoclonal antibody against CD3 (100 μ g/L, Antibody Diagnostic Inc., USA) and IL-1 α (1.0×10^5 U/L, Promega, USA) were added. After another 48h of culture, the supernatant was removed by aspiration and the cells were cultured in the medium without IFN- γ . The medium was then changed every 3 days.

Analysis of cytotoxicity activity

SGC-7901 and AGS cells from LV-PD-L1-siRNA and LV-NC groups were used as the target cells, while CIK cells were used as the effector cells. The effector and target cells were inoculated into the culture plate in triplicate at ratios of 10:1, 20:1 and 40:1. Some wells containing only effector cells or target cells were set as controls. The culture plates were placed in a humidified incubator at 37°C in 5% CO₂ for 24 hours. Later, 100 μ l medium from the co-cultured cells was mixed with 20 μ l of CCK-8 solution and incubated for another 4 hours. The optical density (OD) was recorded at 450 nm for each well by a microplate reader. The Survival (%) = (effector-target group - effector group)/target cell*100%.

Subcutaneous transplantation model study

Two groups of 5 female Balb/c nude mice (4- to 6-weeks old) were bred in an aseptic-specified pathogen-free (SPF) condition and kept at a constant humidity and temperature (25-28°C). Animal experiments were carried out according to protocols approved by the Animal Care and Use Committee and were in compliance with the Guidelines on Animal Welfare of the China National Committee for Animal Experiments. The SGC-7901 cells (1×10^7) from LV-PD-L1-siRNA and LV-NC groups in 0.15 ml PBS were injected subcutaneously in the right inguinal region of nude mice separately. Later, the size of tumors was measured twice a week with caliper, and the volume was calculated using the formula Length $\times$ Width² $\times$ 0.5. Growth curves were constructed, and the data were presented as mean $\pm$ SEM. Finally, the tumors were harvested from mice after 30 days of tumor cell injection. The expression of PD-L1, E-Cadherin and Vimentin proteins were detected by immunohistochemistry of the tumor xenograft sections from each group.

Immunohistochemistry

The paraffin-embedded xenograft tumor tissue blocks were cut into serial 3- μ m-thick sections, and then dewaxed in xylene, rehydrated and graded in ethanol solutions. Antigen was retrieved by heating the tissue sections at 100°C for 30 min in a citrate solution (10 mmol/L, PH 6.0). After cooling of the sections, they were immersed in 0.3% hydrogen peroxide solution for 15 min to block endogenous peroxidase activity. After rinsing in PBS for 5 min, the sections were blocked with 3% BSA solution at room temperature for 20 min. Next the sections were incubated with rabbit anti-human PD-L1 polyclonal antibody, mouse anti-human E-cadherin monoclonal antibody, and mouse anti-human Vimentin monoclonal antibody, respectively, at 4°C overnight. A negative control was performed by omitting the primary antibodies. The sections were later incubated with HRP-labeled goat anti mouse/rabbit secondary antibody, and the diaminobenzene was used as the chromogen and hematoxylin as the nuclear counterstain. Finally, the sections were dehydrated, cleared and mounted.

Statistical analyses

All results are presented as the mean $\pm$ SEM and the data were analyzed by GraphPad Prism 5.0 software package (GraphPad Software, Inc., San Diego, USA). A *P* value of < 0.05 represented statistical significance.

Results

Knockdown of PD-L1 expression in human gastric cancer cell lines SGC-7901 and AGS

In order to further investigate the regulation of PD-L1 on the biological features of human gastric cancer cell lines, we firstly constructed the recombinant lentivirus of siRNA

Fig. 1. Knockdown of PD-L1 expression in human gastric cancer cell lines SGC-7901 and AGS. (A) The efficiency of infection was confirmed by detecting GFP expression by using fluorescence microscopy in SGC-7901 gastric cancer cell line. (B) The efficiency of infection was confirmed by detecting GFP expression by using fluorescence microscopy in AGS gastric cancer cell line. (C) The down-regulation of PD-L1 mRNA expression after knockdown by using RNAi method in human gastric cancer cell lines SGC-7901 and AGS were verified by real-time RT-PCR, which showed that the PD-L1 mRNA expression in LV-PD-L1-siRNA group was significantly lower than that in LV-NC group (both *P*<0.001). (D) The down-regulation of PD-L1 protein expression after knockdown by using RNAi method in human gastric cancer cell lines SGC-7901 and AGS was verified by western blot method, which showed that the PD-L1 protein expression was decreased in LV-PD-L1-siRNA group in contrast to LV-NC group. (E) The down-regulation of PD-L1 protein expression after knockdown by RNAi method in human gastric cancer cell lines SGC-7901 and AGS were also verified by using flow analysis, which showed that the PD-L1 protein expression was decreased in LV-PD-L1-siRNA group in contrast to LV-NC group.

Fig. 2. Knockdown of PD-L1 expression suppresses the proliferation of human gastric cancer cell lines SGC-7901 and AGS. We examined the down-regulation of PD-L1 on the cell proliferation rate *in vitro* by using CCK-8 assay gastric cancer cell lines in both LV-PD-L1-siRNA and LV-NC groups. (A) At 96 hours after seeding, the proliferation rate of SGC-7901-LV-PD-L1-siRNA group was significantly lower than that of SGC-7901-LV-NC group, $P < 0.001$. (B) At 72 hours and 96 hours after seeding, the proliferation rate of AGS-LV-PD-L1-siRNA group was significantly lower than that of AGS-LV-NC group, $P < 0.05$ and $P < 0.01$, respectively.

Fig. 3. Knockdown of PD-L1 expression inhibits the migration of gastric cancer cell lines. The wound healing assay on the two human gastric cancer cell lines in LV-PD-L1-siRNA group and LV-NC group showed that, the cell-free area of LV-PD-L1-siRNA group was significantly wider than that of LV-NC group at 24 hours (both $P < 0.01$) after drawing the scratch line on the monolayer cells.

targeting PD-L1 (LV-PD-L1-siRNA virus) and the non-targeted control mock lentivirus (LV-NC virus), and then transfected them to the human gastric cancer cell lines SGC-7901 and AGS. Furthermore, we sorted LV-PD-L1-siRNA virus transfected SGC-7901 or AGS cells (LV-PD-L1-siRNA group) and LV-NC virus transfected SGC-7901 or AGS cells (LV-NC group), by using Aria II flow sorter (BD, USA) based on GFP via FL1 channel. The efficiency of infection was also confirmed by detecting GFP expression by using fluorescence microscopy (Fig. 1A and B). Then, we used real-time RT-PCR to further confirm the PD-L1 gene expression level in LV-PD-L1-siRNA as well as LV-NC groups, and the results showed that the PD-L1 mRNA expression in LV-PD-L1-siRNA group was significantly decreased in contrast to LV-NC group, respectively (decreased 88.64% in SFC-7901 cells and 73.38% in AGS cells, both $P < 0.001$, Fig. 1C). The western blot analysis and the flow analysis also showed that the PD-L1 protein expression level in LV-PD-L1-siRNA group was significantly decreased in contrast to LV-NC group, respectively (decreased 50.50% in SFC-7901 cells and 49.10% in AGS cells, Fig. 1D and E).

Fig. 4. Knockdown of PD-L1 expression inhibits the invasion of gastric cancer cell lines. (A) The transwell invasion assay showed that the number of invaded cells stained with Cristal Violet was significantly less in LV-PD-L1-siRNA group in contrast to LV-NC group. (B) The statistical analysis showed that the number of invaded cells was significantly less in LV-PD-L1-siRNA group in contrast to LV-NC group, both in SGC-7901 and AGS ($P < 0.05$).

Knockdown of PD-L1 expression suppressed the proliferation, migration and invasion of human gastric cancer cells

In order to verify the role of PD-L1 in the growth of human gastric cancer cells, we next examined the down-regulation of PD-L1 on the cell proliferation rate *in vitro* by using CCK-8 assay in LV-PD-L1-siRNA group as well as LV-NC group cells. As shown in Fig. 2A, we found that at 96 hours, the proliferation rate of SGC-7901-LV-PD-L1-siRNA group was significantly lower than that of SGC-7901-LV-NC control group ($P < 0.001$). As shown in Fig. 2B, we also found that both at 72 hours and 96 hours, the proliferation rate of AGS-LV-PD-L1-siRNA group was significantly lower than that of AGS-LV-NC control group ($P < 0.05$ and $P < 0.01$, respectively). In order to study the role of knockdown PD-L1 expression in regulation of the cellular migration and invasion, we further carried out wound healing assay and transwell assay on the gastric cancer cell lines SGC-7901 and AGS in LV-PD-L1-siRNA group as well as LV-NC group, respectively. The wound healing assay results showed that the migration ability of LV-PD-L1-siRNA group cells was significantly lower than that in LV-NC group. As shown in Fig. 3, both in SGC-7901 and AGS cell lines, the cell-free area of LV-PD-L1-siRNA group was significantly wider than that of LV-NC group at 24 hours (the relative migration rate decreased 42.39% and 37.92% in SGC-7901 and AGS cell lines, both $P < 0.01$) after drawing the scratch line on the monolayer cells respectively, which suggested that the down-expression of PD-L1 could significantly decrease the cell migration of gastric cancer cells in the wound scrape model. As shown in Fig. 4, in the transwell invasion assay, both in SGC-7901 and AGS cell lines, the number of invaded cells stained with Cristal Violet was significantly less in LV-PD-L1-siRNA group, then the invasion rates of LV-PD-L1-siRNA group as well as LV-NC group gastric cancer cells were also detected by counting the number of cells that invaded through matrigel and confirmed the results observed by inverted microscope (the invaded cell numbers decreased 35.34% and 28.89% in SGC-7901 and AGS cell lines, $P < 0.05$, respectively). Thus, our present results showed that the down-expression of PD-L1 could suppress the cellular abilities of proliferation, migration and invasion in human gastric cancer cells.

Fig. 5. Knockdown of PD-L1 expression promotes the cell apoptosis in SGC-7901 and AGS cell lines. (A) The flow analysis showed that there were 5.983% apoptotic cells in SGC-7901-LV-PD-L1-siRNA cells in contrast to 3.423% in SGC-7901-LV-NC cells, and 7.717% apoptotic cells in AGS-LV-PD-L1-siRNA cells in contrast to 5.803% in AGS-LV-NC cells. (B) The statistical analysis showed that the ratio of apoptotic cells was significantly less in LV-PD-L1-siRNA group in contrast to LV-NC group, both in SGC-7901 and AGS ($P < 0.01$, $P < 0.05$, respectively).

Effect of knockdown of PD-L1 expression on cell apoptosis and cell cycle of SGC-7901 and AGS cell lines

In order to explore the effects of knockdown PD-L1 expression on cell apoptosis and cell cycle, we analyzed apoptotic rates and cell cycle distribution by using flow cytometry. As shown in Fig. 5A and B, our results showed that there were 5.983% apoptotic cells in SGC-7901-LV-PD-L1-siRNA cells in contrast to 3.423% in SGC-7901-LV-NC cells, and 7.717% apoptotic cells in AGS-LV-PD-L1-siRNA cells in contrast to 5.803% in AGS-LV-NC cells ($P < 0.01$, $P < 0.05$, respectively), which suggests that the tumor cell expressed PD-L1 could present an anti-apoptosis role in cancer progression. Additionally, the percentage of cells in G0/G1 phase was 68.893% in SGC-7901-LV-PD-L1-siRNA cells and 64.840% in SGC-7901-LV-NC cells, 65.797% in AGS-LV-PD-L1-siRNA cells and 58.030% in AGS-LV-NC cells (Fig. 6A and B), which suggested that knockdown of PD-L1 expression in SGC-7901 and AGS cell lines could induce the cell cycle arrest in G0/G1 phase.

Effect of knockdown of PD-L1 expression on tumor growth in the subcutaneous transplantation mouse model

In order to explore the effect of knockdown PD-L1 expression in the regulation of tumor growth *in vivo*, we further examined the effect of knockdown of PD-L1 expression on the regulation in the subcutaneous transplantation mouse model of gastric cancer by using SGC-7901 cell line. The SGC-7901-LV-PD-L1-siRNA and SGC-7901-LV-NC cells were injected subcutaneously into nude mice. The tumor volumes were measured every 2 days for 30 days. As shown in Fig. 7B, knockdown of PD-L1 expression significantly inhibited tumor growth in nude mice. After 30 days, the tumor volume and weight of LV-PD-L1-siRNA group were

Fig. 6. Knockdown of PD-L1 expression could induce the cell cycle arrest in G0/G1 phase in SGC-7901 and AGS cell lines. The flow analysis showed that the percentage of cells in G0/G1 phase was 68.893% in SGC-7901-LV-PD-L1-siRNA cells and 64.840% in SGC-7901-LV-NC cells, 65.797% in AGS-LV-PD-L1-siRNA cells and 58.030% in AGS-LV-NC cells, which suggests that knockdown of PD-L1 expression in SGC-7901 and AGS cell lines could induce the cell cycle arrest in G0/G1 phase.

smaller and lighter than those of LV-NC group (Fig. 7A and C, $P < 0.001$). The average weight of the resected tumor was 0.5216 g and 0.0801 g in SGC-7901-LV-NC and SGC-7901-LV-PD-L1-siRNA group. These data suggested that knockdown of PD-L1 expression in gastric cancer cells suppressed tumor growth in nude mice in gastric cancer model. As shown in Fig. 7D, our immunohistochemistry results also showed that the PD-L1 protein expression as well as E-cadherin in tumor tissues in LV-PD-L1-siRNA group was down-regulated in contrast to the LV-NC group, while we haven't found any significant difference of the Vimentin protein expression between LV-PD-L1-siRNA group and LV-NC group.

Knockdown of PD-L1 expression in gastric cancer cells could improve the cytotoxic sensitivity to CIK therapy

The analysis results showed that CIK cells have powerful cytotoxicity on both SGC-7901 and AGS cells when the effector-target ratio was in the range 10:1 to 40:1. As can be seen from Fig. 8, the cytotoxic activity was obviously related to the effector-target ratio and increased with increase in the effector-target ratio. The cytotoxic activity of the CIK cells against SGC-7901-LV-PD-L1-siRNA group was markedly higher than that of SGC-7901-LV-NC group under the same conditions (Fig. 8A, $P < 0.01$), and the same in AGS cells (Fig. 8B, $P < 0.01$, $P < 0.05$).

Discussion

It has been suggested that B7 co-inhibitory molecules contributed importantly to the induction of immune suppressive tumor microenvironment [1]. The PD-L1 among B7 molecules, as demonstrated by many studies, has been broadly expressed in various tissues and cell types, including lymphocytes, activated vascular endothelial cells, mesenchymal stem

Fig. 7. Knockdown of PD-L1 expression inhibits the tumor growth in the subcutaneous transplantation mouse model. (A) We examined the effect of knockdown of PD-L1 expression on the regulation in the subcutaneous transplantation mouse model of gastric cancer by using SGC-7901 cell line. The SGC-7901-LV-PD-L1-siRNA and SGC-7901-LV-NC cells were injected subcutaneously into nude mice ($n = 5$, respectively). The tumor volumes were measured every 2 days for 30 days. (B) The statistical analysis showed that knockdown of PD-L1 expression significantly inhibits tumor growth in nude mice. (C) After 30 days, the tumor weight of LV-PD-L1-siRNA group was lighter than that of LV-NC group ($P < 0.001$). (D) The immunohistochemistry results showed that the PD-L1 protein expression as well as E-cadherin in tumor tissues in LV-PD-L1-siRNA group was down-regulated in contrast to LV-NC group, while we haven't found any significant difference of the Vimentin protein expression between LV-PD-L1-siRNA group and LV-NC group. Scale bar = 50 μm .

Fig. 8. Knockdown of PD-L1 expression in gastric cancer cells could improve the cytotoxic sensitivity to CIK therapy. (A) The analysis results showed that CIK cells have powerful cytotoxicity on SGC-7901 cells when the effector-target ratio was in the range 10:1 to 40:1 ($P < 0.01$, respectively). (B) The analysis results showed that CIK cells have powerful cytotoxicity on both AGS cells when the effector-target ratio was in the range 20:1 and 40:1 ($P < 0.01$ and $P < 0.05$, respectively).

cells and cultured bone marrow-derived mast cells. However, the abnormal PD-L1 expression has also been identified in many human solid tumors, including liver, ovary, colorectal, lung, pancreatic, gastric, kidney and breast cancer [24, 25]. The PD-L1 expression on cancer cells helps to evade immune detection, as it has been shown to inhibit T cell mediated anti-tumor immunity, and finally lead to cancer immune tolerance and escape [1, 26, 27]. Our previous study has shown that 42.2% gastric cancer tissues had positive staining for PD-L1 and its

expression levels were significantly associated with tumor size, invasion, nodal metastasis, and overall survival [28].

Currently, it has been widely accepted that extrinsic IFN- γ could up-regulate the PD-L1 expression via JAK/STAT signal pathway. Other signaling pathways such as MEK/ERK/STAT1 and PKD2 were also found to be involved in the regulation of PD-L1 expression [29]. Meanwhile, the abnormal expression of PD-L1 in tumor cells was identified to be regulated by intrinsic mechanisms, such as PTEN/PI3K/Akt pathway and epigenetic regulation by p53/miR34, which provided an additional therapeutic opportunity [30, 31]. However, it has also been accepted in parallel that PD-L1 expression on tumor cells, not only negatively regulated T-cell mediated anti-tumor immunity, but could also regulated biological behaviors of these tumor cells, such as proliferation, apoptosis, migration, invasion, and resistance to radiotherapy and chemotherapy, and again highlight the potential of it being an important immune therapeutic target. In the current study, we focused on the knockdown of PD-L1 expression in human gastric cancer cells and tried to understand its contribution to the tumor progression of these cells and their cytotoxic sensitivity to CIK (cytokine induced killer cells) therapy.

Herein, we have successfully constructed the stable knockdown expression of PD-L1 in SGC-7901 and AGS cell lines by using RNAi method, and investigated the role of knockdown expression of PD-L1 in the regulation of cell proliferation, migration, invasion, apoptosis, cell cycle, xenograft growth in nude mice and the cytotoxic sensitivity to CIK therapy. Our data clearly demonstrated that knockdown PD-L1 expression in human gastric cancer cells significantly inhibited their proliferation, migration, invasion, along with induction of cell cycle arrest and apoptosis, which was consistent with the findings in other human cancers. For example, it has been reported that the level of PD-L1 expression in tumor cell was significantly associated with the ratio of high proliferating cancer cells in human breast cancer [32]. Moreover, the study by Shi et al. also demonstrated that higher PD-L1 expression was associated with poorer prognosis in colorectal carcinoma and could regulate the proliferation and invasion of HCT116 colorectal cancer cells [33]. Thus our results suggested a new paradigm for the biological function of abnormal PD-L1 expression in gastric cancer progression.

Recent studies have also linked the higher expression of PD-L1 on cancer cells with promotion of their epithelial to mesenchymal transition [15-17, 34, 35]. For instance, a study by Wang et al. reported that PD-L1 induced epithelial-to-mesenchymal transition via activating SREBP-1c in renal cell carcinoma [36]. Interestingly, our study showed that knockdown of PD-L1 expression in SGC-7901 cell lines significantly suppressed the tumor growth in the subcutaneous transplantation mouse model. And the immunohistochemistry results indicated that SGC-7901 cells from LV-PD-L1-siRNA group had decreased expression of E-cadherin, but the Vimentin expression showed no change.

Recently, with the development of therapeutic treatments of gastric cancer, antitumor drugs and immunotherapy strategies, especially the immune checkpoint intervention, have made breakthrough progress [37, 38]. The cancer immunotherapy based on PD-L1 targeting has provided major breakthroughs in clinical trials in multiple human cancers [11, 39]. Moreover, PD-L1 expression status has also been used as an important indicator for certain immune therapeutic strategies such as anti-PD-1 checkpoint blockade [40-42]. We have recently reported that radio frequency ablation therapy could induce local release of tumor antigen and pro-inflammatory factors, and in turn could trigger T-cell mediated anti-tumor response. Thus, combination of radio frequency ablation therapy with anti PD-1/PD-L1 therapy could further enhance the radio frequency ablation induced T-cell mediated anti-tumor response and improve the overall therapeutic effect [43]. Consistent with this notion, Dai et al. recently reported about the implications of combined PD-L1/PD-1 blockade with cytokine-induced killer cells and their use as a synergistic immunotherapy strategy for gastrointestinal cancer patients, thereby emphasizing the importance of PD-L1 intervention in adoptive cell therapy. In our study, we have shown that the cytotoxic activity of the CIK cells against LV-PD-L1-siRNA group was markedly higher than that of LV-NC group, under

the same conditions, in both human gastric cancer cell lines. This led us to suggest that PD-L1 detection could also have a role in being used as an essential marker for the selection of CIK therapy in gastric cancer patients [14].

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Disclosure Statement

The authors declare no conflicts of interest.

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