Tumor necrosis factor-α-induced protein 8-like-2 is involved in the activation of macrophages by *Astragalus* polysaccharides *in vitro*

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Abstract. In previous years, studies have shown that *Astragalus* polysaccharides (APS) can improve cellular immunity and humoral immune function, which has become a focus of investigations. Tumor necrosis factor-α-induced protein 8-like 2 (TIPE2) is a negative regulator of immune reactions. However, the effect and underlying mechanisms of TIPE2 on the APS-induced immune response remains to be fully elucidated. The present study aimed to examine the role of TIPE2 and its underlying mechanisms in the APS-induced immune response. The production of nitric oxide (NO) was detected in macrophages *in vitro* following APS stimulation. In addition, the present study interfered with the expression of TIPE2 in macrophages, and examined the production of cytokines, NO and components of the mitogen-activate protein kinase (MAPK) signaling pathway following APS stimulation. The results showed that APS was able to activate macrophages by inducing the production of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-6 and NO. Furthermore, RAW264.7 cells were stimulated with APS when TIPE2 was silenced, and it was found that the production of TNF-α, IL-6, IL-1β and NO were upregulated, and the signaling pathway of MAPK was activated. Taken together, these results demonstrated that TIPE2 had an important negative effect on the APS-induced production of inflammatory cytokines and NO via the MAPK signaling pathway.

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Abbreviations: APS, *Astragalus* polysaccharides; TIPE2, tumor necrosis factor-α-induced protein 8-like-2; NO, nitric oxide; MAPK, mitogen-activated protein kinase

Key words: *Astragalus* polysaccharides, macrophages, activation, downregulation, tumor necrosis factor-α-induced protein 8-like-2

Introduction

Macrophages are important immune cells, which are involved in the stimulation of the innate immune system, including the release of inflammatory cytokines and inflammatory molecules, including tumor necrosis-factor (TNF)-α, interleukin (IL)-1β, IL-6 and nitric oxide (NO) (1,2). These cytokines and inflammatory molecules are often regulated by the mitogen-activated protein kinase (MAPK) signaling pathway (3,4).

TNF-α-induced protein-8 like 2 (TNFAIP8L2, also known as TIPE2), is a novel member of the TNFAIP8 (TIPE) family. TIPE2 was originally identified as a negative regulator of immune homeostasis, being expressed mainly in lymphoid tissues, inflammatory tissues and immune cells, including macrophages (5).

Astragalus, a well-known Chinese traditional medicine and edible food, has multiple effects in pharmacological and biological processes. One of its bioactive components is *Astragalus* polysaccharides (APS), which can improve cellular and humoral immunity, and regulate certain cytokines (6-8). Although it is well known that APS is an immune regulator, few studies have reported on the molecular mechanism underlying the effect of APS on the immune system. MAPKs are a family of serine/threonine protein kinases, which are responsible for the majority of cellular responses to cytokines and are crucial for regulating the production of inflammation mediators. A small number of natural extracts have been shown to inhibit the expression of inflammatory genes by regulating the phosphorylation of MAPK pathways (3-4,9). The present study aimed to determine what effect TIPE2 has on activating macrophages induced by APS *in vitro*.

Materials and methods

Drugs. APS (cat. no. 151001B) for clinical application with an endotoxin content of <0.1 Eu/mg was purchased from Pharmagenesis, Inc. (Palo Alto, CA, USA); it is a polysaccharide with a molecular weight of 20,000-60,000, and the polysaccharides consist of α-1, 4 (1,6) glucan, arabinose-galactose polysaccharides, hamnose-galacturonic acid polysaccharides and arabinose-galactose protein polysaccharides.

Cell line and culture. Murine macrophage RAW264.7 cells (Institute of Biochemistry and Cell Biology, Shanghai, China)

were grown in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with penicillin 100 IU/ml, streptomycin 100 IU/ml and 2 mM/l-glutamine supplemented with 10% bovine serum albumin (PPA, Ferlach, Austria). The cells were maintained subconfluent at 37˚C in humidified air containing 5% CO_2. The RAW264.7 cells were harvested by gentle scraping, washed in PBS, resuspended in culture medium and plated. Non-adherent cells were removed by repeated washing following incubation at 37˚C for 4 h. Each experimental unit comprised $5x10^5$/ml cells.

RNA interference. The TIPE2-specific small interfering (si)RNA-517 (GenePharma Co., Ltd., Shanghai, China) sequences were as follows: Sense 5'-GCAUCAGGCACGUGU UUGATT-3' and antisense 5'-UCAAACACGUGCCUGAUG CTT-3'. The TIPE2-specific siRNA-166 (GenePharma Co., Ltd.) sequences were as follows: Sense 5'-CCGUGGCGCAUCUCU CUUUAUTT-3' and antisense 5'-AUAAAGAGAUGCGCC ACGGTT-3'. The TIPE2-specific siRNA-351 (GenePharma Co., Ltd.) sequences were as follows: Sense 5'-GCUACACGA UUUCGUCAGATT-3' and antisense 5'-UCUGACGAAAUC GUGUAGCTT-3'. The control siRNA sequences (GenePharma Co., Ltd.) were as follows: Sense 5'-UUCUCCGAACGUGUC ACGUTT-3' and antisense 5'-ACGUGACACGUUCGGAGA ATT-3'. For each well, 8.4 ng of siRNA duplexes was diluted into 100 μl of medium and mixed. INTERFERin (4 μl) was added to the mixture, which was immediately homogenize by vortexing for 10 sec. It was then incubated for 10 min at room temperature to allow transfection complexes to form between siRNA duplexes and INTERFERin. During complex formation, the growth medium was removed and 0.5 ml of fresh pre-warmed complete medium added per well. Then, 100 μl of transfection mix was added to the cells and homogenized by gently swirling the plate. The final volume was 600 μl, and the siRNA concentration 20 nM. The plate was incubated at 37˚C and gene silencing measured after 48 h.

NO assay. The quantity of stable NO generated by activated macrophages was determined using Griess reagent (Promega Corp., Madison, WI, USA). The TIPE2 siRNA- or control siRNA-transfected RAW264.7 cells ($5x10^5$/ml) were treated with different concentrations of APS (10, 100, 200 and 400 μg) and LPS (10 μg/ml) at room temperature for different durations (4, 8, 16, 24, 32, 40 and 48 h). Subsequently, 50 μl of the cell culture supernatant was mixed with an equal volume of Griess reagent and incubated at room temperature for 15 min. The absorbance was measured at 550 nm using a microplate reader, and a standard curve was plotted using a serial known concentration of NO.

Detection of TNF-α, IL-6 and IL-1β. The TIPE2 siRNA- or control siRNA-transfected RAW264.7 cells treated with APS (100 μg/ml) were cultured for 16 h. The levels of TNF-α (cat. no. BMS607), IL-6 (cat. no. BMS603) and IL-1β (cat. no. BMS6002) in the supernatant were measured using the ELISA method (Bender Medsystems, Vienna, Austria) according to the manufacturer's protocol.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA was extracted from cells using TRIzol (Takara Bio, Inc., Otsu, Japan) according to the manufacturer's protocol. Single-strand cDNA (100 ng) was generated from total RNA using reverse transcriptase (Toyobo Co., Ltd., Osaka, Japan). RT-qPCR analysis, using 5 μl SYBR-Green detection chemistry mix (Roche Diagnostics, Basel, Switzerland), was performed on a 7500 Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). For mouse TIPE2, 1 μl qPCR primers were: Forward, 5'-TCTCAGAAACATCCAAGGCC-3' and reverse, 5'-TTTGAGCTGAAGGACTCCATG-3'. For mouse β-actin, the qPCR primers were: Forward, 5'-AGTGTGACG TTGACATCCGT-3' and reverse, 5'-GCAGCTCAGTAA CAGTCCGC-3'. PCR amplification program was performed under the following two steps. Stage 1: 50˚C for 2 min, 95˚C for 10 min (1 cycle). Stage 2: 95˚C for 15 sec, 60˚C for 35 sec (39 cycles). Relative expression fold change was calculated by the $2^{\Delta\Delta Cq}$ method, and β-actin was used as the endogenous reference gene to normalize the expression level of target gene.

Western blot analysis. The cells were washed twice with cold PBS and lysed with cell lysis buffer (Cell Signaling Technology, Inc., Boston, MA, USA) supplemented with protease inhibitor mixture (Beyotime Institute of Biotechnology, Shanghai, China). The protein concentrations of the cell lysis extracts were measured using a BCA assay (Pierce; Thermo Fisher Scientific, Inc.) and equalized with the extraction reagent. Protein extracts (30 μg) were loaded and subjected to separation on an 10% SDS-PAGE gel, following which they were transferred onto a PVDF membrane. The membrane was blocked with 5% skim milk in TBST for 1 h at 25˚C and then incubated with rabbit anti murine ERK (cat. no. 9102), JNK (cat. no. 9252), P38 (cat. no. 8690), phosphorylated ERK (cat. no. 9101), phosphorylated JNK (cat. no. 9251), and phosphorylated P38 antibody (cat. no. 9215; Cell Signaling Technology, Inc.) for 1 h at 25˚C. Following washing three times in TBST for 10 min each time, the membrane was incubated with HRP conjugated goat anti rabbit-IgG (cat. no. 18-8816-33; Rockland, Gilbertsville, Philadelphia, PA, USA) for 1 h and the antibody-specific protein was visualized by using an ECL kit (Biological Industries, Kibbutz Beit Haemek, Israel) in the enhanced chemiluminescence detection system as previously described (10). The intensities of the protein bands were analyzed by Gel-Pro Analyzer software. All of the above antibodies were diluted to 1:1,000.

Statistical analysis. The results are expressed as the mean ± standard deviation of the indicated number of experiments. The statistical significance of difference was estimated using a t-test for unpaired observations. All statistical analysis was performed using SPSS software version 15.0 (SPSS, Chicago, IL, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

APS induces the production of NO in a dose-dependent manner. The production of NO in the RAW264.7 cells treated with different concentrations of APS (10, 100, 200 and 400 μg/ml), LPS (10 μg/ml) or negative control for 16 h was detected. As shown in Fig. 1, the production of NO (18.9±1.5 μmol/l;

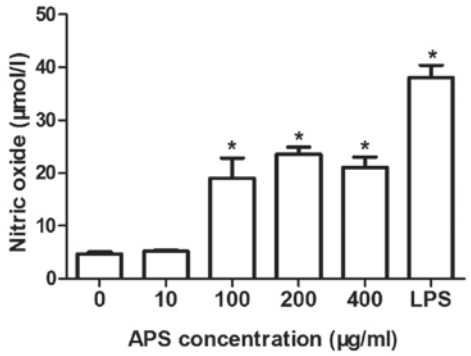

Figure 1. APS induces the production of NO in a dose-dependent manner. Production of NO was measured in RAW264.7 cells treated with different concentrations of APS (10, 100, 200 and 400 μg/ml), LPS (10 μg/ml) as a positive control, and negative control for 16 h were measured. Data are presented as the mean ± standard deviation (n=3) of three independent experiments. *P<0.01, vs. 0 μg/ml. NO, nitric oxide; APS, *Astragalus* polysaccharides; LPS, lipopolysaccharide.

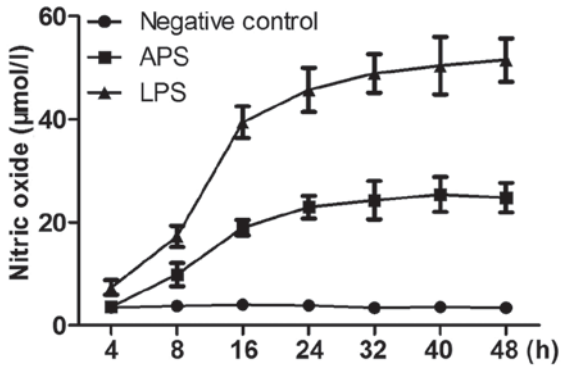

Figure 2. APS induces the production of NO in a time-dependent manner. Production of NO was measured production in RAW264.7 cells treated with APS (100 μg/ml), LPS (10 μg/ml) as a positive control, and negative control for in different durations. Data are presented as the mean ± standard deviation (n=3) of three independent experiments. NO, nitric oxide; APS, *Astragalus* polysaccharides; LPS, lipopolysaccharide.

P<0.01) was induced significantly 16 h following treatment with APS (100 μg/ml). When treated with 200 μg/ml of APS, the production of NO in the RAW264.7 cells reached a peak and then began to weaken.

APS induces the production of NO in time-dependent manner. The production of NO in RAW264.7 cells treated with APS (100 μg/ml), LPS (10 μg/ml) or negative control for different durations are shown in Fig. 2. It was found that the production of NO was significantly induced (9.87±2.23 μmol/l; P<0.01) 8 h following treatment with APS. After 16 h, the rate of NO production slowed down (18.9±1.5 μmol/l; P<0.01). In the negative control group, only minimal NO was released and there were no significant difference at any time point.

Downregulated expression of TIPE2 is induced by APS in vitro. The expression of TIPE2 in RAW264.7 cells treated with APS (100 μg/ml), LPS (10 μg/ml) or negative control for different durations were examined using RT-qPCR analysis, as shown in Fig. 3. It was found that the expression of TIPE2 was significantly reduced following treatment of the cells with

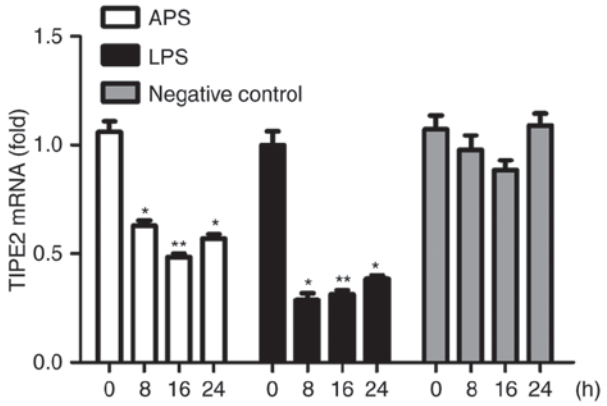

Figure 3. APS reduces the expression of TIPE2 in RAW264.7 cells. Expression levels of TIPE2 in RAW264.7 cells treated with APS (100 μg/ml), LPS (10 μg/ml) as a positive control, and negative control for different durations were measured using reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard deviation (n=3) of three independent experiments. *P<0.05 and **P<0.01, vs. control. TIPE2, tumor necrosis factor-α-induced protein 8-like-2; APS, *Astragalus* polysaccharides; LPS, lipopolysaccharide.

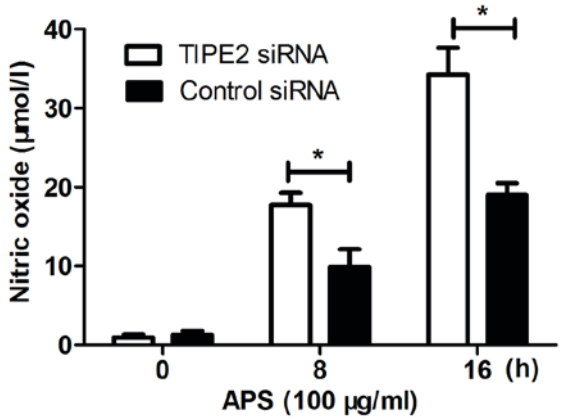

Figure 4. Silencing of TIPE2 promotes the production of NO in RAW264.7 cells following stimulation with APS for indicated durations. The RAW264.7 cells were transfected with control siRNA or TIPE2 siRNA, as indicated, at a final concentration of 20 nM. After 48 h, the cells were stimulated with or without APS (100 μg/ml) for the indicated durations. NO was measured following stimulation. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05. TIPE2, tumor necrosis factor-α-induced protein 8-like-2; NO, nitric oxide APS, *Astragalus* polysaccharides; siRNA, small interfering RNA.

APS and LPS. The downregulation of TIPE2 upon APS stimulation suggested that TIPE2 may function as a regulator of the APS-induced immune response in macrophages.

Effect of TIPE2 siRNA on the production of NO induced by APS. In the present study, RAW264.7 cells were transfected with TIPE2 siRNA or control siRNA and, 48 h later, the cells were stimulated with APS (100 μg/ml). The supernatants were collected at different time points to detect the production of NO. As shown in Fig. 4, the production of NO was significantly increased in the TIPE2 siRNA group, compared with that in the control siRNA group at the different time points. These results showed that TIPE2 negatively regulated the APS-induced production of NO in macrophages.

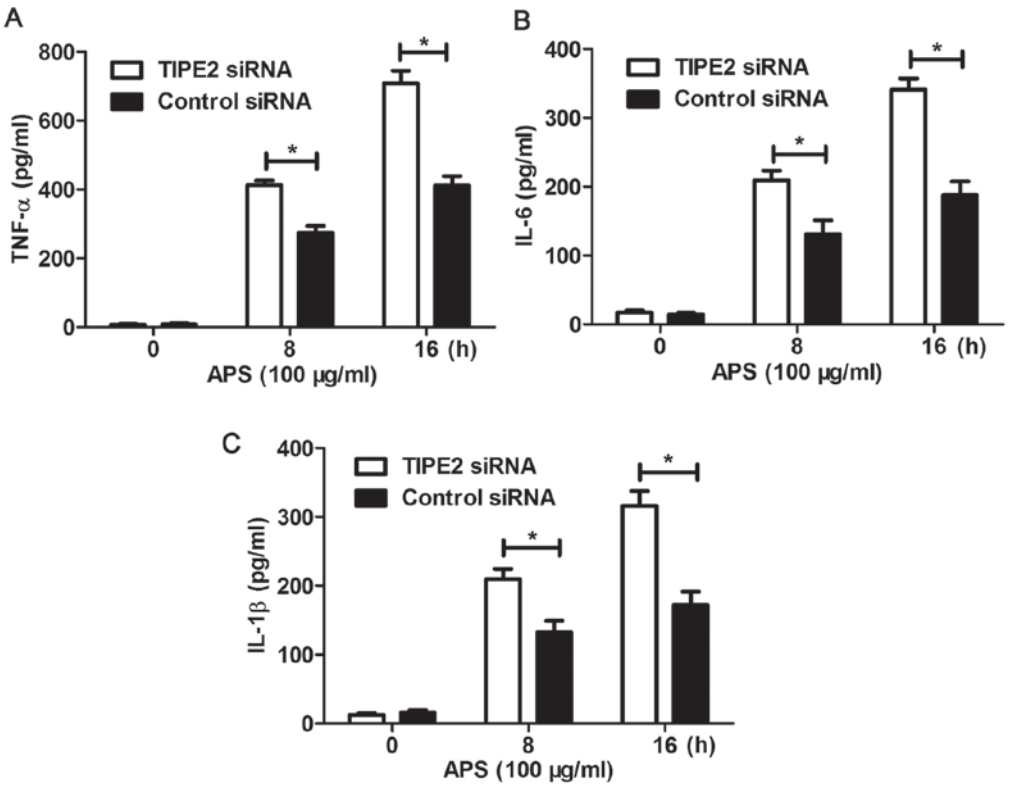

Figure 5. Silencing of TIPE2 promotes the production of inflammatory cytokines in RAW264.7 cells following stimulation with APS for indicated durations. RAW264.7 cells were transfected with control siRNA or TIPE2 siRNA, as indicated, at a final concentration of 20 nM. After 48 h, the cells were stimulated with or without APS (100 μg/ml) for the indicated durations. Levels of (A) TNF-α, (B) IL-6 and (C) IL-1β were measured using ELISA following stimulation. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.05. TIPE2, tumor necrosis factor-α-induced protein 8-like-2; APS, *Astragalus* polysaccharides; siRNA, small interfering RNA; TNF-α, tumor necrosis factor-α, IL, interleukin.

Effect of TIPE2 siRNA on the production of inflammatory cytokines induced by APS. The RAW264.7 cells were transfected with TIPE2 siRNA or control siRNA and, 48 h later, the cells were stimulated with APS (100 μg/ml). The supernatants were collected at different time points to detect the production of inflammatory cytokines, including TNF-α, IL-6 and IL-1β. It was found that the expression of inflammatory cytokines TNF-α (Fig. 5A), IL-6 (Fig. 5B) and IL-1β (Fig. 5C) were upregulated in the TIPE2-silenced RAW264.7 cells infected with APS at different time points. Taken together, these results demonstrated that TIPE2 negatively regulated the APS-induced production of inflammatory cytokines in macrophages.

Effect of TIPE2 on the MAPK signaling pathway in RAW264.7 cells treated with APS. To further examine the possible mechanism of TIPE2 on the regulation of inflammatory cytokine expression induced by APS, western blot analysis was performed to determine the effect of TIPE2 on the MAPK signaling pathway. As shown in Fig. 6, the results revealed found that the phosphorylation of P38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK) were significantly activated following APS challenge following interference of endogenous TIPE2 in macrophages. Therefore, it was hypothesized that TIPE2 may inhibit the secretion of inflammatory cytokines when stimulated with APS by inhibiting the activation of the MAKP signaling pathway.

Discussion

Several preliminary studies have shown that polysaccharides isolated from *Astragalus* possess a wide range of biological functions (11-13). In the present study, APS with a molecular weight of 20,000-60,000 was selected due to its specificity in binding to macrophages. Macrophages are important in immune defense, immune stability and immune surveillance. Activated macrophages can identify and kill pathogenic microorganisms, and clean up apoptotic cells and mutant cells, and can also initiate acquired immunity by their efficient antigen-presenting ability to activate T cells and B cells. NO is considered to be a key molecule in regulating the immune response (14), which is associated with the cytolytic function of macrophages against a variety of tumor cells, and the increase of NO synthesis can interfere with tumor cell growth (15). One of the most prominent characteristics of TNF-α is its ability to induce the apoptosis of tumor-associated endothelial cells, resulting in the necrosis of tumor cells (16). IL-6 and IL-1β can be secreted by macrophages, which act as inflammatory and anti-inflammatory cyokines (17). The data obtained in the present study showed that the basal levels of NO, TNF-α, IL-6 and IL-1β were relatively low in resting macrophages; however, APS induced the activation of macrophages, resulting in significant increases in the production of IL-1β, TNF-α, IL-6 and NO, which may be beneficial for resisting pathogen invasion. Of note, the present study also found that the production of

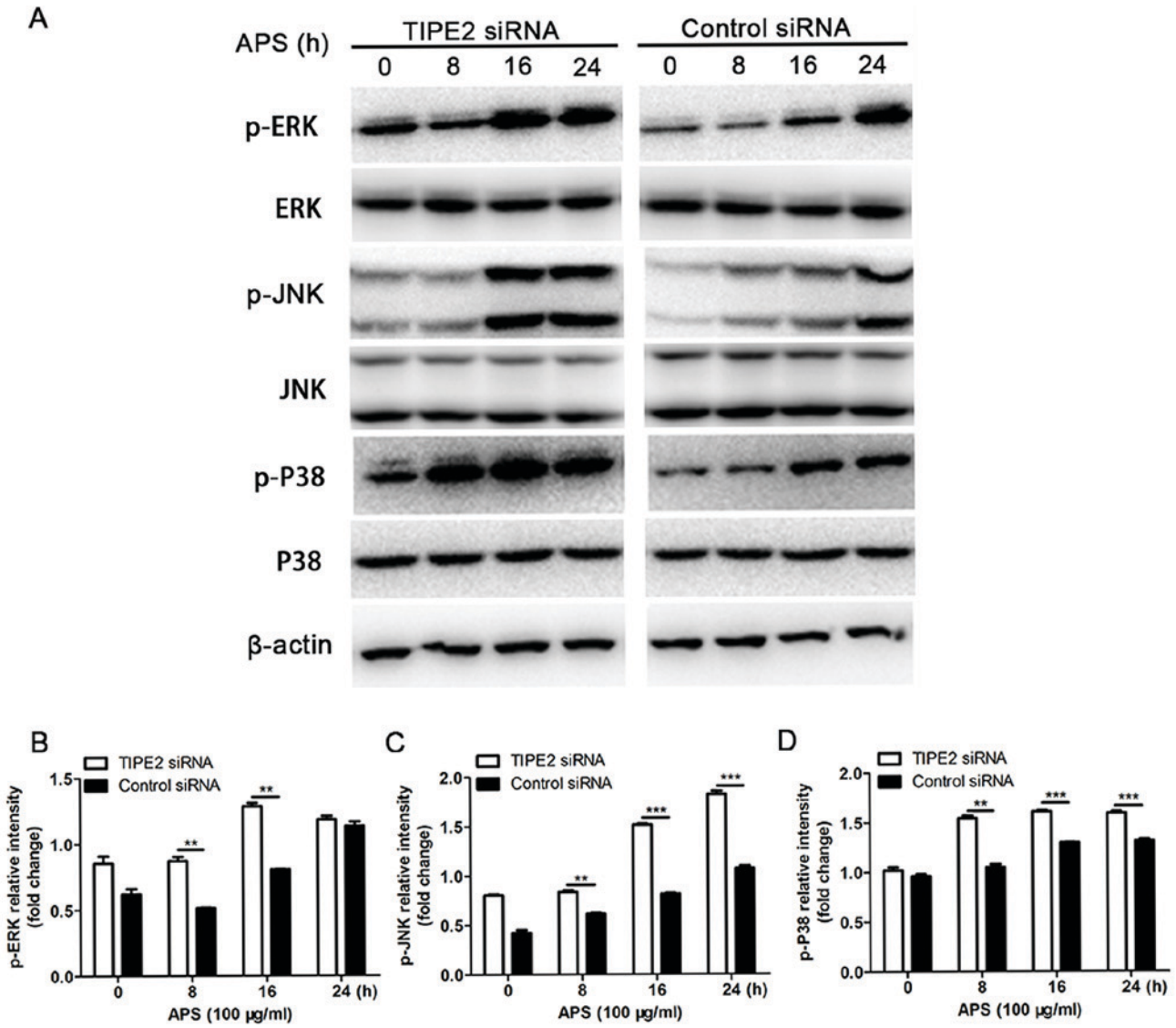

Figure 6. (A) Effect of silenced TIPE2 on APS-induced signaling pathway activation in RAW264.7 cells. RAW264.7 cells ($1x10^6$) were transfected with control siRNA or TIPE2 siRNA, as indicated, at a final concentration of 20 nM. After 48 h, the cells were stimulated with APS (100 μg/ml) for different durations, as indicated. Cell lysates were analyzed using western blot analysis for phosphorylated mitogen-activated protein kinase (ERK, JNK and P38). β-actin was used as the loading control. Data shown are from one representative experiment of three. Fold change in the expression of (B) p-ERK, (C) p-JNK and (D) p-P38. Data are presented as the mean ± standard deviation of three independent experiments. **P<0.05 and ***P<0.01. TIPE2, tumor necrosis factor-α-induced protein 8-like-2; APS, *Astragalus* polysaccharides; siRNA, small interfering RNA; p-, phosphorylated; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase.

NO induced by APS occurred in a dose-dependent manner and a time-dependent manner. These results suggested the possibility that APS may be administrated to the human body to activate macrophages for immunological activities.

TIPE2, a novel protein of the TNFAIP8 family, has attracted increasing attention (18). TIPE2 was initially identified as an essential protein, which is important in maintaining immune homeostasis by negatively regulating innate and adaptive immunity (5,19-23). TIPE2-knockout and knockdown macrophages are hypersensitive to toll-like receptor stimulation, and TIPE2 can inhibit the secretion of inflammatory cytokine IL-6 in mouse macrophages (11). In the present study, the expression of TIPE2 was markedly reduced in RAW264.7 cells. TIPE2 siRNA was used to inhibit the endogenous expression of TIPE2 in RAW264.7 cells, following which they were stimulated with APS. It was found that TIPE2 negatively regulated the APS-induced production of inflammatory cytokines (IL-1β, TNF-α and IL-6) and NO in macrophages. However, the molecular mechanisms, particularly the signaling pathway of TIPE2 involved in macrophage activation by APS, remain to be fully elucidated. It has been reported that the expression of TIPE2 is closely associated with MAPK signal transduction (24,25). Therefore, the present study focused on the effect of TIPE2 on the MAPK signaling pathway activated by APS. It was shown that stimulation of the RAW264.7 cells with APS resulted in activation of the MAPK signaling pathway, and TIPE2 inhibited the MAPK signaling pathway in the RAW264.7 cells activated by APS. These results suggested that TIPE2 negatively regulated the production of inflammatory cytokines and NO, which were induced by APS, in macrophages via inhibiting the MAPK signaling pathway.

There were severe limitations in the present study. First, the experiments did not examine the secretion of various inflammatory factors following the activation of macrophages by APS under the overexpression of TIPE2. Second, TIPE2-deficient mice were not included in the present study, leading to the lack of relevant investigations in primary peritoneal macrophages. The investigation of these factors is likely to provide more direct evidence for the role of TIPE2. Finally, further investigations are required to determine which molecules were directly bound with TIPE2.

APS has been widely used clinically as an injection in patients in China to improve immune functions. The present study provided a clearer understanding of the possible mechanism involved, compared with previous reports. According to the results of the present study, APS was effective in inducing the activation of macrophages and TIPE2 was identified as a novel molecule with a negative effect on the APS-induced immune response via the MAPK signaling pathway. These findings provide insights into the novel function of TIPE2 in APS-induced immunity and its associated clinical significance.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JZ designed the study and interpreted experiment data. YZ was responsible for cell culture and RNA interference. FF was responsible for the NO assay. GW was responsible for detection of TNF-α and IL-1β. ZX was responsible for western blot analysis. HZ was responsible for preparation of the study.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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