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Astragalus Polysaccharide Mediates Immunomodulatory Effects on Crosstalk between Human Peripheral Blood Mononuclear Cells and Ovarian Cancer Cell Line

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ABSTRACT

Astragalus polysaccharide (APS) is a functional component of *Astragalus membranaceus* with antitumor and immunomodulatory properties. This study evaluated the effect of APS on the peripheral blood mononuclear cell (PBMC) proliferation, cytokine secretion, and regulatory T cell (Treg) induction in an in vitro coculture model of human PBMCs and A2780 human ovarian cancer cells.

PBMC proliferation and Treg frequency were measured by flow cytometry. Cytokine levels were assessed by enzyme-linked immunosorbent assay.

APS significantly enhanced the PBMC proliferation, reduced Treg frequency, decreased anti-inflammatory cytokines including interleukin [IL]-10, transforming growth factor beta (TGF-β), and vascular endothelial growth factor-A (VEGF-A), and increased the pro-inflammatory cytokine IL-6.

These findings suggest that APS may be an effective immunomodulatory supplement for cancer therapy, particularly for ovarian cancer by enhancing antitumor immune responses.

Keywords: *Astragalus* polysaccharides; Inflammatory cytokines; Ovarian cancer; Regulatory T cells, Tumor microenvironment

INTRODUCTION

Ovarian cancer (OC) is one of the most common cancers in women. About 21,750 new cases of OC were diagnosed, and 13,940 women died of OC in the United States in 2020. OC is usually diagnosed in advanced stages because early stages are typically symptom-free.[1]

Although surgery, chemotherapy, and radiotherapy are some of the widely used treatment options for cancer patients, but they have limited efficacy side effects.[2] Therefore, there is a need for effective strategies to enhance these therapies and reduce their toxicity. Various traditional medicinal herbs including Curcumin, Ginseng, and Ginger have been proposed as complementary therapies as they contain a wide variety of immunomodulatory components. Phytochemicals such as polysaccharides have been reported to be responsible for the plants' immunomodulatory properties.[3]

Astragalus propinquus (syn. *Astragalus membranaceus*, Fisch), commonly known as Huangqi in Chinese, is one of the 50 fundamental herbs used in traditional Chinese medicine for various disorders.

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A membranaceus contains 14 kinds of polysaccharides and 161 saponins, also known as astragalosides. Among them, *Astragalus* polysaccharides (APSs) have anti-inflammatory, immunomodulatory, and anticancer characteristics.[4-6]

Various studies have reported that APS could inhibit tumor growth in H22 hepatocarcinoma mouse models,[7] suppress innate and acquired immune responses,[5] stimulate the release of different cytokines,[8] and increase natural killer (NK) cell activity.[9]

In this study, we investigated the effects of APS on the peripheral blood mononuclear cells (PBMC) proliferation, cytokine secretion, and regulatory T cell (Treg) induction that were examined in a coculture model of PBMCs and a human OC cell line (A2780).

MATERIALS AND METHODS

Reagents

The A2780 human OC cell line (ATCC#C461) was purchased from Pasteur Institute of Iran (Tehran, Iran). The Roswell Park Memorial Institute Medium 1640 (RPMI 1640), penicillin/streptomycin, fetal bovine serum (FBS), and trypsin were purchased from Gibco, USA. Propidium iodide (PI) was obtained from Sigma; the (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl bromide (MTT) dye was obtained from Sigma-Aldrich, Germany. Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific, LE, UK. The ELISA kit (BD Biosciences, San Diego, CA, USA) was purchased for the cytokine assay. Carboxyfluorescein succinimidyl ester (CFSE) dye was purchased from eBioscience (USA) and stored at −20°C in dark bottles at a final concentration of 5 mM.

Preparation of APS Reagent

The water extract of *A membranaceus* root was purchased from NOW Foods (Bloomingdale, Illinois, USA). The root extract contained flavonoids, saponins, and polysaccharides with a purity of at least 70%. The main components of *Astragalus* polysaccharides have a molecular weight of 10 to 50 kDa and consist of heteropolysaccharide, dextran, acidic polysaccharide glucose, arabinose, and other monosaccharides.[9,11] APS reagent was prepared by dissolving the content of each APS capsule (containing 500 mg of root extract) in 100 mL of distilled phosphate-buffered saline (PBS). It was incubated at 4°C overnight. After centrifuging at 3000*g* for 20 minutes, homogenized supernatants were collected to generate the last stock solution of 5 mg/mL for in vitro experiments. The stock solution was filtered through a 0.22-µm filter and kept at −20°C. Final dilutions were prepared using a complete medium (1000, 500, 250, 125, 60, 30, and 15 µg/mL).

MTT Cytotoxicity Assay

The cytotoxicity of the APS on the PBMCs and A2780 cells was measured by the MTT assay. PBMCs and A2780 cells (15×10^4 and 1×10^4 cells/well, respectively) were seeded in the 96-well plates. Then, different concentrations of APS (1000, 500, 250, 125, 60, 30, and 15 µg/mL) were added to each well and incubated for 24, 48, 72, and 96 hours at 37°C. Two sets of 3 wells, with and without treatment with 5 µg/mL of phytohemagglutinin (PHA), were considered positive and negative controls, respectively. Next, 20 µL of the MTT solution at 5 mg/mL was added to each well, and the plate was incubated for 4 hours in darkness at 37°C. Then, by adding 100 µL DMSO to each well, absorbance was measured at 570 nm. The concentration of APS that showed no toxicity on PBMCs was used to assess the proliferation of PBMCs.

Proliferation Assay of PBMCs

To evaluate whether APS could affect the proliferation of PBMCs, the CFSE-labeled PBMCs were cultured in the presence of various concentrations of APS. First, PBMCs from 10 healthy donors were isolated via Ficoll-Hypaque density gradient centrifugation (Lymphodex, Germany). Freshly isolated PBMCs (1×10^6 cells/well) were incubated with 5 µM CFSE for 5 minutes at 37°C in dark. After completing the process by adding PBS containing 5% FBS, the PBMCs were seeded in 24-well plates at a density of 5×10^5 cells/well, with or withour the various concentrations of APS. The proliferation of PBMCs was evaluated on day 4 using flow cytometry (BD FACS caliber, USA).

Coculture of PBMCs and A2780 Cells

The A2780 cells were cultured at 2.5×10^5 cells in each well in the complete medium at 37°C for 24 hours. First, to assess the effect of A2780 on the proliferation of PBMCs, CFSE-labeled PBMCs (1×10^6 cells/well) were cocultured with A2780 cells at different ratios (1:1, 1:5, 1:10) without treatment for 3 days. Then, to evaluate the effect of APS on PBMC proliferation, CFS-labeled PBMCs (1×10^6 cells per well) were cocultured with

A2780 cells at a 1:5 ratio (based on previous results), and APS (1000, 500, 250, 125, 60, 30, and 15 µg/mL) was added. It was then incubated for 3 days at 37°C. PHA-treated PBMCs and untreated PBMCs served as positive and negative controls, respectively. After 3 days, PBMCs were stained with PI, and the proliferation of PBMCs was examined by flow cytometry.

Identification of Induced Treg Frequency

The effect of APS on Treg frequency was evaluated in 4 different settings. First, freshly isolated PBMCs were stained for CD4, CD25, and CD127 markers with a panel of monoclonal antibodies (Table 1).

Second, to determine the optimal PBMC:A2780 ratio for coculture, PBMCs were cocultured with A2780 at several ratios (1:10, 1:50, and 1:100) without any treatment for 3, 7, and 8 days. Then, the induction of Tregs was assessed by flow cytometry. According to the significantly higher frequency of Treg induction after 7 days, this coculture time was chosen for this stage. The experiment was also repeated after 8 days of coculture, but cell death started to occur when they were cocultured for more than 7 days. As a result, the optimum number of days for coculture at this stage was set at 7 days.

In the third setting, freshly isolated PBMCs were cultured in 24-well plates in the absence of APS or in the presence of 15 µg/mL APS for 7 days. This concentration was selected according to the proliferation of PBMCs.

In the fourth setting, freshly isolated PBMCs (1×10^6 cells/well) were cocultured with A2780 cells at a 1:100 ratio (significantly higher frequency of Treg induction was seen in this ratio) without any treatment as a negative control or with 1000 µg/mL APS. Based on the results of proliferation in the previous stage, a concentration of 1000 µg/mL APS was designated for coculturing of PBMCs and A2780 cells.

On day 3, half of the medium was replaced with the fresh medium containing 1000 µg/mL APS, and the coculture continued for an additional 4 days. On day 7, PBMCs were stained for surface markers CD4, CD25, and CD127,and the frequency of $CD4^+CD25^+CD127^{low/-}$ T cells was assessed using flow cytometry (BD FACS caliber, USA).

Table 1. Conjugated monoclonal antibodies that were used for flow cytometry

Antibody	Flourochrome	Clone	Manufacturer	Cat#
Anti-human CD4	FITC	RPA-T4	BD Biosciences	555346
Anti-human CD25	APC	M-A251	BD Biosciences	555434
Anti-human CD127	PE	HIL-7R-M21	BD Biosciences	557938
Mouse IgG1κ, Isotype Control	FITC	MOPC-21	BD Biosciences	556649
Mouse IgG1κ, Isotype Control	APC	MOPC-21	BD Bioscience	550854
Mouse IgG1κ, Isotype Control	PE	MOPC-21	BD Bioscience	551436

Cytokine Concentration Assay

The levels of TGF-β, IL-10, IL-6, and VEGF-A inculture supernatants were determined by ELISA. PBMCs (1×10^5 cells/well) were cocultured with A2780 cells (5×10^5 cells/well) treated in the presence or absence of 1000 µg/mL of APS or PHA (1% v/v). In addition, PBMCs and A2780 cells were cultured in separate wells and treated with 15 and 1000 µg/mL of APS (for PBMCs and A2780 cells, respectively) as additional controls. The supernatants were collected after 72 hours of incubation, and analyzed for cytokine concentrations.

Statistical Analysis

The Mann-Whitney U test was used to compare the differences between datasets. A p value of <0.05 was considered statistically significant. All statistical

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analyses were performed using GraphPad Prism software (version 8.0.2; San Diego, CA, USA).

RESULTS

The Impact of APS on PBMCs and A2780 Cell Viability

As shown in Figure 1, the cell viability of PBMCs was significantly improved following treatment with 15 µg/ml APS for 96 hours (p<0.01). PBMCs treated with APS at 15 and 60 µg/mL for 24 hours showed a cell viability of 190% and 144%, respectively (Figure 1A), compared with the control group (n=3; p<0.01 and p<0.05, respectively).

As shown in Figure 1B, the cell viability of A2780 cells significantly dropped after treatment with 1000 µg/mL of APS for 24 and 48 hours (n=3; p<0.01 and p<0.05, respectively). The APS concentration ranging from 15 to 1000 µg/mL was selected to investigate the ability of APS to stimulate the proliferation of CFSE--labeled PBMCs since none of the APS concentrations exerted cytotoxic effects on PBMCs.

The Proliferation of PBMCs by APS

To evaluate whether APS could affect the proliferation of PBMCs, the CFSE-labeled PBMCs were cultured in the presence of different concentrations of APS. As shown in Figures 2A and 2B, treatment of PBMCs with 15 µg/mL of APS inccreased their proliferation significantly compared to the control group (PBMCs were cultured alone in a complete medium) (n=4, p<0.001). However, increasing the concentration of APS (30 to 1000 µg/mL), did not significantly increase the proliferation of PBMCs(p<0.05).

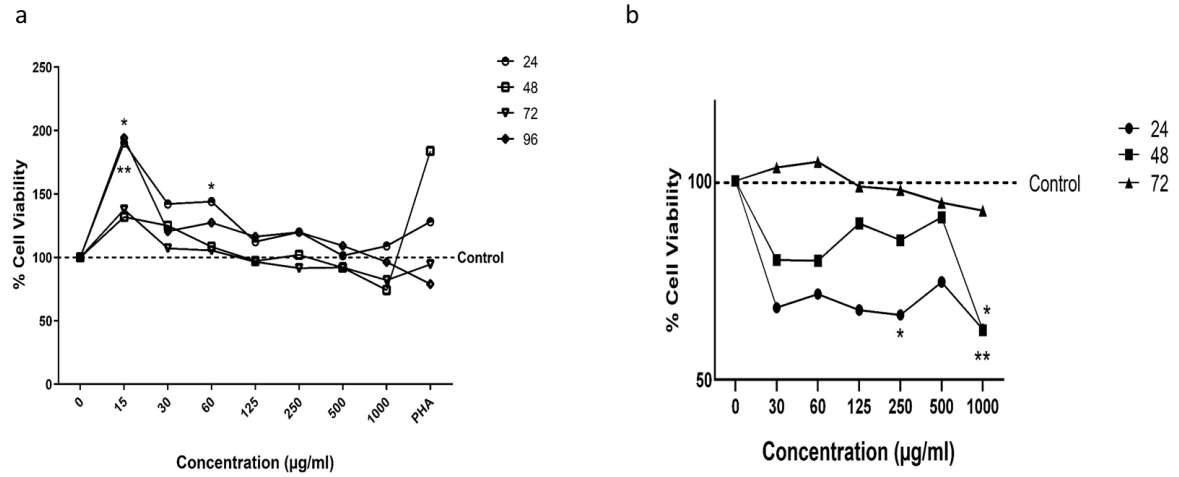

Figure 1. MTT assay was used to analyze cell viability.
Effects of different concentrations of APS on cytotoxicity of A) PBMCs B) A2780 cells and the percentage of viable cells were illustrated. The results were expressed as mean±SEM and then analyzed through Mann-Whitney U test.
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide; APS: Astragalus polysaccharides; PBMCs: peripheral blood mononuclear cells. *p<0.05 and **p<0.01

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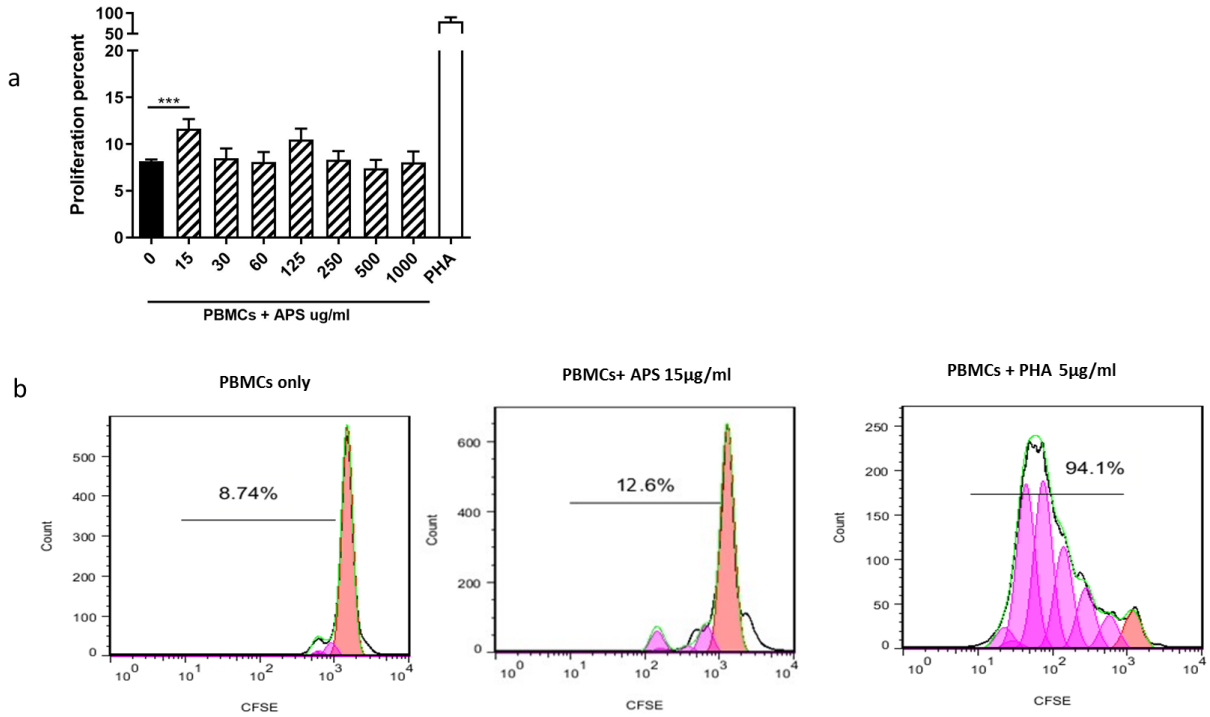

Figure 2. The proliferation of PBMCs with and without APS treatment. CFSE-labeled PBMCs were cultured alone or with different concentrations of APS. Lymphocytes were recognized by forward and side scatter, and the proliferation of CFSE-labeled cells were calculated. APS: Astragalus polysaccharides; PBMCs: peripheral blood mononuclear cells; CFSE: carboxyfluorescein succinimidyl ester; PHA: polyhydroxyalkanoates. ***p<0.001

Effect of APS on the Proliferation of PBMCs Cocultured with A2780 Cells

To evaluate whether APS could exert a proliferation effect on PBMCs cocultured with the A2780 cell line, the impact of A2780 cells on the proliferation of PBMCs was initially determined. Figure 3A shows that A2780 cells significantly affected the proliferation of PBMCs at a ratio of 1:5 (A2780:PBMCs) (n=4, p<0.05). Then, to examine whether APS impacted the proliferation of PBMCs cocultured with A2780 cells, APS-treated PBMCs were cocultured with A2780 cells. Figures 3B and 3C show that no significant difference was seen when PBMCs were cocultured with A2780 cells and treated with 15 to 1000 µg/mL of APS for 4 days (n=4, p>0.05). Although PBMCs cocultured with A2780 cells and treated with 1000 µg/mL of APS showed a higher proliferation rate compared to those cocultured with A2780 cells without any treatment, this difference did not reach a significant level (n=4, p>0.05).

Immunomodulatory Effect of APS on Treg Induction

To assess the immunomodulatory effect of APS on Treg induction, PBMCs were treated with 15 µg/mL of APS in the absence of A2780 cells for 7 days. In addition, the $CD4^+CD25^+CD127^{low/-}$ Treg frequency of healthy donors in the population of PBMCs was analyzed (Day 0). Figures 4A and 4C show a fall in the induction of Tregs when PBMCs are treated with 15 µg/mL (n=3, p<0.05). PBMCs were then cocultured with A2780 cells in a contact-dependent manner to evaluate the effect of A2780 cells on the induction of Tregs. In the coculture with the ratio of 1:100 (A2780:PBMC), a significantly higher frequency of Treg cells was observed compared to those without coculturing (n=3, p<0.05; Figures 4B and 4C). Next, PBMCs were cocultured with A2780 cells and treated with 1000 µg/mL of APS, and Treg induction was examined. No significant decrease in the induction of $CD4^+CD25^+CD127^{low/-}$ Treg cells was observed when PBMCs were cocultured with A2780 cells and treated with 1000 µg/mL APS. (n=3, p>0.05 Figure 4C).

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Impact of APS on Cytokine Secretion

The alterations in cytokine production by PBMCs cocultured with A2780 cells were measured with and without APS treatment. As shown in Figure 5, the level of IL-6 was significantly higher when PBMCs were cocultured with A2780 and treated with 1000 µg/mL APS (p<0.05, Figure 5B). However, the concentrations of IL-10 (p< 0.05, Figure 5A), VEGF-A (p<0.05, Figure 5D), and TGF-β (p<0.05, Figure 5C) were lower in the same condition. In addition, the concentration of IL-6 increased after PBMCs were treated with 15µg/ml APS compared to the control group (p<0.05). On the other hand, there was no difference in the concentration of IL-10 and TFG-β in a similar condition (p>0.05). After 72 hours of exposure to APS, A2780 cells secreted fewer TGF-β, VEGF-A, and IL-6 levels than untreated A2780 cells (p>0.05, p<0.05, and p<0.05, respectively). The current survey suggests that APS may have immunomodulatory activity by changing the secretion of cytokines in the tumor microenvironment (TME).

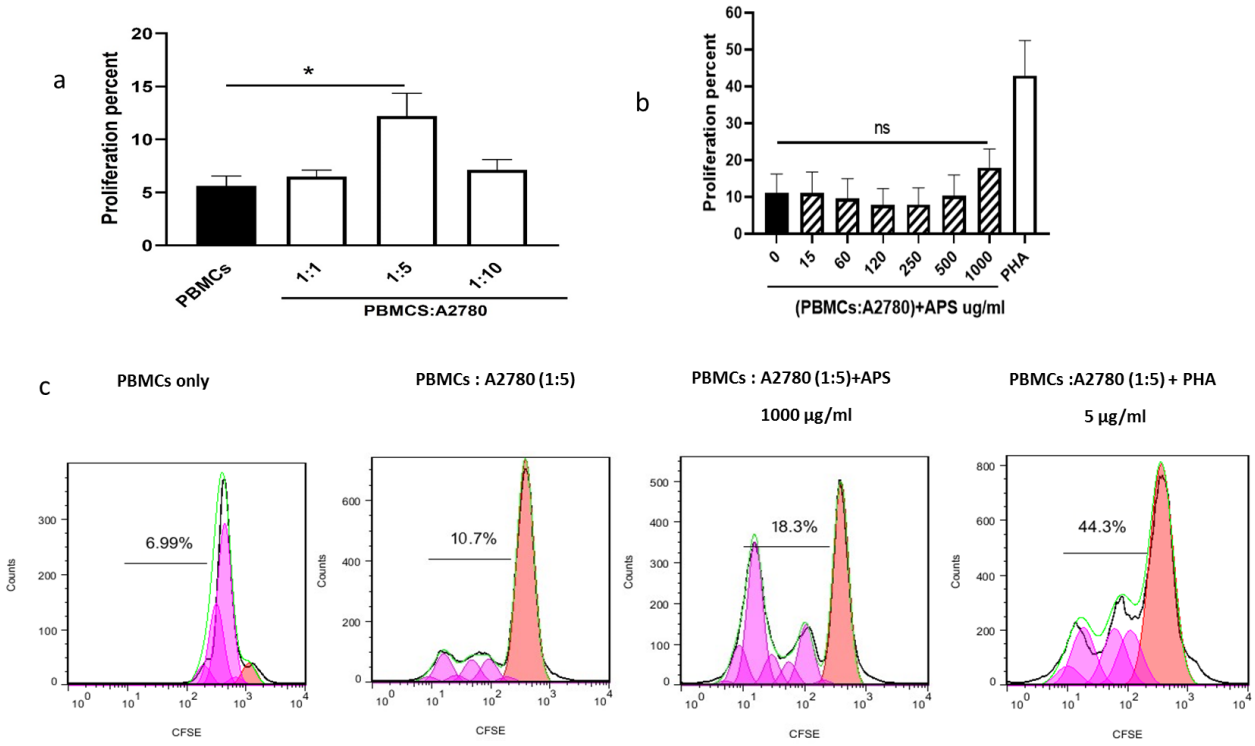

Figure 3. Effect of APS on the proliferation of PBMCs co-cultured with A2780 cells. The proliferation of CFSE-labeled PBMCs was analyzed using flow cytometry after 4 days of being co-cultured with A2780 cells with 1000µg/ml APS. APS: Astragalus polysaccharides; PBMCs: peripheral blood mononuclear cells; CFSE: carboxyfluorescein succinimidyl ester; ns: non-significant; PHA: polyhydroxyalkanoates. *p<0.05

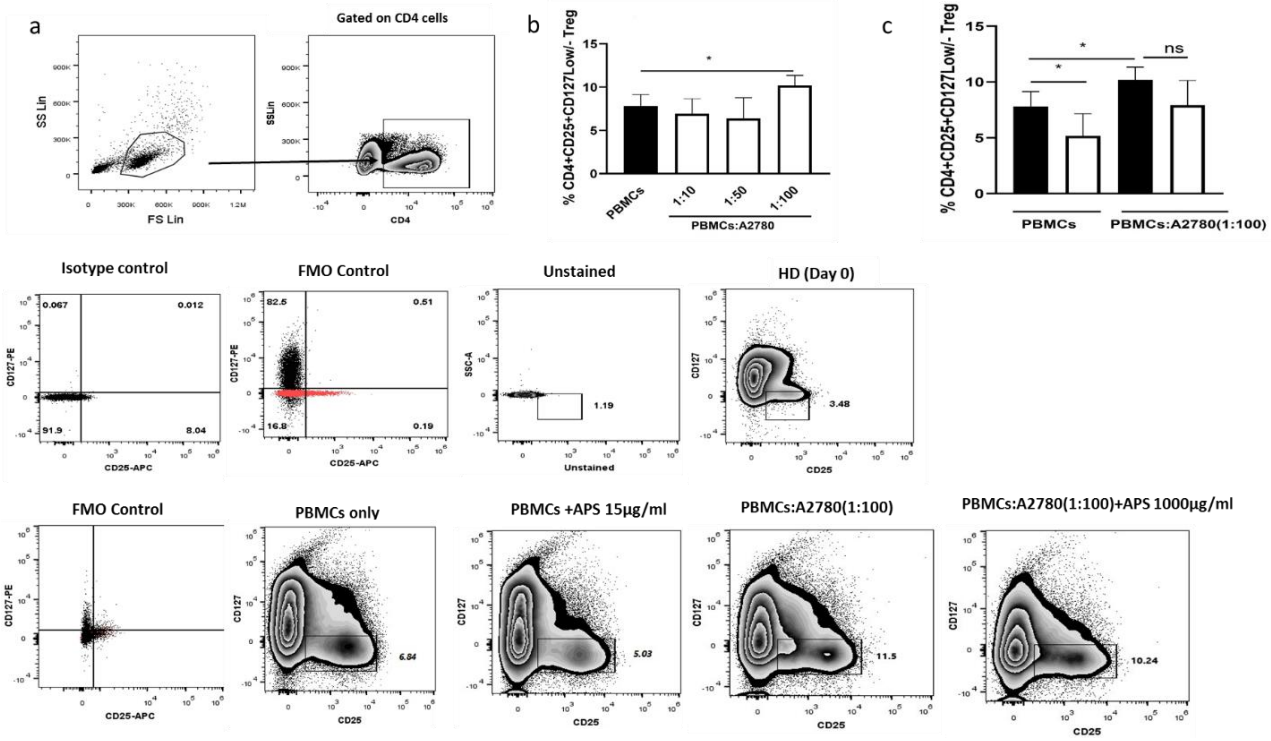

Figure 4. APS could decrease the induction of Tregs in vitro. PBMCs were stained with a mixuture of monoclonal antibodies including anti-CD4, anti-CD25, and anti-CD127. CD4+ cells from live PBMCs were gated for further analysis and CD25+CD127-/low cells were gated as Tregs. APS: Astragalus polysaccharides; PBMCs: peripheral blood mononuclear cells; ns: non-significant; HD: Healthy Donors; FMO: fluorescence minus one. *p<0.05

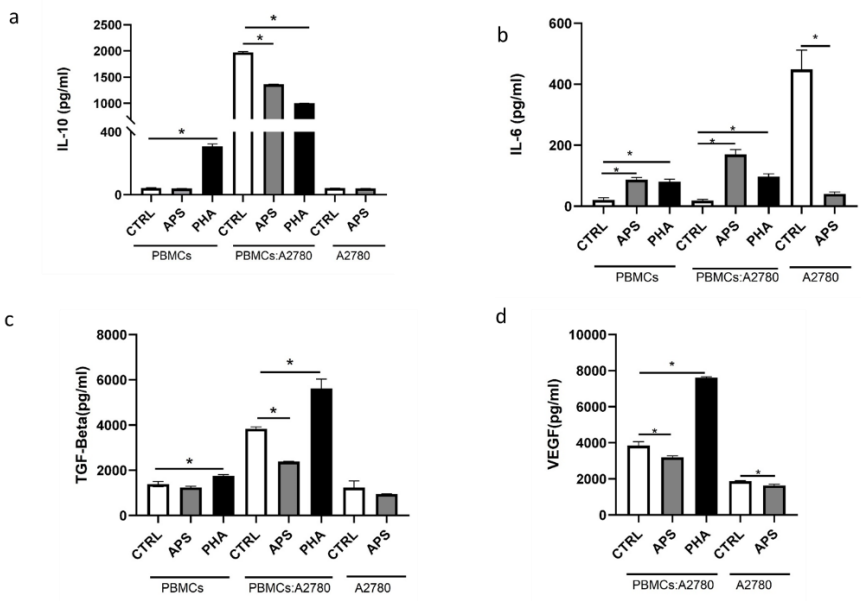

Figure 5. Effect of APS on IL-6, IL-10, TGF-β, and VEGF-A secretion. Measurement of cytokine concentrations in the supernatant of PBMCs cultured alone or with A2780 cells without treatment after 72 hours by ELISA. Data are presented as mean ± standard error of the mean. *p<0.05 versus unstimulated control group.

PBMC: peripheral blood mononuclear cell; APS: *Astragalus polysaccharides*; PHA: polyhydroxyalkanoates; ELISA: enzyme-linked immunosorbent assay; IL:interleukin; TGF-β: transforming growth factor-beta; VEGF: vascular endothelial growth factor.

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DISCUSSION

A membranaceus is typically composed of more than 10 monosaccharides attached by a glycosidic bond, a type of bioactive macromolecule exerting several pharmacological effects.[11,12] Recently, some studies have shown that APS plays a vital role in improving the immune response to attack the tumor cells in vivo, suggesting its potential for clinical antitumor therapy.[7,11]

In addition to its efficacy in antitumor activities, APS has multiple immunomodulatory functions, such as enhancing the production of immunomodulatory factors by macrophages through activating the MyD88-dependent signaling pathway mediated by toll-like receptor 4 and stimulating the activity of cytotoxic lymphocytes.[13-15] However, before the present study, the immunomodulatory effect of APS had not been investigated using a coculture model of PBMCs and ovarian cancer cell lines that mimics the TME. Little has been reported about how APS enhances the immune system's ability to fight tumors.

Regarding the effect of APS on PBMCs, we observed that APS could increase the viability of these cells, in a time- and dose-dependent manner. In contrast, APS could decrease the viability of A2780 cells at a higher concentration. Moreover, the inhibitory effect of APS has been reported in ovarian cancer cell lines, whose proliferation could be suppressed by overexpression of microRNA-27a.[16]

Recent studies have shown that APS can thwart the proliferation of human gastric cancer cell lines by inducing cancer cell apoptosis.[17]Furthermore, APS treatment could also suppress the viability and proliferation of breast cancer cells[18] and human OS MG63 cells.[19]

We further investigated the effect of APS on the proliferation of PBMCs in monoculture or coculture with A2780 cells. The results demonstrated that APS could potentiate the proliferation of PBMCs at low concentrations. Previous studies have shown that APS could increase the proliferation of CD8+ T lymphocytes in HIV-infected patients.[5] APS also facilitates the maturation of dendritic cells.[8] Furthermore, APS treatment increased the proliferation of lymphocytes[20] and multiplied the frequency of $CD11c^{high}CD45RB^{low}$ dendritic cells significantly.[21] APS also activated PBMCs.[22]

We found that APS-treated PBMCs secreted more IL-6 when cultured alone or with A2780 cells. Similarly, oral administration of APS inhibited tumor weight, and the levels of IL-6 increased in EAC- bearing mice.[15] APS treatment also increased mRNA expression of IL-6 in RAW264.7 cells.[8] IL-6 induced B cell expansion and stimulated T cell proliferation.[8,23,24]. APS increased T-cell proliferation and promoted splenocyte production of IL-6.[8] Notably, these results support our finding that APS can boost PBMC proliferation and IL-6 secretion.

In addition, we showed that the impact of APS on the proliferation of PBMCs can be associated with its ability to increase IL-6 expression. The coculture of PBMCs and ovarian cancer cells from patients increased IL-6 production.[25] However, other factors such as coculture time, transwell plate use, and cancer cell type might also affect PBMC proliferation in vitro.

Tumor cells gain the capacity to escape from immune response through diverse immune evasion mechanisms by the induction of immunosuppressive cells such as Tregs, M2 macrophages, and myeloid-derived suppressor cells (MDSC). In addition, Treg cells can overwhelm the antitumor immune responses and help to develop immunosuppressive TME in different cancer types.[26]

We provided evidence that the percentage of Treg cells increased in the coculture of PBMCs and A2780 cells. Multiple myeloma cells can generate $CD4^{+}CD25^{+}FoxP3^{+}$ Tregs by increasing Forkhead box P3 (*FOXP3*) expression in a contact-dependent manner in vitro.[27] This result was consistent with ours.

We cocultured cells with different concentrations of APS to investigate its effect on Treg, PBMC, and A2780 frequencies. APS suppressed Treg induction when PBMCs were cultured alone and with a low concentration of APS. Moreover, in a similar study by Xiaogang et al., APS augments the suppression of Treg cells.[30] APS also inhibited Treg frequency and *FOXP3* expression both in vivo and in vitro.[28] Therefore, APS can modulate the immune system response against tumors by suppressing Tregs and thereby improving antitumor immune responses.

TGF-β1 and IL-10 have a vital role as inhibitory cytokines in the development of Tregs and induction of *FOXP3* expression.[29] Here, we observed that the PBMCs cocultured with A2780 cell line secreted less TGF-β1 and IL-10. Similarly, another study revealed that APS could suppress tumor growth by decreasing

IL-10 levels in H22 tumor-bearing mice.[32,33] Xiaogang Du et al. [28,32] also reported that APS administration downregulated TGF-β1 production in vivo. In contrast, our study showed that A2780 cells increased Treg induction significantly in an in vitro model, while reducing TGF-β, IL-10, and VEGF-A production from PBMCs. We need to elucidate whether soluble factors or cell-cell contact mediated Treg induction following coculturing of PBMCs and A2780 cells. We suggest that APS treatment reduces Treg frequency due to lower inhibitory cytokines release at the protein level. However, no current reports support this hypothesis. Therefore, we propose to investigate the complete molecular mechanism and the effect of APS on Treg function using transwell plates instead of direct coculture.

In addition, we studied the capacity of APS-treated PBMCs cocultured with A780 cells to produce VEGF. The expression of VEGF in ovarian cancer represses tumor immunity through the induction of MDSCs, contributing to poor prognosis.[33] A prior survey showed that APS could improve immune function in a lung-cancer mouse model by inhibiting the expression of VEGF and the metastasis of cancer cells.[34]

The present research demonstrated that APS could reduce VEGF-A production in vitro. Therefore, APS seems to be able to ameliorate the antitumor responses by bypassing the immune-inhibitory effects of Tregs and adjusting the secretion of cytokines in vitro. However, we do not fully understand the precise mechanisms by which APS can inhibit VEGF production in this context. Therefore, further studies are required to investigate the role of APS in suppressing the functions of Tregs in the coculture of PBMCs and A2780 cells.

In summary, we found that APS could improve immune responses through suppression of Treg induction and immunosuppressive cytokines such as TGF-β and IL-10 in vitro. Therefore, APS appears to have the potential to stimulate an immune-mediated response against tumor cells. This finding adds to the existing knowledge on the anticancer effects of APS and suggests that it could be a promising therapeutic approach in cancer therapy, especially for OC. This study provided a rationale for further in vivo studies in animal models and clinical trials that are crucial to confirm the efficacy and safety of APS in vivo.

STATEMENT OF ETHICS

This research was approved by the Student Research Committee of Iran University of Sciences (reference number: IR.IUMS.REC.1395.9311127005).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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