Biochemical and Biophysical Research Communications 561 (2021) 187–194

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Identification of vitamin B6 as a PD-L1 suppressor and an adjuvant for cancer immunotherapy

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ARTICLE INFO

Article history:
Received 9 May 2021
Accepted 10 May 2021
Available online 21 May 2021

Keywords:
PD-L1
Metabolite
Combination immunotherapy
Pyridoxal
Degradation

ABSTRACT

Interaction of programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) inhibits T cell activation. Tumor tissues can evade immune surveillance by expressing higher levels of PD-L1. Identification of potential regulators of PD-L1 through natural metabolites may contribute to discovering new drugs for immunotherapy. By using a metabolite library screen, we showed that pyridoxal (PL) significantly suppresses PD-L1 expression. Mechanistically, PL accelerates PD-L1 degradation in a proteasome-dependent manner, and STUB1 serves as an E3 ligase during the process. Functionally, PL enhances T cell killing activity by blocking the PD-1/PD-L1 signaling pathway. Thus, we have identified PL as an inhibitor of PD-L1, which provides a feasible option for combination immunotherapy.

1. Introduction

The immune system has evolved to surveil, recognize and eliminate abnormal cells, including pathogen-infected cells and cancer cells [1]. Unfortunately, cancer cells can often escape immune surveillance cleverly by aberrant activation of immune checkpoints to exhaust antigen-specific $CD8^+$ T cells [2]. Programmed death-ligand 1 (PD-L1) and programmed death-1 (PD-1) are two such checkpoint molecules [3]. Extracellular interaction between PD-L1 and PD-1 abolishes antitumor immunity activity by slowing proliferation rates and triggering apoptosis of T cells [4]. Immune checkpoint blockade (ICB) therapies targeting PD-1/PD-L1 signaling pathway have been approved for treating a large number of malignancies due to its substantial clinical benefits [5].

Even so, developing a new drug always takes a significant amount of time, substantial costs, and steady efforts. Identifying new uses for existing medications, known as drug repurposing, seems to be an attractive alternative due to its advantages of low risk of failure, confirmed clinical safety, and short study period [6]. Metabolites are intermediates or end products of cellular metabolism catalyzed by different enzymes. It has many potential functions in regulating tumorigenesis, tumor progression, and metastasis. For example, the antioxidants *N*-acetylcysteine and vitamin E could reduce free heme levels and stabilize BACH1, resulting in lung cancer metastasis [7]. Few researchers focus on existing metabolites to identify the regulator of PD-L1. Here, we determined to screen a new regulator of PD-L1 through an established metabolite library in our lab. Strikingly, pyridoxal (PL) stood out due to its pronounced effect in downregulating PD-L1 among all the metabolites, suggesting that a combination of pyridoxal and immunotherapy seems to be a feasible approach to improve the efficiency of PD-1/PD-L1 signaling pathway blockade.

PL is a member of vitamin B6. Vitamin B6 comprises six interconvertible vitamers, pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and their phosphorylated counterparts, pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP), and pyridoxamine 5′-phosphate (PMP) [8]. The mainly bioactive form of vitamin B6 is PLP, which serves as a co-factor and catalyzes more than 150 biochemical reactions for cellular and organismal metabolism [9]. The antitumor effect of vitamin B6 has been reported for a long time in animal studies, but no researchers attempt to elaborate the role of vitamin B6 in tumor immune escape. In the study, we showed that PL decreased PD-L1 expression remarkably. Furthermore, we proved that PL promoted PD-L1 degradation in a

Abbreviations: CHX, cycloheximide; ICB, immune checkpoint blockade; PBMC, peripheral bloodmononuclear cells; PD-1, programmed death-1; PD-L1, programmed death-ligand 1; PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine.

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https://doi.org/10.1016/j.bbrc.2021.05.022
0006-291X/

proteasome-dependent manner. Blocking the PD-1/PD-L1 signaling pathway by PL enhanced T cell killing activity against cancer cells. The appropriate supplement of vitamin B6 has the potential to increase the efficacy of immunotherapy.

2. Material and methods

2.1. Cell lines and cell culture

All cell lines, HCT116, HEK293T, HepG2 and SW1990 were from the National Infrastructure of Cell Line Resource of China. All cells were grown in RPMI 1640 medium and DMEM medium (Invitrogen) respectively, supplemented with 10% FBS, 1% non-essential amino acids, and 1% penicillin-streptomycin and were cultured at 37 °C with 5% CO_2.

2.2. Generation of stable cell lines

Using plenti-PD-L1-Flag plasmid, we established SW1990 and HepG2 cell lines stably expressing PD-L1-Flag. In brief, stably expressing PD-L1-Flag cell lines were obtained by lentivirus transduction of the plenti-PD-L1-Flag transgene and followed by puromycin selection. Infected cells were selected with puromycin for at least five days, and whole-cell extracts were then used for immunoblot analysis to confirm the integration of the transgenes in the genome.

2.3. Chemicals and reagents

Anti-Flag M2 magnetic beads were from Sigma-Aldrich. Cycloheximide (CHX) was from Cayman. DAPI was from Sangon Biotech. MG132 was from Beyotime. Opti-MEM was from Gibco. Penicillin-streptomycin and non-essential amino acids were from Solarbio. Pyridoxal, Pyridoxine and Pyridoxamine were from Sangon Biotech.

2.4. Western blotting and antibodies

Cells were lysed in RIPA buffer with PMSF and protease inhibitor cocktail. Protein samples were resolved by SDS-PAGE and transferred to PVDF membrane (Bio-Rad). After blocking, membranes were incubated with primary antibodies overnight at 4 °C with gentle shaking, followed by incubation with secondary antibodies for 1 h at room temperature. Protein bands were visualized with chemiluminescence reagents. The primary and secondary antibodies are listed below, and the experiments were repeated at least three times.

The following antibodies were used in this study: AMPK (Phospho-Thr183/Thr172) (D1512121, Sangon Biotech); β-Actin (A3854, Sigma-Aldrich); Flag (PA1-984B, Invitrogen); Goat anti rabbit-Alexa Fluor 633 (A21071, Invitrogen); Goat anti-rabbit Alexa Fluor 488 (A11008, Invitrogen); HA (12013819001, Roche); HRP-linked goat anti-rabbit (7074, Cell Signaling Technology); HRP-linked horse anti-mouse (7076, Cell Signaling Technology); PD-L1 (13684, Cell Signaling Technology); PD-L1 (66248-1-Ig, proteintech).

2.5. T cell-mediated tumor cell killing assay

The blood samples were purchased from Tianjin Blood Center and approved by the Ethics Committee of Tianjin Blood Center. The human peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation in Lymphoprep (STEMCELL). To acquire $CD8^+$ T cells, PBMCs were performed using EasySep Human CD8 Pos Sel Kit (STEMCELL). To activate $CD8^+$ T cells, $CD8^+$ T cells were cultured in RPMI 1640 medium with CD3/CD28 (2 μg/ml, STEMCELL) and IL-2 (10 ng/ml, Biolegend) for 96 h. Activated $CD8^+$ T cells were co-cultured with tumor cells for 24 h with or without PL (100 μM), and the ratio of tumor cells to $CD8^+$ T cells was 1: 3 or 1: 5. At the end of incubation, $CD8^+$ T cells and tumor cell debris were removed by PBS wash, and living tumor cells were stained with crystal violet.

2.6. qRT-PCR assays

Total RNA was extracted from cells by TRIzol reagent (ThermoFisher) according to the manufacturer's protocol. cDNA was synthesized using TransScript® II First Strand cDNA Synthesis SuperMix (Transgen). qRT-PCR was conducted with SYBR Master Mix (Yeasen). The sequences of primers used for qPCR are shown below:

Human PD-L1 forward:TCCGGTGGTATGGATGAGAAA.
Human PD-L1 reverse:ACCAAGGCCAGTAGCATTCTT.
Human β-Actin forward: CGTACCACTGGCATCGTGAT.
Human β-Actin reverse: AGGTAGTCAGTCAGGTCCCG.
Human HRD1 forward: GCTCACGCCTACTACCTCAAA.
Human HRD1 reverse: GCCAGACAAGTCTCTGTGACG.
Human SPOP forward: GCCCCGTAGCTGAGAGTTG.
Human SPOP reverse: ACTCGCAAACACCATTTCAGT.
Human STUB1 forward: AGCAGGGCAATCGTCTGTTC.
Human STUB1 reverse: CAAGGCCCGGTTGGTGTAATA.

2.7. Co-immunoprecipitation assay

Cells were lysed in PBS buffer supplemented with protease inhibitor cocktail and 1% Triton X-100. The cells were then further disrupted by repeated freeze-thaw cycles and vortexed with glass beads. And the lysates were centrifuged at 10000 g and 4 °C for 15 min. The supernatant was transferred to new tubes and quantified with a NanoDrop spectrophotometer. The supernatants were incubated with anti-Flag M2 magnetic beads at 4 °C for 1 h on a rotating wheel. After incubation, the beads were washed with PBS at 4 °C for 5 times. The co-immunoprecipitation complex was eluted using 40 μl of elution buffer (3 × Flag peptide in PBS) at 4 °C for 30 min and subjected to SDS-PAGE, western blotting.

2.8. RNA interference

siRNA targeting human HRD1, SPOP, STUB1, PDXK, PD-L1 and a non-silencing control were purchased from Synbio Technologies. siRNA targeting human AMPKα1/2 were purchased from Santa Cruz Biotechnology (sc-45312).

The sequences of the siRNA are shown below:
siCtrl: UUCUCCGAACGUGUCACGUTT
siHRD1: CAGAGGAGCUCCAGGCAAU
siPD-L1: CAAAAUCAACCAAAGAAUU
siSPOP: AGAUCAAGGUAGUGAAAUU
siSTUB1: CCAAGCACGACAAGUACAU
siPDXK:GGCUGAACAACAUGAAUAA.

2.9. Immunofluorescence

Cells grown on glass-bottom plates were washed and fixed with 4% paraformaldehyde at 4 °C for 30 min. The cells were then treated with blocking and permeabilization buffer (3% BSA and 0.2% Triton X-100 in PBS) for 1 h at 37 °C. After blocking and permeabilization, the cells were incubated with primary antibodies in antibody dilution buffer (1% BSA and 0.02% Triton X-100 in PBS) overnight at 4 °C, followed by incubation with an appropriate fluorophore-labeled secondary antibody in the same buffer for 1 h at 4 °C and then for 5 min with 1 μg/ml DAPI at room temperature.

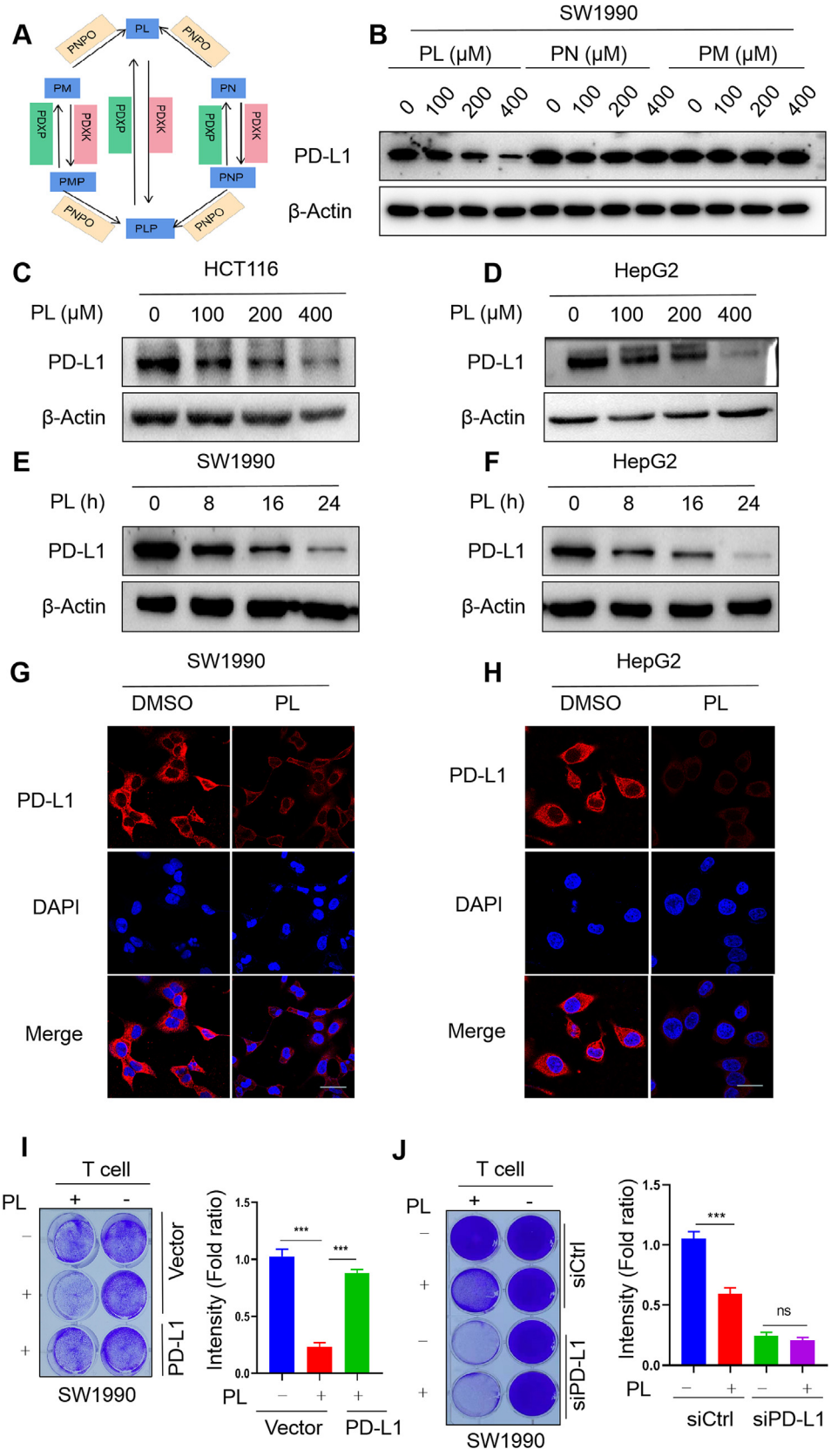

Fig. 1. Pyridoxal rather than other forms of vitamin B6 reduces PD-L1 expression. (A) Diagram showing the relationships among pyridoxal (PL) and five other vitamin B6 comprising pyridoxine (PN), pyridoxamine (PM), pyridoxal 5′-phosphate (PLP), pyridoxine 5′-phosphate (PNP), and pyridoxamine 5′-phosphate (PMP). (B) western blotting was conducted to determine the effect of PL, PN, and PM on PD-L1 of SW1990 cells after different concentrations (100, 200, 400 μM) treatment for 24 h. (C–D) The protein expression levels of PD-L1 in the HCT116 cells and HepG2 cells were evaluated by western blotting after different concentrations (100, 200, 400 μM) PL treatment for 24 h. (E–F) The protein expression levels of PD-L1 in the SW1990 cells and HepG2 cells were evaluated by western blotting after 400 μM PL treatment for different times (8, 16, 24 h). (G–H) PD-L1 antibody and DAPI (blue)

2.10. Statistical analysis

Data are presented as the mean ± standard deviation. Differences in different groups were compared using an unpaired, two-tailed Student's t-test. Statistical analyses were performed using GraphPad Prism 8. $P < 0.05$ was considered statistically significant.

3. Results

3.1. PL is identified to be a negative regulator of PD-L1

To identify the potentially existing regulator of PD-L1, we performed a metabolite repurposing screening through an established library in our lab. Consequently, PL greatly aroused our interest due to its pronounced effect on the regulation of PD-L1. PL is a member of vitamin B6, and six forms of vitamin B6 are interconvertible (Fig. 1A). Only three non-phosphorylated vitamin B6 were chosen to examine due to its cell-permeant property. To determine whether vitamin B6 has effects on PD-L1 expression levels, we treated pancreatic SW1990 cells with various concentrations of PL, PN and PM. The result showed us that only PL significantly suppresses the protein expression levels of PD-L1 in a dose-dependent manner. In contrast, other forms of vitamin B6, PM and PN have no apparent effect on PD-L1 expression (Fig. 1B). Next, we investigated the impact of different concentrations of PL on other tumor cell lines, including HepG2 and HCT116. After being incubated with 100, 200, or 400 μM PL for 24 h, the protein expression levels of PD-L1 in HepG2 and HCT116 decreased remarkably (Fig. 1C and D). To examine the time-dependent effect of PL, we then cultured SW1990 and HepG2 cells with or without 400 μM PL for 8, 16, 24 h. Consistently, the results showed that PL inhibited the protein expression levels of PD-L1 in a time-dependent manner (Fig. 1E and F). The immunofluorescence (IF) assay also revealed a remarkable reduction of PD-L1 in the presence of PL (Fig. 1G and H). Taken together, these data intensely indicated that PL decreases the protein expression levels of PD-L1 in various cell lines. Based on the above study, we therefore performed experiments to test whether PL enhanced T cell-mediated cancer cell death. To this end, we performed T cell-mediated tumor cell killing assay. Consistent with these results, PL did increase the sensitivity of tumor cells to T cell killing (Fig. 1I, S1A). Meanwhile, knocking down PD-L1 neutralized PL-induced T cell-mediated tumor cell killing (Fig. 1J, S1B, and S1C). So, vitamin B6 enhances T cell killing activity by blocking the PD-L1/PD-1 signaling pathway.

3.2. PL promotes PD-L1 degradation

Through qRT-PCR analysis, PL altered the abundance of PD-L1 without a change in mRNA level (Fig. 2A). Cycloheximide (CHX) chase assay showed that PL promoted PD-L1 degradation (Fig. 2B). Meanwhile, we subsequently incubated SW1990 cells with CHX for the different indicated times in the presence of PL or DMSO. We found that PL reduced the half-life of PD-L1 (Fig. 2C and D). Theoretically, exogenous PD-L1 will also be reduced if PL inhibits PD-L1 level at post-translational level. We generated stable SW1990 and HepG2 cell lines expressing Flag-tagged PD-L1. Indeed, exogenous PD-L1 can be downregulated remarkably upon 400 μM PL treatment for 24 h (Fig. 2E and F).

3.3. PL promotes PD-L1 degradation in a proteasome-dependent manner

To determine whether PL promoted PD-L1 degradation in a proteasome-dependent manner, we treated cells with proteasome inhibitor MG132. Interestingly, MG132 could rescue degradation of PD-L1 induced by PL in SW1990 cells (Fig. 3A). Similar results could also be obtained in HepG2 cells (Fig. 3B). Exogenous PD-L1 degradation by PL could also be abrogated in the presence of MG132 (Fig. 3C and D). Since PL induced PD-L1 proteasome pathway degradation, we asked whether PL affected the localization of PD-L1 in cells. To this end, we performed IF staining of endogenous and exogenous PD-L1 in the presence of PL or DMSO upon MG132 treatment. Interestingly, PD-L1 was located primarily on the cell membrane under normal conditions. In contrast, PD-L1 was detected mainly in the cytosol with reduced signals on the membrane when treated with PL (Fig. 3E and F). After trypsin digestion of PD-L1 located on the membrane, PL increased PD-L1 level in the cytoplasm when pretreatment with MG132 (Fig. 3G). These findings consistently indicated that PL promoted PD-L1 degradation in a proteasome-dependent manner. The intracellular bioactive form of vitamin B6 is PLP, which can be converted through PL by pyridoxal kinase (PDXK) (Fig. 1A). The phenomenon of PD-L1 reduction by PL still existed when PDXK was knocked down by siRNA.(Fig. S2A, B). Therefore, it is PL rather than PLP, which decreases PD-L1 levels. Phosphorylated PD-L1 at S195 by activated AMPK induces abnormal glycosylation and promotes PD-L1 degradation through the ERAD pathway [10]. In our study, AMPK was not activated by PL, and knocking down AMPK did not neutralize PD-L1 degradation induced by PL (Fig. S2C, D). PD-L1 is a highly glycosylated protein with four *N*-glycosylation sites, N35, N192, N200, and N219 [11]. We therefore substituted each of the four asparagines (N) to glutamines (Q) respectively or mutated the four glycosylation sites altogether. The result suggested that the degradation of PD-L1 by PL is in a glycosylation-independent manner (Fig. S2E).

3.4. STUB1 serves as an E3 ligase during the process of PL induced PD-L1 degradation

Since PL promoted PD-L1 degradation via proteasome machinery, we measured the ubiquitination level of PD-L1 upon PL treatment. Ubiquitination of exogenous PD-L1 was enhanced after PL treatment (Fig. 4A). When the K48R mutant ubiquitin was used for the assay, no changes of ubiquitination were observed upon PL treatment (Fig. 4B). Several E3 ligases of PD-L1 has been reported, including HMG-CoA reductase degradation protein 1 (HRD1), Speckle-type POZ protein (SPOP) and STIP1 homology and U-box containing protein 1 (STUB1) [10,12,13]. We treated cells with PL under condition of knocking down the each of the three E3 ligases. Only STUB1 depletion abrogated reduction of endogenous PD-L1 by PL (Fig. 4C and D). In addition, co-immunoprecipition (co-IP) assay showed strong physical interaction between STUB1 and PD-L1 (Fig. 4E). PL enhanced the interaction of STUB1 and PD-L1(Fig. 4F). All the data indicated that STUB1 is the E3 ligase responsible for PD-L1 degradation upon PL treatment.

were used to stain the PD-L1 and DNA of SW1990 cells and HepG2 cells, respectively. Cells were treated with 400 μM PL for 24 h. Scale bars, G, 20 μm, H, 10 μm. (I) T cell-mediated tumor cell killing assay. WT or overexpression of PD-L1 SW1990 cells co-cultured with activated $CD8^+$ T cells for 24 h with or without PL (100 μM). SW1990-to-T cell ratio, 1:5. n = 3 for the quantification. (J) T cell-mediated tumor cell killing assay. SW1990 cells co-cultured with activated $CD8^+$ T cells for 24 h with or without PL (100 μM). Control or PD-L1 siRNA was transfected into SW1990 cells. SW1990-to-T cell ratio, 1:3. n = 3 for the quantification. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

4. Discussion

In this work, we provided evidence for the effect of PL on anti-tumor immunity. Briefly, PL promotes PD-L1 degradation in a proteasome-dependent manner. The binding of STUB1 to PD-L1 has been enhanced after PL administration, which reinforces K48-linked polyubiquitination of PD-L1, resulting in its degradation. In addition, degradation of PD-L1 by PL augments T cell killing activity against cancer cells.

Our work firstly identified a naturally occurring regulator of PD-L1 from the perspective of metabolites. Metabolomics has become a hot spot of research in the fields of life science due to its significant importance in metabolic and non-metabolic functions. Tumourigenesis, cancer progression, and metastasis are always accompanied by metabolic reprogramming, following altered metabolite levels. Increasing studies reported that additional supplement of metabolites changes metabolic pathways of tumor. Hence, metabolite library could be considered a naturally occurring drug library with highly potential value and huge advantages of lower cost and shorter development cycle.

Increasing evidence indicated that high doses of vitamin B6 treatment or disruption of the vitamin B6 metabolism exerts influences on tumor cell proliferation, tumorigenesis as well as tumor progression [14,15]. For example, Chen et al. recently demonstrated that blockade of the vitamin B6 metabolic pathway exhibited anti-leukemic activity, PLP and PDXK kinase activity was specifically required for leukemic cell proliferation [16]. Vitamin B6 sensitizes a large panel of cancer cells to apoptosis by exacerbating cisplatin-mediated DNA damage [17]. Notably, vitamin B6 has been reported to possess the function of improving immunogenicity [18]. Low vitamin B6 level affects cell-mediated immunity as well as humoral immunity in both animal and human studies [8,19]. Besides, abundant epidemiology studies also validated that dietary vitamin B6 intake correlates with cancer risk [20].

However, there is not yet any study focused on the molecular mechanism responsible for vitamin B6-mediated antitumor immunity. Our work demonstrated that PL decreased PD-L1 levels in a ubiquitin-proteasome degradation pathway. As a 33 kDa type 1 transmembrane protein with a half-life of more than 16 h [11], PD-L1 is primarily degraded in a lysosome-dependent manner under normal conditions. A small fraction of PD-L1 with abnormal glycan structure has been reported to be degraded via the ubiquitin-proteasome pathway. Metformin-induced S195 phosphorylation of PD-L1 impairs normal glycosylation, blocking its translocation

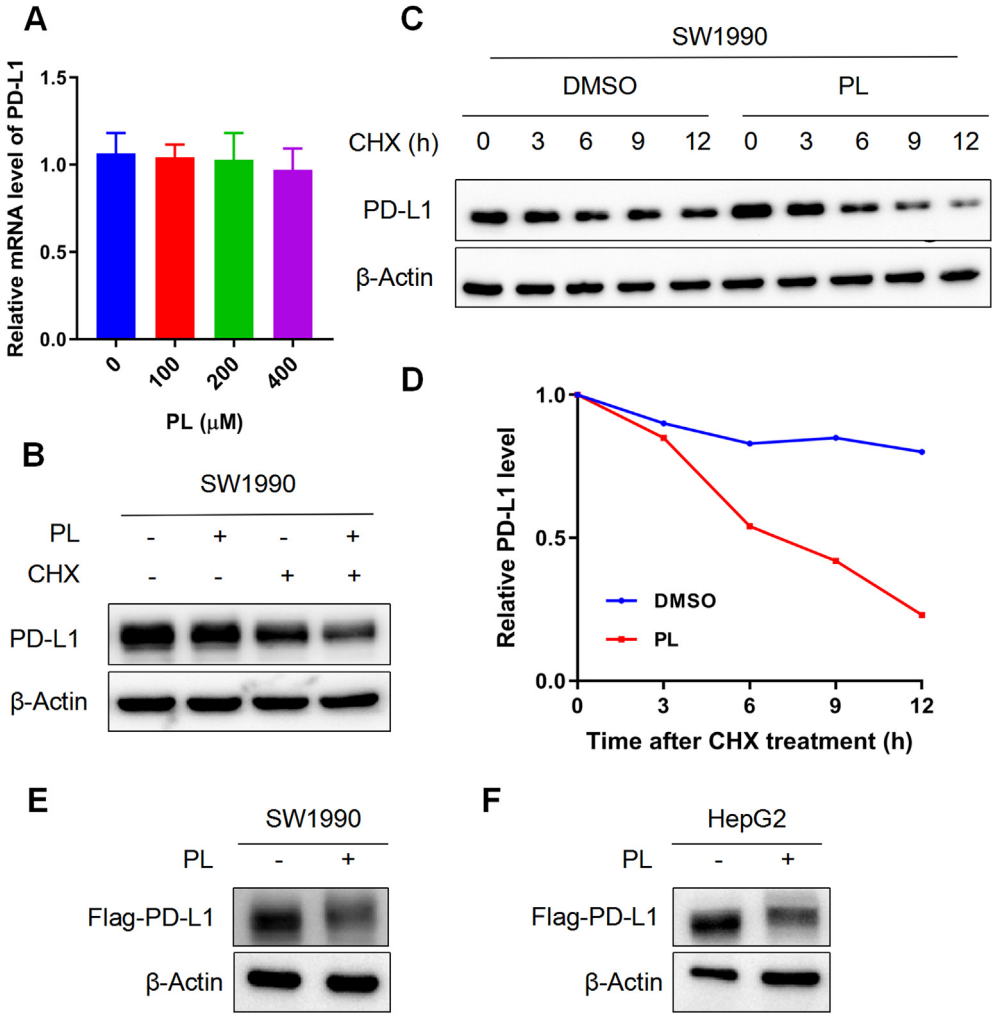

Fig. 2. PL promotes PD-L1 degradation. (A) The mRNA expression levels of PD-L1 in the SW1990 cells were evaluated by qRT-PCR after different concentrations (100, 200, 400 μM) PL treatment for 24 h. (B) SW1990 cells were cultured for 8 h with or without CHX (50 μM) and PL (400 μM). (C–D) Following treatment with CHX (50 μM), SW1990 cells were collected for western blotting analysis of PD-L1 protein expression at the indicated times (3, 6, 9, 12 h). Representative blots (C) and quantification of the blots (D) were shown. n = 3 for the quantification. (E–F) SW1990 cells and HepG2 cells stably expressing PD-L1-Flag were cultured for 24 h with or without 400 μM PL.

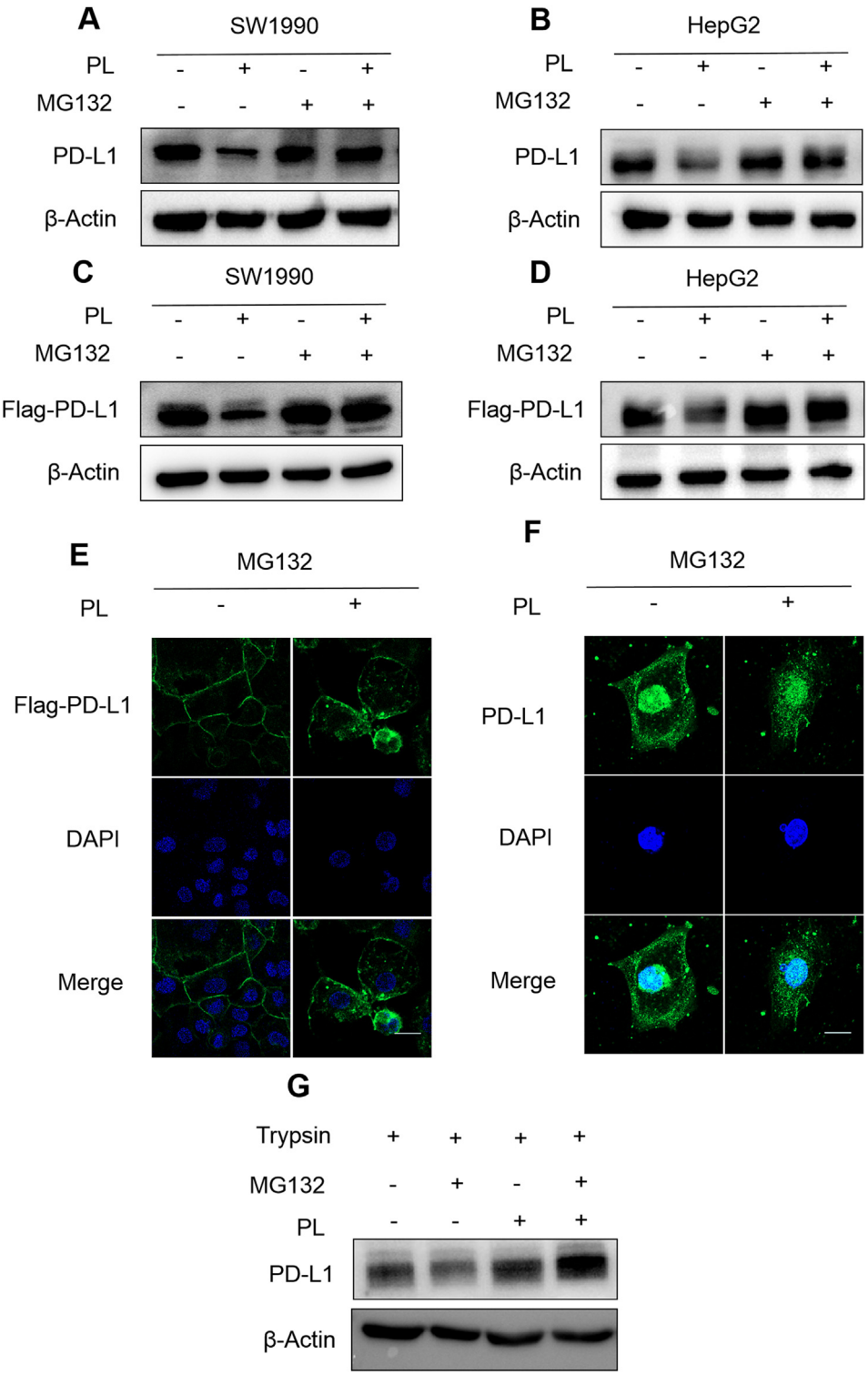

Fig. 3. PL promotes PD-L1 degradation in a proteasome-dependent manner. (A–B) SW1990 cells and HepG2 cells were cultured for 8 h with or without 400 μM PL and 20 μM MG132. (C–D) SW1990 cells and HepG2 cells stably expressing PD-L1-Flag were cultured for 16 h with or without 400 μM PL and 5 μM MG132. (E) Trypsin digestion of SW1990 cells with or without PL and MG132. Scale bars, 10 μm. (F) Immunofluorescence staining of endogenous PD-L1 in SW1990 cells. SW1990 cells were cultured for 8 h with 400 μM PL in the presence of 20 μM MG132. Scale bars, 10 μm. (G) Immunofluorescence staining of exogenous PD-L1 in SW1990 cells. SW1990 cells were cultured for 16 h with 400 μM PL in the presence of 5 μM MG132. SW1990 cells stably expressing PD-L1-Flag.

from the endoplasmic reticulum (ER) to the Golgi apparatus, which leads to PD-L1 degradation by the endoplasmic-reticulum associated protein degradation (ERAD) complex [10]. In addition, non-glycosylated PD-L1 interacting with glycogen synthase kinase 3β (GSK3β) could be degraded by b-TrCP in a proteasome-dependent manner [11]. Meanwhile, the fluctuation of PD-L1 protein abundance during cell cycle progression is regulated by SPOP directly through the proteasome pathway, which does not need the

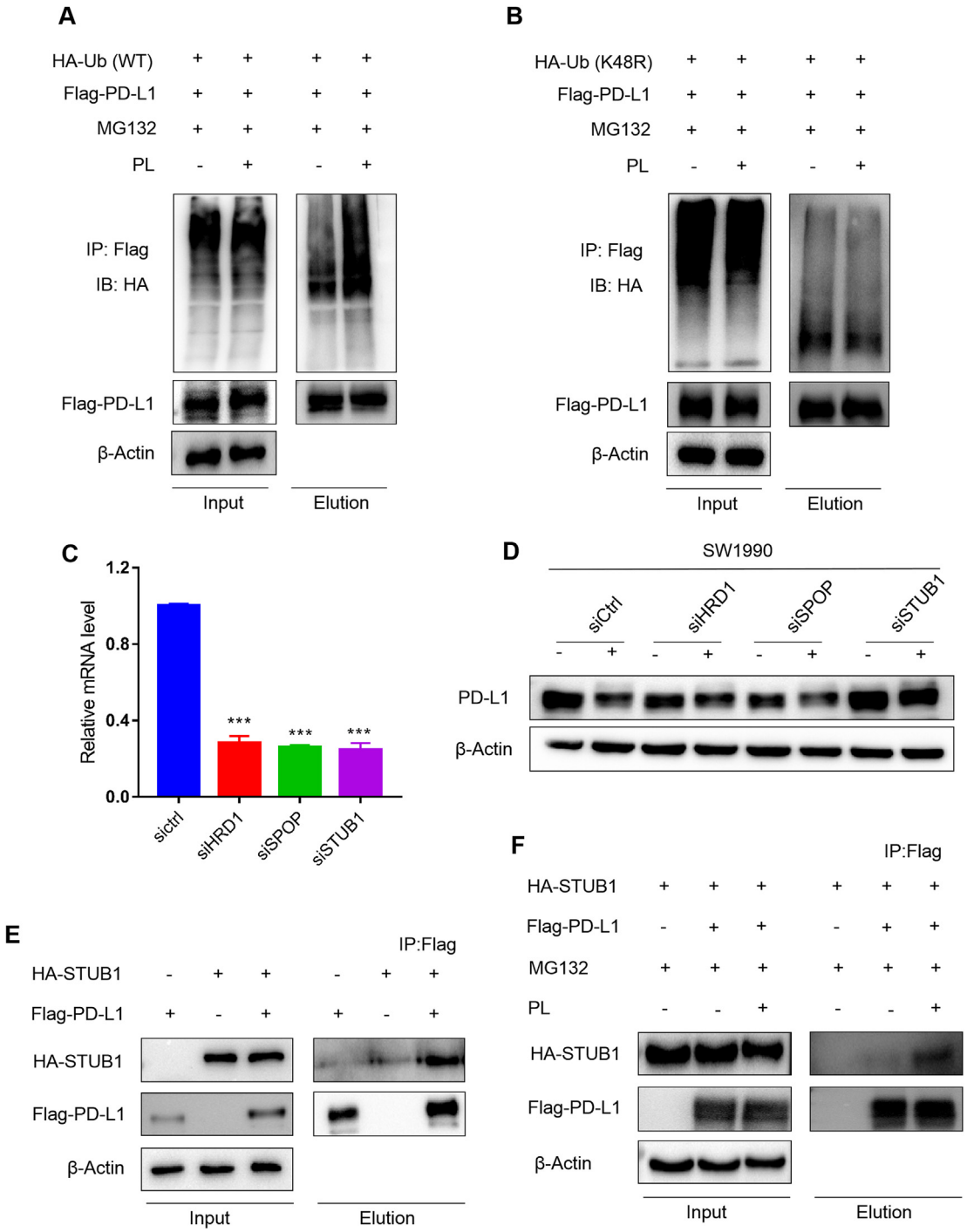

Fig. 4. STUB1 serves as an E3 ligase during the process of PL induced PD-L1 degradation. (A–B) PL promotes PD-L1 polyubiquitin via K48-ubiquitin linkage. HEK-293T cells transiently expressing Flag-PD-L1 and WT or K48R mutant HA-tag ubiquitin were treated with or without PL (400 μM) and MG132 (5 μM) for 16 h. Ubiquitination of Flag-PD-L1 was examined by HA immunoblotting after IP with Flag M2 magnetic bead. (C–D) Control and siRNA targeting SPOP, HRD1, and STUB1 were transfected into SW1990 cells. western blotting analysis of the protein expression of PD-L1 in SW1990 cells with or without PL (400 μM). (E) Co-immunoprecipitation (co-IP) measuring the interaction of PD-L1 and STUB1 in HEK293T cells. (F) co-IP measuring the interaction of PD-L1 and STUB1 in HEK293T cells with or without PL (400 μM).

structural change of PD-L1 [12]. In our work, STUB1 has been proved to be the E3 ligase promoting PD-L1 degradation upon PL treatment. But we failed to in-depth analyze the cause for PD-L1 degradation. The underlying molecular mechanism by which vitamin B6 promotes PD-L1 degradation needs to be further investigated precisely.

Immune checkpoint blockade (ICB) aiming to reactivate exhausted T cells and arouse the immune system by blocking PD-1/PD-L1 signaling pathway has been developed to be effective therapies, which substantially benefits cancer patients. However, the treatment meets a series of challenges, such as low response rate and nondurable clinical effects [21,22]. Various combination immunotherapies have been investigated to enhance the efficacy of ICB and improve the response rate of cancer patients, including a

combination with anti-CTLA-4 and anti-PD-1 in melanoma [23,24]. The combination therapy achieved great success and increased survival rates. Combination of immune checkpoint inhibitors with traditional antitumor treaments such as radiation therapy and chemotherapy also to a large extent benefits patients relative to monotherapy [25]. Thus, according to our findings, PL seems to be a plausible alternative for combining immunotherapy without detective side effects.

Acknowledgments

This work was supported by the National Nature Science Foundation of China [grant numbers 81874055] and [grant numbers 82073051].

Declaration of competing interest

We declare that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2021.05.022.

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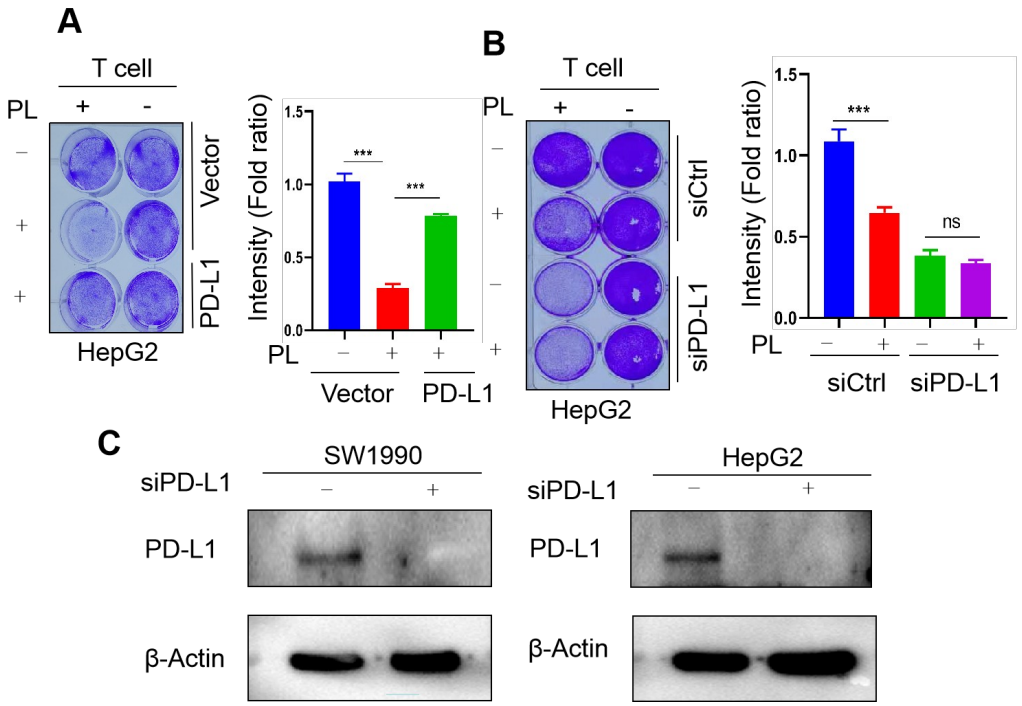
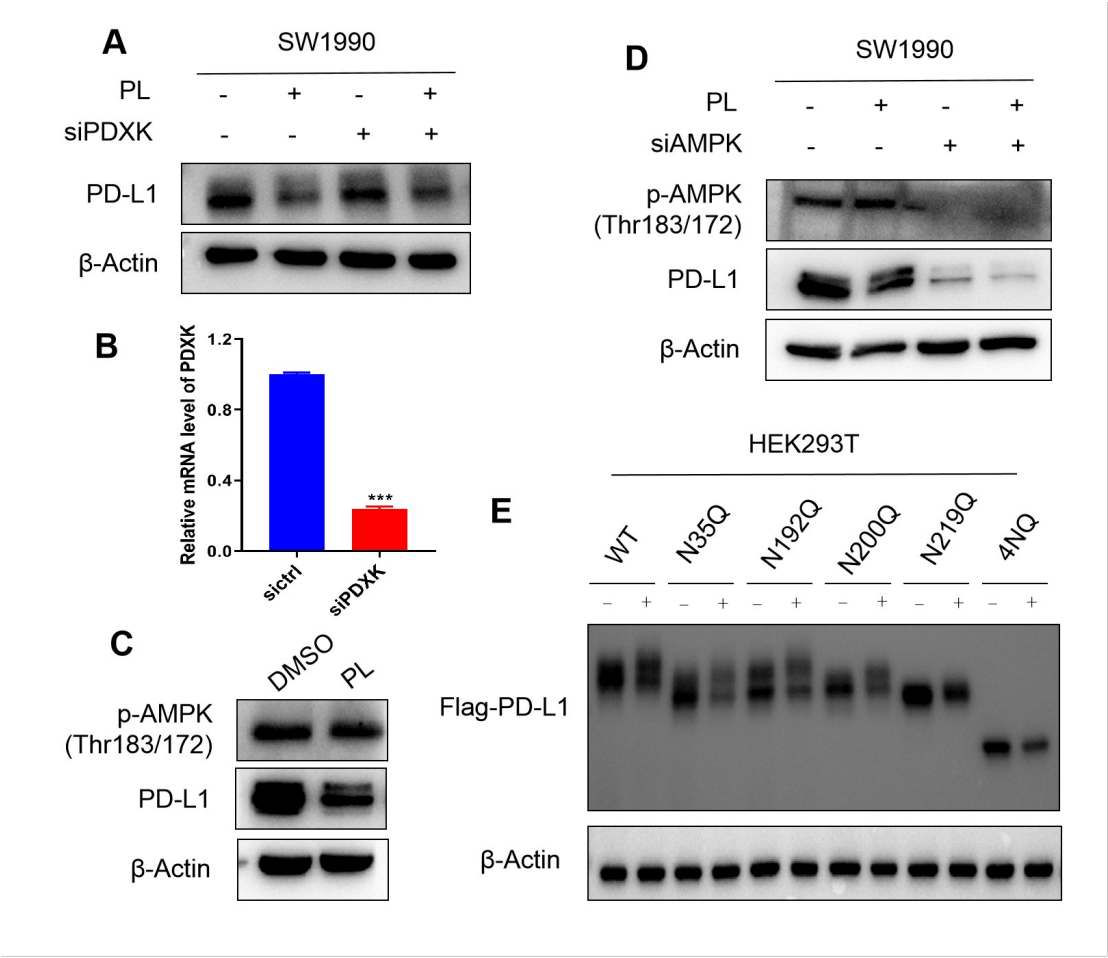
Supplementary Data Fig. 1



(A) T cell-mediated tumor cell killing assay. WT or overexpression of PD-L1 HepG2 cells co-cultured with activated CD8+ T cells for 24 h with or without PL (100 μM). HepG2-to-T cell ratio, 1:5. n=3 for the quantification. (B) T cell-mediated tumor cell killing assay. HepG2 cells co-cultured with activated CD8+ T cells for 24 h with or without PL (100 μM). Control or PD-L1 siRNA was transfected into HepG2 cells. HepG2-to-T cell ratio, 1:3. n=3 for the quantification. (C) PD-L1 was knocked-down by siRNA in SW1990 and HepG2 cells.

Supplementary Data Fig. 2



(A) Control and PDXK siRNA were transfected into SW1990 cells. SW1990 cells were collected for western blotting analysis of PD-L1 protein expression with or without PL (400 μM). (B) The efficiency of PDXK knockdown was examined by qRT-PCR. (C) Western blotting analysis of the protein expression of p-AMPK in SW1990 cells with PL treatment (400 μM). (D) Control and AMPK siRNA were transfected into SW1990 cells. SW1990 cells were collected for western blotting analysis of PD-L1 protein expression with or without PL (400 μM). (E) Western blotting analysis of the protein expression of PD-L1 WT and its NQ mutants in HEK293T cells with or without PL treatment.