Tumor cells-derived exosomal PD-L1 promotes the growth and invasion of lung cancer cells *in vitro via* mediating macrophages M2 polarization

Xiangjun Lu,[1]* Jian Shen,[2]* Siyuan Huang,[2]* Dongdong Liu,[2] Haitao Wang[2]

[1]Department of Radiology; [2]Department of Thoracic Surgery, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, Hangzhou, Zhejiang, China

*These authors share first authorship

ABSTRACT

Lung cancer originating from the bronchial epithelium is the most common lung malignancy. It has been reported that programmed cell death 1 ligand 1 (PD-L1) and tumor-associated macrophages are closely related to the development of lung cancer. However, whether tumor-derived exosomal PD-L1 could mediate the regulation of macrophage polarization in lung cancer remains unclear. For this research, the level of PD-L1 in normal tissues and lung cancer tissues was evaluated using RT-qPCR. Next, the apoptosis of lung cancer cells was evaluated using flow cytometry assay. Then, the structure and morphology of vesicles were observed using transmission electron microscopy and nanoparticle tracking analysis. Later on, the internalization of exosomes by macrophage was observed using fluorescence microscopy. Our results showed that the level of PD-L1 was upregulated in tumor tissues and lung cancer cells. Knockdown of PD-L1 notably inhibited the viability, migration and invasion of lung cancer cells. In addition, lung cancer cells-derived exosomal PD-L1 could be absorbed by macrophages. Meanwhile, exosomal PD-L1 was able to promote macrophages M2 polarization. Moreover, macrophages M2 polarization induced by exosomal PD-L1 further remarkably promoted the viability, migration, invasion, and epithelial-mesenchymal transition process of lung cancer cells. Collectively, knockdown of PD-L1 notably inhibited the viability, migration and invasion of lung cancer cells. Tumor cell-derived exosomal PD-L1 could promote the growth of lung cancer cells by mediating macrophages M2 polarization. Thus, inhibiting macrophages M2 polarization might be a promoting therapy for the treatment of lung cancer.

Key words: lung cancer; exosome; programmed cell death receptor-ligand 1 (PD-L1); tumor-associated macrophages; macrophages M2 polarization.

Correspondence: Haitao Wang, Department of Thoracic Surgery, Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, 158 Shangtang Road, Hangzhou 310014, Zhejiang, China.
E-mail: wanghaitaosrm@163.com

Contributions: XL, JS, SH, major contributions to the conception, design and manuscript drafting and revision, data acquisition, analysis and interpretation; DL, HW substantial contributions to the conception and design of the study, manuscript revision. All the authors read and approved the final version of the manuscript and agreed to be accountable for all aspects of the work.

Conflict of interest: the authors declare that they have no competing interests, and all authors confirm accuracy.

Ethics approval and consent to participate: this study was approved by Ethics Committee of the Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College, with approval document no. 2021008. The written informed consent has been obtained from patients donators.

Availability of data and materials: the datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Funding: this work was supported by the Foundation of Zhejiang Provincial Scientific Research on Traditional Chinese Medicine (2019ZA002); the General Project Funds from the Health Department of Zhejiang Province (2020KY009); the Foundation for Natural Science of Zhejiang Province (LY20H010005).

Introduction

Lung cancer originating from the bronchial epithelium is a malignant tumor.[1-3] Lung cancer is divided into non-small and small cell lung cancer according to the histological cell types.[4,5] The pathogenesis of lung cancer includes smoking.[1,6] It has been reported that smokers are about 10-20 times more likely to develop lung cancer than non-smokers.[7,8] In addition, due to the concealment of the development of lung cancer and the negligence of patients, most of the patients with lung cancer are in the middle and late stages, and the curative effect is not good.[9] Current treatments for lung cancer include targeted therapy, drug therapy, radiotherapy and checkpoint immunotherapy, while the effects remain unsatisfactory.[4,10,11] At present, immunotherapy has been recognized as the preferred treatment for lung cancer.[11] Immunotherapy is a treatment method that modulates the activity of immune cells in the body, which can reactivate T cells and indirectly kill tumor cells.[12] In addition, immunotherapy includes programmed cell death receptor 1 (PD-1) inhibitors and anti-programmed cell death receptor-ligand 1 (PD-L1) antibodies.[13] PD-1 can bind to its ligand PD-L1, which is able to inhibit the activation of T cells.[14] Evidence has shown that PD-L1 is notably upregulated in various cancers including lung cancer.[15] Therefore, inhibition of PD-L1 is one of the most promising approaches for the treatment of lung cancer.

Tumor growth is regulated by tumor-associated macrophages (TAM), which play a critical role during the interaction between immune system and tumor.[16,17] Macrophages undergo two different types of activation in different environments, mainly including classically activated macrophages (macrophages M1 polarization) and alternatively activated macrophages (macrophages M2 polarization).[18,1]. Besides, macrophages M2 polarization was able to secrete a variety of cytokines that promoted the survival of tumor cells including lung cancer.[20,21] In other words, macrophages M2 polarization plays a vital role in the metastasis of tumor cells.[22,23] Furthermore, exosomes are small extracellular vesicles, which could mediate cell-to-cell communication.[24] Tumor cells could secrete exosomal PD-L1 to repress immune response.[25] We aimed to explore the relationship between exosomal PD-L1 and macrophages in lung cancer in the present research.

Materials and Methods

Clinical sample collection

The adjacent normal tissues and tumor tissues from patients with lung cancer (n=15) were collected from Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College. All samples were frozen in liquid nitrogen immediately and stored at −80°C.

The study was approved by the Ethics Committee of the Zhejiang Provincial People's Hospital, People's Hospital of Hangzhou Medical College. The written informed consent has been obtained from patient donators.

Cell culture

BEAS-2B cells (human normal lung epithelial cell lines), NCI-H1650, NCI-H1299 and A549 cells (lung cancer cell lines), and THP-1 (human acute monocytic leukemia cell lines) were obtained from American Type Culture Collection (ATCC). These cells were maintained at 37°C and 5% CO_2 in RPMI-1640 Medium (Thermo Fisher Scientific, Waltham, MA, USA) containing with 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA) and 10% fetal bovine serum (FBS). To obtain macrophages (M0 type), THP-1 cells were differentiated with phorbol-12 myristate-13 acetate (PMA) for 18 h. To obtain M2-polarized macrophages, THP-1 cells were treated with PMA for 12 h and then exposed to PMA, IL-4 and IL-13 for another 48 h (as a positive control).[26]

Cell transfection

NCI-H1299 and A549 cells were transfected with PD-L1 small interfering RNA (siRNA)1, PD-L1 siRNA2, PD-L1 siRNA3 and PD-L1 siRNA-negative control (NC) using Lipofectamine® 2000 according to the manufacturer's instructions for 6 h. Next, the transfected cells were cultured in fresh complete medium for another 48 h. PD-L1 siRNAs were available from Genepharma. SiRNA target sequences: 5'-CCAAGGACCTATATGTGGTAGAGTA-3' (PD-L1 siRNA1); 5'-CCGACTACAAGCGAATTACTGTGAA-3' (PD-L1 siRNA2); 5'-CGACTACAAGCGAATTACTGTGAAA-3' (PD-L1 siRNA3); 5'-CCAAGCCATATGTGTTGGAAGAGTA-3' (siRNA control/siRNA-NC).

For PD-L1 overexpression, empty pcDNA3.1 vector (pcDNA3.1-ctrl) or pcDNA3.1-PD-L1 (sense: 5'-CGGGATCCATGAGGATATTTGCTGTCTT-3', antisense: 5'-CCGCTCGAGGCCGTCTCCTCCAAATGTGTAT-3') overexpression (OE) were transfected into A549 cells using Lipofectamine® 2000. PcDNA3.1-ctrl and PD-L1 OE were available from Genepharma.

RT-qPCR

Total RNA in collected lung cancer cells and macrophages was isolated using the Trizol reagent (ELK Biotechnology, Wuhan, China). EntiLink™ 1st Strand cDNA Synthesis Kit (ELK Biotechnology) was conducted to reverse transcription. Next, qPCR was conducted using EnTurbo™ SYBR Green PCR SuperMix (ELK Biotechnology). The relative level of PD-L1 was calculated using the $2^{-\Delta\Delta Ct}$ method. The information of primers: PD-L1 forward, 5'-CCCCATACAACAAAATCAACCAA-3' and reverse, 5'-TCTCCTAAAAGTGCAGTAGAAAATC-3'; GAPDH forward, 5'-TCAAGAAGGTGGTGAAGCAGG-3' and reverse, 5'-TCAAAGGTGGAGGAGTGGGT-3'.

CCK-8 assay

The viability of lung cancer cells was calculated by CCK-8 assay kit (Beyotime Biotechnology Company, Shanghai, China). NCI-H1299 and A549 cells (5×10^4 cells/well) were inoculated in 96-well plates overnight. Then, cells were transfected with siRNA-NC or PD-L1 siRNA1 for 6 h using Lipofectamine® 2000. After that, the culture medium was changed, and cells continued to be cultured in RPMI-1640 Medium for 0, 24, 48 and 72 h. Next, CCK-8 solution (10 μL) was added into each well. After 2 h of incubation at 37°C, the OD value at 450 nm was calculated using a microplate reader (MULTISKAN MK3, Thermo Fisher Scientific).

EdU staining

The proliferation of lung cancer cells was calculated by the EdU Detection kit (Beyotime). Lung cancer cells were inoculated in 24-well plate (2.5×10^5 cells/well), and then transfected with siRNA-NC or PD-L1 siRNA1 using Lipofectamine® 2000. Next, 50 μM EdU was added to lung cancer cells. After 2 h of incubation at 37°C, cells were incubated with DAPI (1 μg/mL) for 10 min. Finally, EdU-positive cells were observed using a microscope.

Flow cytometric assay for apoptotic cells

Lung cancer cells were inoculated in 6-well plate (5×10^4 cells/well), and then transfected with siRNA-NC or PD-L1 siRNA1 using Lipofectamine® 2000. Next, lung cancer cells were stained with Annexin V-FITC (5 μL) and PI (5 μL) reagents (Keygen Biotech, Nanjing, China) for 15 min in darkness. Subsequently, the apoptosis of lung cancer cells was calculated

using flow cytometry (FACSCalibur, BD Biosciences, Franklin Lakes, NJ, USA).

Transwell assay

Non-Matrigel and Matrigel-coated transwell chambers (Corning Inc., Corning, NY, USA) were applied to detect cell migration and invasion respectively. Firstly, lung cancer cells were transfected with siRNA-NC or PD-L1 siRNA1 using Lipofectamine® 2000. Then, lung cancer cells were seeded onto the upper chambers supplemented with serum-free RPMI-1640 Medium. Meanwhile, RPMI-1640 medium containing 10% FBS was added to the lower chambers. After 24 h of incubation at 37°C, cells in the upper chambers migrated or invaded to the lower chambers and were stained with 0.1% crystal violet. Subsequently, a microscope was applied to observe the stained cells.

Exosomes extraction

A549 cells were transfected with pcDNA3.1-ctrl or PD-L1 OE plasmids using Lipofectamine® 2000. Next, BEAS-2B, A549 cells, pcDNA3.1-ctrl- or PD-L1 OE-transfected A549 cells were collected and then centrifuged at 300 *g* for 15 min according to previous reports.[27] To remove cell debris, cells were centrifuged at 2500 *g* for 15 min. Next, the cells were centrifuged at 4000 g to concentrate the supernatant. Subsequently, the supernatant was centrifuged at 100,000 g for 1 h. Finally, the vesicles were collected.

Transmission electron microscopy analysis

The vesicles were dropped on the carbon-supporting membrane. Then, the excess liquid was sucked up with filter paper. Subsequently, 2% phosphotungstic acid was dropped on the carbon-supporting membrane. Next, the structure and morphology of vesicles were validated using transmission electron microscopy (HT7700, Hitachi, Chiyoda City, Tokyo, Japan) at 80 keV according to the previous report.[28]

Nanoparticle tracking analysis

The size of the vesicles was employed by nanoparticle tracking analysis (NTA). The sample tank was washed using deionized water. Then, polystyrene microspheres were used to calibrate the ZetaView analyzer. Subsequently, the sample tank was washed using 1 x PBS buffer. Next, the size of the vesicles was validated using NTA NanoSight instruments.

Western blot assay

Total protein was isolated using the RIPA buffer (Beyotime), and then quantified using the BCA protein assay kit (Beyotime). Next, protein samples were separated with 10% SDS-PAGE and then transferred to a PVDF membrane. PVDF membrane was then blocked using TBST containing 5% fat-free milk for 1 h. After that, the PVDF membrane was incubated with primary antibodies of anti-TSG101 (1:1000; Proteintech, Rosemont, IL, USA), anti-CD63 (1:500, Affinity Biotech, Houston, TX, USA), anti-CD9 (1:1000; Proteintech), anti-E-cadherin (1:1000; Affinity), anti-N-cadherin (1:2000; Proteintech), anti-Vimentin (1:2000; Proteintech) and anti-β-actin (1:10000; Proteintech). After being incubated with primary antibodies, the PVDF membrane was incubated with HRP-conjugated goat anti-rabbit secondary antibody

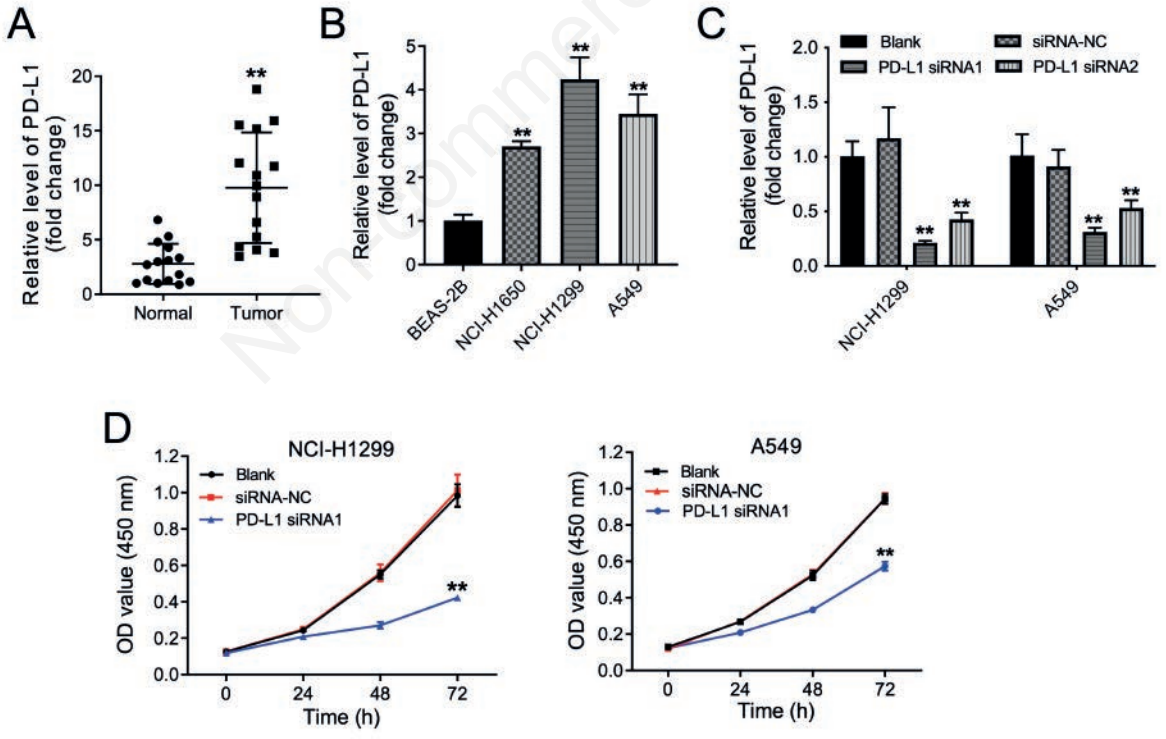

Figure 1. Knockdown of PD-L1 inhibits the viability of lung cancer cells. **A**) The relative level of PD-L1 in normal tissues and tumor tissues was evaluated using RT-qPCR. **B**) The relative level of PD-L1 in BEAS-2B and lung cancer cells was evaluated using RT-qPCR. **C**) PD-L1 siRNA1, PD-L1 siRNA2, PD-L1 siRNA3 and PD-L1 siRNA-NC were transfected into lung cancer cells using Lipofectamine® 2000; the relative level of PD-L1 in lung cancer cells was evaluated using RT-qPCR. **D**) PD-L1 siRNA1 and siRNA-NC were transfected into lung cancer cells; the viability of lung cancer cells was evaluated using CCK-8 at 0, 24, 48 and 72 h. **p<0.01 *vs* siRNA-NC group; n=3.

(1:10000; Aspen Biosciences, San Diego, CA, USA) for 1 h at 37°C and followed by washed using TBST. Afterward, the relative expressions of proteins were calculated by efficient chemiluminescence (ECL) kit (Applygen Technology, Beijing, China).

Exosome labeling and uptake

Firstly, exosomes isolated from A549 cells (A549 Exo) and exosomes isolated from A549 cells overexpressing PD-L1 ($A549^{PD\text{-}L1\ OE}$ Exo) were labeled using PKH26 dye (2 μM) for 2 h at 37°C in darkness. Then, macrophages were co-cultured with PKH26-labeled exosomes. Next, DAPI was used to stain cell nucleus. Finally, the staining result was observed using a fluorescence microscopy (IX51, 20x objective; mercury lamp; Olympus, Tokyo, Japan).

Flow cytometry assay for M1-type and M2-type macrophages

Markers for M1-type (CD86) macrophages and M2-type (CD206) macrophages were assessed by flow cytometry. Macrophages were incubated with anti-CD86 (20 μL/10^6 cells, FITC Conjugate; Abcam, Cambridge, UK) and anti-CD206 (10 μL/10^6 cells, PE-A Conjugate; Proteintech) for 30 min at 4°C in the dark. After that, CD86 positive cells and CD206 positive cells were detected using a CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, IN, USA).

ELISA

The levels of IL-1β or IL-10 in the supernatant of macrophages were evaluated using Human IL-10 ELISA Kit and Human TGF-β ELISA Kit following the manufacturer's instructions. These kits were available from ELK Biotechnology.

Statistical analysis

All data were repeated in triplicate. Experiment data were analyzed using GraphPad Prism (version 7.0). The comparisons were detected by ANOVA and Tukey's test. These data values were presented as mean ± SD. A $p<0.05$ was regarded to be significant.

Results

Knockdown of PD-L1 inhibits the viability of lung cancer cells

To evaluate the role of PD-L1 during the progression of lung cancer, the level of PD-L1 in lung cancer tissues and adjacent tissue was evaluated using RT-qPCR. The expression of PD-L1 was significantly upregulated in lung cancer tissues (Figure 1A). Consistently, PD-L1 level was significantly higher in NCI-H1650, NCI-H1299 and A549 cells than in BEAS-2B cells (Figure 1B). Since PD-L1 level in NCI-H1299 and A549 cells was higher than that in NCI-H1650 cells, NCI-H1299 and A549 cells were selected in the following study. Next, PD-L1 siRNAs were applied to knockdown the level of PD-L1 in NCI-H1299 and A549 cells. As indicated in Figure 1C, PD-L1 siRNA1 remarkably reduced the mRNA level of PD-L1 in lung cancer cells. In addition, PD-L1 siRNA1 obviously inhibited the viability of NCI-H1299 and A549 cells compared with the siRNA-NC group (Figure 1D). To sum up, knockdown of PD-L1 could inhibit the viability of lung cancer cells.

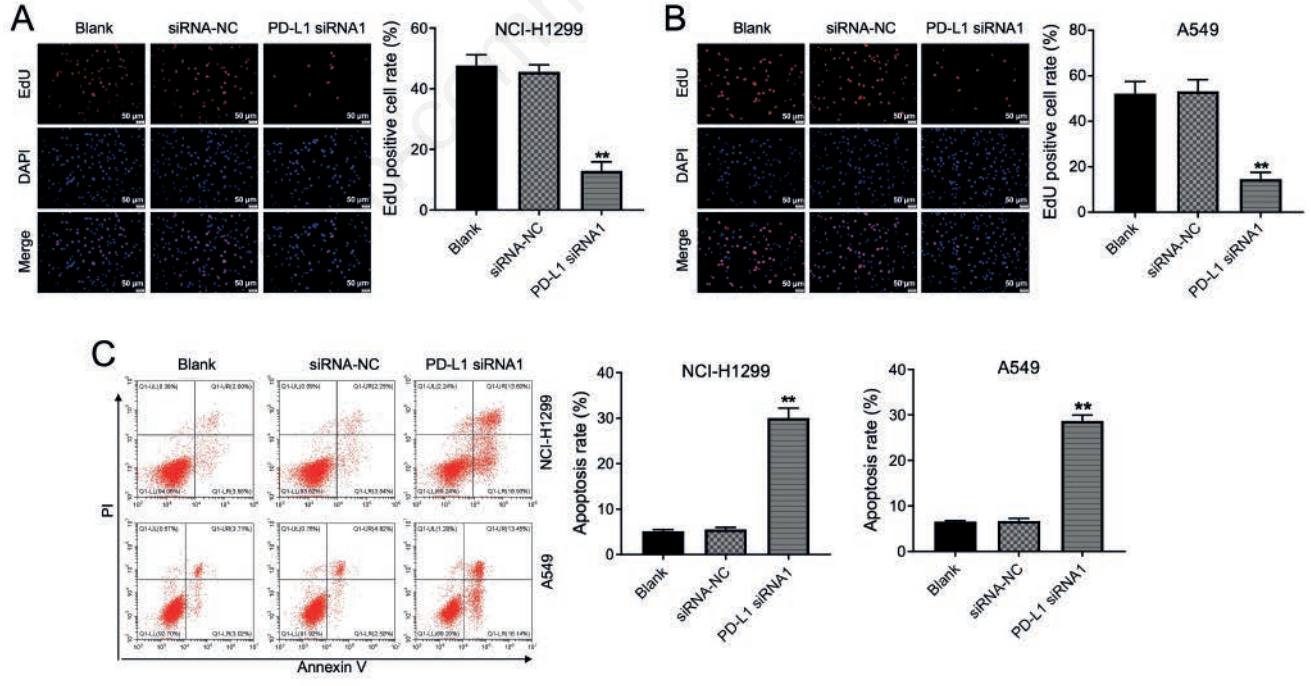

Figure 2. Knockdown of PD-L1 inhibits the proliferation and induces the apoptosis of lung cancer cells. PD-L1 siRNA1 and siRNA-NC were transfected into lung cancer cells. **A,B**) The proliferation of lung cancer cells was evaluated using EdU staining assay. **C**) The apoptosis of lung cancer cells was evaluated using Annexin V/PI staining assay. **p<0.01 *vs* siRNA-NC group; n=3.

Knockdown of PD-L1 reduces the proliferation, migration and invasion, and induces the apoptosis of lung cancer cells

With the aim to investigate the biological effect of PD-L1 siRNA1 on the proliferation of lung cancer cells, EdU staining assay was conducted. The results illustrated that PD-L1 siRNA1 clearly inhibited the proliferation of lung cancer cells (Figure 2 A,B). Additionally, PD-L1 siRNA1 markedly induced the apoptosis of lung cancer cells (Figure 2C). Moreover, PD-L1 siRNA1 clearly repressed the migratory and invasive abilities of NCI-H1299 and A549 cells (Figure 3 A,B). To sum up, knockdown of PD-L1 could suppress the proliferation, migration and invasion of lung cancer cells, while inducing apoptosis.

Lung cancer cells-derived exosomal PD-L1 can be absorbed by macrophages

To our knowledge, NSCLC cell-derived exosomal PD-L1 can promote the tumorigenesis and metastasis of NSCLC.[29] In addition, macrophages M2 polarization could promote the malignant behavior of lung cancer.[30] Based on the above background, the relationship between lung cancer cell-derived exosomal PD-L1 and macrophages was explored in the current study. Firstly, exosomes (BEAS-2B Exo and A549 Exo) were extracted from BEAS-2B and A549 cells. As illustrated Figure 4 A,B, vesicles derived from BEAS-2B and A549 cells had a disc-shaped structure with diameters between 40 and 150 nm. Meanwhile, these vesicles were able to express exosomes-specific markers (TSG101, CD63 and CD9) (Figure 4C). Collectively, these isolated vesicles were exosomes. In addition, the level of PD-L1 in A549 Exo was higher than that in BEAS-2B Exo (Figure 4D). At the same time, PD-L1 OE obviously promoted the level of PD-L1 in A549 cells and A549 Exo (Figure 4E). Furthermore, with the aim to investigate whether A549 Exo and A549$^{PD\text{-}L1\ OE}$ Exo could be internalized by macrophages, A549 Exo and A549$^{PD\text{-}L1\ OE}$ Exo were labeled using PKH26 dye and co-cultured with macrophages. As showed in Figure 4F, PKH26-labeled A549 Exo and A549$^{PD\text{-}L1\ OE}$ Exo could be absorbed by macrophages. Expectedly, A549$^{PD\text{-}L1\ OE}$ Exo clearly elevated the expression of PD-L1 in macrophages (Figure 4G). To sum up, lung cancer cell-derived exosomal PD-L1 could be absorbed by macrophages.

Lung cancer cell-derived exosomal PD-L1 promotes macrophages M2 polarization

To explore the role of exosomal PD-L1 on macrophages polarization, macrophage-related markers were evaluated. As implied in Figure 5 A-C, A549$^{PD\text{-}L1\ OE}$ Exo or IL4/IL13 (as a positive control) notably increased the levels of CD206, arginase-1, IL-10 and TGF-β (M2 macrophage-related makers) in macrophages, and decreased the levels of CD86, iNOS (M1 macrophage-related makers) in macrophages, compared with the control group.[31] In summary, A549$^{PD\text{-}L1\ OE}$ Exo could promote macrophages M2 polarization.

Tumor-derived exosomal PD-L1 the migration, invasion and epithelial-mesenchymal transition (EMT) of lung cancer cells by mediating macrophages M2 polarization

Next, to research the effect of macrophages M2 polarization on the development of lung cancer, A549 Exo-treated macrophages or A549$^{PD\text{-}L1\ OE}$ Exo-treated macrophages were co-cultured with A549 cells. The results of CCK-8 and transwell assays showed that M2 polarized macrophages induced by A549$^{PD\text{-}L1\ OE}$ Exo markedly promoted the viability, migration, and invasion of A549 cells com-

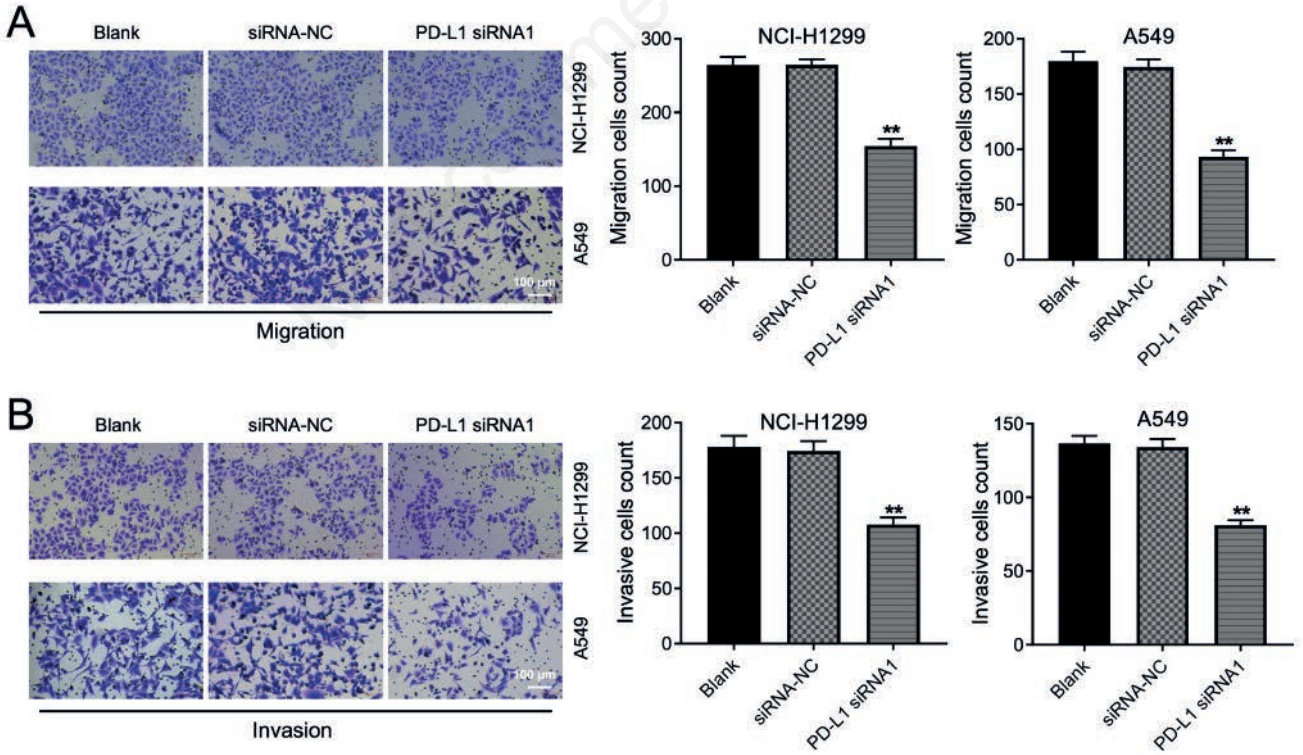

Figure 3. Knockdown of PD-L1 suppresses the migration and invasion of lung cancer cells. PD-L1 siRNA1 and siRNA-NC were transfected into lung cancer cells. **A,B**) The migration and invasion of lung cancer cells were evaluated using transwell assays. **p<0.01 *vs* siRNA-NC group; n=3.

pared with the control group (Figure 6 A-C). In addition, M2 polarized macrophages induced by A549$^{PD\text{-}L1\ OE}$ Exo obviously downregulated the level of E-cadherin and upregulated the levels of N-cadherin and vimentin in A549 cells compared with the control group (Figure 7 A-D). To sum up, exosomal PD-L1 could promote the migration, invasion and EMT process of lung cancer cells by mediating macrophages M2 polarization.

Discussion

In recent years, tumor immunology therapy has become a research hotspot in the treatment of lung cancer.[32] PD-1/PD-L1 is a classic molecular marker of tumor immunotherapy.[33-36] For example, PD-L1 is highly expressed in lung cancer.[15] In addition,

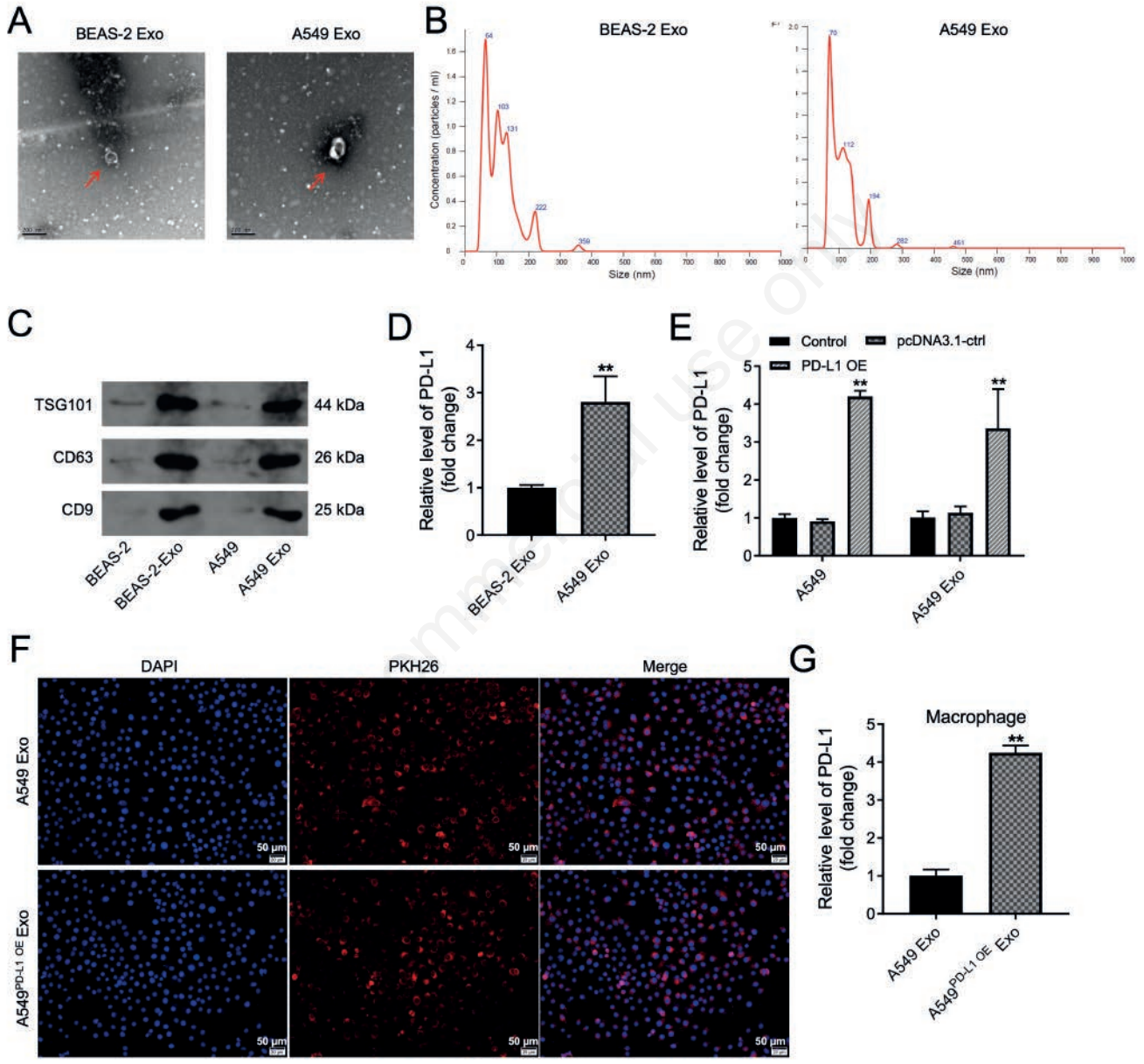

Figure 4. Lung cancer cells-derived exosomal PD-L1 can be absorbed by macrophage. **A**) The structure and morphology of vesicles was validated using a TEM. **B**) The size of vesicles was validated using the NTA. **C**) Western blot was performed to evaluate the relative expressions of TSG101, CD63 and CD9 in exosomes extracted from BEAS-2B and A549 cells. **D**) The relative level of PD-L1 in exosomes extracted from BEAS-2B and A549 cells was evaluated using RT-qPCR; **p<0.01 *vs* BEAS-2B-Exo group; n=3. **E**) A549 cells were transfected with PD-L1 OE and pcDNA3.1-ctrl. The relative level of PD-L1 in transfected A549 cells and exosomes extracted from transfected A549 cells was evaluated using RT-qPCR; **p<0.01 *vs* pcDNA3.1-NC group; n=3. **F**) Exosomes were labeled using PKH26 dye; then, macrophages were co-cultured with PKH26-labeled exosomes; the internalization of exosomes by macrophage was observed using fluorescence microscopy; cell nucleus was stained with DAPI. **G**) Macrophages were treated with indicated exosomes; the relative level of PD-L1 in macrophages was evaluated using RT-qPCR; **p<0.01 *vs* A549 Exo group; n=3.

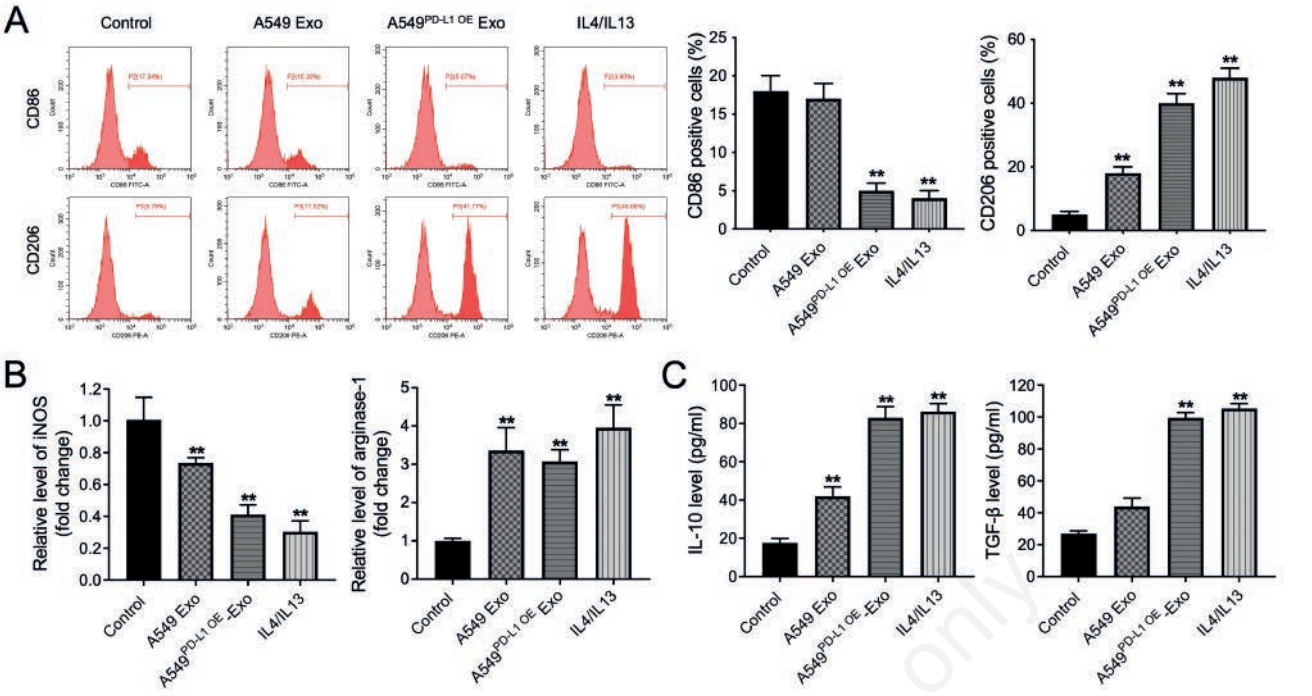

Figure 5. A549^{PD-L1} OE Exo promotes macrophages M2 polarization. Macrophages were treated with indicated exosomes or IL4/IL13. **A**) Flow cytometry was performed to evaluate macrophage-related markers CD86 and CD206 levels in macrophages. **B**) The relative levels of iNOS (M1 macrophage-related makers) and arginase-1 (M2 macrophage-related makers) in macrophages were evaluated using RT-qPCR. **C**) ELISA was performed to evaluate the expressions of M2 macrophage-related makers (IL-10 and TGF-β) in macrophages. **p<0.01 *vs* control group; n=3.

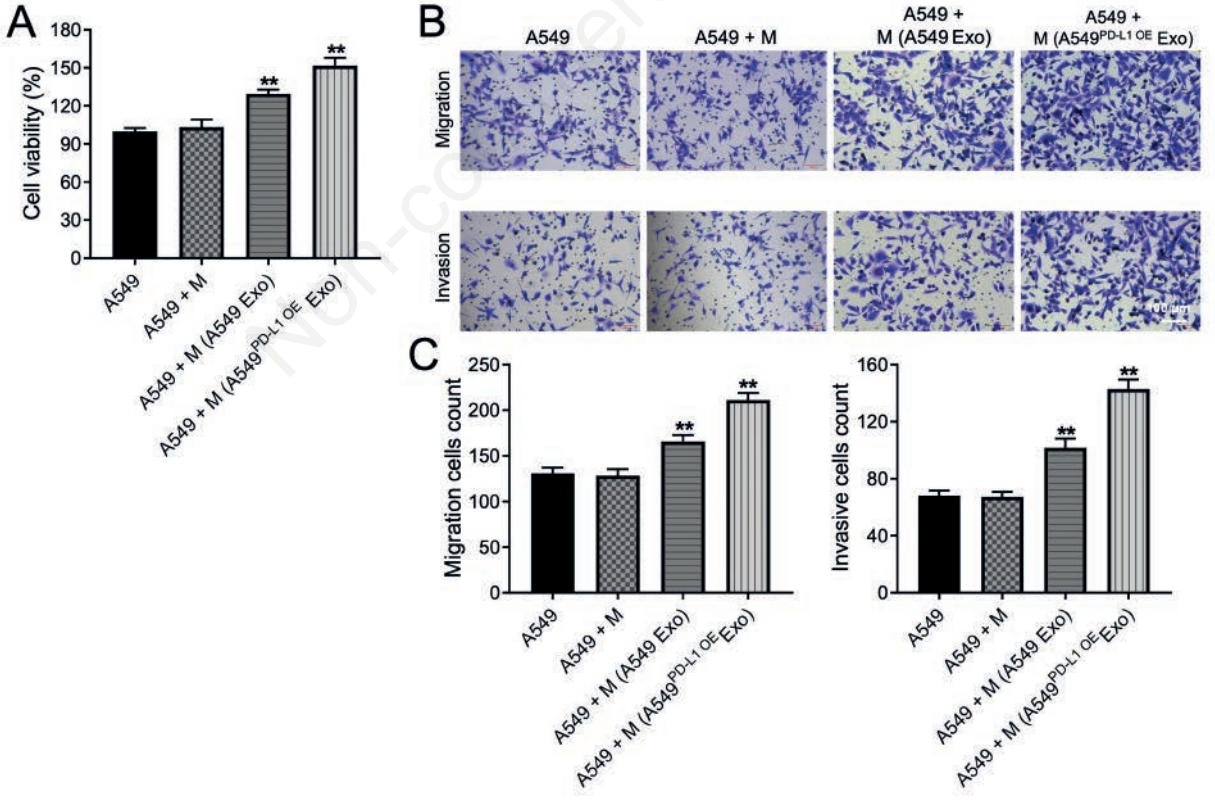

Figure 6. Tumor-derived exosomal PD-L1 promotes the migration and invasion of lung cancer cells by mediating macrophages M2 polarization. M (A549 Exo) or M (A549PD-L1 OE Exo) was co-cultured with A549 cells. **A**) The viability of A549 cells was evaluated using CCK-8 assay. **B,C**) The migration and invasion abilities of A549 cells were evaluated using transwell assays. **p<0.01 *vs* A549 group; n=3.

Increased PD-L1 expression is associated with poor prognosis in patients with NSCLC.[37] In the current study, we found that the expression of PD-L1 was increased in lung cancer tissues as well as in lung cancer cells. In addition, knockdown of PD-L1 notably inhibited the viability, migration and invasion of lung cancer cells. These results are commonly shown that blocking the PD-1/PD-L1 signaling pathway has become an important way of anti-tumor immunity. This finding was consistent with previous literature.[38,39] In this study, we firstly explored the relationship between tumor-derived exosomal PD-L1 and macrophages in lung cancer.

It has been confirmed that macrophages M2 polarization is closely related to the malignant behavior of cancers.[20,3]. On the one hand, the tumor microenvironment (TME) can promote macrophages M2 polarization.[30,40] For instant, A549R and H460R cells, which are non-small cell lung cancer cell lines, were able to stimulate macrophages M2 polarization.[30] In addition, lung cancer cell-derived exosomes could facilitate macrophages M2 polarization.[40] In this study, lung cancer cell-derived exosomal PD-L1 could be absorbed by macrophage. Meanwhile, exosomal PD-L1 could promote macrophages M2 polarization. These results indicated that cancer-derived exosomes were able to promote macrophages M2 polarization. Unlike previous literature, lung cancer cells-derived exosomal PD-L1 was focused. On the other hand, macrophages M2 polarization could promote tumorigenesis as well.[41] For instance, macrophages M2 polarization was able to enhance the ability of metastasis in lung cancer cells.[22] In addition, macrophages M2 polarization could stimulate the migration and invasion of colorectal cancer cells.[41] Consistently, in the present research, we found that macrophages treated with exosomal PD-L1 remarkably facilitated lung cancer cell migration and invasion. Additionally, EMT has been found to play an important role in tumor development, which is characterized by N-cadherin and vimentin upregulation and E-cadherin downregulation.[42-44] Promoting EMT process could contribute to lung cancer cell invasion and metastasis.[45] Our results showed that macrophages treated with exosomal PD-L1 significantly reduced E-cadherin level and elevated vimentin and N-cadherin levels in A549 cells, resulting in the activation of EMT in lung cancer. These results suggested that

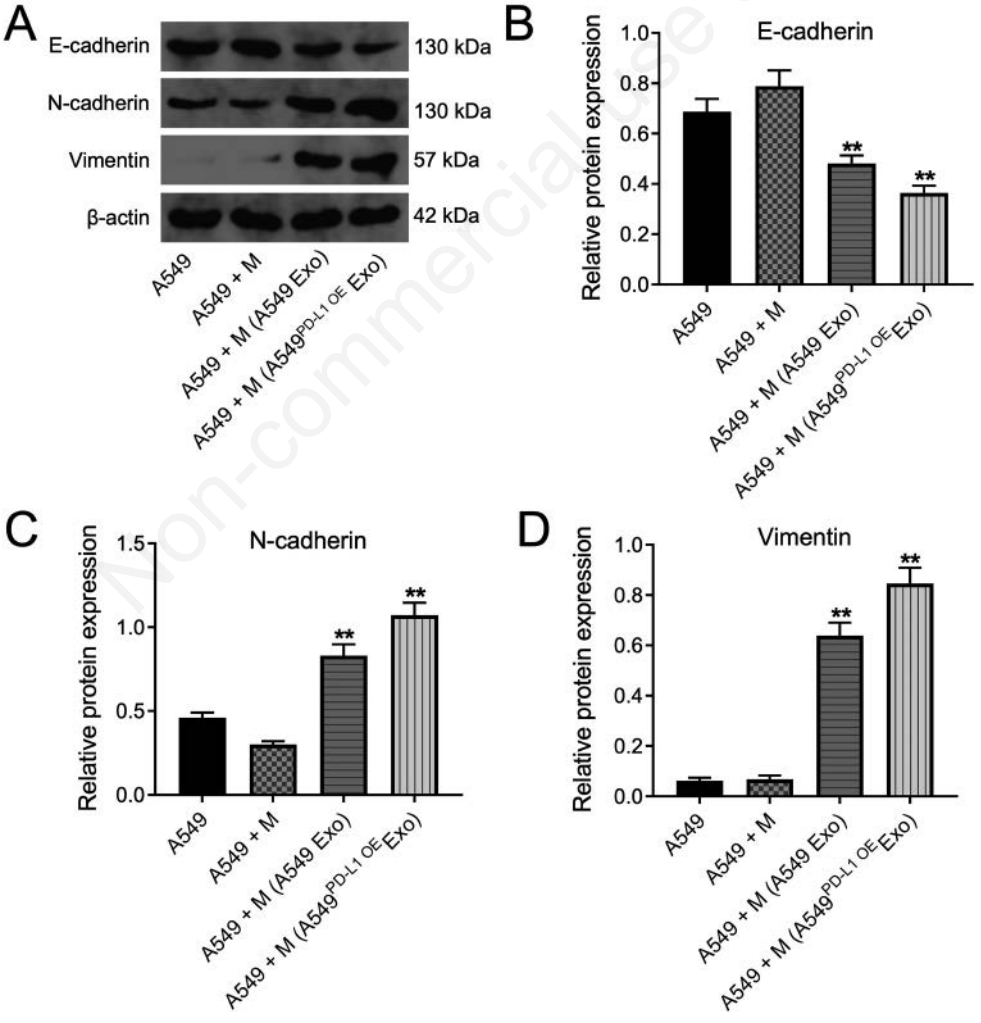

Figure 7. Tumor-derived exosomal PD-L1 mediates macrophages M2 polarization to promote EMT process of lung cancer cells. M (A549 Exo) or M (A549$^{PD\text{-}L1\ OE}$ Exo) was co-cultured with A549 cells. **A-D**) Western blot was performed to evaluate the expressions of E-cadherin, N-cadherin and Vimentin in A549 cells. **p<0.01 *vs* A549 group; n=3.

exosomes with overexpressed PD-L1 could promote macrophages M2 polarization to facilitate migration, invasion and EMT in lung cancer cells.

Inevitably, this research also had some limitations. We just investigated and found that tumor-derived exosomal PD-L1 could promote lung cancer progression by mediating macrophages M2 polarization. It is still unknown whether other target genes cause similar changes. Furthermore, the mechanism by which PD-L1 regulates the development of lung cancer must be investigated. As a result, more research should be conducted in the future.

In conclusion, knockdown of PD-L1 notably inhibited the viability, proliferation, migration and invasion of lung cancer cells *in vitro*. It may be hypothesized that tumor-derived exosomal PD-L1 could promote the growth and metastasis of lung cancer cells by mediating macrophages M2 polarization. Thus, inhibiting macrophages M2 polarization might be a promoting therapy for the treatment of lung cancer.

References

1. Yoshida K, Gowers KHC, Lee-Six H, Chandrasekharan DP, Coorens T, Maughan EF, et al. Tobacco smoking and somatic mutations in human bronchial epithelium. Nature 2020;578:266-72.
2. Yang D, Liu Y, Bai C, Wang X, Powell CA. Epidemiology of lung cancer and lung cancer screening programs in China and the United States. Cancer Lett 2020;468:82-7.
3. Bersimbaev R PA, Bulgakova O, Asia K, Aripova A, Izzotti A. Radon biomonitoring and microRNA in lung cancer. Int J Mol Sci 2020;21:2154.
4. Ruiz-Cordero R, Devine WP. Targeted therapy and checkpoint immunotherapy in lung cancer. Surg Pathol Clin 2020;13:17-33.
5. Oser MG, Niederst MJ, Sequist LV, Engelman JA. Transformation from non-small-cell lung cancer to small-cell lung cancer: molecular drivers and cells of origin. Lancet Oncol 2015;16:e165-72.
6. de Groot P, Munden RF. Lung cancer epidemiology, risk factors, and prevention. Radiol Clin North Am 2012;50:863-76.
7. Jung KJ, Jeon C, Jee SH. The effect of smoking on lung cancer: ethnic differences and the smoking paradox. Epidemiol Health 2016;38:e2016060.
8. Dubin S, Griffin D. Lung cancer in non-smokers. Mo Med 2020;117:375-9.
9. Nwagbara UI, Ginindza TG, Hlongwana KW. Lung cancer awareness and palliative care interventions implemented in low-and middle-income countries: a scoping review. BMC Public Health 2020;20:1466.
10. Vinod SK, Hau E. Radiotherapy treatment for lung cancer: Current status and future directions. Respirology 2020;25:S61-71.
11. Steven A, Fisher SA, Robinson BW. Immunotherapy for lung cancer. Respirology 2016;21:821-33.
12. Gou Q, Dong C, Xu H, Khan B, Jin J, Liu Q, et al. PD-L1 degradation pathway and immunotherapy for cancer. Cell Death Dis 2020;11:955.
13. Ai L, Xu A, Xu J. Roles of PD-1/PD-L1 pathway: signaling, cancer, and beyond. Adv Exp Med Biol 2020;1248:33-59.
14. Li C, Zhang N, Zhou J, Ding C, Jin Y, Cui X, et al. Peptide blocking of PD-1/PD-L1 interaction for cancer immunotherapy. Cancer Immunol Res 2018;6:178-88.
15. Yu H, Boyle TA, Zhou C, Rimm DL, Hirsch FR. PD-L1 expression in lung cancer. J Thorac Oncol 2016;11:964-75.
16. Kim J, Bae JS. Tumor-associated macrophages and neutrophils in tumor microenvironment. Mediators Inflamm 2016;2016:6058147.
17. Jaynes JM SR, Ronzetti M, Bautista W, Knotts Z, Abisoye-Ogunniyan A, et al. Mannose receptor (CD206) activation in tumor-associated macrophages enhances adaptive and innate antitumor immune responses. Sci Transl Med 2020;12:eaax6337.
18. Yunna C, Mengru H, Lei W, Weidong C. Macrophage M1/M2 polarization. Eur J Pharmacol 2020;877:173090.
19. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: Different gene signatures in M1(LPS+) vs. classically and M2(LPS-) vs. alternatively activated macrophages. Front Immunol 2019;10:1084.
20. Zhao S, Mi Y, Guan B, Zheng B, Wei P, Gu Y, et al. Tumor-derived exosomal miR-934 induces macrophage M2 polarization to promote liver metastasis of colorectal cancer. J Hematol Oncol 2020;13:156.
21. Yang Y, Ye YC, Chen Y, Zhao JL, Gao CC, Han H, et al. Crosstalk between hepatic tumor cells and macrophages via Wnt/β-catenin signaling promotes M2-like macrophage polarization and reinforces tumor malignant behaviors. Cell Death Dis 2018;9:793.
22. Tariq M, Zhang JQ, Liang GK, He QJ, Ding L, Yang B. Gefitinib inhibits M2-like polarization of tumor-associated macrophages in Lewis lung cancer by targeting the STAT6 signaling pathway. Acta Pharmacol Sin 2017;38:1501-11.
23. Ren W, Hou J, Yang C, Wang H, Wu S, Wu Y, et al. Extracellular vesicles secreted by hypoxia pre-challenged mesenchymal stem cells promote non-small cell lung cancer cell growth and mobility as well as macrophage M2 polarization via miR-21-5p delivery. J Exp Clin Cancer Res 2019;38:62.
24. Gurung S, Perocheau D, Touramanidou L, Baruteau J. The exosome journey: from biogenesis to uptake and intracellular signalling. Cell Commun Signal 2021;19:47.
25. Fan Z, Wu C, Chen M, Jiang Y, Wu Y, Mao R, et al. The generation of PD-L1 and PD-L2 in cancer cells: From nuclear chromatin reorganization to extracellular presentation. Acta Pharm Sin B 2022;12:1041-53.
26. Xu F, Cui WQ, Wei Y, Cui J, Qiu J, Hu LL, et al. Astragaloside IV inhibits lung cancer progression and metastasis by modulating macrophage polarization through AMPK signaling. J Exp Clin Cancer Res 2018;37:207.
27. Xia Q, Wang Q, Lin F, Wang J. miR-125a-5p-abundant exosomes derived from mesenchymal stem cells suppress chondrocyte degeneration via targeting E2F2 in traumatic osteoarthritis. Bioengineered 2021;12:11225-38.
28. Tan D, Li G, Zhang P, Peng C, He B. LncRNA SNHG12 in extracellular vesicles derived from carcinoma-associated fibroblasts promotes cisplatin resistance in non-small cell lung cancer cells. Bioengineered 2022;13:1838-57.
29. Kim DH, Kim H, Choi YJ, Kim SY, Lee JE, Sung KJ, et al. Exosomal PD-L1 promotes tumor growth through immune escape in non-small cell lung cancer. Exp Mol Med 2019;51:1-13.
30. Huang WC, Kuo KT, Wang CH, Yeh CT, Wang Y. Cisplatin resistant lung cancer cells promoted M2 polarization of tumor-associated macrophages via the Src/CD155/MIF functional pathway. J Exp Clin Cancer Res 2019;38:180.
31. Espinosa Gonzalez M, Volk-Draper L, Bhattarai N, Wilber A, Ran S. Th2 cytokines IL-4, IL-13, and IL-10 promote differentiation of pro-lymphatic progenitors derived from bone marrow myeloid precursors. Stem Cells Dev 2022;31:322-33.
32. Naylor EC, Desani JK, Chung PK. Targeted therapy and immunotherapy for lung cancer. Surg Oncol Clin N Am 2016;25:601-9.
33. Xia L, Liu Y, Wang Y. PD-1/PD-L1 Blockade therapy in

advanced non-small-cell lung cancer: current status and future directions. Oncologist 2019;24:S31-S41.
34. Hashimoto K, Nishimura S, Shinyashiki Y, Ito T, Kakinoki R, Akagi M. Clinicopathological assessment of PD-1/PD-L1 immune checkpoint expression in desmoid tumors. Eur J Histochem 2023;67:3688.
35. Hashimoto K, Nishimura S, Ito T, Kakinoki R, Akagi M. Immunohistochemical expression and clinicopathological assessment of PD-1, PD-L1, NY-ESO-1, and MAGE-A4 expression in highly aggressive soft tissue sarcomas. Eur J Histochem 2022;66:3393.
36. Hashimoto K, Nishimura S, Ito T, Akagi M. Characterization of PD-1/PD-L1 immune checkpoint expression in soft tissue sarcomas. Eur J Histochem 2021;65:3203.
37. Zhang M, Li G, Wang Y, Wang Y, Zhao S, Haihong P, et al. PD-L1 expression in lung cancer and its correlation with driver mutations: a meta-analysis. Sci Rep 2017;7:10255.
38. Huang Q, Wu X, Wang Z, Chen X, Wang L, Lu Y, et al. The primordial differentiation of tumor-specific memory CD8(+) T cells as bona fide responders to PD-1/PD-L1 blockade in draining lymph nodes. Cell 2022;185:4049-66.e25.
39. Chen X, Gao A, Zhang F, Yang Z, Wang S, Fang Y, et al. ILT4 inhibition prevents TAM- and dysfunctional T cell-mediated immunosuppression and enhances the efficacy of anti-PD-L1 therapy in NSCLC with EGFR activation. Theranostics 2021;11:3392-416.
40. Pritchard A TS, Wang Y, Hough K, Khan S, Strenkowski J, et al. Lung tumor cell-derived exosomes promote M2 macrophage polarization. Cells 2020;9:1303.
41. Liu Q, Yang C, Wang S, Shi D, Wei C, Song J, et al. Wnt5a-induced M2 polarization of tumor-associated macrophages via IL-10 promotes colorectal cancer progression. Cell Commun Signal 2020;18:51.
42. Wei J, Wu L, Yang S, Zhang C, Feng L, Wang M, et al. E-cadherin to N-cadherin switching in the TGF-β1 mediated retinal pigment epithelial to mesenchymal transition. Exp Eye Res 2022;220:109085.
43. Taki M, Abiko K, Ukita M, Murakami R, Yamanoi K, Yamaguchi K, et al. Tumor immune microenvironment during epithelial-mesenchymal transition. Clin Cancer Res 2021;27:4669-79.
44. Kuburich NA, den Hollander P, Pietz JT, Mani SA. Vimentin and cytokeratin: Good alone, bad together. Semin Cancer Biol 2022;86:816-26.
45. Menju T, Date H. Lung cancer and epithelial-mesenchymal transition. Gen Thorac Cardiovasc Surg 2021;69:781-9.

Received: 27 May 2023. Accepted: 3 July 2023.

Licensee PAGEPress, Italy
European Journal of Histochemistry 2023; 67:3784
doi:10.4081/ejh.2023.3784