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Platycodin D triggers the extracellular release of programed death Ligand-1 in lung cancer cells

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ABSTRACT

Programmed death ligand-1 (PD-L1) is an important immune checkpoint for cancer immunotherapy in clinic. In this study, we reported that platycodin D, a natural product isolated from an edible and medicinal plant *Platycodon grandiflorus* (Jacq.) A. DC., down-regulated the protein level of PD-L1 in lung cancer cells. Flow cytometry and immunofluorescence assay showed a weaker surface PD-L1 signal in NCI–H1975 cells after the incubation with platycodin D (10 μM) for 15 min compared to the control group. Jurkat T cells showed enhancive interleukin-2 secretion when co-cultured with platycodin D-treated NCI–H1975 cells, suggesting that platycodin D-induced PD-L1 reduction increases the activation of Jurkat T cells. An augmentation of PD-L1 protein was detected in the cell culture medium from platycodin D treatment group. Chlorpromazine (60 μM) almost abolished the platycodin D-mediated PD-L1 extracellular release and restored the membrane PD-L1. Finally, hemolysis assay exhibited that platycodin D-triggered PD-L1 extracellular release was independent of the hemolytic mechanism. Taken together, our study demonstrates that platycodin D reduces the protein level of PD-L1 in lung cancer cells via triggering its release into the cell culture medium, which sheds new light for the application of natural products in cancer immunotherapy.

1. Introduction

Programmed death ligand-1 (PD-L1) is one of the most famous immunotherapy checkpoints, which has been a research focus in the field of cancer therapy recently (Pardoll, 2012; Chen and Han, 2015; Homet Moreno and Ribas, 2015). PD-L1 is a type I transmembrane protein and the binding of PD-L1 to its receptor, programmed cell death protein 1 (PD-1), exhibits immunosuppression leading to immune evasion in cancer (Li et al., 2016; Zou, 2005). Thus, inhibition of PD-1/PD-L1 axis has been developed as an important cancer therapy approach in clinic. Currently, antibodies against PD-1 or PD-L1 have been widely investigated. Several PD-1/PD-L1 antibodies, such as nivolumab, pembrolizumab and atezolizumab, have been successfully approved for the clinical immunotherapy in variety of cancers including melanoma, bladder cancer, non-small cell lung cancer, *etc.* (Borghaei et al., 2015; Raedler, 2015; Robert et al., 2015; Rittmeyer et al., 2017; Massard et al., 2016; Apolo et al., 2017). Meanwhile, researchers also attempt at exploiting small molecules to disrupt the PD-1/PD-L1 interactions. Compared with monoclonal antibodies, small molecule PD-1/PD-L1 antagonists might have the advantages of easy diffusion across physiologic barriers and plasma membranes, resulting in an better tissue uptake and easier metabolic clearance from systemic circulation of human body (Zarganes-Tzitzikas et al., 2016). However, the PD-1/PD-L1 target consists of a very hydrophobic, large and flat protein-protein interaction, which would increase the difficulty of the drug design (Zarganes-Tzitzikas et al., 2016; Collin, 2016). In this case, we considered whether any natural product could specific decrease PD-L1 on the cell membrane to exert immunotherapy effect. Recently, curcumin, isolated from *Curcumae Longae Rhizoma*, was reported to promote PD-L1 degradation by disrupting the COP9 signalosome 5 (CSN5) signal and then sensitized the breast cells to anti-cytotoxic T-lymphocyte-associated antigen 4 (anti-CTLA-4) therapy, which supported our

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hypothesis (Lim et al., 2016).

Platycodon grandiflorus (Jacq.) A. DC., mainly distributed in Northeast Asia, is considered as a functional food because it contains many kinds of nutritious compounds and it also can be processed into delicious dishes in many countries as an important food source (Zhang et al., 2015). Meanwhile, it is a main source of famous Chinese medicinal herb *Platycodonis Radix*, which has been widely used to treat cough, excessive phlegm, sore throat for a long history in China (Zhang et al., 2015). Platycodin D (PD) is a triterpenoid saponin isolated from *Platycodon grandiflorus* (Jacq.) A. DC and has been used as an active quality control maker (Fu et al., 2018). *In vitro* and *in vivo* experimental evidences have revealed that PD had multiple pharmacological properties including anti-oxidative, anti-inflammatory, anti-obesity and hepatoprotective effects, *etc*. (Cho et al., 2018; Wang et al., 2017; Xu et al., 2018; Lee et al., 2019). Meanwhile, previous studies from our group showed PD possessed potential anti-cancer effect via inducing cell apoptosis, inhibiting adhesion, migration and invasion (Li et al., 2014, 2015; Tang et al., 2014). Recently, PD was also identified as a novel heat shock protein 90 (Hsp90) inhibitor, which could disrupt Hsp90/Cdc37 complex and enhanced the anti-cancer effect of the mammalian target of rapamycin (mTOR) inhibitor or AKT inhibitor (Li et al., 2016, 2017).

Herein, various natural products isolated from Chinese medicinal herbs have been screened and PD is the candidate which significantly decreased the protein level of PD-L1 in cancer cells. To our knowledge, this is the first time that PD was reported with the function of decreasing the protein level of PD-L1 in lung cancer cells via triggering its extracellular release, which reveals a new aspect of the multiple pharmacological effects of PD. Meanwhile, PD-treated cancer cells restored the activation of Jurkat T cells in the co-culture system, indicating the potential application of this natural product in cancer immunotherapy.

2. Materials and methods

2.1. Regents

Roswell Park Memorial Institute (RPMI) 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, phosphate-buffered saline (PBS), and trypsin-EDTA solution were obtained from Gibco (Carlsbad, CA, USA). Dimethyl sulfoxide (DMSO), paraformaldehyde (PFA), Triton-X100, CPZ, and Invitrogen Human IL-2 Uncoated ELISA Kit were acquired from Sigma (Saint Louis, MO, USA). Hoechst 33342 was obtained from Molecular Probes (Grand Island, NE, USA). Phytohaemagglutinin (PHA) and phorbol-12-myristate-13-acetate (PMA) were attained from InvivoGen Ltd. (San Diego, CA, USA). Chloroquine (CQ), bafilomycin A1 (Baf-A1), MG-132 and bortezomib (BORT) were obtained from Selleck Chemicals (Houston, TX, USA). PD was brought from Best-Reagent (Chengdu, Sichuan, China) and its purity is 99%. Platycodin D2 (PD2), saikosaponins A (SA), timosaponin A (Timo-A) and diosin (DIO) were purchased from Aoke Biology Research Co., Ltd (Beijing, China).

2.2. Cell culture

Human NCI–H1975 cells, NCI–H358 cells and Jurkat T cells were purchased from Shanghai Cell Bank of Chinese Academy of Sciences (Shanghai, China). All cells were cultured in RPMI 1640 medium containing 10% FBS and 1% penicillin–streptomycin (100 units/mL of penicillin and 100 μg/ml of streptomycin). All cells were incubated under 5% CO_2 at 37 °C.

2.3. Western blot

Cells were seeded into 6-well plate at a density of 1.8×10^5 per well and allowed to adhere for 24 h before treatment. The Western blot was performed according to our previous study (Jiang et al., 2017). Samples was separated by SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and then blocked with 5% nonfat milk in PBST over 1 h at room temperature. The membranes were separated and probed with specific primary antibodies against PD-L1 (#13684) and GAPDH (#2118) overnight at 4 °C. Then they were washed by PBST three times and incubated with anti-rabbit IgG HRP-conjugated secondary antibodies for another 2 h at room temperature. Thenceforward, signals were detected by an ECL selected Western blot detection reagent (GE healthcare, Buckinghamshire, UK). All antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA).

2.4. Immunofluorescence

Cells were seeded into the confocal dishes at a density of 1.5×10^5 per well and allowed to adhere for 24 h. After the PD (10 μM) treatment, all cell samples were washed with PBS slightly, and fixed with 4% PFA for 30 min at 37 °C. Then cells were permeabilized with 0.5% Triton X-100 for 20 min and then blocked with 5% BSA for 1 h. Next, primary antibody against PD-L1 (#86744, Cell Signaling Technology, Beverly, MA, USA) were added into the cell samples overnight at 4 °C. After rinsing with PBS three times, samples were incubated with anti-rabbit IgG (H + L), F(ab')2 Fragment (Alexa Fluor® 488 Conjugate/ Alexa Fluor® 594 Conjugate) (#A-11008/#A-11012, Thermo Fisher Scientific, Waltham, MA, USA) for 1 h at room temperature. The cell nucleuses were stained with Hoechst 33342 solution for 5 min. Immunofluorescence images were captured by the confocal laser scanning microscope (Leica TCS SP8, Solms, Germany).

2.5. Cell surface PD-L1 detection by flow cytometry

Cells were plated at a density of 1.8×10^5 per well into 6-well plate and allowed to adhere for 24 h. After 10 μM PD treatment, cells were trypsinized and washed with PBS, then collected by centrifugation at 1500 rpm for 5 min the cells were resuspended with 4% PFA for 10 min at 37 °C. After being washed with PBS, cells were incubated with 0.5% BSA for 10 min at 37 °C. The specific primary antibody against PD-L1 (#86744) and a matched isotype control antibody were added into the cell samples for 1 h at room temperature, respectively. After being washed with PBS, cells were incubated with anti-rabbit IgG (H + L), F(ab')2 fragment (Alexa Fluor 488® Conjugate) (#A-11008, Thermo Fisher Scientific, Waltham, MA, USA) for 30 min in the dark. Cell surface PD-L1 was detected by flow cytometry (Becton Dickinson FACS Canto, Franklin Lakes, NJ, USA) and data was analyzed by using FlowJo VX software (Tree Star, Inc, San Carlos, CA, USA).

2.6. IL-2 detection by ELISA in co-culture system

NCI–H1975 cells were seeded into a 24-well plate at a density of 3×10^4 per well and allowed to adhere for 24 h. NCI–H1975 cells were treated with 10 μM PD for 15 min, then the cell culture medium containing PD was aspirated and replaced with 10% FBS 1640. Jurkat T cells were pre-activated with 1 μg/ml of PHA and 25 ng/ml of PMA for 24 h, then mixed with PD pre-treated NCI–H1975 cells at a density of 6×10^4 per well for co-culture. After 24 h, the cell culture medium was collected from the co-culture system. The level of IL-2 was detected by the Invitrogen Human IL-2 Uncoated ELISA Kit (#88-7025-88) and the absorbance was analyzed by a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA) (Burr et al., 2017).

2.7. Quantitative real-time PCR

Total RNA was extracted from cells by using the TRIzol reagent from Life Technologies (Shanghai, China). The cDNA was synthesized by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Penzberg, OBB, Germany). Quantitative real-time PCR (qPCR) was performed and analyzed according to the previous studies (Jiang et al.,

2017; Zhang et al., 2018). The sequences of the PD-L1 primers: 5′-CAATGTGACCAGCACACTGAGAA-3' (forward) and 5′-GGCATAATA AGATGGCTCCCAGAA-3' (reverse); GAPDH primers: 5′-GCGACACCCA CTCCTCCACCTTT-3' (forward) and 5′-TGCTGTAGCCAAATTCGTTGTC ATA-3' (reverse).

2.8. Protein salting out

The cells were plated at a density of 1.8×10^5 per well and the culture medium was collected after the treatment with PD (10 μM) for 15 min, followed by incubation with saturated ammonium sulfate ($(NH_4)_2SO_4$) solution at 4 °C overnight. Then samples were precipitated at 15000 rpm for 20 min. After centrifugation, the supernatant was removed and the precipitate was dissolved with RIPA lysis buffer containing 1% protease and phosphatase inhibitor cocktail. The samples are then prepared for Western blot.

2.9. Hemolysis assay

The erythrocytes were obtained from rabbits, which purchased from Animal Experimental Center, Guangdong Academy of Medical Sciences. All the tested compounds were dissolved in DMSO at a concentration of 20 mM. Then, PBS was added to dilute the solution to the testing concentrations. The erythrocytes were diluted with PBS to obtain a 4% erythrocytes suspension. The 500 μL 4% erythrocyte suspension was added into 500 μL of different saponin solutions respectively and all samples were slightly stirred and incubated at 37 °C with for 60 min. The final concentration of saponins was 10 μM. The samples were then centrifuged at 3000 rpm for 5 min. Absorbance of the supernatant was measured at 540 nm using a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale, CA, USA). The hemolysis (%) was calculated by comparison with the 100% hemolysis caused by distilled water (positive control) and 0% hemolysis caused by PBS (negative control). The animal experiments were performed in accordance with institutional guidelines of the Animal Care and Experimentation Committee in Zunyi Medical University.

2.10. Statistical analysis

Data were expressed as the mean ± SD from three independent experiments. All statistical analyses were performed using the Student's unpaired *t*-test of variance from the GraphPad Prism software 6 (GraphPad Software, Inc, CA, USA). A P value < 0.05 was considered statistically significant. $^{*}P$ value < 0.05 and $^{**}P$ value < 0.01.

3. Results

3.1. PD decreases the protein level of PD-L1 in lung cancer cells

Previous study showed 24 h treatment with 10 μM PD significantly decreased the protein level of PD-L1 in NCI–H1975 cells. Then, a time-effect experiment of PD on cancer cells was performed at the concentration of 10 μM. A remarkable reduction of PD-L1 was observed in both NCI–H1975 and NCI–H358 cell lines when treated with PD for 15 min compared with the control group (Fig. 1A). Meanwhile, cells were treated with PD of gradient concentrations (0, 2.5, 5, 10 μM) for 1 h, and the cellular PD-L1 was detected by Western blot. As shown in Fig. 1B, PD decreased the protein level of PD-L1 in both cell lines in a concentration-dependent manner. Therefore, a concentration of 10 μM and a treatment time of 15 min were chosen for the following study. PD-L1 is a high glycosylated protein mainly located on the cell membrane. The cell surface PD-L1 was then determined by immunofluorescence assay. As shown in Fig. 1C, PD-L1 labeled with Alexa Fluor® 488 conjugated antibody in the control group was located on cell membranes and formed green fluorescent rings surrounding the cell surfaces. While cell membranes showed weaker PD-L1 signal when the cells were incubated with PD for 15 min compared with the control group. Consistently, PD caused a distinct left-shift of PD-L1 signal relative to the non-treated group as showed by the flow cytometry result, indicating a reduction of cell membrane PD-L1 (Fig. 1D). Collectively, these results indicate that PD rapidly and significantly reduces the protein level of PD-L1 in lung cancer cells.

3.2. Jurkat T cells co-cultured with PD-treated NCI–H1975 cells shows enhanced IL-2 secretion

PD-L1 interacts with its receptor PD-1, leading to the escape of cancer cells from immune surveillance. The therapeutic response of targeting PD-1/PD-L1 result from the reactivation of immune system. To evaluate the biological effect of PD-mediated PD-L1 reduction to the T cells activation, we established a Jurkat T cells and NCI–H1975 cells co-culture model, in which the change of IL-2 production can represent the activation level of Jurkat T cells (Fig. 2A). Jurkat T cells showed the increased PD-1 expression after being activated by PHA/PMA, and responded to the PD-1 inhibition. As showed in Fig. 2B, PD pre-treated NCI–H1975 cells increased the IL-2 secretion of Jurkat T cells compared with the non-treated group, partially indicating that PD-triggered PD-L1 reduction restores the Jurkat T cells activation.

3.3. PD triggers the extracellular release of PD-L1

To figure out whether PD down-regulated the transcriptional level of PD-L1, the qPCR analysis was performed to evaluate the mRNA level of PD-L1 in NCI–H1975 and NCI–H358 cells. However, PD did not reduce the mRNA level of PD-L1 in NCI–H1975 cells. By contrast, it slightly enhanced the mRNA level of PD-L1, whose mechanism has not been studied yet (Fig. 3A). Meanwhile, qPCR experiment on NCI–H358 cells showed there was no significant difference between the control group and PD treated group (Fig. 3A). These results showed that PD had different regulation to PD-L1 mRNA level in two cell lines while triggered the same PD-L1 extracellular release, supporting that PD-triggered PD-L1 extracellular release is independent of the expression of PD-L1 at the mRNA level. Since intercellular PD-L1 is generally degraded through lysosome or ubiquitin–proteasome pathways (Burr et al., 2017; Mezzadra et al., 2017). We suspected whether PD-induced reduction of PD-L1 was mediated by protein degradation. To test this hypothesis, NCI–H1975 cells were treated by PD combined with the lysosome or proteasome inhibitors, and the protein level of PD-L1 was determined by Western blot. As shown in Fig. 3B, although the lysosome inhibitors (CQ and Baf-A1) significantly disrupted lysosome function as supported by the up-regulation of LC3-II (data not shown), they still could not restore the reduction of PD-L1 induced by PD. Meanwhile, co-treatment with proteasome inhibitors (MG-132 and BORT) also could not attenuate the effect of PD-induced PD-L1 reduction (Fig. 3B). These results indicated that PD could not promote the protein degradation of PD-L1. Taken together, these findings reveal that PD decreases the protein level of PD-L1 without inhibiting mRNA level or promoting its protein degradation. Based on these results, we also suspected that the protein of PD-L1 might be released into the cell culture medium from cell membranes under PD treatment. To verify this idea, the total protein in NCI–H1975 and NCI–H358 cell culture medium was extracted by $(NH_4)_2SO_4$ salting out method, and then Western blot was used to determine the protein of PD-L1. Consistent with our hypothesis, an augmentation of PD-L1 protein was detected in the cell culture medium from PD group in comparison to control group (Fig. 3C). Collectively, these findings demonstrate that PD triggers the extracellular release of PD-L1.

3.4. CPZ abolishes the effect of PD-triggered extracellular release of PD-L1

Previous study showed that PD reduced the cell surface PD-L1 significantly and rapidly via triggering its extracellular release. Meanwhile

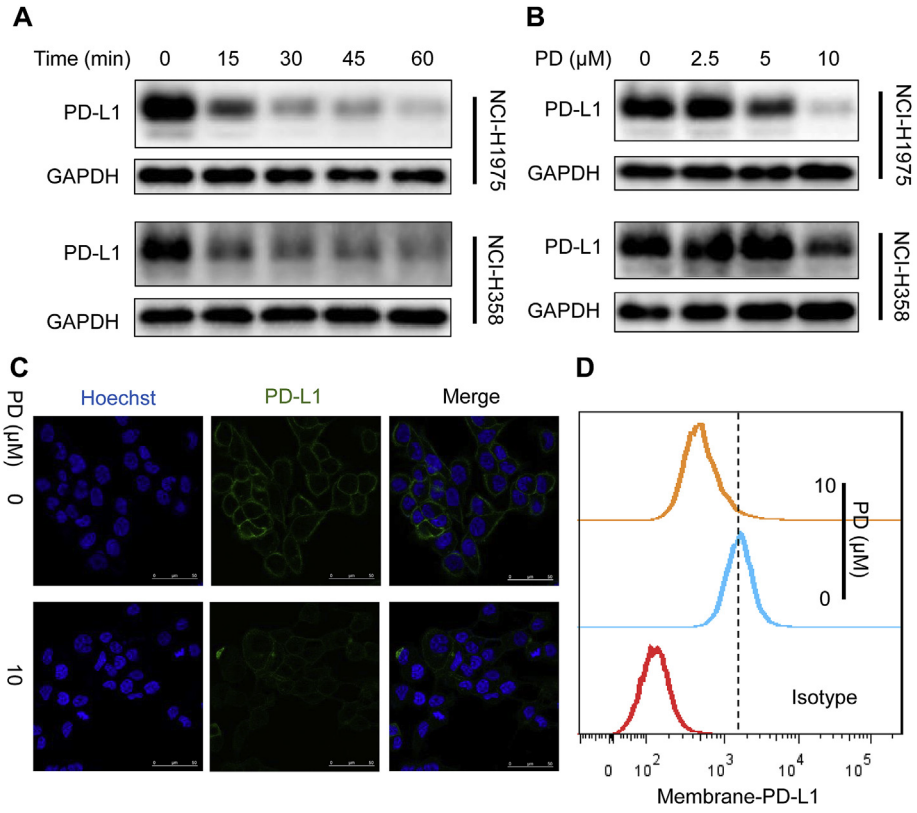

Fig. 1. PD down-regulates the protein level of PD-L1 in lung cancer cells. (A) Western blot was used to detect the protein level of PD-L1 after the treatment with 10 μM PD for 0, 15, 30, 45, and 60 min, respectively. (B) The protein level of PD-L1 was detected by Western blot after the treatment with PD (0, 2.5, 5, and 10 μM) for 1 h. (C) The location of PD-L1 on the cell membrane was detected by immunofluorescence assay after the treatment with 10 μM PD for 15 min (scale bar: 50 μm). (D) The cell surface PD-L1 was evaluated by flow cytometry in the presence of 10 μM PD for 15 min.

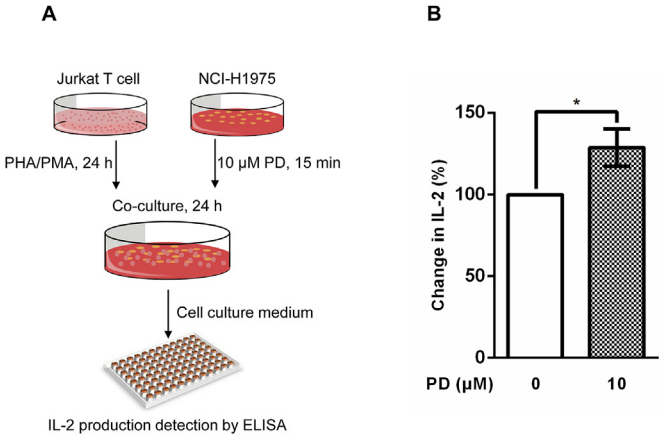

Fig. 2. Jurkat T cells co-cultured with PD-treated cancer cells shows enhanced IL-2 secretion. (A) The co-culture model of NCI–H1975 cells and Jurkat T cells. Jurkat T cells were pre-activated overnight with PMA (25 ng/ml) and PHA (1 μg/ml) and then co-cultured with PD pre-treated NCI–H1975 cells or non-treated NCI–H1975 cells. IL-2 levels in the culture supernatant were measured by ELISA after 24 h co-culture of Jurkat cells and NCI–H1975 cells. (B) Percent change in IL-2 secretion compared with the control group. *P value < 0.05, compared with control group.

PD did not decrease the mRNA level of PD-L1 or promote protein degradation. Then, we screened several compounds (20 μM amiloride, 25 μM monensin sodium salt, and 60 μM chlorpromazine) that may influence the cellular vesicular trafficking process and found that co-treatment with 60 μM chlorpromazine (CPZ) almost totally restored the reduction of PD-L1 caused by PD in both NCI–H1975 and NCI–H358 cells compared with the PD group (Fig. 4A). Meanwhile, CPZ also abolished PD-induced extracellular release of PD-L1 in both cell lines (Fig. 4B). Co-treatment with CPZ extensively restored the PD-mediated reduction of PD-L1 signals on cell membrane as evidenced by the immunofluorescence (Fig. 4C). Similarly, CPZ massively offset PD-induced left-shift of PD-L1 signal, further confirming that CPZ could prevent the reduction of membrane PD-L1 (Fig. 4D). In total, CPZ abolishes the effect of PD-induced extracellular release of PD-L1.

3.5. PD-triggered extracellular release of PD-L1 is independent of the hemolytic mechanism

PD is a triterpenoid saponin, which was reported with hemolytic effect on erythrocytes. In this case, we wondered whether the mechanism of PD-triggered PD-L1 extracellular release was associated with the mechanism of PD-induced hemolysis. Thus, we chose several saponins to detect their hemolytic effect on rabbit erythrocytes and their capacity of promoting PD-L1 extracellular release in NCI–H1975 cells. As shown in Fig. 5A, the hemolysis ratios of saponins like SA and DIO were quite high at the same concentration, but they did not cause PD-L1 extracellular release. Timo-A showed almost 0% hemolysis and had no effect on PD-L1 release. By contrast, PD-L1 was detected in the cell culture medium from PD and its analogue PD2 treatment group, which only caused a slight hemolytic effect on 2% rabbit erythrocyte suspension. Collectively, our results show that the effect of PD-induced PD-L1 extracellular release may be independent of the hemolytic mechanism.

4. Discussion

PD-L1 serves as one of the important cancer therapy targets in clinic (Pardoll, 2012). The studies of PD-L1 regulation mainly focused on its transcriptional level and protein stability (Sun et al., 2018). As far as we known, it is the first time to show that PD triggered the extracellular release of PD-L1. PD showed weak regulation on the mRNA level of PD-L1 in both NCI–H1975 and NCI–H358 cells (Fig. 3A). Meanwhile, PD decreased the intracellular PD-L1 independent of protein degradation (Fig. 3B). Based on our data, PD may not change the total protein expressional levels of PD-L1 (both inside and outside cells) at least in our

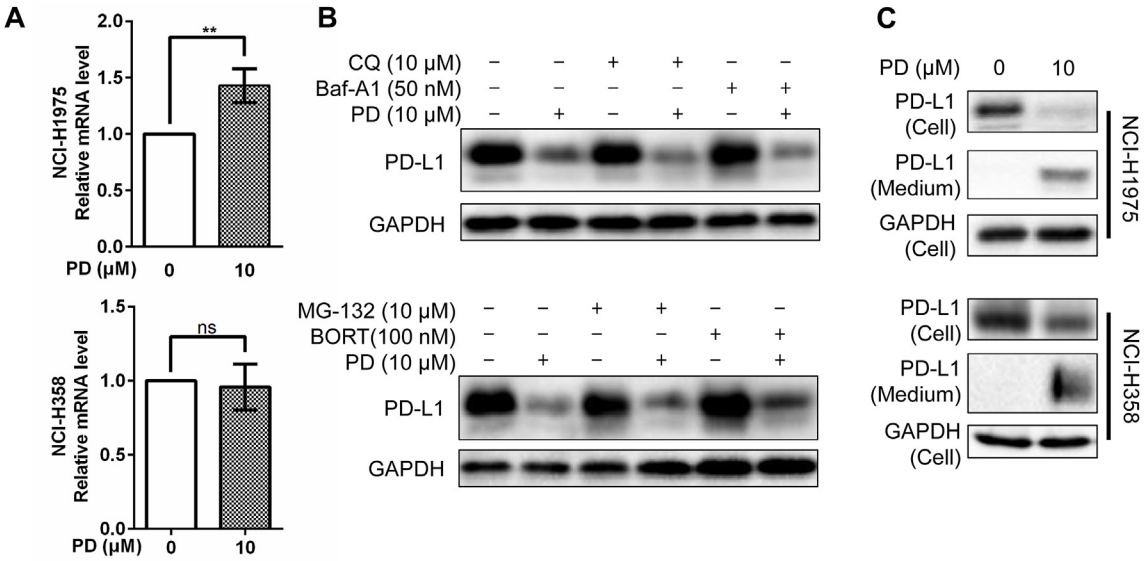

Fig. 3. PD triggers the extracellular release of PD-L1. (A) The mRNA level of PD-L1 in NCI–H1975 and NCI–H358 cells was analyzed by qPCR after the treatment with 10 μM for 15 min. $^{**}P$ value < 0.01, compared with control group. (B) Western blot was used to detect the protein level of PD-L1 in NCI–H1975 cells after the pre-treatment with lysosome inhibitors (chloroquine, CQ and bafilomycin A1, Baf-A1) or proteasome inhibitors (MG-132 and bortezomib, BORT) for 1 h, and then followed by the treatment with or without PD for 15 min. (C) NCI–H1975 and NCI–H358 cells were treated with 10 μM PD for 15 min and the total protein in cell culture medium was extracted by $(NH_4)_2SO_4$ salting out method. The protein level of PD-L1 was detected by Western blot.

experimental condition. Besides, our study showed that other membrane receptors, such as EGFR and IGFR, could be also detected in the cell culture medium with PD treatment (data not shown), indicating that PD might trigger an extensive release of cell membrane proteins. Previously, our group has found that PD reduced the expression of multiple Hsp90 client proteins like EGFR via disrupting the protein–-protein interaction of Hsp90/Cdc37 complex (Li et al., 2017). However, the proteasome inhibitors MG132 only partially restored the PD-induced EGFR down-regulation, which did not exclude the possibility of EGFR extracellular release. Based on current results, we suspect that PD-mediated release of membrane proteins may serve as one of the mechanisms behind its multiple pharmacological effects. In another aspect, we can have a deeper understanding about the diversity of PD-triggered biological function, which accords with the complicated characteristic of natural products (Huang et al., 2018).

It is reported that PD disrupted the formation of lipid rafts by depleting cholesterol (Hu et al., 2016). Cholesterol is a very important component of cell membrane structure, which can affect the membrane fluidity (Yeagle, 1985). Depletion of membrane cholesterol would enhance the release of extracellular membrane vesicles in epithelial cells (Marzesco et al., 2009). Therefore, we suspected the relationship between the PD-induced PD-L1 extracellular release and cell membrane structures. Previously, we also observed that cell morphology was changed roundly with short time PD (10 μM) treatment and recovered after 6 h treatment (data not shown). On one hand, although our study showed that 15 min PD treatment seemed have no effect on the membrane lipid rafts in NCI–H1975 cells (data not shown), PD's effect on cholesterol is still unclear. On the other hand, we considered that saponins caused hemolytic effect via forming complex with sterols on the erythrocytes membrane, leading to an increase of permeability in cells and the subsequent loss of hemoglobin (Bissinger et al., 2014; Baumann et al., 2000). Nevertheless, PD did not show obviously hemolytic effect at the concentration used in current study. By contrast, some saponins induced severe hemolytic effect without promoting the extracellular release of PD-L1 in cancer cells. These results illustrated PD-mediated the release of PD-L1 may be independent of the hemolytic effect. Accidently, we found that CPZ, an inhibitor of clathrin-mediated endocytosis, could abolish the release of PD-L1. However, silencing clathrin, a key protein of clathrin-mediated endocytosis, could not restore PD-induced PD-L1 release. Thus, the mechanism of CPZ involved in this process may be not associated with its effect of inhibiting endocytosis, which still needs to be further studied.

Crucially, we would like to figure out whether PD-induced decreased PD-L1 could recovery the loss immunity induced by cancer cells. Current results showed the PD-treated cancer cells increased the IL-2 production for the activated Jurkat T cells, which indicated that PD-induces PD-L1 reduction might restore the activation of Jurkat T cells. Perhaps extracellular release is one of the approaches to decrease cellular PD-L1, but it should be verified whether the released PD-L1 has capacity to interact with PD-1 or not. Recently, exosomal PD-L1 was reported with immunosuppression in metastatic melanoma (Chen et al., 2018). In this regard, although we have made efforts on the study for the released PD-L1, its function on the immune cells is still unclear. Meanwhile, PD was reported with the function of regulating immune cells (Chen et al., 2015). Therefore, a comprehensive *in vivo* study on whether the PD-triggered PD-L1 extracellular release can enhance anti-tumor immune response is needed.

In conclusion, our results demonstrate that PD triggers the extracellular release of PD-L1 and PD-treated cancer cells can restore the Jurkat T cells activation. Our study sheds new light for the application of natural products for cancer immunotherapy in the future.

Conflicts of interest

The authors declare no potential conflicts of interest.

Acknowledgements

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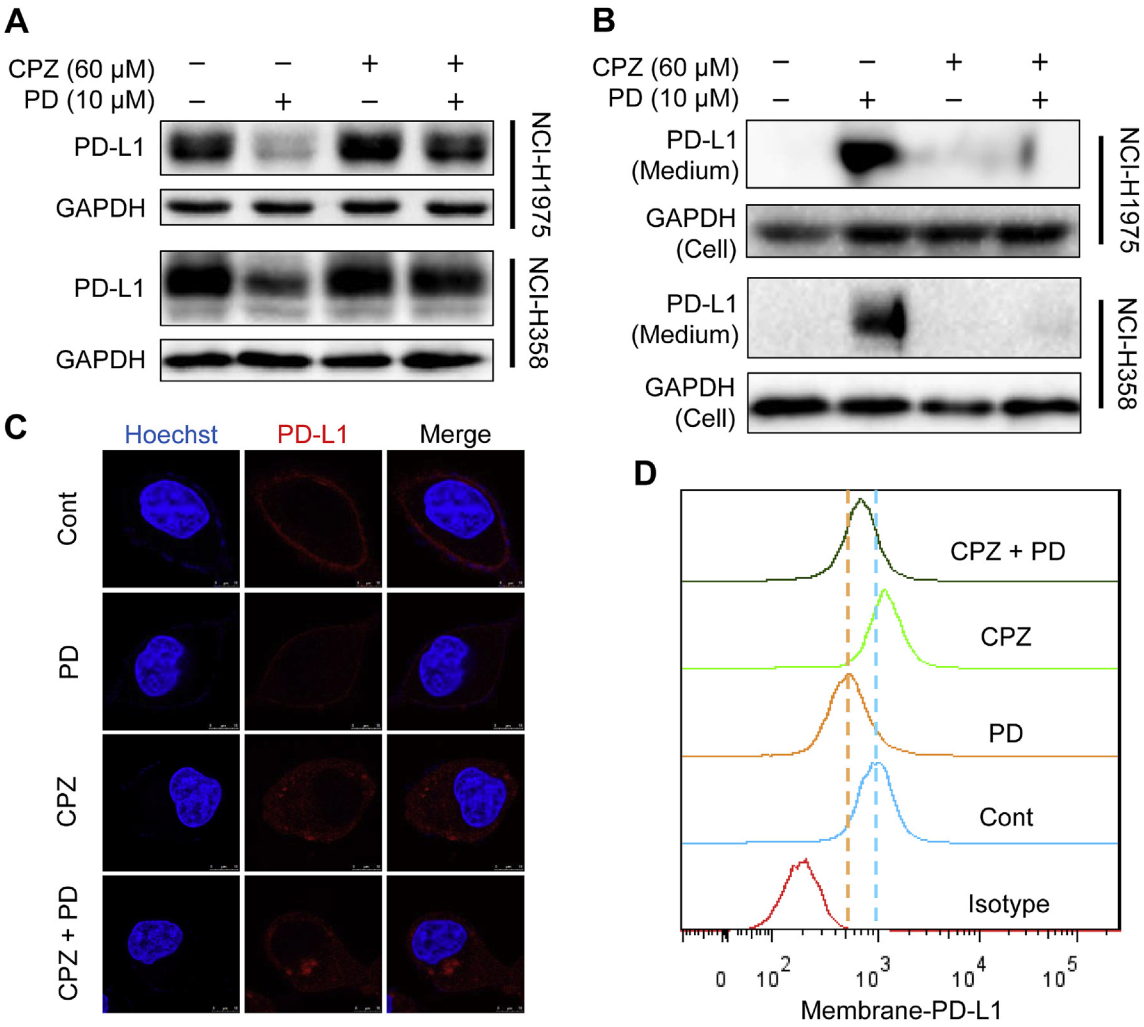

Fig. 4. CPZ abolishes the effect of PD-triggered extracellular release of PD-L1. (A) NCI–H1975 and NCI–H358 cells were treated with 10 μM PD after 1 h pre-treatment with 60 μM CPZ, and then the protein level of PD-L1 was detected by Western blot. (B) After the co-treatment with PD and CPZ, the total of protein in cell culture medium was extracted by the $(NH_4)_2SO_4$ salting out method. The protein level of PD-L1 was detected by Western blot. (C) NCI–H1975 cells were treated with PD after pre-treatment with CPZ for 1 h, and the localization of PD-L1 was observed by immunofluorescence (scale bar: 10 μm). (D) Flow cytometry assay was performed to determine the cell surface PD-L1 in NCI–H1975 cells that were pre-treated by CPZ for 1 h, followed by treatment with PD for 15 min.

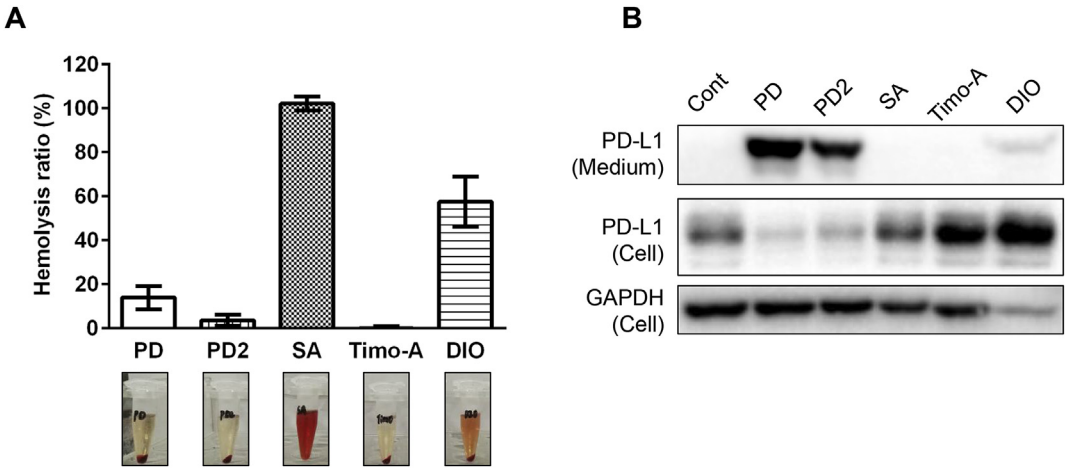

Fig. 5. PD-triggered the extracellular release of PD-L1 is independent of the hemolytic mechanism. (A) Platycodin D (PD), platycodin D2 (PD2), saikosaponins A (SA), timosaponin A (Timo-A) and diosin (DIO) were mixed with 2% rabbit erythrocyte suspension at the final concentration of 10 μM. After incubation at 37 °C for 1 h, the absorbance of supernatant was detected at 415 nm. (B) NCI–H1975 cells were treated with different saponins with 10 μM for 15 min, the total protein in cell culture medium was enriched by $(NH_4)_2SO_4$ salting out method. The protein level of PD-L1 was detected by Western blot.

Abbreviations

Baf-A1	bafilomycin A1
BORT	bortezomib
CPZ	chlorpromazine
CSN5	COP9 signalosome 5
CTLA-4	cytotoxic T-lymphocyte-associated antigen 4
CQ	chloroquine
DIO	diosin
Hsp90	heat shock protein 90
IL-2	interleukin-2
mTOR	the mammalian target of rapamycin
PD	platycodin D
PD-L1	programmed death ligand-1
PD-1	programmed cell death protein 1
PHA	phytohaemagglutinin
PMA	phorbol-12-myristate-13-acetate
PD2	platycodin D2
qPCR	Quantitative real-time PCR
SA	saikosaponins A
Timo-A	timosaponin A

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