Contribution of PD-L1 to oncogenesis of lymphoma and its RNAi-based targeting therapy

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Abstract

Lymphomas are common malignancies that are prevalent worldwide. In recent years, some types of lymphoma have become curable, but relapsed and refractory lymphomas remain common and challenging. Impaired host immunity and therapeutic resistance are thought to play pivotal roles in the pathogenesis and progression of lymphomas. PD-L1 (programmed death ligand 1) is a newly discovered member of the B7 family of molecules, and its receptor is PD-1. It is known that the costimulatory signal of PD-1 and PD-L1 can help tumor escape from cellular immunity. However, little is known of the impact of PD-L1 on tumor cells, especially lymphomas. Our investigation shows for the first time that: (1) PD-L1 has a vital role in lymphoma oncogenesis; (2) the down-regulation of PD-L1 combined with chemotherapy can significantly suppress lymphoma growth, promote antitumor activity and prolong the survival rate; and (3) targeted therapy of PD-L1 may provide a new and promising approach to the treatment of lymphomas.

Keywords: PD-L1, lymphoma, costimulatory signal, therapy

Introduction

Lymphoma is a common malignancy. In the USA and most other westernized countries, the incidence of lymphoma is about 20/100,000 people per year [1]. According to the cancer statistics of Jemal et al., the incidence of lymphoma is increasing, and it is among the 10 leading cancer types in relation to new cancer cases and deaths [2]. Although some types of lymphoma are curable, relapsed and refractory lymphomas remain common and challenging. Drug resistance is a persistent clinical problem, and limits the successful treatment of disseminated malignancies. The molecular mechanisms of tumor pathogenesis and therapeutic resistance remain poorly understood. Nowadays, the development of new chemotherapeutic drugs is technically limited, and most scientists are interested in biotherapy.
and PD-L1 targeting therapy, hoping to provide a reference for clinical therapy.

Materials and methods

Cell lines and animal experiments

Jurkat, Molt4, Karpos299, Raji, Z138, 721.221 and OCI-Ly-10 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Unless otherwise specified, tumor cells were cultured in RPMI 1640 (Gibco Invitrogen, Grand Island, NY) medium containing 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Gibco Invitrogen), at 37°C in a 5% CO₂ incubator. OCI-Ly-10 cells were cultured in Iscove’s modified Dulbecco’s medium (IMDM) (Gibco Invitrogen).

Female NOD-SCID (non-obese diabetic–severe combined immunodeficiency) mice (NOD.CB17-Prkdcscid/NcrCrl), 6–8 weeks of age, approximately 15 g, were obtained from the Experimental Animal Center, Peking University Health Science Center, Beijing, China. All of the experimental procedures and handling of animals were performed with approval of the Institutional Authority for Laboratory Animal Care of Peking University.

A total of 1 × 10⁶ cells (Jurkat/Raji cells, resuspended in phosphate buffered saline [PBS]) were injected in a total volume of 100μL into the single flanks of mice subcutaneously. When tumors reached a volume of 30 mm³, the mice were allocated randomly into three groups (30 mice per group): (a) PBS treatment, (b) vector + cisplatin (DDP) treatment and (c) short hairpin RNAs targeting PD-L1 (shRNAs) + DDP treatment. DDP was administered intraperitoneally at a dose of 8 mg/kg (or an equal volume of PBS as a buffer control). Plasmid DNA (5 μg) and Lipofectamin 2000 (15 μg; Invitrogen) were gently mixed in 100 μL PBS, and then the complex was injected intratumorally three times per week for 40 days. Tumor size was measured twice per week during the treatment period. Tumor volume was calculated using the following formula: volume = length × (width²)/2.

Immunohistochemistry and flow cytometry

The lymphoma tissue array T203a was purchased from US BioMax, Inc. (Rockville, MD) and used in an immunohistochemistry (IHC) staining study [21]. Specimens from normal and pathologic tissues were formalin-fixed and paraffin-embedded (FFPE). Histologic sections were subjected to heat-induced epitope retrieval using a steamer at 95°C for 25 min in 0.01 mol/L citrate buffer, pH 6.0. Then the sections were incubated with rabbit monoclonal antibodies (mAbs) to PD-L1 (ProSci, Poway, CA); appropriate biotin-conjugated secondary antibodies were applied followed by horseradish peroxidase (HRP)-conjugated streptavidin (Sigma, Germany). Isotype-matched IgGs were used as negative controls. After 10 min of incubation with 3,3’-diaminobenzidine (DAB), sections were counterstained with hematoxylin.

Primary lymphoma specimens were obtained from lymph node biopsies following written informed consent. For fluorescence activated cell sorting (FACS) analysis, single cell suspensions were stained with primary antibodies on ice for 30 min. After three washes in PBS containing 0.1% bovine serum albumin (BSA), cells were analyzed using a flow cytometry system (FACS Calibur; BD Biosciences, San Jose, CA). Monoclonal antibodies used to measure the expression of cell surface markers by flow cytometry included phycocerythrin (PE)-conjugated anti-human PD-L1/B7-H1 (clone MIH1), fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 (clone UCHT1), alkaline phosphocynasin (APC)-conjugated anti-human CD19 (clone HIB19) and appropriate isotype controls, all from eBioscience (San Diego, CA).

Lentivirus-based RNA interference transfection, RT-PCR and Western blotting

PD-L1 knockdown in Jurkat or Raji cells was performed using lentivirus transduction to stably express short hairpin RNAs (shRNAs) targeting PD-L1. The PD-L1 shRNA target sequence 5’-GATAATTGCTGTTTATAT-3’ and a control vector were used to generate recombinant lentiviral particles [22,23]. Knockdown of PD-L1 was confirmed by reverse transcription-polymerase chain reaction (RT-PCR), FACS and Western blotting analysis.

RT-PCR was performed according to standard protocols [24]. Total RNA was prepared using the RNeasy extraction kit (Qiagen, Valencia, CA). Sequences for matrix metalloproteinase-2 (MMP-2), MMP-9 and β-actin primer sets used for RT-PCR analysis were as follows: MMP-2: 5’-TTG AGT GCA GAC AGG AGC GAC TC-3’ (sense) and 5’-ACT TGC AGT ACT CCC CAT CG-3’ (anti-sense); MMP-9: 5’-TTG ACA GGC AGA AGT GG-3’ (sense) and 5’-CCC TCA GTG AAG CGG TAC AT-3’ (anti-sense); β-actin: 5’-AGG GCA AAA TCG TGC GTG ACA-3’ (sense) and 5’-GTG GAC TTT GGT GAG GAC TGG-3’ (antisense) [25]. Cell lysates were analyzed by Western blotting using anti-human Cyclin D1, CDK4 and β-actin antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell proliferation assay

Cell number was assessed using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan) for cell proliferation according to the manufacturer’s instructions. Cell suspension (2 × 10⁴ cells in 100 μL/well) was inoculated into a 96-well plate and the plate was incubated in a humidified incubator for 72 h. Some 10 μL of CCK-8 solution was added to each well of the plate, and the plate was incubated for 4 h in the incubator. Absorbance was measured at 450 nm using a microplate reader.

A colony forming assay of lymphoma cells in soft agar medium was carried out. A 2.5 × 10³ single-cell suspension was resuspended in 1 mL growth medium with an equal volume of saline (buffer control) and 0.5% low melting temperature agarose (Promega, Madison, WI). Samples were plated on 24-well plates, and the plates were incubated for 14–15 days until colonies formed. A colony with more than 30 cells was counted as one positive colony.

Cell cycle and invasion assay

Elimination of serum from the culture medium for 24 h resulted in the accumulation of cells at G1 phase. This effect of nutritional deprivation has been shown to lead to cell-cycle synchronization [26]. After the addition of serum for 72 h, cells were collected and fixed with ethanol; cellular
DNA was stained with propidium iodide (5 μL/10⁶ cells) and incubated at room temperature for 15 min. DNA content and cell number were determined by FACS analysis, and cell cycle profiles were analyzed using the ModFit program (Verify Software House, Inc.).

Invasion assays were carried out as described [27]. Matrigel invasion assays were performed using 24-well 6.5 mm Transwell plates and 8.0 μm pore polycarbonate membrane inserts (Corning). Transwells were coated with Matrigel solution at 100 μg/cm², and were rehydrated with 100 μL medium. Cells were washed, and resuspended in serum-free medium. The polycarbonate membrane inserts were then placed in the upper chamber with 1 × 10⁶ lymphoma cells (200 μL). The lower chambers contained medium with 10% FBS. Cells with invasive properties are able to penetrate the Matrigel collagen and migrate into the lower chamber. After incubation at 37°C and in 5% CO₂ for 24 h, the membrane inserts were removed, and the lymphoma cells that had migrated to the bottom of each well were counted by light microscopy using a hemocytometer with trypan blue exclusion in three optic fields [28].

Analysis of apoptosis
We initially treated 1 × 10⁶ lymphoma cells with 15 μM chemotherapy drug (DDP; Sigma-Aldrich) for 24 h. FITC-conjugated annexin V and propidium iodide were added to the suspended cells according to the manufacturer’s instructions (Biosea, China). After incubation for 20 min at room temperature, 400 μL of binding buffer was added, and samples were immediately analyzed on a FACSCalibur flow cytometer with FL1-H (525 nm) and FL2-H (575 nm) filters, respectively. CellQuest software (Becton Dickinson) was used for data acquisition and analysis.

Hoechst 33342 staining was used to assess changes in nuclear morphology following DDP treatment. Cells were harvested and fixed with 4% paraformaldehyde for 30 min at 25°C, then washed with pre-chilled PBS three times and exposed to 10 μg/mL Hoechst 33342 (Sigma) at room temperature in the dark for 10 min. Samples were observed under a fluorescence microscope (Olympus RX 400). Viable cells displayed diffuse fluorescence in cellular nuclei. Apoptotic cells showed concentrated, dense, granular fluorescence and shrunken nuclei.

Statistical analysis
Statistical analysis was performed using SPSS software version 13.0 (Chicago, IL). Differences between groups were evaluated using one-way analysis of variance (ANOVA). Results were expressed as mean ± SD of triplicate values for each experiment. Statistical comparisons involved use of Student’s t-test. To examine data on mortality, we used Kaplan–Meier survival analysis (log-rank). A value of p < 0.05 was considered to be statistically significant.

Results
PD-L1 is highly expressed in lymphomas
An immunohistochemical monoclonal antibody specific for PD-L1 was used to detect the molecule in tissues of patients with lymphomas (Table I). Notably, PD-L1 was detected in the following lymphoma types: nodular sclerosis classical Hodgkin lymphoma (NSHL), diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), peripheral T-cell lymphoma (PTCL) and lymphoplasmacytic lymphoma (LPL), whereas PD-L1 was not detectable in normal lymph nodes. The results demonstrate that PD-L1 was strongly and widely expressed in various types of human lymphoma. Such reactivity was not found in isotype-matched control mAb staining [Figures 1(A) and 1(B)].

Furthermore, we screened a panel of human lymphoma cell lines for PD-L1 expression by flow cytometry (Table II). PD-L1 was widely expressed by lymphoma cell lines. T cell lines – Jurkat, Molt4 and Karpas299 – expressed PD-L1 strongly, as did B cell lines – Raji, 721.221, OCI-Ly-10 and Z138. In the remainder of this study, the two most common and representative cell lines, Jurkat and Raji, were used as research objects, representing T-cell lymphomas and B-cell lymphomas, respectively [Figures 1(C) and 1(D)]. Several primary lymphoma specimens (five B-cell lymphoma cases and three T-cell lymphoma cases) were analyzed using FACS. PD-L1 was positive in both B-cell lymphoma and T-cell lymphoma; representative cases are shown in Figures 1(E) and 1(F).

PD-L1 knockdown inhibits tumor proliferation in vitro and in vivo
PD-L1 knockdown in Jurkat or Raji cells was performed using lentivirus transduction to stably express shRNAs that targeted PD-L1. We detected the level of PD-L1 by FACS, RT-PCR and Western blotting [Figures 2(A) and 2(B)]. The expression of PD-L1 was silenced by over 70%. PD-L1 knockdown reduced the proliferation of tumor cells markedly compared with the empty vector transfected control [Figure 3(A)]. It also decreased the colony formation of Jurkat/Raji cells compared with empty vector transfected control cells. To study the in vivo effects of PD-L1 knockdown on lymphoma growth, we established animal models by subcutaneously injecting Jurkat/Raji cells into the backs of NOD-SCID mice. Silenced PD-L1 expression inhibited tumor growth significantly compared with the control groups injected with empty vector cells [Figure 3(B)]. At the end of observation (40 days), the rate of inhibition of tumor growth was considered to be statistically significant.
down-regulating the secretion of MMP-2 and MMP-9. To test whether PD-L1 expression had an effect on cell cycle progression, we used DNA flow cytometry to compare the cell cycle in pre-synchronized populations of Jurkat/Raji either expressing or not expressing PD-L1. Figure 4(C) shows that the proliferation index of Jurkat PD-L1 silenced cells (26.5%) was lower than that of the vector (38.4%), indicating that PD-L1 could promote cell proliferation and arrest lymphoma cells in G1/S phase. The same conclusion can be drawn for Raji cells (28.4% vs. 39.7%). We also analyzed the cell cycle-related proteins, and found Cyclin D1 and CDK4 expression to be lower in PD-L1 silenced cells than in controls [Figure 4(D)]. These data support the idea that PD-L1 plays an important role in tumor invasion and cell cycle progression.

**PD-L1 knockdown reverses drug-resistance and increases apoptosis of tumor cells**

In our previous study, we demonstrated that PD-L1 silencing represses tumor proliferation, decreases tumor invasion and down-regulates cell cycle progression. To investigate whether PD-L1 knockdown affected chemotherapeutic drug-induced apoptosis, we selected a drug most commonly used in lymphoma therapy, DDP. We evaluated growth with PD-L1 knockdown was 59.2% and 56.4% for Jurkat and Raji, respectively.

**PD-L1 knockdown decreases tumor invasion ability and inhibits cell cycle progression**

Observations showed that PD-L1 silencing significantly inhibited cell invasion ability and decreased migration to the lower chamber [more than five-fold reduction, Figure 4(A)]. Furthermore, we measured invasion-related proteins, and found that MMP-2 and MMP-9 were lower in PD-L1 silenced cells than in the vector [Figure 4(B)]. These results showed that PD-L1 affected cell invasive properties through down-regulating the secretion of MMP-2 and MMP-9. To test whether PD-L1 expression had an effect on cell cycle progression, we used DNA flow cytometry to compare the cell cycle in pre-synchronized populations of Jurkat/Raji either expressing or not expressing PD-L1. Figure 4(C) shows that the proliferation index of Jurkat PD-L1 silenced cells (26.5%) was lower than that of the vector (38.4%), indicating that PD-L1 could promote cell proliferation and arrest lymphoma cells in G1/S phase. The same conclusion can be drawn for Raji cells (28.4% vs. 39.7%). We also analyzed the cell cycle-related proteins, and found Cyclin D1 and CDK4 expression to be lower in PD-L1 silenced cells than in controls [Figure 4(D)]. These data support the idea that PD-L1 plays an important role in tumor invasion and cell cycle progression.

Table II. Expression of PD-L1 in seven human lymphoma cell lines.

<table>
<thead>
<tr>
<th>Lymphoma type</th>
<th>Cell line</th>
<th>Lymphoma subtype</th>
<th>PD-L1*</th>
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<tbody>
<tr>
<td>B cell</td>
<td>Raji</td>
<td>Burkitt lymphoma</td>
<td>+</td>
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<tr>
<td></td>
<td>Z138</td>
<td>Mantle cell lymphoma</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>721.221</td>
<td>B lymphoblast cell</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>OCI-1y-10</td>
<td>Diffuse large B-cell lymphoma</td>
<td>++</td>
</tr>
<tr>
<td>T cell</td>
<td>Jurkat</td>
<td>T acute lymphoblastic leukemia</td>
<td>++</td>
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<tr>
<td></td>
<td>Molt4</td>
<td>T acute lymphoblastic leukemia</td>
<td>++</td>
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<tr>
<td></td>
<td>Karpas299</td>
<td>Anaplastic large cell lymphoma</td>
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*Expression of PD-L1 was measured by flow cytometry, + indicates ≥2-log mean fluorescent intensity (MFI) above isotype control; + indicates < 2-log MFI above isotype control.
the apoptotic effects of PD-L1 vector and silenced cells in combination with DDP (15 μM) for 24 h. Observations suggested that PD-L1 silencing significantly promoted apoptosis in Jurkat cells (apoptosis rate 27.6% vs. 6.0%), as well as in Raji cells (25.9% vs. 5.2%) [Figure 5(A)]. In Hoechst 33342 staining, after exposure to DDP, Jurkat/Raji cells underwent typical morphologic changes of apoptosis. Marked apoptosis was observed in the PD-L1 silenced cells [Figure 5(B), 20% over control]. These data indicate that PD-L1 has a function in drug-resistance and anti-apoptosis. Data were taken from at least three separate experiments.

PD-L1 RNAi combined with DDP can profoundly suppress tumor growth, promote antitumor activity and prolong survival rate of tumor-bearing mice

As shown in Figures 3 and 4, PD-L1 knockdown significantly repressed cell proliferation, colony formation and invasion of lymphomas. It also promoted apoptosis of DDP treatment (Figure 5). To assess the therapeutic potential of PD-L1 targeting against lymphoma, we examined whether PD-L1 down-regulation enhanced the antitumor activity of DDP in vivo. Initially, we constructed a tumor-bearing mouse model. When the tumor volume reached 30 mm³, mice

Figure 2. Knockdown of PD-L1 by shRNA. (A) PD-L1 was knocked down in Jurkat and Raji cells, determined by FACS; (B) PD-L1 was knocked down in Jurkat and Raji cells, determined by Western blotting. β-actin was probed as a positive control.

Figure 3. PD-L1 knockdown inhibits tumor proliferation in vitro and in vivo. (A) PD-L1 silencing inhibited Jurkat/Raji cell proliferation analyzed by CCK-8 assay; data are from at least three separate experiments. (B) Silencing of PD-L1 significantly inhibited tumor growth in vivo compared with control groups. At the end of observation (40 days), the inhibition rate of tumor growth for PD-L1 knockdown was 59.2% and 56.4% for Jurkat and Raji, respectively.
malignant transformation and oncogenesis of lymphomas have not been elucidated. The B7 family plays a vital role in regulating tumor immunity. The growing B7 family now comprises seven members [5]. In our previous study, we investigated B7.1 and B7.2 in lymphomas, and found that B7.1 provides crucial positive signals to stimulate and support T-cell activation and promote recognition between T cells and tumor cells. In many tumors, including lymphomas, B7.1 is expressed at low levels, leading to tumoral immune escape [30]. Compelling evidence indicates that the B7 family can also offer negative signals that control and suppress T-cell responses. These negative signals are largely provided by the newly identified B7 family member PD-L1 [8]. Here, we used NOD-SCID mice to characterize human lymphoma cell lines, without fear of elimination of tumor cells by the endogenous mouse immune system and avoiding the interference of PD-1 expressed on lymphocytes.

The results of our experiments show that PD-L1 is highly expressed in various kinds of lymphomas, either in tumor tissues or lymphoma cell lines. The expression of PD-L1 varies in different cell lines. An increased level is accompanied by a high proliferation index of tumor cells (data not shown). The study of Andorsky et al. also showed that most T-cell and B-cell lymphomas highly express biologically active PD-L1 [31]. Moreover, knockdown of PD-L1 can
In recent years, targeted immunotherapy combined with chemotherapy has proven to be preferably curative. PD-L1/PD-1 forms a molecular shield to prevent destruction by cytotoxic lymphocytes and implicates a new approach for immunotherapy of human cancers. Specific monoclonal antibodies have been used to block PD-L1/PD-1 interaction, which can markedly inhibit the proliferation of activated T cells. The blockade of PD-L1 or PD-1 could reverse drug resistance and profoundly enhance therapeutic efficacy [34,35].

DDP is a mainstay chemotherapeutic in lymphoma, and can sense DNA damage, interfere with cell cycle processes and induce apoptosis. In the examination of drug-induced apoptosis, PD-L1 silenced cells showed a high death rate. The chemotherapy effect was promoted when the expression of PD-L1 was down-regulated. Azuma et al. also found that PD-L1 could transmit an anti-apoptotic signal to cancer cells [36]. In our study, we used shRNAi-PD-L1 to down-regulate the expression of PD-L1 on tumor cells, not only preventing tumor escape from killing by activated T cells, but also inhibiting the oncogenesis of lymphoma. This therapy could double the efficacy and reduce the side effects of chemotherapeutics. All of these data support that PD-L1 is closely correlated with lymphoma oncogenesis and the degree of malignancy. However, the exact signaling pathways of proliferation and anti-apoptosis remain unclear, and further studies should be carried out regarding this aspect in the future. Previous studies have shown that PD-L1 silencing can enhance chemotherapy or anti-cancer immune responses using RNA interference or antibody blocking [22,32,33]. However, the contribution of PD-L1 silencing directly in lymphoma cells has not been thoroughly assessed. This is the first time that the effects of PD-L1 silencing on lymphoma functions have been characterized in the absence of a fully competent immune system.

In recent years, targeted immunotherapy combined with chemotherapy has proven to be preferably curative. PD-L1/PD-1 forms a molecular shield to prevent destruction by cytotoxic lymphocytes and implicates a new approach for immunotherapy of human cancers. Specific monoclonal antibodies have been used to block PD-L1/PD-1 interaction, which can markedly inhibit the proliferation of activated T cells. The blockade of PD-L1 or PD-1 could reverse drug resistance and profoundly enhance therapeutic efficacy [34,35]. DDP is a mainstay chemotherapeutic in lymphoma, and can sense DNA damage, interfere with cell cycle processes and induce apoptosis. In the examination of drug-induced apoptosis, PD-L1 silenced cells showed a high death rate. The chemotherapy effect was promoted when the expression of PD-L1 was down-regulated. Azuma et al. also found that PD-L1 could transmit an anti-apoptotic signal to cancer cells [36]. In our study, we used shRNAi-PD-L1 to down-regulate the expression of PD-L1 on tumor cells, not only preventing tumor escape from killing by activated T cells, but also inhibiting the oncogenesis of lymphoma. This therapy could double the efficacy and reduce the side effects of chemotherapeutics. The in vivo study showed that anti-PD-L1 RNAi combined with DDP had a dramatic antitumor effect in reducing the growth of established lymphoma, and the combination was significantly more effective than DDP alone. Taken together,
accumulating evidence suggests that PD-L1 plays a pivotal role in lymphoma oncogenesis. The present study shows that the combination of a chemotherapeutic drug with targeting PD-L1 by RNA interference can significantly sensitize lymphoma cells to chemotherapy treatment, which could help to decrease chemotherapeutic drug dosages, minimizing acute and late toxicity for patients.

Above all, our study shows that: (1) PD-L1 is highly expressed in various kinds of lymphomas, either in tumor tissues or lymphoma cell lines; (2) knockdown of PD-L1 can help to down-regulate tumor proliferation, tumor growth and cell cycle progression, as well as tumor invasion ability; and (3) PD-L1 contributes to resistance to DDP, and the blockade of PD-L1 or PD-1 could reverse drug resistance and profoundly enhance therapeutic efficacy. Although current treatment protocols have improved the overall outcome of patients with lymphoma, patients remain at high risk of relapsed and refractory disease. New mechanisms and molecular targets are needed and targeted therapy of PD-L1 may provide a novel and promising therapy approach to lymphoma.

Figure 6. PD-L1 RNAi combined with DDP can suppress tumor growth, promote antitumor activity and prolong survival rate of tumor-bearing mice. (A) Effect of different treatments on volume of Jurkat/Raji xenograft tumors. Antitumor growth of shPD-L1 combined with DDP was significantly better than that of the group with DDP alone. (B) Excised xenograft tumors at end of treatment (40 days). (C) Comparison of survival curves between different treatments: shPD-L1 combined with DDP significantly prolonged survival rate of lymphoma bearing mice compared with control groups.

Acknowledgements
We are very grateful to Professor Xiao-Feng Yang of Temple University School of Medicine in Philadelphia, USA and Dr. Zu-Sen Fan of the Institute of Biophysics, Chinese Academy of Sciences, for statistical analysis and assistance.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

This work was supported by a grant from the National Natural Science Foundation of China (81172245).

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