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ORIGINAL RESEARCH

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Recombinant human *IL-17D* promotes the progression of human ovarian cancer SKOV3 cells and the expression of PD-L by activating the NF-κB pathway

Yuanchun Fan[1], Lei Feng[1], Yueping Liu[2], Shihao Liu[1], Hui Zhang[1,*]

[1]The Gynecology Department, the Fourth Hospital of Hebei Medical University, 050011 Shijiazhuang, Hebei, China
[2]The Pathology Department, the Fourth Hospital of Hebei Medical University, 050011 Shijiazhuang, Hebei, China

***Correspondence**
zhanghui@hebmu.edu.cn
(Hui Zhang)

Abstract

Ovarian cancer (OC) is often diagnosed in its advanced stages with many patients suffering from recurrence. Relapse is frequent after standard treatment leading to platinum resistance and poor prognosis in patients. Several studies have reported that immune status and inflammatory factors, such as Interleukin-17D (*IL-17D*), are major players in the prognosis of malignant tumors. IL-17 family is an important pro-inflammatory cytokine family, which mediates immunosuppression and promotes tumor progression. Our previous exploration revealed the promoting role of *IL-17D* expression in the occurrence and development of ovarian carcinoma, but the specific mechanism remains unclear. In this study, we investigated the effects of rhIL-17D on the proliferation, migration and invasion of human ovarian carcinoma cell SKOV3 *in vitro*. In addition, we also detected the effect of rhIL-17D on programmed death ligand-1 (PD-L1) expression of SKOV3 cells and possible mechanism.We found that rhIL-17D accelerated SKOV3 cell proliferation rate, enhanced the migration and penetration ability, and promoted the expression of PD-L1. After knockdown P65 expression,the activated form of NF-κB, in SKOV3 cells, the proliferation activity, migration, and invasion of ovarian cancer cells were significantly reduced. And the expression of PD-L1 decreased. In summary, we believe that *IL-17D* can accelerate cell proliferation and enhance cell migration and invasion abilities of ovarian cancer and induce PD-L1 expression by regulating the activation of NF-κB pathway. Down-regulation of *IL-17D* in tumor microenvironment may be a new approach to inhibit ovarian cancer.

Keywords

Ovarian cancer; *IL-17D*; NF-κB signaling pathway; *p65*; PD-L1

1. Introduction

Among all gynecological malignant tumors, ovarian cancer poses the most severe threat to women's health. According to the Cancer Statistics Report 2022, it is estimated that there will be 19,880 newly diagnosed cases of ovarian cancer and 12,810 deaths in the United States [1]. The 5-year overall survival rates are 32.1% for stage III/IV high-grade serous ovarian carcinoma (HGSOC), a common ovarian cancer [2]. The poor prognosis is largely due to the late diagnosis of cancer in its advanced stages (FIGOIII/IV), high recurrence rates even after primary cytoreductive surgery combined with postoperative platinum-based chemotherapy. The high recurrence and progression to platinum resistance are significantly detrimental to prognosis in patients with ovarian cancer; traditional treatment strategies are often unsatisfactory in such cases.

Maintenance therapies, such as bevacizumab, poly-ADP-ribose polymerase inhibitors (PARPi), and immunotherapy are gradually changing the landscape of advanced or recurrent ovarian cancer treatment [3]. Biomarkers for early screening are urgently needed. Also, mechanisms involved in the development of ovarian cancer need to be explored to identify effective therapeutic targets and improve the survival rate in patients.

Immunosuppressants have been increasingly used in tumor treatment [4] over the last several decades, *e.g.*, The blockade of PD-1 and PD-L1. PD-L1 and PD-1 suppress the activation of T lymphocytes and weaken the host immune response against the tumor. PD-L1 expressed by multiple cancer cells, including melanoma, prostate cancer, gastrointestinal cancer, lung cancer, and renal cell carcinoma [5–9]. However, there aren't enough studies with a high enough level of evidence to support it PD-1/PD-L1 blocking antibodies still suffer from several limitations as a treatment strategy for ovarian cancer [3, 10]. PD-L1 is essential for immune escape. Many transcription factors including NF-κB are involved in the regulation of PD-L1 expression, *p65* is one of the most studied members of family NF-κB. Studies have shown that *p65* regulates the expression of PD-L1, and the level of PD-L1 mRNA in non-small cell lung cancer (NSCLC) cells with *p65* knockdown is

reduced [11].

The tumor microenvironment (TME), especially inflammatory cells and inflammatory mediators, plays a key role in immunotherapy [12]. Inflammation-mediated immunosuppression promotes tumor progression [13]. IL-17 family, including 6 members (IL-17A to IL-17F), are involved in regulating inflammation and immune responses and affect the progression of malignant disease. IL-17A (commonly known as IL-17) was associated with the high expression of PD-L1 in prostate, colon, and breast cancers [14, 15]. Based on previous observations, Aotsuka *et al*. [16] found that IL-17 induce PD-L1 expression across NF-κB pathway and associated factors. It is further inferred that IL-17 is a promising predictor of prognosis and PD-L1 expression in ovarian cancer patients. It's unknown whether *IL-17D* regulates PD-L1 expression.

IL-17D promoted ovarian cancer in a transplanted murine model [17]. Here, we test whether *IL-17D* affects the biological characteristics of human ovarian cancer SKOV3 cells and induces the expression of PD-L1. We aim to identify new therapeutic targets and discover a theoretical basis for immunotherapy of ovarian cancer to improve the prognosis of ovarian cancer patients.

2. Materials and methods

2.1 Reagents

McCoy's 5A medium, penicillin, and streptomycin were purchased from GIBCO (NY, USA). Recombinant human *IL-17D* was purchased from PEPROTECH (New Jersey, USA). Rabbit anti-human PD-L1 antibody and rabbit anti-human *p65* antibody were all purchased from Affinity Biosciences (OH, USA). The homologous secondary goat anti-rabbit antibody was purchased from KPL, USA. MTS kit was purchased from Promega (G3580, Madison, Wisconsin, USA). Transwell cabin was purchased from Corning, USA.

2.2 Cell culture

The ovarian cancer SKOV3 (CSTR: 19375.09.3101HUMTCHu 185) cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). SKOV3 ovarian cancer cell was cultured in McCoy's 5A medium, supplemented with 10% fetal bovine serum, purchased from Wisent Bio Products (085-150, Montreal, QC, Canada), and 1% penicillin/streptomycin, and cultured in a sterile incubator at 37 °C, 5% carbon dioxide (CO_2), and saturated humidity.

2.3 siRNA and transient transfection

The chemically synthetic siRNA (Shanghai Jima Biotechnology Company) targeting NF-κB *p65* was transfected into SKOV3 cells by Lipofectamine 2000 according to the manufacturer's instructions. The target sequence of si-*p65* as follows: Forward: 5′-CCAUCAACUAUGAUGAGUUdTdT-3′, Reverse: 3′-dGdTGGUAGUUGAUACUACUCAA-5′. A si-NC was used as the negative control, and qPCR and WB were used to evaluate the silencing efficiency of si-*p65*.

2.4 Quantitative RT-PCR

SKOV3 was inoculated with 10% fetal bovine serum containing different concentrations of rhIL-17D; the concentrations were 0 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL. After treatment with rhIL-17D for 48 hours, total RNA was extracted using Trizol Reagent (15596018, Thermo, Waltham, MA, USA). Following the manufacturer's instructions, cDNA was reverse-transcribed by the Reverse Transcription System. Then, quantitative PCR was performed with the qPCR Mix using the 7500 RT-PCR System. Real-time PCR assays were performed to quantify the mRNA levels of *p65* and PD-L1. All experiments were repeated three times. The primer sequences were as follows:

β-Actin, Forward: 5′-GTCTCCTCTGACTTCAACAGCG-3′,

Reverse: 5′-ACCACCCTGTTGCTGTAGCCAA-3′.

p65, Forward: 5′-TGAACCGAAACTCTGGCAGCTG-3′,

Reverse: 5′-CATCAGCTTGCGAAAAGGAGCC-3′.

PD-L1, Forward: 5′-TGCCGACTACAAGCGAATTACTG-3′,

Reverse: 5′-CTGCTTGTCCAGATGACTTCGG-3′.

2.5 Western blot

WB assay was used to detect the protein expression of *p65* and PD-L1 in SKOV3 cells. The total protein was extracted from ovarian cancer cells by using RIPA buffer (Solarbio, Beijing, China). The protein concentration was measured using a Bicinchoninic acid (BCA) protein quantification kit (Bioss, Beijing, China). Equal amounts of proteins were loaded and separated onto a 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Solarbio, Beijing, China) and transferred to a polyvinylidene difluoride (PVDF) membrane (Hongruite, Shijiazhuang, China). The membrane was then blocked with 5% nonfat milk for 1 h at room temperature and incubated with the corresponding primary antibodies at 4 °C overnight. Next, the membrane was incubated with homologous secondary goat anti-rabbit antibodies (KPL, USA) at RT for 1 h. The target protein bands were visualized using a G: BOX gel imaging system (Syngene, Cambridge, England).

2.6 Cell Proliferation assay

SKOV3 cells, si-*p65*-SKOV3 cells, and si-NC-SKOV3 cells were plated in 96-well plates (1×10^3 cells per well), rhIL-17D was added at a concentration of 10ng/mL. No rhIL-17D was added to the control group. Next, 20 μL of MTS reagent (Sigma, USA) was added to each well and incubated at 37 °C for 4 h. The microplate reader was used to measure the optical density (OD) value at 490 nm to measure cell proliferation.

2.7 Wound scratch assay

SKOV3 cells, si-*p65*-SKOV3 cells, and si-NC-SKOV3 cells were plated in six-well plates (5×10^5 cells/well). When the cells covered the bottom of the plate completely, wounds were scratched vertically with the tip of a suction tube. Next, the plates were washed with PBS, and 10 ng/mL rhIL-17D in serum-free medium was added with a untreated group. The spacing of cell scratches was observed at 0 h, 12 h, and 24 h

under the Nikon inverted microscope, and cell mobility was calculated. Mobility was defined as follows: (original scratch width-scratch width at a time)/original scratch width.

2.8 Transwell invasion assay

Matrigel glue (50 μL) was added to the upper chamber of a transwell plate and covered the bottom of the transwell chamber. Serum-deprived (overnight) SKOV3 cells, si-*p65*-SKOV3 cells, and si-NC-SKOV3 cells, previously treated with rhIL-17D for 48 h, were plated onto the upper chamber at a density of 1×10^5 cells/well. A complete medium was added to the lower chamber and incubated for 24 h. Next, the cells were fixed with 4% formaldehyde, followed by staining with 0.1% crystal violet solution. The migrated cells were counted in 5 randomly selected fields by an inverted microscope.

2.9 Statistical analyses

All experiments were repeated three times. The results were presented as mean $\pm$ standard deviation. This study was analyzed by SPSS 19.0 software (SPSS Inc., Chicago, IL, USA) and GraphPad Prism 5.0 software (La Jolla, CA). The differences between any two groups were calculated using an independent *t*-test. Multiple group comparisons were carried out using a one-way analysis of variance (ANOVA). A *p*-value < 0.05 was considered statistically significant.

3. Results

3.1 rhIL-17D induces the expression of p65 and PD-L1 in a dose-dependent manner

We investigated whether rhIL-17D induces the expression of *p65* and PD-L1 in SKOV3 cells. We found that *p65* and PD-L1 mRNA levels started to increase in SKOV3 cells when they were treated with 1 g/mL of rhIL-17D for 48 h, and their expression reached the highest level when the cells were treated with 10 g/mL of rhIL-17D. *p65* mRNA: 0 ng/mL *vs.* 10 ng/mL $p = 0.000054$; 0 ng/mL *vs.* 100 ng/mL $p = 0.00008$. PD-L1 mRNA: 0 ng/mL *vs.* 10 g/mL $p = 0.003$; 0 ng/mL *vs.* 100 ng/mL $p = 0.002$. (Fig. 1A–B). rhIL-17D stimulated protein expression of these two targets was also dose-dependent (Fig. 1C). Based on these results, we selected 10 ng/mL rhIL-17D as the optimum dose for the following studies.

3.2 Assessment of silencing efficiency of si-p65

We compared the mRNA expression of *p65* between si-NC-SKOV3 cells or untreated group cells and si-*p65*-SKOV3 cells and found that *p65* mRNA expression was significantly decreased compared with the si-NC-SKOV3 cells ($p = 0.023$) and the untreated group cells ($p = 0.021$) (Fig. 2A). The protein expression of *p65* was also decreased in si-*p65*-SKOV3 cells compared with the control groups (Fig. 2B).

3.3 Knockdown of *p65* down-regulates *IL-17D*-induced *p65* and PD-L1 expression

Three groups of cells were cultured with rhIL-17D (10 ng/mL) for 48 h.The expression of *p65* mRNA was down-regulated in si-*p65*-SKOV3 cells compared with si-NC-SKOV3 cells ($p = 0.003$) and untreated group cells ($p = 0.006$) (Fig. 3A). The PD-L1 mRNA expression in the si-*p65*-SKOV3 group significant and downregulation after treatment with rhIL-17D ($p = 0.012$) (Fig. 3B). Both *p65* and PD-L1 protein expressions were decreased in the si-*p65*-SKOV3 group compared with the control groups (Fig. 3C).

3.4 IL-17D promotes the proliferation of SKOV3 cells *in vitro*

We added rhIL-17D to SKOV3 cells and used the MTS assay to test the induction of proliferation of SKOV3 cells by *IL-17D*. The proliferation rate of SKOV3 cells was significantly accelerated after treatment with rhIL-17D for 48, 72 and 96 h (Fig. 4A).

3.5 IL-17D promotes the SKOV3 cell migration and invasion *in vitro*

We used the wound scratch assay to test whether *IL-17D* affects SKOV3 cell migration. rhIL-17D stimulation significantly increases the migration of SKOV3 cells (Fig. 5A). Transwell assay was used to determine the effect of *IL-17D* on ovarian cancer cell invasion ability. rhIL-17D treatment significantly increased the invasion of SKOV3 cells (Fig. 6A).

3.6 IL-17D-induces SKOV3 cells proliferation *via* the NF-κB pathway

To determine the role of the NF-κB pathway in *IL-17D* mediated SKOV3 cell proliferation, we knocked down *p65* by transfecting the SKOV3 cell line with si-RNA and then used rhIL-17D to stimulate si-*p65*-SKOV3 cells. MTS experiment showed that knocking down *p65* reduced the IL17D-iduced proliferation rate of SKOV3 cells at 48, 72 and 96 h, as compared to si-NC cells (Fig. 4B).

3.7 IL-17D-induces SKOV3 cells migration and invasion via the NF-κB pathway

The wound scratch assay showed that knocking down *p65* decreased the rhIL-17D-induced cell migration of SKOV3 cells (Fig. 5B). Compared with The si-NC-SKOV3 group, the cell invasion of si-*p65*-SKOV3 group reduced the amount of penetration through the artificial basement membrane ($p < 0.01$) (Fig. 6B). In conclusion, the NF-κB pathway was found to be implicated in the malignant biological characteristics of ovarian cancer cells induced by *IL-17D*.

4. Discussion

Ovarian cancer has been described as the “silent killer” since it is difficult to detect and is asymptomatic in its early stages. More than 70% of ovarian cancer patients are diagnosed with advanced-stage cancer, studies have shown that the 5-year

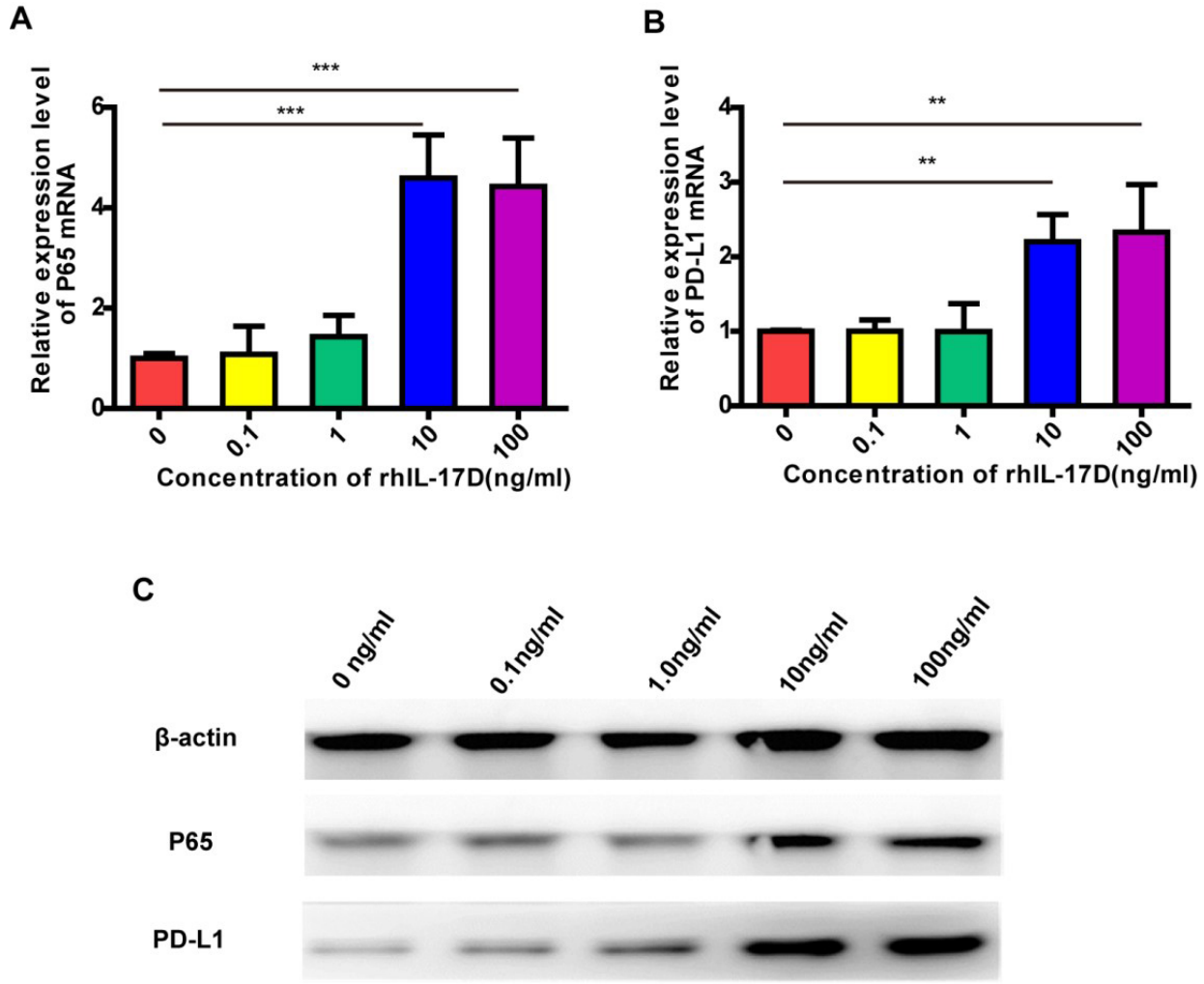

FIGURE 1. Effects of rhIL-17 on *p65* and PD-L1 expression in SKOV3 cells. (A,B) The ovarian cancer cells were treated with different concentrations of rhIL-17D for 48 hours. qRT-PCR shows that rhIL-17D treatment increases the *p65* and PD-L1 mRNA expression compared with the control treatment (0 ng/mL). $^{**}p < 0.01$, $^{***}p < 0.001$. (C) WB analyzed the *p65* and PD-L1 protein expression. The results were consistent with the mRNA expression. rhIL-17: recombinant human Interleukin-17D; PD-L1: programmed death ligand-1.

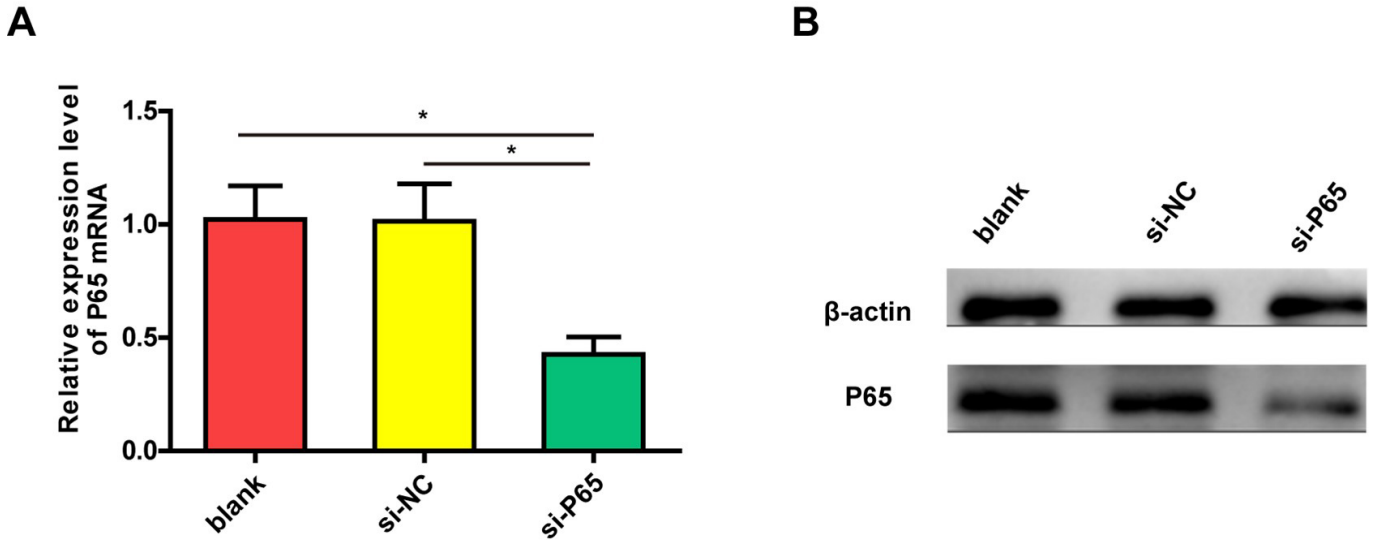

FIGURE 2. Knockdown of *p65* expression by si-RNA targeting *p65*. (A) Validation of *p65* knockdown efficiency of silencing-*p65* (si-*p65*) in SKOV3 cell lines compared with the untreated group by qRT-PCR. $^{*}p < 0.05$. (B) Validation of *p65* knockdown efficiency of si-*p65* in SKOV3 cell lines by Western blot. si-NC: silencing-negative control.

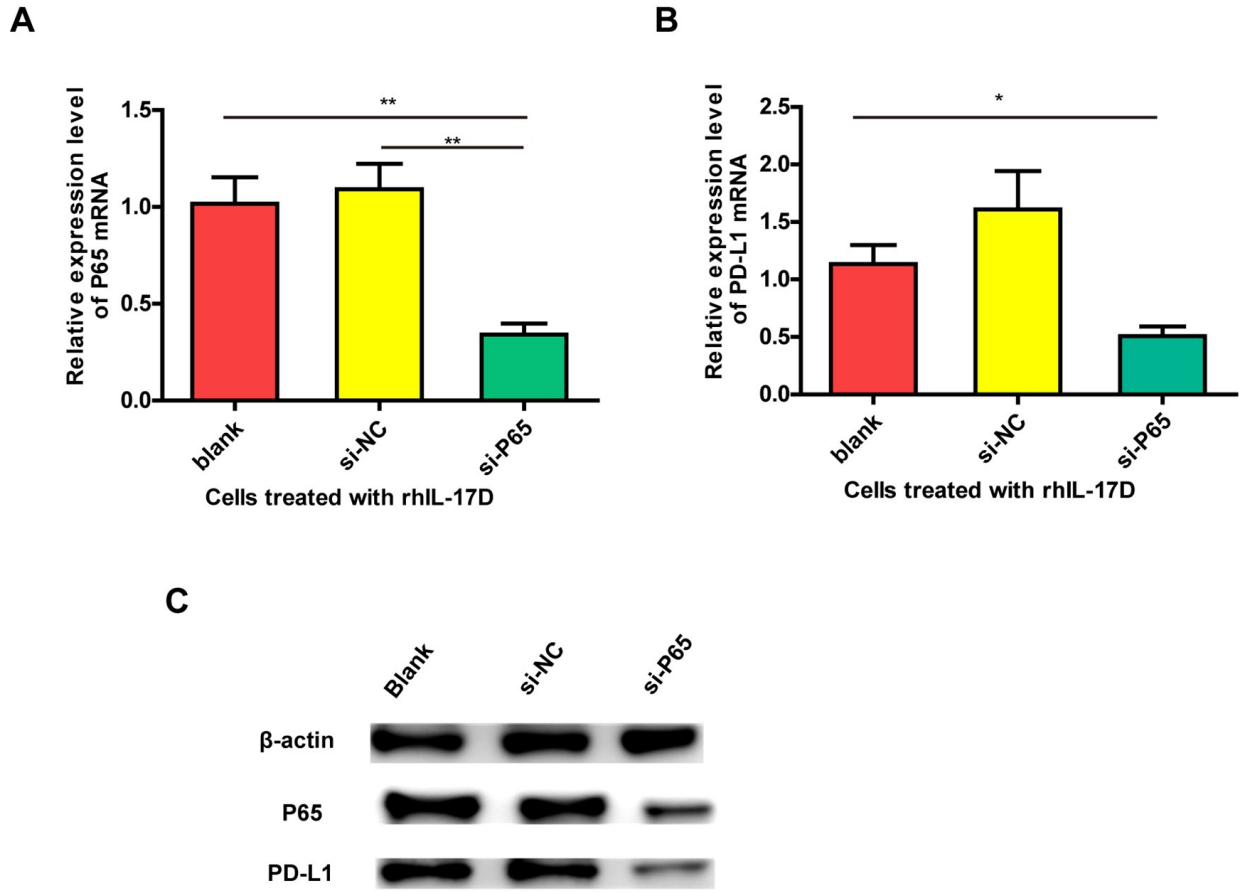

FIGURE 3. *IL-17D* did not induce up-regulation of *p65* and PD-L1 in si-*p65*-SKOV3 cells. (A,B) qRT-PCR assays measured the mRNA expression of *p65* and programmed death ligand-1 (PD-L1) in the following three groups: si-*p65*-SKOV3 group, si-NC-SKOV3 control group, and the untreated group. All groups were treated with recombinant human Interleukin-17D (rhIL-17) (10 ng/mL). $*p < 0.05$, $**p < 0.01$. (C) *p65* and PD-L1 protein expression were detected in the three groups by WB. rhIL-17: recombinant human Interleukin-17D; PD-L1: programmed death ligand-1; si-*p65:*silencing-*p65*; si-NC: silencing-negative control.

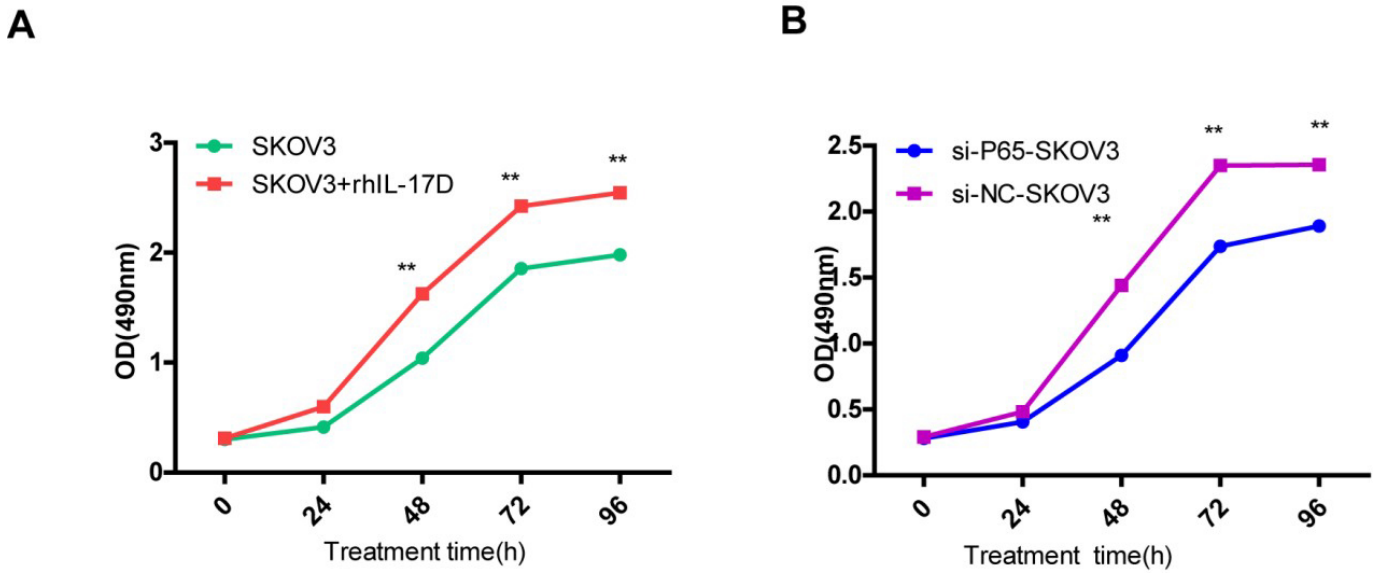

FIGURE 4. Knockdown of *p65* inhibits the rhIL-17D-induced SKOV3 cell proliferation *in vitro*. (A) MTS assay: rhIL-17D increased the proliferation of SKOV3 cells. (B) The decrease of *p65* expression affected rhIL-17D-mediated (10 ng/mL) cell proliferation of si-*p65*-SKOV3 cells compared with si-NC-SKOV3 cells. $**p < 0.01$. OD: optical density. rhIL-17: recombinant human Interleukin-17D; PD-L1: programmed death ligand-1; si-*p65*: silencing-*p65*; si-NC: silencing-negative control.

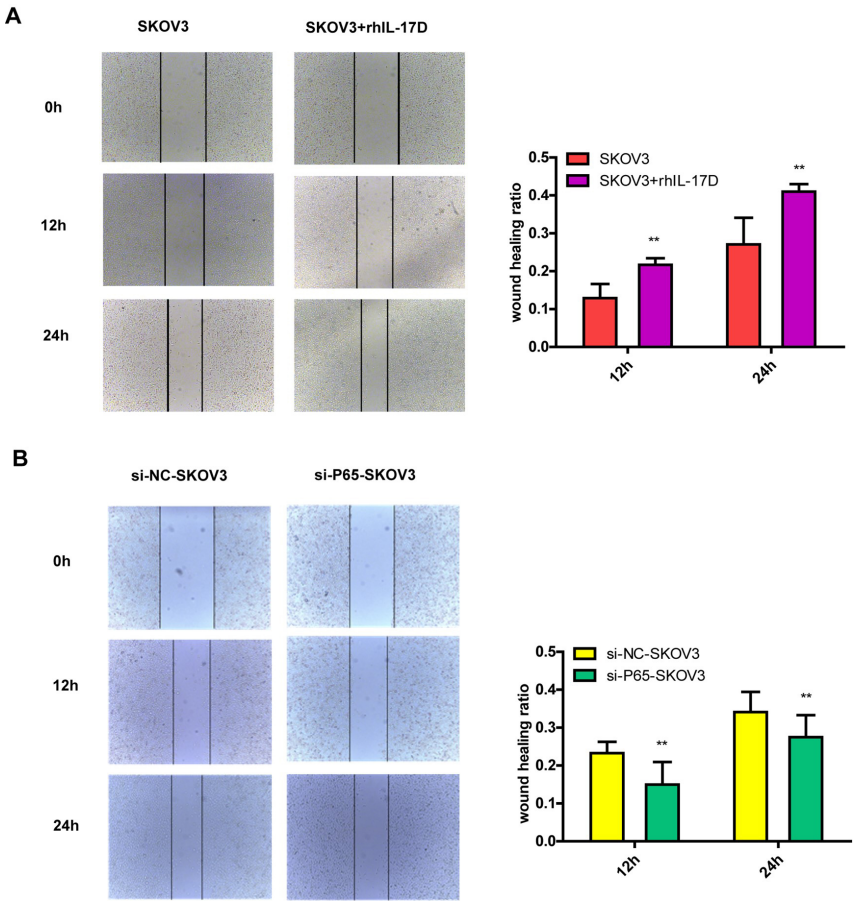

FIGURE 5. Wound scratch assay, *p65*-knockdown reduced SKOV3 cell migration induced by rhIL-17D *in vitro*. (A) The role of rhIL-17D in cell migration.$^{**}p < 0.01$. (B) The role of the NF-κB pathway in cell migration induced by *IL-17D*. Mobility was defined as follows: (original scratch width − scratch width at a time)/original scratch width. (200×), $^{**}p < 0.01$. rhIL-17: recombinant human Interleukin-17D; si-*p65*: silencing-*p65*; si-NC: silencing-negative control.

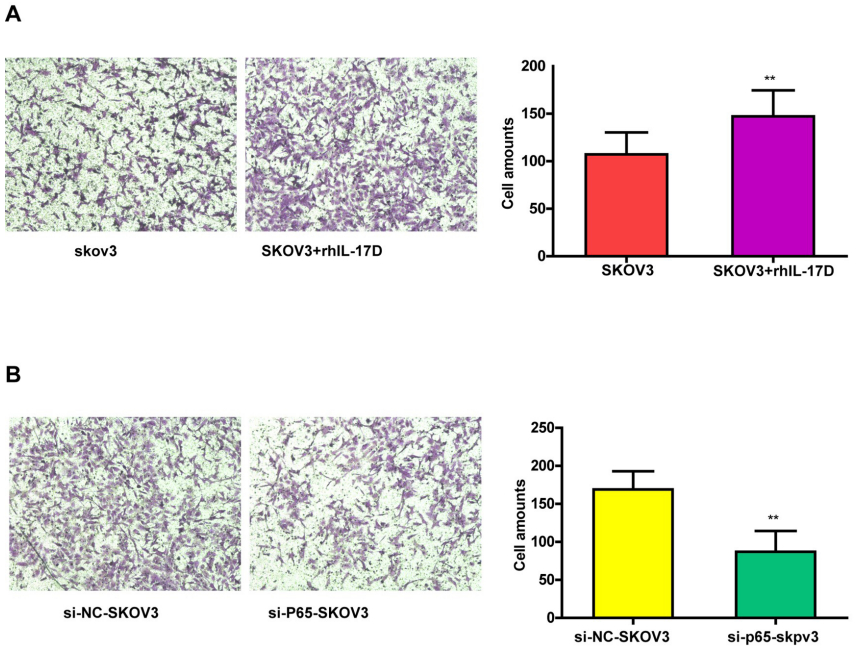

FIGURE 6. Transwell invasion assays (200×), knockdown of *p65* inhibits the SKOV3 cell invasion induced by rhIL-17D *in vitro*. (A) The role of rhIL-17D in cell invasion, $^{**}p < 0.01$. (B) The role of NF-κB pathway in cell invasion induced by *IL-17D*. $^{**}p < 0.01$. rhIL-17: recombinant human Interleukin-17D; si-*p65*: silencing-*p65*; si-NC: silencing-negative control.

survival rate for ovarian cancer is 47.4% [18]. Extensive metastasis and chemo-resistance contribute to the poor survival rate in ovarian cancer patients. Recently, new drugs, including bevacizumab and PARP inhibitors have somewhat improved the prognosis of ovarian cancer patients, but most patients still experience cancer recurrence and eventually die due to drug resistance. Future treatment strategies for ovarian cancer include the combination of traditional standard therapies, targeted therapy, and immunotherapy.

The risk factors for ovarian cancer include reproductive, obesity, smoking, lifestyle and environmental, family history, genetic, and chronic pelvic inflammation [19]. Inflammation is a marker of cancer [20], and chronic inflammation promotes tumor progression, metastasis, and drug resistance by triggering immunosuppression and inhibiting anti-tumor immunity through inflammatory cells, cytokines, chemokines, and growth factors in the tumor micro-environment. However, the exact mechanism remains unclear.

IL-17 family is an important inflammatory cytokine family and mediates innate and adaptive immunity. The IL-17 family was comprised of six cytokines (IL-17A to IL-17F). The IL-17 recruits multifunctional signaling proteins such as Act1 to the IL-17R; Act1 then recruits and ubiquitinates TRAF and activates various downstream pathways, such as NF-κB and MAPK, to activate the target gene expression [21]. The IL-17 family also is involved in the progression of malignant disease, inflammation, and immune responses [15, 22, 23].

IL-17D was added much later to the IL-17 family and rarely studied. *IL-17D* was reported to exhibit an anti-tumor function by recruiting NK cells to the tumor microenvironment, polarizing M1 macrophages, and resisting tumor development in murine models [24]. Another study also confirmed the host protection of *IL-17D* in tumor cells; mice deficient in *IL-17D* had a higher incidence of tumor compared with wild-type (WT) mice [25]. On the other hand, *IL-17D* promotes the secretion of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF) in endothelial cells under stress [26], indirectly regulating the immune response of the host and promoting tumor progression.

We previously showed that the *IL-17D* expression was up-regulated in ovarian cancer patients, especially in advanced disease, compared with benign ovarian tumor patients [27], suggesting that *IL-17D* may help in tumor progression. In this study, we tested the effect of rhIL-17D on the biological characteristics of ovarian cancer cells. The results confirmed the tumor-promoting effect of *IL-17D*. We found that *IL-17D* accelerated the proliferation, cell motility, and the cell invasiveness of human ovrian cancer SKOV3 cells. The cell proliferation rate reflects the ability of tumor progression, and cell motility or cell invasiveness are necessary for tumor metastasis; the enhancements of these abilities by *IL-17D* confirmed its tumor-promoting role in ovarian cancer.

PD-L1 (known as B7-H1) encoded by the cluster of differentiation 274 (CD274) gene. PD-L1 is a type I transmembrane protein and belongs to the B7 family and is expressed in a variety of cancers [28]. Combined with PD-1, PD-L1 inhibits the tumor-cytotoxicity of T cells and mediates immune escape of tumor cells leading to immunosuppression in the TME and a poor prognosis of cancer. PD-1/PD-L1 inhibitors have been recommended for many advanced malignant tumors, but the overall response rate still less than 40% [29]. Therefore, the regulation of PD-L1 expression requires further investigation. Pro-inflammatory factors in the tumor micro-environment, such as tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ), lipopolysaccharide (LPS), GM-CSF, and interleukins [30–36] promote PD-L1 expression. IL-17A synergistically induces PD-L1 expression with IFN-γ and TNF-α in Mesenchymal Stem Cells (MSCs) *via* the accumulation of nitric oxide [37]. Wang *et al*. [38] reported that IL-25 promotes PD-L1 expression by activating the JNK and STAT3 pathways in hMSCs. Another study demonstrated that IL-17 and TNF-α up-regulate PD-L1 expression in prostate cancer cells, which was induced by the NF-κB signaling pathway [14]. In this study, we used qRT-PCR and WB to analyze PD-L1 expression in the SKOV3 cell line. We found that *IL-17D* up-regulates the expression of PD-L1 in SKOV3 cells.

NF-κB is a critical transcription factor widely expressed by almost all cells. The NF-κB family contains five transcription proteins: p50, p52, *p65*, RelB and cRel. The classical NF-κB pathway includes the activation of p50, RelA (*p65*), and c-Rel [39]. NF-κB dysregulation has been implicated in several diseases, including cancer, chronic inflammation, and autoimmune diseases. The up-regulation of NF-κB has been reported in many malignant tumors, such as renal cancer, pancreatic cancer, gastric cancer, colorectal cancer, and ovarian cancer [40–44]. NF-κB pathway mediates cell proliferation and regulates the development of ovarian cancer and chemo-resistance [45, 46].

In the present study, the effects of the NF-κB pathway on *IL-17D*-mediated PD-L1 expression and tumor promotion were explored. We found that rhIL-17D promotes *p65* mRNA and protein expression. We also showed that NF-κB activation regulates cell proliferation, migration, and invasion in SKOV3 cells. Knockdown of *p65* inhibited the effect of rhIL-17D on cell proliferation, cell invasion, and migration in SKOV3 cells. Neither *p65* nor PD-L1 was induced by rhIL-17D in the si-*p65*-SKOV3 cell line. We hypothesize that *IL-17D* induces PD-L1 expression through the activation of the NF-κB pathway, and PD-L1 inhibits the host anti-tumor immunity, leading to the immune escape.

5. Conclusions

In conclusion, we found that *IL-17D* facilitates cell invasion and metastatic abilities of ovarian cancer cells and promotes PD-L1 expression, possibly, *via* NF-κB activation.

IL-17D may be critically involved in the immune regulation of the ovarian cancer microenvironment and may be a potential immunotherapeutic target in ovarian cancer.

AVAILABILITY OF DATA AND MATERIALS

The data are contained within this article.

AUTHOR CONTRIBUTIONS

HZ—contributed to conception and design of the study. YCF, LF, YPL and SHL—performed experimental procedures and collected the data. HZ and YCF—analyzed the experimental data. All authors participated in the writing and final approval of the manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study design was approved by the Ethics Committee of the Fourth Hospital of Hebei Medical University (IACUC-4thHos Hebmu-2021007).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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