

## Research Article

# Astragalus Polysaccharides Inhibit Pancreatic Cancer Progression by Downregulation of TLR4/NF- $\kappa$ B Signaling Pathway

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**Background.** Pancreatic cancer (PC) leads to high human malignancy mortality worldwide. This study explored the role of Astragalus polysaccharide (APS) on human PC PANC-1 cells and its underlying mechanisms. **Method.** Cell viability, proliferation, apoptosis, invasion, and migration were measured by CCK-8, EdU incorporation, flow cytometry, Transwell, and wound healing assay, respectively. ELISA assay was utilized to detect the IL-1 $\alpha$ , IL-4, IL-6, IL-8, and TNF- $\alpha$  levels. The western blot assay was performed to measure the level changes of cell function-related proteins. The transportation of NF- $\kappa$ B P65 protein was detected through immunofluorescence assay. **Results.** Compared with the control group, APS treatment could significantly inhibit cell proliferation. APS treatment could also suppress cell migration and invasion ability and induce apoptosis and inflammation in PANC-1 cells. Furthermore, APS inhibited the activation of TLR4/NF- $\kappa$ B signaling pathway via suppressing the phosphorylation and transportation of NF- $\kappa$ B P65 into the PANC-1 cell nucleus. **Conclusion.** APS suppresses PANC-1 cell viability, proliferation, migration, and invasion while inducing inflammation and apoptosis. APS might regulate PC cell motility via downregulating TLR4/NF- $\kappa$ B signaling pathway.

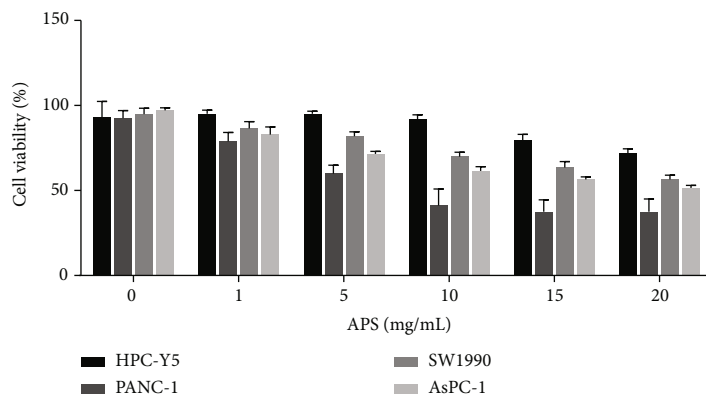
## 1. Introduction

Pancreatic cancer (PC) has the characteristic of rapid progression, insidious onset, and low survival rate [1], thus resulting in high human malignancies mortality worldwide [2]. Nowadays, the survival rate of PC has been reduced with the development of surgical removal and target therapy [3]. However, the PC survival rate has a minor progression since PC metastasis often develops dramatically, and surgical removal cannot cure [4]. The prognosis of PC remains poor since around 50% of the PC patient recurrence in the following year after their first surgery [5]. Therefore, a specific and effective therapeutic agent is required to improve the prognosis of PC patients.

Astragalus polysaccharides (APS) is isolated from the root of *A. membranaceus* and serves as a traditional medicine. Many recent studies indicated that APS promotes various bioactivities including immunomodulation, anti-inflammation, and anticancer [6]. Furthermore, APS pos-

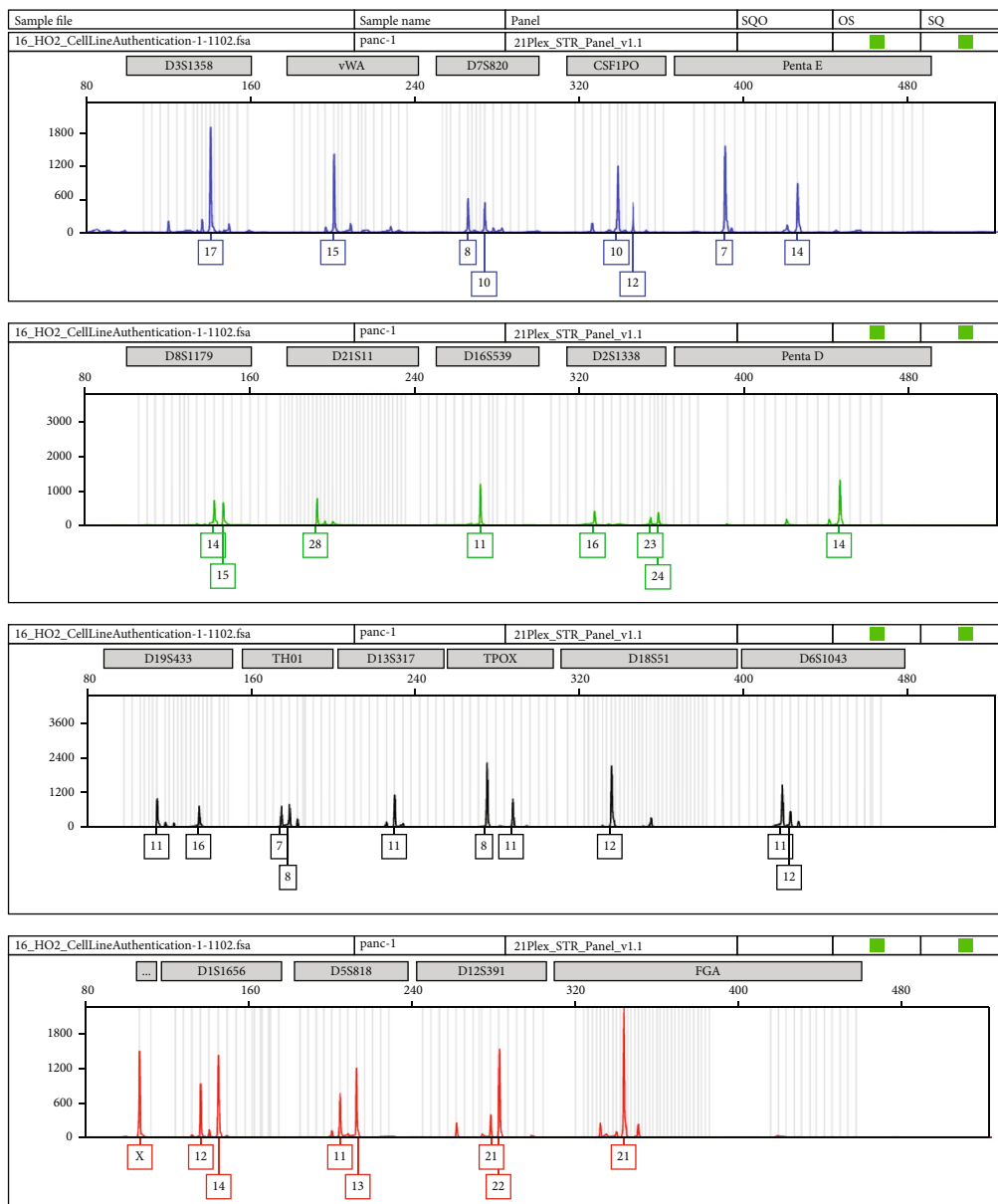
sesses immunomodulatory properties and promotes the production of antibodies [7]. The early stages of obstructive pancreatitis apoptosis results in cell death of pancreatic acinar cells [8]. The apoptosis process is implemented through multiple pathways and intersected and has three major biological pathways, including the death receptor, the mitochondrial, and the endoplasmic reticulum pathway. The cascade reaction is the central link for both the death receptor pathway and the mitochondrial pathway, in which caspase-3 acts as the ultimate executor of apoptosis. Recent studies reported that APS could reduce cell viability and promote cell apoptosis, indicating a strong anticancer effect of APS treatment [9, 10]. However, the role and underlying mechanisms of APS in PC still need more research.

In this study, we aimed to investigate the roles of APS in PC cells' viability, proliferation, migration, invasion, inflammation, and apoptosis and its association with the TLR4/NF- $\kappa$ B signaling pathway.



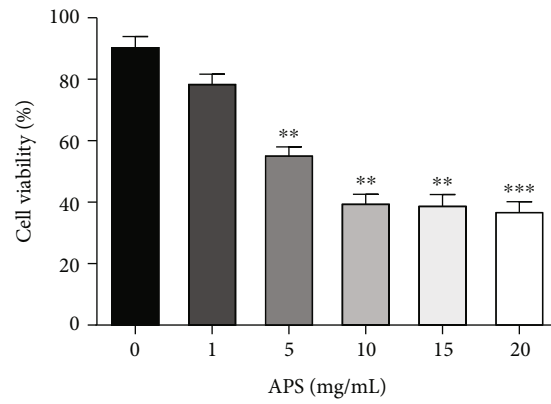
(a)

PANC-1

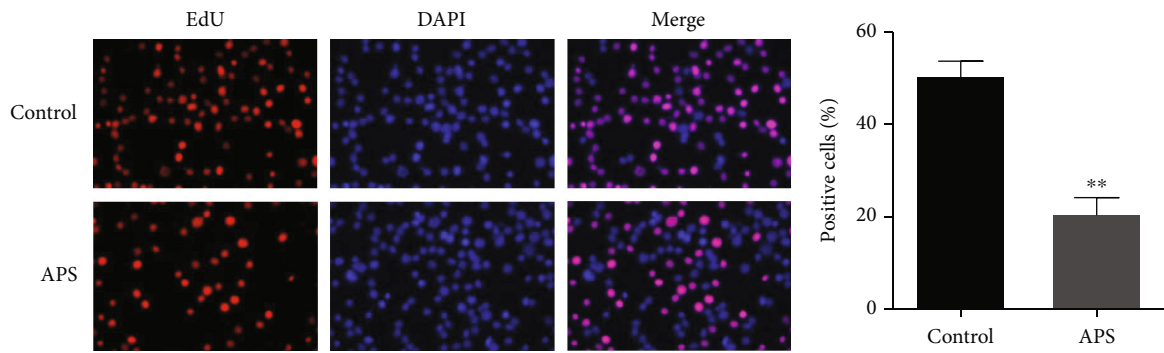


(b)

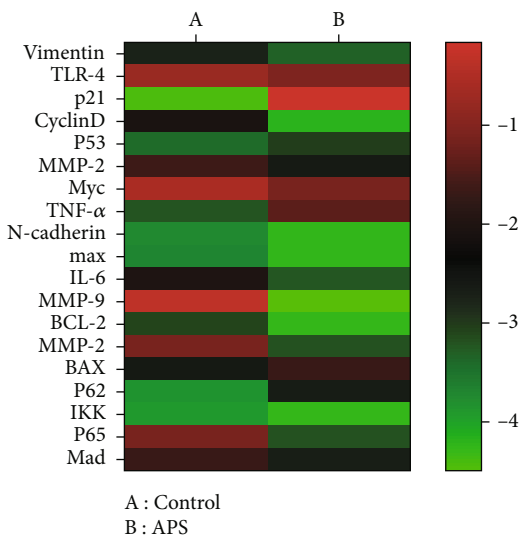
FIGURE 1: Continued.



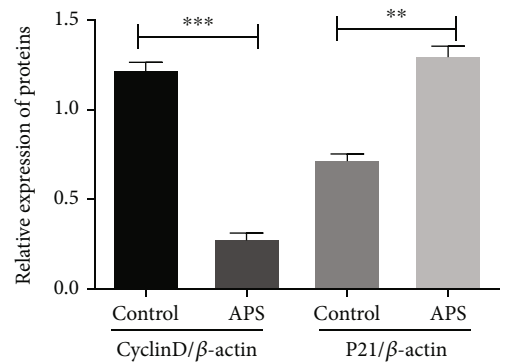
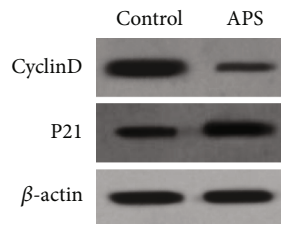
(c)



(d)



(e)



(f)

FIGURE 1: Continued.

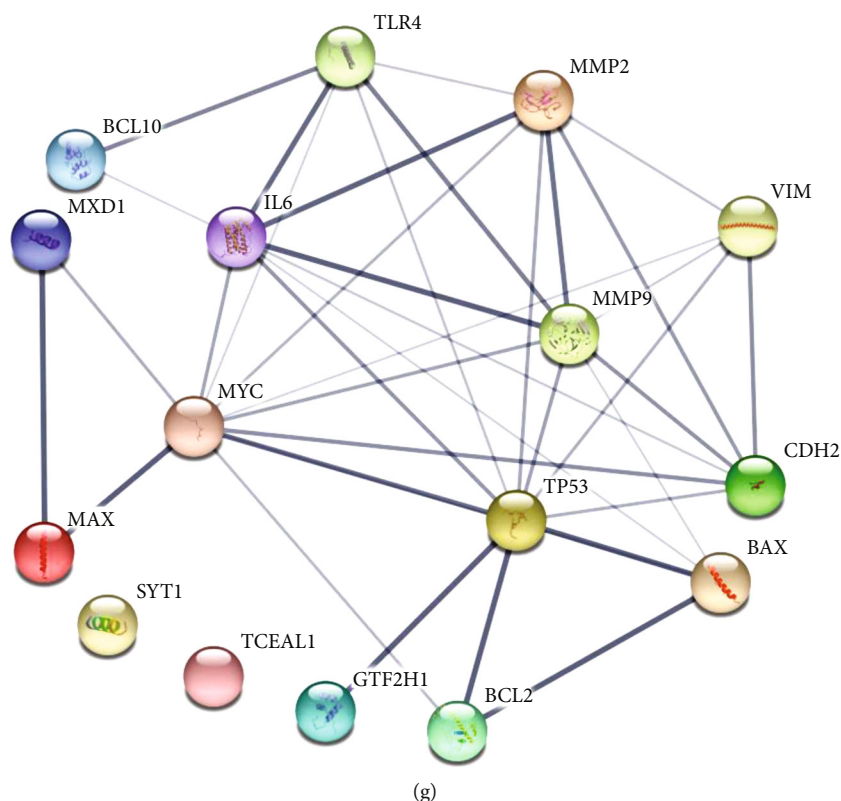


FIGURE 1: APS inhibited viability and proliferation of PANC-1 cells. (a) CCK-8 assay was used to detect the effects of Astragalus polysaccharides (APS) on cell viability and proliferation in human normal pancreatic cell line HPC-Y5 and pancreatic cancer cell lines including PANC-1, SW1990, and AsPC-1. Various doses of APS (0, 1, 5, 10, 15, and 20 mg/mL) were treated to the cells and cultured for 24 h. (b) PANC-1 cell line STR identification. (c) CCK-8 assay was applied to explore the cell viability of different concentrations (0 (control), 1, 5, 10, 15, and 20 mg/mL) of APS treatment in PANC-1 cells. (d) EdU assay was used to examine the proliferation of APS on PANC-1 cells. (e) RNA-Seq analysis was used to detect the differentially expressed genes in PANC-1 cells treated with APS. (f) Western blot assay was performed to detect the expression of proliferation related proteins. (g) Protein interaction network of differentially expressed genes after APS treatment. The thicker the line, the stronger the interaction. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , compared with the control group. Scale bar = 100  $\mu\text{m}$ .

## 2. Material and Methods

**2.1. Cell Culture.** The pancreatic cancer cell line PANC-1 was obtained from Shanghai Fuheng Biotechnology (China). The PANC-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) containing 10% fetal bovine serum (FBS) (Gibco, USA) and 1% penicillin-streptomycin (Gibco, USA). The cells were cultured in a humidified incubator (Thermo Fisher, USA) with 5%  $\text{CO}_2$  at 37°C.

**2.2. Cell Viability Assay.** The PANC-1 cells were seeded in the 96-well plates ( $2 \times 10^3$  cells/well). Then, 200  $\mu\text{L}$  normal culture medium containing various doses of APS (0, 1, 5, 10, 15, and 20 mg/mL) was added to PANC-1 cells for 24 h [11]. Then, 10  $\mu\text{L}$  of CCK-8 (Dojindo, Japan) was added, and the cells were cultured for another 1 h. Subsequently, a microplate reader (Bio-Rad, USA) was used to detect the absorbance at the wavelength of 450 nm.

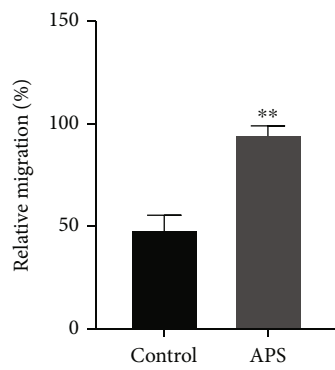
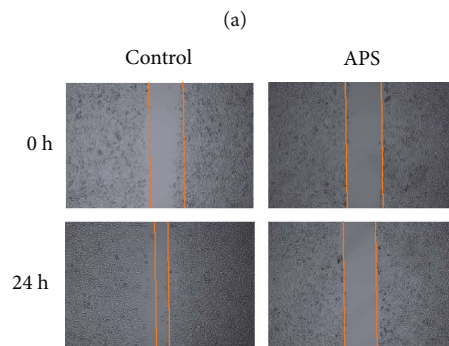
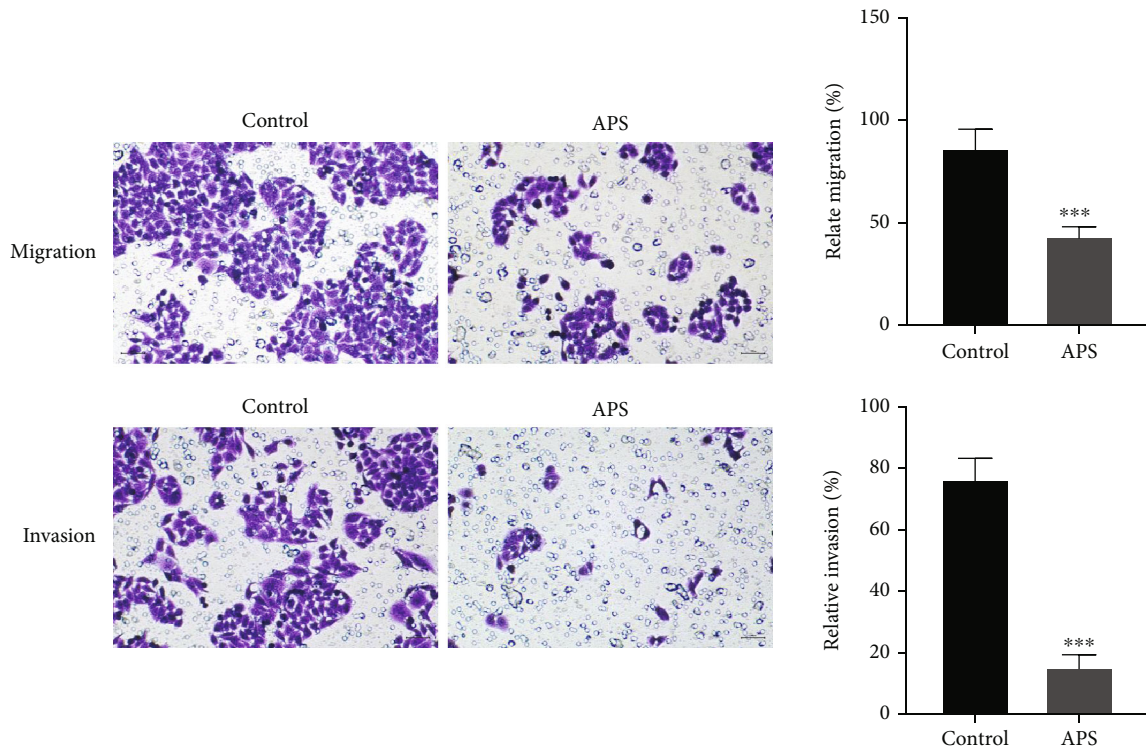
**2.3. Cell Proliferation Assay.** 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay (Beyotime Biotechnology, China)

was utilized to examine cell proliferation. The cells were seeded and treated according to the instruction. The staining was captured by a fluorescence microscope.

**2.4. Migration and Invasion Assay.** To measure the cell migration ability, PANC-1 cells were cultured in the top chamber; the normal medium was added into the lower compartment. The plates were cultured in a humidified incubator for 24 h. After fixing, the cell was stained in crystal violet. To detect the cell invasion ability, the Transwell membranes were precoated with Matrigel (BD Biosciences, USA).

**2.5. Wound Healing Assay.** After seeding cells for 24 h, a pipette tip was utilized to scratch three parallel lines on the bottom of plates, and cell debris was rinsed with DMEM. After the corresponding treatment for 24 h, a light microscope was used to assess the ability of the cells to migrate into a cell-free area.

**2.6. Flow Cytometric Analysis of Cell Apoptosis.** The annexin V-FITC/PI double-stained kit (Invitrogen, USA) was utilized



(b)

FIGURE 2: Continued.

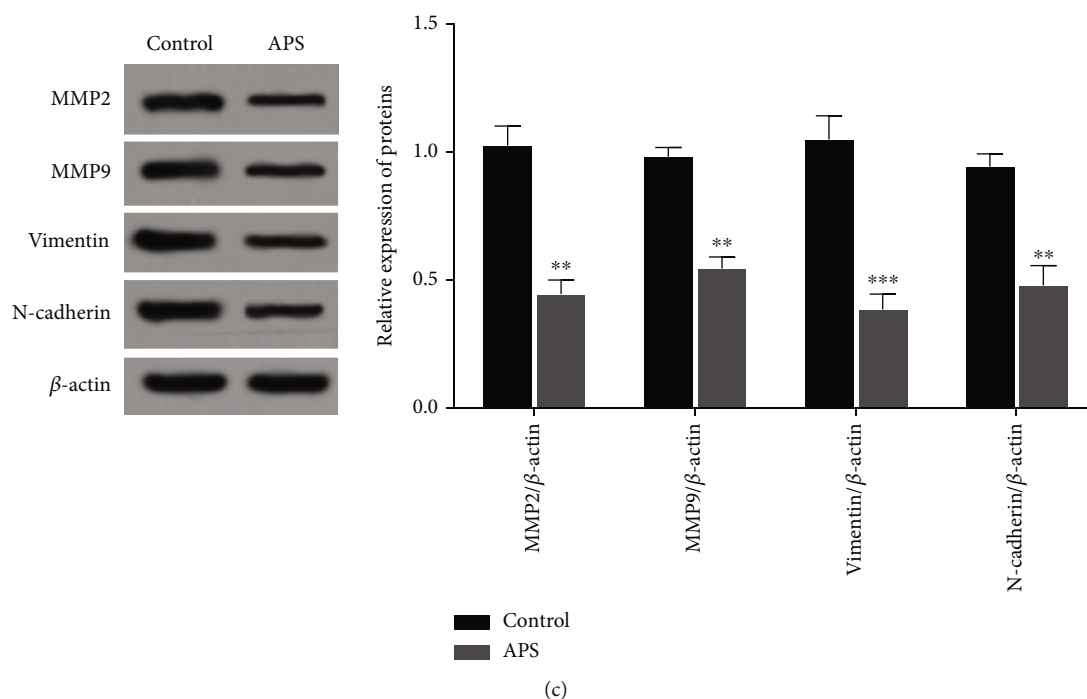


FIGURE 2: APS inhibited migration and invasion of PANC-1 cells. (a) Transwell experiment was conducted to study the effects of APS on cell migration and invasion. (b) Wound healing assay was used to study the effects of APS on cell migration. (c) Western blot assay was used to measure the protein levels of migration- and invasion-related proteins including MMP2, MMP9, vimentin, and N-cadherin. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , compared with the control group.

to detect cell apoptosis. After resuspending in binding buffer, the cells were stained with the fluorescent dye for 15 min at temperature without light. The treated cells were detected through flow cytometry. FlowJo software (Tree Star, USA) was utilized to analyze the portion of apoptotic cells.

**2.7. ELISA Analysis.** IL-1 $\alpha$ , IL-4, IL-6, IL-8, and TNF- $\alpha$  were detected using enzyme-linked immunosorbent assay kits (Boster Biological Technology, China) at a wavelength of 405 nm.

**2.8. Real-Time Quantitative PCR.** TRIzol reagent was used to extract the total cell RNA (Thermo Fisher, USA). After synthesizing cDNA, real-time PCR was performed with the SYBR Green master mix (Takara, Japan).

**2.9. Western Blot.** RIPA (Thermo Fisher, USA) was utilized to extract total cell proteins. After running a 12% SDS-PAGE electrophoresis (Beyotime Biotechnology, China), the proteins were transferred to a PVDF membrane (Millipore, USA). The primary antibodies against CyclinD1 (1:1000, ab134175, Abcam, UK), P21 (1:1000, ab109520, Abcam, UK), MMP2 (1:2000, ab92536, Abcam, UK), MMP9 (1:2000, ab76003, Abcam, UK), vimentin (1:1000, ab92547, Abcam, UK), N-cadherin (1:1000, AB76001, Abcam, UK), Bcl-2 (1:1000, ab196495, Abcam, UK), Bax (1:2000, ab182733, Abcam, UK), P62 (1:2000, ab109012, Abcam, UK), TLR4 (1:2000, ab13556, Abcam, UK), Myd88 (1:2000, ab133739, Abcam, UK), phosphorylated NF- $\kappa$ B P65 (p-P65, 1:1000, ab76302, Abcam, UK), P65 (1:2000, ab32536, Abcam, UK), and  $\beta$ -actin (1:3000,

ab8226, Abcam, UK) were used. After being cultured with the corresponding second antibodies (Beyotime Biotechnology, China), the signal of the protein band was detected in a ChemiDoc XRS<sup>+</sup> system (Bio-Rad, Berkeley, CA, USA).

**2.10. Immunofluorescence (IF) Experiment.** The TLR4/NF- $\kappa$ B signaling pathway agonist, human tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), was purchased from the Cell Signaling Technology Company (USA). 24 h before the experiment, PANC-1 cells were paved with Cellvis board (Shanghai Kavason Biotech, no. D29-20-1 N, China). Once the cell density reached 80%, the corresponding drugs, TNF- $\alpha$  and APS, were treated for 4 h, followed by 24 h cell culture after the liquid change. The cells were then fixed at room temperature with 4% polyformaldehyde for 30 min, followed by washing with PBS three times, 10 min at a time. The cells were then incubated at 0.1% Triton-100 (Beyotime Biotechnology, China) at room temperature for 30 min. PBS (Solarbio, China) was added to wash the cells three times, 10 min each time. An antidilution solution of p65 antibodies (1:200) was added and incubated throughout the night. The antibody was collected, and the cells were washed three times by PBS. After 1 h incubation with goat anti-rabbit fluorescent secondary antibody (Invitrogen, China) at room temperature at the concentration of 1:200, the cells were washed three times with PBS, 15 min each. The distribution of p65 was then observed under the Leica fluorescence microscope (Germany).

**2.11. Statistical Analysis.** All quantitative data were expressed as mean  $\pm$  standard deviation (SD). SPSS 17.0

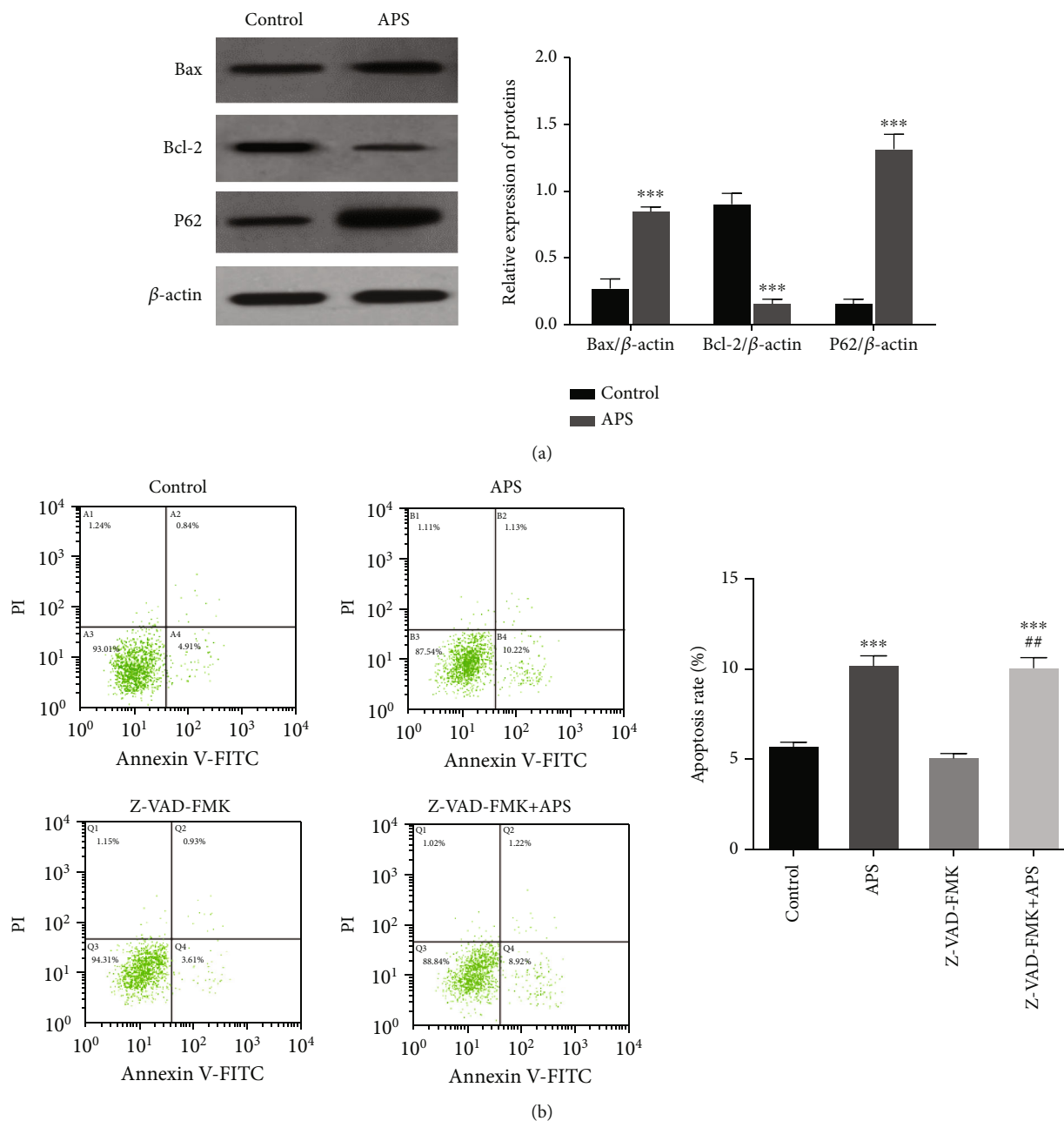


FIGURE 3: APS induced the apoptosis of PANC-1 cells. (a) Western blot assay was used to detect the apoptosis-related protein expression including Bax, Bcl-2, and P62. (b) Flow cytometry assay was performed to detect cell apoptosis rate. \*\*\* $P < 0.001$ , compared with the control group; ## $P < 0.01$ , compared with the Z-VAD-FMK group.

software (SPSS, USA) was used to analyze the data. One-way analysis of variance (ANOVA) was applied for the significant differences between the groups by Tukey's multiple range test. The  $P < 0.05$  was considered statistically significant.

### 3. Results

**3.1. APS Inhibited Viability and Proliferation of PANC-1 Cells.** As shown in Figure 1(a), we chose several PC cell lines including PANC-1, SW1990, and AsPC-1 and human normal pancreatic cell line HPC-Y5 to detect the cytotoxicity

of APS treatment during the preexperiments. We found that PANC-1 cells were the most sensitive cell line; thus, we used PANC-1 cells in the following formal experiment. The PANC-1 cell line verification was shown in Figure 1(b). In Figure 1(c), APS treatment inhibited cell viability and proliferation in a dose-dependent manner (all  $P < 0.05$ ). In the following APS treatment experiments, 10 mg/mL APS was selected. Compared with the control group, the EdU assay showed that APS treatment significantly suppressed cell proliferation ( $P < 0.05$ , Figure 1(d)). As shown in Figure 1(e), the intracellular transcription factors CyclinD, p21, p65, and MMP-2 changed significantly after APS treatment. As

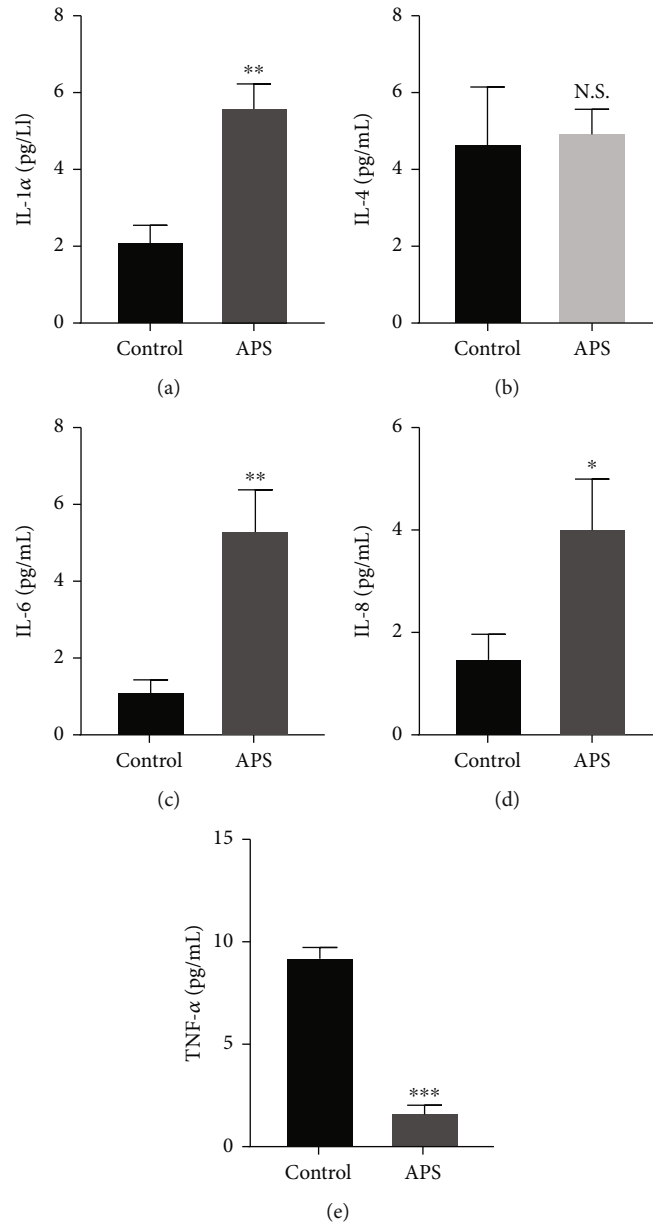


FIGURE 4: Effects of APS on inflammatory cytokines expression in PANC-1 cells. (a–e) The expression levels of IL-1 $\alpha$ , IL-4, IL-6, IL-8, and TNF- $\alpha$  were measured by ELISA assay. \* $P < 0.05$ , \*\* $P < 0.01$ , and \*\*\* $P < 0.001$ , compared with the control group.

shown in Figure 1(f), western blot assay illustrated that APS treatment could decrease the protein levels of CyclinD1 ( $P < 0.05$ ) and increase the P21 protein expression ( $P < 0.05$ ). These results suggested that APS inhibited viability and proliferation of PANC-1 cells. Additionally, we also constructed the protein interaction network of APS downstream genes. According to the number of interacting genes, IL-6, Myc, TP53, and MMP9 interact most widely and are at the core of the network (Figure 1(g)). These genes may be the key link of APS downstream mechanism.

**3.2. APS Suppressed Migration and Invasion of PANC-1 Cells.** In Figure 2(a), the migration and invasion of cells were significantly inhibited after APS treatment ( $P < 0.05$ ). Besides, wound healing assay showed consistent results

( $P < 0.05$ , Figure 2(b)). We also detected the migration- and invasion-related proteins including MMP-2, MMP-9, vimentin, and N-cadherin. In Figure 2(c), APS treatment significantly decreased the protein levels of MMP-2, MMP-9, vimentin, and N-cadherin (all  $P < 0.05$ ), which indicated that APS could suppress migration and invasion in PANC-1 cells.

**3.3. APS Induced Apoptosis of PANC-1 Cells.** We measure the protein levels of apoptosis-related proteins including Bax, Bcl-2, and P62. As shown in Figure 3(a), APS treatment obviously downregulated the expression levels of Bcl-2 ( $P < 0.05$ ) and upregulated the levels of Bax and P62 (all  $P < 0.05$ ). Furthermore, as shown in Figure 3(b), compared with the control group, cell apoptosis rate in the APS group



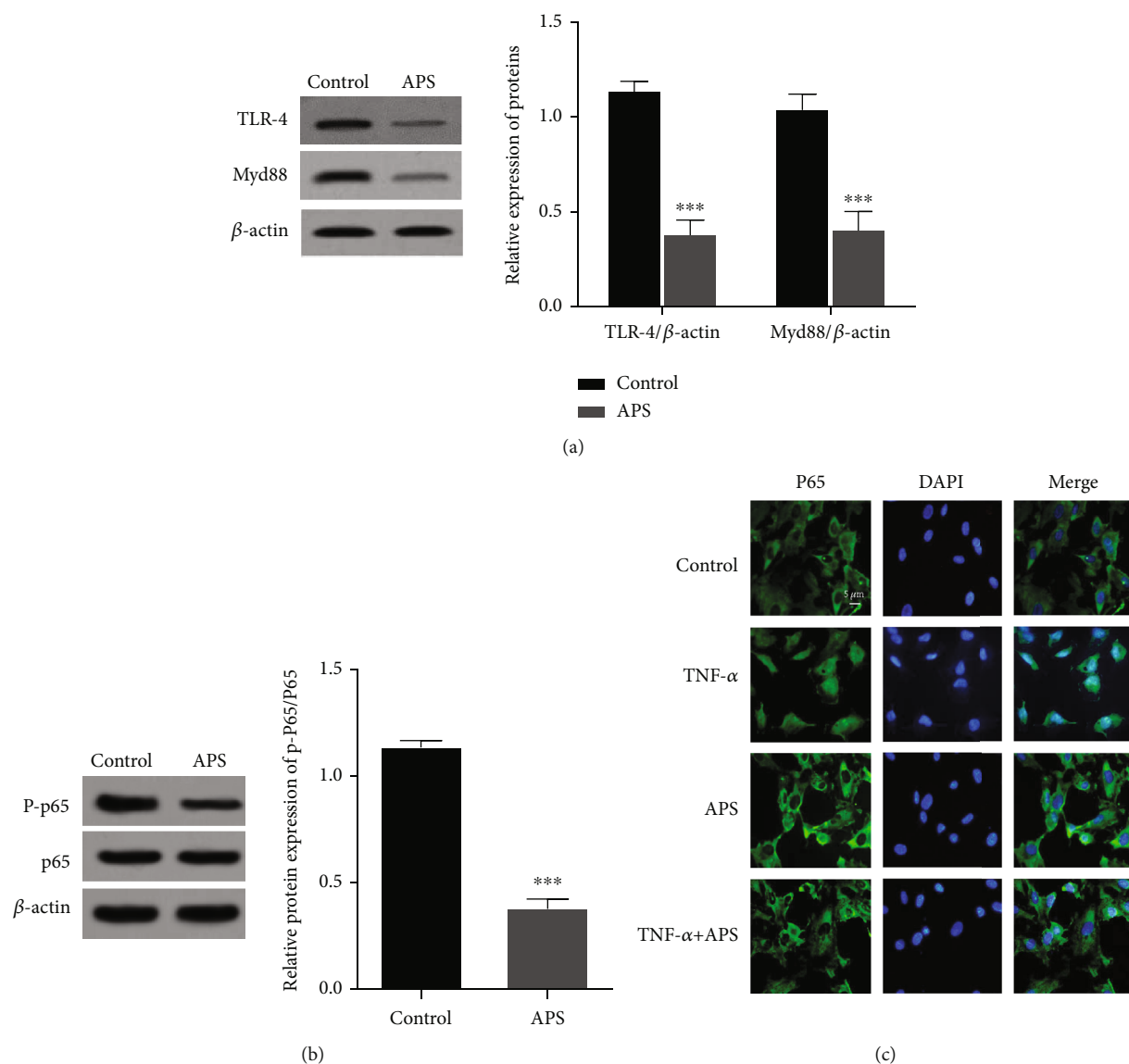


FIGURE 5: APS suppressed the activation of TLR4/NF- $\kappa$ B signaling pathway in PANC-1 cells. (a and b) The protein expression of TLR4, Myd88, nuclear phosphorylated p65 (P-p65), and p65 was measured by western blot assay. (c) The distribution of p65 was detected by IF experiment. \*\*\* $P < 0.001$ , compared with the control group. Scale bar = 5  $\mu$ m.

was significantly higher ( $P < 0.05$ ). Meanwhile, after treating cells with apoptosis inhibitors, Z-VAD-FMK, we found that APS could still induce apoptosis, but its induction efficiency was inhibited ( $P < 0.05$ ). The above results suggested that APS could induce PANC-1 cell apoptosis.

**3.4. APS Induced Inflammation in PANC-1 Cells.** To explore the roles of APS in the PANC-1 cell inflammatory injury, we examined the effects of APS on the concentrations of IL-1 $\alpha$ , IL-4, IL-6, IL-8, and TNF- $\alpha$  using ELISA kit in PANC-1 cells. As shown in Figures 4(a)–4(e), we found that compared to the control group, APS significantly increased the levels of IL-1 $\alpha$ , IL-6, and IL-8 (all  $P < 0.05$ ). However, we found an inverse change of TNF- $\alpha$  ( $P < 0.05$ ) and did not observe any significant change of IL-4 in both groups. These results suggested that APS could induce PANC-1 cells inflammatory injury.

**3.5. APS Suppressed TLR4/NF- $\kappa$ B Signaling Pathway in PANC-1 Cells.** To study the underlying mechanism of APS on cell motility, we further detected the effects of APS on TLR4/NF- $\kappa$ B signaling pathway in PANC-1 cells. In Figures 5(a) and 5(b), we found that APS decreased the protein levels of TLR4, Myd88, and phosphorylation p65 (all  $P < 0.05$ ), which suggested that APS suppressed the activation of the TLR4/NF- $\kappa$ B pathway. In Figure 5(c), IF experiments showed that the transcription factor P65 could be activated and enter the nucleus, thus enhancing the expression of downstream target genes in the presence of TLR4/NF- $\kappa$ B signaling pathway agonist TNF- $\alpha$ . When both TNF- $\alpha$  and APS were presented, p65 remained in the cytoplasm and could not activate the TLR4/NF- $\kappa$ B signaling pathway (Figure 5(b)). Besides, we also found that APS downregulated the levels of TNF- $\alpha$ , which could further inhibit the

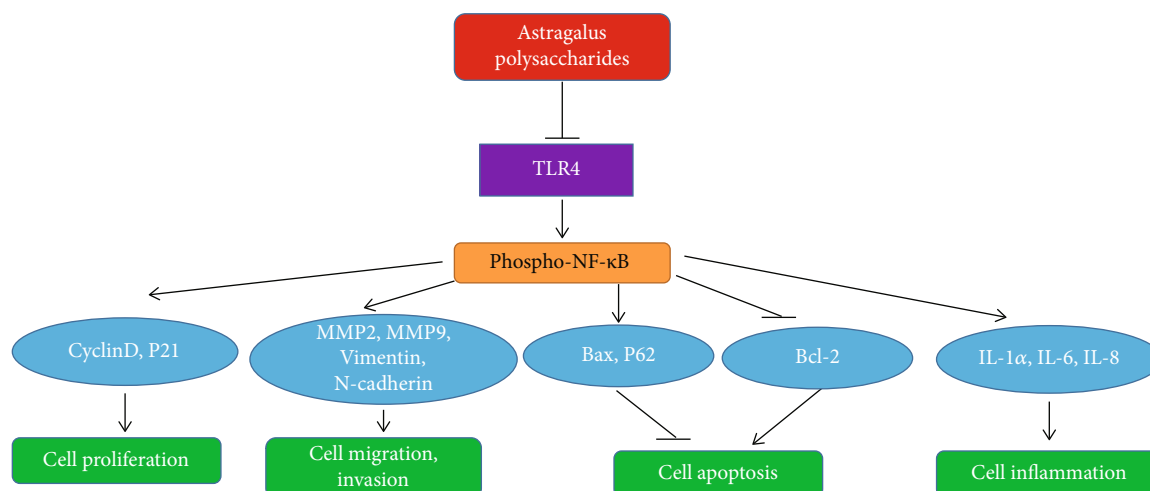


FIGURE 6: Schematic diagram of signaling pathway.

transportation of p65 and then inhibit the activation of TLR4/NF- $\kappa$ B signaling pathway. These results suggested that APS suppressed the activation of TLR4/NF- $\kappa$ B signaling pathway in PANC-1 cells.

#### 4. Discussion

Characteristics of tumor cells promote proliferation and suppress apoptosis in the occurrence and development of cancer [12–14]. In this study, the decrease in EdU-positive portion in APS-treated group suggested that APS could inhibit PANC-1 cell proliferation. CyclinD1 and P21 are the key cell cycle proteins that promote mitosis [15, 16]. We found that APS treatment could downregulate the protein levels of CyclinD1 and P21. Furthermore, the results of flow cytometry and ELISA assay suggested the proapoptotic and proinflammatory roles of APS. After using antiapoptotic agent Z-VAD-FMK (anticaspase), we found that APS could still induce apoptosis, but its induction efficiency was inhibited. The above results suggested that APS could induce apoptosis via caspase-dependent pathways.

The metastatic potential and metastasis of PC usually cause adverse outcomes [17, 18]. However, APS on invasion and migration of PANC-1 cells was unexplored [19]. It is known that extracellular matrix (ECM) contributes to EMT since cancer cells begin to invade and migrate [20, 21] by first degrading ECM. MMP-2 and MMP-9 can damage ECM and basement membrane [22]. In this study, APS inhibited the migration and invasion of PANC-1 cells and reduced MMP-2 expression in PANC-1 cells. Besides, APS also downregulated the migration- and invasion-related proteins including vimentin and N-cadherin protein levels, which was consistent with the results of MMPs.

TLR is a key receptor to initiate inflammation process. Some studies have found that TLR4 can activate SIRS. TLR4 plays a vital role in the pathophysiology and severity of acute biliary pancreatitis [23]. However, whether TLR4 is related to human PC and the role it plays in PC is not clear. TLR is a specific product of innate immune system that recognizes

invading pathogenic microorganisms and destroys tissue or cell degradation products and reacts to them. TLR4 is a member of the TLR family [24, 25]. In addition to the identification of specific product bacteria/viral pathogen-associated molecular patterns (PAMP), TLR4 also can identify the damage tissue or cell matrix after degradation product of small molecules, such as heat shock protein 60, 70, Gp96, polysaccharide hyaluronic acid low molecular fragments, fibrinogen, and HMGB1 [26]. Activation of TLR4 signaling pathways will activate an important regulatory factor, NF- $\kappa$ B, to activate inflammatory response genes and ultimately lead to the synthesis and secretion of IL-6 and IL-8 in inflammatory cells [27–29]. Furthermore, TLR4 had a suppressive effect in the regulatory T cells via activating the NF- $\kappa$ B pathway [30]. This study showed that APS treatment could upregulate the levels of IL-1 $\alpha$ , IL-6, and IL-8, which suggested that APS might induce inflammation in PC cells. However, we did not observe significant change of IL-4, and the levels of TNF- $\alpha$  were even decreased. Based on bioinformatics analysis, TLR4 may be involved in the APS downstream signaling, and we found that APS decreased the expression of TLR4 and nuclear p-65, suggesting that APS suppressed the NF- $\kappa$ B signaling pathway. If experiment showed that APS treatment inhibited the transportation of p65 into cell nucleus. The downregulation of TNF- $\alpha$  could enhance this inhibitory effect as an agonist of the TLR4/NF- $\kappa$ B signaling pathway. The above results suggested that APS could regulate the suppressed activation of TLR4/NF- $\kappa$ B pathway in PANC-1 cells (Figure 6).

There are some limitations in our study. At first, we did not use multiple cell lines in our experiments. However, we have verified that APS treatments could significantly induce cell damage in some PC cell lines including PANC-1, SW1990, and AsPC-1 and human normal pancreatic cell line HPC-Y5. Among those cells, PANC-1 cells were the most sensitive. Thus, we used this cell lines to perform the following experiments. Besides, although we found that APS could inhibited cancer cell functions and promote apoptosis and inflammation through TLR4/NF- $\kappa$ B pathway, the underlying mechanism and related functional consequences still need further investigation in animal models.

To summarize, we found that APS showed a suppressive activity in PC cells via decreasing cell viability, proliferation, invasion, and migration and inducing cell apoptosis and inflammation in PANC-1 cells. Furthermore, APS could inhibit the activation of TLR4/NF- $\kappa$ B signaling pathway through the p65 phosphorylation and transportation into cell nucleus. This study suggested a cell experiment basis for developing the clinical application of APS-related therapy for PC patients.

## Data Availability

The data used to support the findings of this study are included within the article.

## Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

## Acknowledgments

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