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Wei Wei  
*Hong Kong Baptist University*

Hai-Tao Xiao  
*Hong Kong Baptist University*

Wan-Rong Bao  
*Hong Kong Baptist University*

Dik Lung Ma  
*Hong Kong Baptist University, edmondma@hkbu.edu.hk*

Chung-Hang Leung  
*University of Macau*

*See next page for additional authors*

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Authors
Wei Wei, Hai-Tao Xiao, Wan-Rong Bao, Dik Lung Ma, Chung-Hang Leung, Xiao-Qiang Han, Chun-Hay Ko, Lau Clara Bik-San, Chun-Kwok Wong, Kwok-Pui Fung, Ping-Chung Leung, Zhaoxiang Bian, and Quanbin Han

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TLR-4 may mediate signaling pathways of Astragalus polysaccharide

RAP induced cytokine expression of RAW264.7 cells

Wei Wei*, Hai-Tao Xiao*, Wan-Rong Bao, Dik-Lung Ma, Chung-Hang Leung, Xiao-Qiang Han, Chun-Hay Ko, Clara Bik-San Lau, Chun-Kwok WONG, Kwok-Pui Fung, Ping-Chung Leung, Zhao-Xiang Bian**, Quan-Bin Han**

*aSchool of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China

bDepartment of Chemistry, Hong Kong Baptist University, Kowloon Tong, Hong Kong, China;

cState Key Laboratory of Quality Research in Chinese Medicine, Institute of Chinese Medical Sciences, University of Macau, Macao, China;

dState Key Laboratory of Phytochemistry and Plant Resources in West China, Institute of Chinese Medicine, The Chinese University of Hong Kong, Shatin, N. T., Hong Kong SAR, China

*Equal contribution

**Corresponding authors:

Q.B. Han

7 Baptist University Road, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China
Tel: 00852-34112906 / Fax: 00852-34112461
E-mail: simonhan@hkbu.edu.hk

Z.X. Bian

7 Baptist University Road, School of Chinese Medicine, Hong Kong Baptist University, Kowloon Tong, Hong Kong SAR, China
Tel: 00852-34112905 / Fax: 00852-34112461
E-mail: bzxiang@hkbu.edu.hk
Abstract:

Ethnopharmacological relevance: Polysaccharides of Radix Astragali (Astragalus membranaceous (Fisch) Bge.; Huangqi) are able to induce cytokine production of macrophages and are considered the main active ingredient for the immune-enhancing effect of this commonly used medicinal herb.

Aim of study: To investigate the molecular mechanism of immunomodulating activities of a reported Astragalus polysaccharide, RAP, which is a hyperbranched heteroglycan with average molecular weight of 1334 kDa.

Materials and methods: The cytokine production of RAW264.7 cells were analyzed by using ELISA assays while cell viability was assessed by MTT method. Western blot analysis was used for determining protein contents of mitogen-activated protein kinases (MAPKs). In addition, the level of IL-6, iNOS, and TNF-α mRNA was determined by RT-PCR.

Results: It has been found that RAP itself did not have any cytotoxic effect on mouse mammary carcinoma 4T1 cells, but it significantly enhanced cytotoxicity of the supernatant of RAW264.7 cells on 4T1 cells. Furthermore, RAP enhanced the production of NO and cytokines in RAW264.7 cells, and significantly up-regulated gene expressions of TNF-α, IL-6, iNOS. All these bioactivities were blocked by the inhibitor of TLR4 (Toll-like receptor 4), suggesting that TLR4 is a receptor of RAP and mediates its immunomodulating activity. Further analyses demonstrated that RAP rapidly activated TLR4-related MAPKs, including phosphorylated ERK, phosphorylated JNK, and phosphorylated p38, and induced translocation of NF-κB as well as degradation of IκB-α. These results are helpful to better understand the immunomodulating effects of Radix Astragali.

Conclusions: RAP may induce cytokine production of RAW264.7 cells through TLR4-mediated activation of MAPKs and NF-κB.

Keywords: Astragalus polysaccharides; RAW264.7 macrophages; MAPKs; NF-κB
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tr>
<td>APC</td>
<td>Antigen presenting cells</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>PRRs</td>
<td>Pattern-recognition receptors</td>
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<td>TLRs</td>
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<td>TLR4</td>
<td>Toll-like receptor 4</td>
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<td>RAP</td>
<td>Radix Astragali polysaccharides</td>
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<td>RT-PCR</td>
<td>Real time-polymerase chain reaction</td>
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<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
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<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
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<tr>
<td>MAPKs</td>
<td>Mitogen-activated protein kinases</td>
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<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>Nuclear factor-κB</td>
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<td>NF-kappa-B inhibitor alpha</td>
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<td>Activating protein-1</td>
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<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<td>NO</td>
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<td>Tumor necrosis factor-alpha</td>
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<tr>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
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1. **Introduction**

Radix Astragali, popularly known as Huangqi in Chinese or Milkvetch in English, is one of the important “Qi tonic” or adaptogenic herbs used in Chinese medicine. It has been classically used for centuries to replenish Qi (vital energy or the instigator of body functions, a concept closely linked to immunity) of patients who have general debility and chronic illnesses and receive fu-zheng therapy (Block and Mead, 2003; Sinclair, 1998). It is also used as a tonic herb in anticancer therapy for increasing the body’s resistance to cancer, enhancing the immune system, and strengthening the physique (Block and Mead, 2003; Tan and Vanitha, 2004). As recorded in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010), Radix Astragali is usually taken to treat all diseases caused by “insufficient Qi” (life energy) that typically includes the following symptoms: feelings of weakness, fatigue, poor appetite, diarrhea, vulnerability to infection, etc.

*Astragalus* polysaccharides, which comprise the majority of the chemical components of Radix Astragali decoction, have been shown to have diverse bio-activities, such as immunomodulatory (Jin et al., 2014), antioxidant (Li et al., 2010; Li et al., 2012), antitumor, antidiabetic (Liu et al., 2010; Mao et al., 2007), antiviral (Huang et al., 2008; Jiang et al., 2010a), hepatoprotective (Jia et al., 2012; Yan et al., 2009), anti-inflammatory (Jiang et al., 2010b; Li and Chen, 2010; Li et al., 2007), and anti-atherosclerotic (Cheng et al., 2011; Wang et al., 2010). Among them, antitumor activity of *Astragalus* polysaccharides *in vivo* is particularly striking. Both
clinical trials and animal studies have showed that *Astragalus* polysaccharide has an
anti-tumor effect (Huang et al., 2010; Song et al., 2013). *Astragalus* polysaccharides
have been applied to improve immune system in treating a variety of diseases in clinic
(Yang and Wang, 2013). *Astragalus* injection combined with chemotherapy is used to
improve the quality of life in patients with advanced cancer (Zou and Liu, 2003). The
antitumor effects of *Astragalus* polysaccharides in vivo could be related to its immune
system-modulating activities (Li et al., 2009; Zhu et al., 2011), e.g. enhancing the
expression of IL-1, IL-2, IL-6 and TNF-α, and decreasing that of IL-10 (Tian et al.,
2012). It has been demonstrated that *Astragalus* polysaccharides possess strong
immunomodulatory effects on lymphocytes (Shao et al., 2004), dendritic cells (Shao
et al., 2006), and especially, on macrophages (Lee et al., 2005; Shao et al., 2004).

In our previous study, a water soluble polysaccharide named RAP was purified
from the water extract of Radix Astragali. Its average molecular weight was 1334 kDa.
It was composed of Rha, Ara, Glc, Gal and GalA in a molar ratio of 0.03: 1.00: 0.27:
0.36: 0.30. It was found to be able to stimulate human peripheral blood mononuclear
cells to produce IL-1β, TNF-α, IL-10, IL-12p40, and GM-CSF (Yin et al., 2012).
Regarding the underlying mechanism, it has been known that the activation of nuclear
factor-kB/Rel (NF-κB/Rel) was involved in the ability of *Astragalus* polysaccharides
to induce cytokine production of mouse peritoneal macrophages and RAW264.7
macrophages (Lee and Jeon, 2005; Zhao et al., 2011). However, the exact molecular
mechanism of this induction has not been clearly elucidated.
In recent years, it has been reported that TLR4 is essential for many natural polysaccharide-induced activations of macrophages (Hsu et al., 2004), dendritic cells (Lin et al., 2005) and B cells (Lin et al., 2006). The polysaccharides requiring TLR4 include *Ganoderma lucidum* polysaccharides, *Polyporus umbellatus* polysaccharides (Li and Xu, 2011), safflower polysaccharides (Ando et al., 2002), and *Platycodon grandiflorum* polysaccharides (Yoon et al., 2003). TLRs are one kind of important pattern-recognition receptors (PRRs) of macrophages. Among TLRs, TLR4 plays an important role in pathogen recognition, activation of antigen-presenting cells (dendritic cells, macrophages and so on), and initiation of adaptive immunity (Janeway and Medzhitov, 2002). Activation of TLR4 was shown to induce expression of TNF-α, IL-6 and NO genes and to elicit conserved inflammatory signaling cascades, resulting in the activation of a key transcription factor, NF-κB, downstream MAPKs and AKT (Kawai and Akira, 2006). Up to now, it is unknown whether TLR4 is also a receptor of *Astragalus* polysaccharides. Therefore we investigated the role of TLR4 as a candidate receptor in RAP-induced immunomodulation of RAW264.7 cells and its related signaling pathways.

2. **Materials and methods**

2.1. **Materials**

LPS (from *Escherichia coli* 0111:B4), Griess reagent (modified),
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and DAPI were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse IL-6 ELISA kit and TNF-alpha ELISA kit were purchased from eBioscience (San Diego, CA, USA). SYBR® Select Master Mix for RT-PCR amplification was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). Abs: monoclonal anti-MAPK, activated (diphosphorylated ERK1/2) Ab, monoclonal anti-JNK kinase, activated (diphosphorylated JNK) Ab, monoclonal anti-p38 MAPK, activated (diphosphorylated p38) Ab, monoclonal anti-p65 Ab, monoclonal anti-actin Ab, anti-rabbit IgG, HRP-linked antibody and anti-biotin, and HRP-linked antibody were obtained from Cell Signaling Technology Inc. (Beverly, Massachusetts, USA); The FITC-labeled anti-rabbit IgG antibody was purchased from Abcam (Cambridge, UK). Primers for pro-IL-6, TNF-α, iNOS, and β-actin were from Invitrogen Life Technologies (Carlsbad, CA, USA). TLR4 inhibitor peptide and control peptide were purchased from IMGENEX (Carlsbad, CA, USA). ERK inhibitors, PD184352, p38 inhibitor SB239063 and JNK inhibitor SP600125 were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. RAP preparation

The roots of Astragalus membranaceus were purchased from herbal store in Hong Kong and identified by Dr. Chun-Feng Qiao. The voucher specimens are deposited at the Institute of Chinese Medicine, the Chinese University of Hong Kong, with voucher specimen number 2010-3268 (Yin et al., 2012).

The isolation and purification procedure is according to the previous study (Yin et al., 2012). Briefly, the air-dried Radix Astragali was powdered and extracted twice with boiling water. The solution was filtered, combined, and concentrated. The solution was precipitated with absolute ethanol. The precipitate was resolved again in
water and deproteined. Then the water solution was dialyzed. Finally, the retentate was lyophilized. The product was dissolved in distilled water again and separated with a HiLoad 26/60 Superdex-200 column, eluted with water. Fractions were collected, dialyzed and finally lyophilized to obtain RAP.

The purified RAP is a water soluble heteropolysaccharide (Yin et al., 2012). RAP takes random coil feature. The molecular weight of RAP is 1334kDa. The total sugar content was 76.5%. It had a high specific rotation of $[\alpha]_D^{20} +125.8$ (0.54, H$_2$O) and weak UV absorption at 280 nm which was consistent with its low protein content (only 0.72%). The uronic acid content was 56.7%. It was composed of Rha, Ara, Glc, Gal and GaLA in a molar ratio of 0.03:1.00:0.27:0.36:0.30. The backbone consisted of 1,2,4-linked Rhap, $\alpha$-1,4-linked Glcp, $\alpha$-1,4-linked GalAp6Me, $\beta$-1,3,6-linked Galp, with branched at O-4 of the 1,2,4-linked Rhap and O-3 or O-4 of $\beta$-1,3,6-linked Galp. The side chains mainly consisted of $\alpha$-T-Araf and $\alpha$-1,5-linked Araf with O-3 as branching points, having trace Glc and Gal. The terminal residues were T-linked Araf, T-linked Glcp and T-linked Galp.

2.3. Cell cultures

The murine macrophage cell line RAW264.7 and mouse mammary carcinoma cell line 4T1 were obtained from American Type Culture Collection (Manassas, VA, USA) and were propagated in DMEM high glucose medium (Invitrogen Life Technologies, Carlsbad, CA, USA) and RPMI 1640 medium (Invitrogen Life Technologies, Carlsbad, CA, USA), respectively. Both kinds of medium were supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 $\mu$g/ml streptomycin. Cells were cultured at 37°C in a 5% CO$_2$ incubator.

2.4. Cell viability assay

The viability of cells was measured using MTT assay as previous study (Yoon et
al., 2009). LPS, a structural part of the outer membrane of Gram-negative bacteria, is one of the most effective stimulators of the immune system and has been widely applied in macrophage model to produce cytokines (Wyns et al., 2015). Many literatures showed that macrophages respond to LPS at nanogram per milliliter concentrations with secretion of cytokines such as IL-6, TNF-α, IL-1, etc (Izeboud et al., 1999). Thus, cells were treated with LPS as the positive control in our study. Briefly, RAW264.7 cells (5×10^3 cells/well) were plated in 96-well microplates overnight and then treated with serial concentrations of RAP or LPS (1 µg/mL and 100 ng/ml) for 24 h. An equal volume of medium was used as vehicle control. After treatment, cells were stained with MTT at a final concentration of 0.5 mg/mL in PBS (pH 7.4), incubated for another 4 h in dark, and then the medium was discarded. The formazan crystals present in cells were dissolved by dimethyl sulfoxide. The absorbance was read at 570 nm in a Benchmark Plus microplate reader (Bio-Rad, Richmond, CA, USA). The results were expressed as ratio of absorbance values between treatment and vehicle control cells.

2.5. Treatment

To examine the potential interaction of TLR4 and RAP leading to the induction of TNF-α, IL-6 and NO production, TLR4 inhibitor peptide was used in pre-incubation for blocking TLR4 activation in RAW264.7 cells, and the TLR4 inhibitor peptide was known to specifically inhibit LPS-induced signaling pathways which is mediated by TLR4 (Putra et al., 2014). RAP (30 µg/ml) or 100 ng/ml of LPS were added to RAW264.7 cells pretreated with or without TLR4 inhibitor peptide, and incubated for 1 h.
In assays to determine the cytotoxicity of RAW264.7 cell supernatants induced by RAP, RAW264.7 cells (1×10^5 cells/well) were seeded in 96-well plates, incubated overnight and then exposed to RAP (30, 100, and 300 µg/ml) and LPS (100 ng/mL) for 24 h. The cell supernatants were collected by centrifugation at 1,000 × g for 10 min. And then these supernatants were added to 4T1 cells. The viability of cells was measured using MTT assay according to Materials and Methods 2.3.

In experiments to determine the effects of SB239063, PD184352 and SP600125 on RAP-induced MAPKs, cells were treated with these kinase inhibitors for 1 h at the indicated concentrations: SB239063 (10, 20, and 40 µM), SP600125 (10, 20, and 40 µM), PD184352 (25, 50, and 100 µM) and then co-exposed to RAP (100 µg/ml) for 24 h. At the end of treatment, the medium was used for NO, IL-6 and TNF-α determination.

2.6. RNA extraction and reverse

For analysis of TNF-α, iNOS and IL-6 mRNA expression, RAW264.7 cells were stimulated with RAP and LPS. The cells were harvested after 24 hours for analysis. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. For cDNA synthesis, 500 ng RNA was used for reverse transcription using PrimeScript™ RT Master Mix kit (TAKARA, Japan) according to the manufacturer’s instructions.
2.7. Real-time quantitative PCR

The expression of TNF-α, iNOS, and IL-6 genes was assessed by real-time RT-PCR using the SYBR® Select Master Mix (Life Technologies, Carlsbad, CA, USA) with ViiA™ 7 Real-Time PCR System. PCR primers for the TNF-α, iNOS, IL-6 and the invariant housekeeping gene β-actin were designed using online website Primer-BLAST of NCBI (National Center for Biotechnology Information) synthesized by Invitrogen Corporation (Carlsbad, CA, USA). The following sequences for primers from 5’ to 3’ end were used: TNF-α: forward, 5’-ATGAGC
ACAGAAAGCATGATC-3’, TNF-α: reverse, 5’-TACAGGCTTGTCACTCGAATT-3’;
IL-6: forward, 5’-GATGCTACCAAACGATATACT-3’, IL-6: reverse,
5’-GGTCCTTAGCCACTCTTTCTGTG-3’; iNOS: forward,
5’-GCCGTGGGCAATCAGTACT-3’, iNOS: reverse,
5’-GGTCTTCCTGGGCTCGATCTG-3’; β-actin: forward,
5’-TGTCACCTTCCAGCAGATGT-3’, β-actin: reverse,
5’-AGCTCAGTAACAGTCCGCTAGA-3’. Optimized runs were started with 10 minutes at 95°C, followed by 45 cycles of 15 seconds each at 95°C, and finally 1 minute at 60°C. PCRs were done using: 1 µl of sample DNA, 1µl of forward and reverse primer, 5 µl of SYBR® select master mix and 2 µl of DEPC-treated water to give a final volume of 10 µl. Samples and no template controls were included in each run as duplicates, and each run was performed three times. The relative gene expression was quantified by the comparative CT method (Livak and Schmittgen, 2001). β-actin gene was used as invariant housekeeping gene internal control.
2.8. Measurement of nitric oxide

NO production was monitored by assessment of nitrite accumulation as previous study (Huang et al., 2011). Briefly, RAW264.7 cells (1×10^5 cells/well) were seeded in 96-well plates overnight, and then stimulated with RAP (30, 100, and 300 μg/ml) or LPS (100 ng/mL) for 24 h; LPS treatment was used as a positive control. After treatment, 100 μL of each supernatant was mixed with an equal volume of Griess reagent (modified); mixtures were incubated in a 96-well plate at room temperature for 15 min. The optical density was determined at 540 nm on Benchmark Plus microplate reader (Bio-Rad, Richmond, CA). Nitrite production was determined by comparing the optical density with the standard curve obtained with NaNO₂.

2.9. ELISA for quantitative analysis of cytokines

RAW264.7 cells (1×10^4 cells/well) were seeded in 96-well plates, incubated overnight and then exposed to RAP (30, 100, and 300 μg/ml) and LPS (100 ng/mL) for 24 h. The cell supernatants were collected by centrifugation at 1,000 × g for 10 min. The amount (pg/mL) of IL-6 and TNF-α secretion in culture supernatants were determined in duplicate by using an ELISA kit according to the manufacturer’s instructions.

2.10. Western blot assays of ERK, JNK and p38 MAPKs in RAP-induced RAW264.7 cells
The procedure of western blot analysis was according to the methods described previously (Ha et al., 2005). Briefly, RAW264.7 cells (8×10^5 cells/well in 6-well plate) were treated with different concentrations of RAP and 100 ng/ml of LPS for 30 min. Then, cells were lysed in buffer containing 50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate and a protease inhibitor cocktail (Sigma- Aldrich, St. Louis, MO, USA) for preparation of cellular extracts. ERK, p38, JNK phosphorylated ERK, phosphorylated p38, and phosphorylated JNK was detected by Western blot analysis with anti-phospho-ERK, anti-phospho-p38, anti-phospho-JNK, anti-ERK, anti-JNK, and anti-p38 antibodies. Immunoreactive bands were normalized to that of control anti-β-actin antibodies. All blots were developed using ECL reagents (Bio-Rad, Richmond, CA).

### 2.11. Immunofluorescence staining

The glass slides were coated with rat tail collagen and put into 12-well plate. Then, RAW264.7 cells (5×10^5 cells/well in 12-well plate) were seeded into 12-well plate and incubated with or without RAP and LPS for 30 min. The slides were washed three times with PBS and permeabilized by adding 0.3% Triton X-100. The monoclonal anti-p65 Ab was added to each slide, and slides were incubated overnight at 4 °C. Then, the slides were incubated with FITC-labeled anti-rabbit IgG antibody and DAPI for 2 h and 30 min, respectively. For scanning, the stained glass slides were mounted with faramount mounting medium (Dako, Carpinteria, CA, USA) and observed using a Leica TCS SP2 microscope (Germany).
2.12. Statistical analysis

Statistical differences between each experimental group were examined by ANOVA, and statistical significance was determined at *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Each experiment was conducted three times or as indicated; all data are expressed as mean±SD (n=3).

3. Results

3.1. RAP enhanced cytotoxicity of supernatants from RAW264.7 cells

As shown in Figure 1A, both RAP and LPS exhibited no cytotoxicity towards RAW264.7 cells except at a very high concentration (RAP at 1000 μg/ml and LPS at 1000 ng/ml). Thus, the concentrations of 30, 100, and 300 μg/ml of RAP and 100 ng/ml of LPS were set for further study. Neither RAP (30, 100, and 300 μg/ml) nor LPS (100 ng/ml) alone directly affected the cell viability of 4T1 cells, compared to the control group (100%), and the results were shown in Figure 1B. However, as shown in Figure 1C, the cell viability of 4T1 cells significantly decreased to 90.6±2.8%, 87.2±3.6%, 85.6±2.7% when treated with the supernatants of RAW264.7 cells stimulated with RAP at concentrations of 30, 100 and 300 μg/ml, respectively, and to 84.2±3.2% when treated with the supernatants of RAW264.7 cells stimulated with 100 ng/ml of LPS. RAP and LPS themselves did not have cytotoxicity on 4T1
cells, but RAP and LPS enhanced the cytotoxicity of supernatants of RAW264.7 cells on 4T1 cells. The enhanced cytotoxicity of supernatants of RAP/LPS-treated RAW264.7 might be due to cytokines in the supernatants (Mac Micking et al., 1997), thus the contents of cytokines will be subsequently determined.

3.2. RAP induced IL-6, NO, and TNF-α production in RAW264.7 cells

ELISA assays and Greiss reagent were used to quantify IL-6, TNF-α and nitrite secretions in the conditioned medium of RAW264.7 cells. As shown in Figure 2, RAP-treated cells produced significantly more TNF-α, IL-6 and NO production (p<0.0001) compared with that of control cells. In Figure 2A1, the NO production of the control group was 4.95±1.09 μM, and that of the positive control LPS (100 ng/ml) group was 18.84±0.97 μM. When treated with RAP at the concentrations of 30, 100, and 300 μg/ml, RAW264.7 cells increased NO production to 16.38±0.50, 19.20±0.66, and 20.21±1.80 μM (p<0.0001).

Similarly, as seen in Figure 2B1, IL-6 secretion increased from 2.86±4.95 pg/ml (control group) to 283.57±33.11, 468.57±53.02, and 587.86±42.86 (p<0.0001) after RAP treatment at the concentration of 30, 100, and 300 μg/ml, respectively, in a dose-dependent manner. Production of TNF-α was also dramatically increased to 411.52±23.93, 800.20±19.03, and 916.43±29.41 pg/ml compared to that of control group (207.93±15.58 pg/ml, Figure 2C1). Production of IL-6 and TNF-α of RAW264.7 cells stimulated with LPS were 547.62±50.33 and 871.18±20.11 pg/ml (p<0.0001). These results showed that the functions of RAP in RAW264.7 cells were similar to those of LPS.
3.3. RAP up-regulated iNOS, TNF-α and IL-6 gene expression

RT-PCR method was used to detect the expression of iNOS, TNF-α and IL-6 genes in order to confirm the above observation. As shown in Figure 2, the gene expression levels of iNOS, TNF-α, and IL-6 also increased in a dose-dependent manner when the cells were incubated with RAP at concentrations of 30, 100, and 300 μg/ml. Gene expression levels of iNOS (Figure 2A2) in the cells of the control group, the three RAP groups and the LPS group were 1.04±0.40, 5.17±0.48, 6.41±0.15, 7.79±0.13, and 6.52±0.21, respectively. RAP treatment brought 5-8-fold increase in gene expression levels of iNOS, compared with the control group (p<0.0001). As demonstrated in Figures 2B2 and 2C2, RAP also generated similar dose-dependent increases in gene expression levels of IL-6 and TNF-α.

3.4. TLR4 mediates RAP-induced gene expressions and may participate in the signaling pathways of RAP

TLR4 inhibitor peptide inhibited gene expression and production of TNF-α, IL-6, and NO induced by LPS (100 ng/ml) and RAP (30 μg/ml) (Figure 3), suggesting that TLR4 might be a receptor of RAP, like LPS. As displayed in Figure 3A1, after treatment with the control peptide (with molecular weight similar to that of TLR4 inhibitor, but without the ability to inhibit TLR4), NO production of RAW264.7 cells induced by LPS (100 ng/ml) and RAP (30 μg/ml) were 23.94±1.54 and 19.76±1.19 μM, respectively. But the NO production of RAW264.7 cells was significantly
decreased to 15.76±0.45 and 14.38±1.01 μM, respectively \( (p<0.001) \), after treatment with TLR4 inhibitor. Significant decrease was also observed in TNF-α and IL-6 production of RAW264.7 cells treated with LPS/RAP and TLR4 inhibitor (Figures 3B₁ and 3C₁) compared with the cells treated with LPS/RAP and control peptide. When compared to the control group (428.57±28.57 pg/ml), RAW264.7 cells produced significantly less IL-6 (206.19±45.54 pg/ml, \( p<0.0001 \), Figure 3B₁) when treated with RAP and TLR4 inhibitor; Production of TNF-α was decreased to 226.79±16.42 pg/ml, \( p<0.0001 \) Figure 3C₁) compared to the control group (492.08±7.93 pg/ml).

The gene expression of iNOS, IL-6 and TNF-α of RAW264.7 cells induced by RAP (30 μg/ml) were increased to 4.46±0.40, 4.75±0.28, and 3.87±0.10 fold, respectively. Treatment with TLR4 inhibitor decreased the values to 2.05±0.80, 2.66±0.14, and 2.52±0.51, respectively. A similar trend was also observed in RAW264.7 cells induced by LPS (100 ng/ml). All of these results indicate that TLR4 is probably involved in the signaling pathways of cells treated with RAP and LPS.

Many studies have reported that MAPKs and NF-κB signaling pathways involved in TLR4 receptor (Kawai and Akira, 2006; Takeda and Akira, 2004). MAPKs and NF-κB signaling pathways may be associated with RAP-mediated gene expression and production of TNF-α, IL-6 and NO. Therefore, our further study focused on TLR4-related MAPKs and NF-κB.

3.5. RAP activated MAPK phosphorylation in RAW264.7 cells
To examine RAP-mediated signal transduction pathways in the regulation of TNF-α, IL-6 and NO gene expression, Western blot assays were used for analyzing the amounts of MAPK proteins, including six important MAPK members: phosphorylated-ERK, total ERK, phosphorylated-JNK, total JNK, phosphorylated-p38, and total p38. At the beginning, cells were stimulated by 100 µg/ml of RAP for different lengths of time, namely, 10 min, 20 min, 30 min, 45 min, 60 min, 90 min, 120 min, 4 h and 6h and detected the protein content with Western blot. The results (data not shown) indicated that phosphorylated proteins of RAW264.7 cells were first detected at 10 min, and the levels reached a maximum in 30 min. Thus, RAW264.7 cells were treated with 30, 100, and 300 µg/ml of RAP for 30 min, followed by Western blot analyses with anti-phospho-ERK Ab, anti-phospho-JNK Ab, anti-phospho-p38 Ab, anti-ERK Ab, anti-JNK Ab and anti-p38 Ab.

As shown in Figure 4, phosphorylated p38 (Figure 4A), phosphorylated ERK (Figure 4C), and phosphorylated JNK (Figure 4E) in RAW264.7 cells stimulated with RAP all increased markedly in a dose-dependent manner. The ratio of protein content between phosphorylated p38 and β-actin was 0.76±0.09, 1.00±0.07, and 1.01±0.11 at the concentrations of 30, 100, and 300 µg/ml of RAP, respectively, but the ratio of the control group was only 0.23±0.12 (Figure 4A, p<0.0001). After stimulation with 30, 100, and 300 µg/ml of RAP, the ratio of protein content between phosphorylated ERK and β-actin increased from 0.19±0.26 (control group) to 1.05±0.10, 1.59±0.07, and
1.60±0.05, respectively (Figure 4C, p<0.001); Moreover, the ratio of protein content
between phosphorylated JNK and β-actin was also significantly increased from
0.39±0.14 (control group) to 1.44±0.20, 2.04±0.08, and 2.05±0.20 (Figure 4E,
p<0.001) when cells were treated with 30, 100, and 300 μg/ml of RAP, respectively.
However, RAP did not influence expression levels of total ERK (Figure 4D), total
JNK (Figure 4F) or total p38 (Figure 4B) in RAW264.7 cells.

A series of three specific inhibitors, SB239063 (an inhibitor of p38 MAPK),
PD184352 (an inhibitor of MEK1/2), and SP600125 (an inhibitor of JNK/SAPK)
were used to confirm the mechanisms underlying NO, TNF-α, and IL-6 regulation of
RAW264.7 cells by RAP. MTT assays showed that these three inhibitors did not have
significant cytotoxicity in RAW264.7 cells in the concentrations in which we used
them (data not shown). ELISA analysis showed that SB239063 (Figure 5B, at
concentrations of 10, 20, and 40 μM), PD184352 (Figure 5C, at concentrations of 10,
20, and 40 μM), and SP600125 (Figure 5A, at concentrations of 25, 50, and 100 μM)
blocked RAP-induced NO production. Moreover, treatment with these three inhibitors
all dramatically decreased TNF-α (Figure 5G, 5H, 5I) and IL-6 (Figure 5D, 5E, 5F)
production of RAW264.7 cells in a dose-dependent manner. Western blot analyses
also showed these three inhibitors blocked RAP-induced protein contents of NO, IL-6,
and TNF-α in RAW264.7 cells (supplementary information Figure 1).

3.6. RAP induced IκB-α degradation and NF-κB translocation into nucleus.
The nuclear factor κB (NF-κB) signaling pathway is an essential pathway in the regulation of a wide variety of cellular genes, especially those involved in immune and inflammatory responses. Degradation of cytoplasmic inhibitor, IκB-α, is a vital step for activating the NF-κB family of transcription factors. Therefore, we firstly examined the effect of RAP on degradation of IκB-α using Western blotting analysis.

As shown in Figure 6A, the protein content ratio between IκB-α and β-actin dramatically decreased from 0.66±0.10 (control group) to 0.09±0.02, 0.09±0.06, and 0.10±0.02 (p<0.001), after RAP treatment at concentrations of 30, 100, and 300 μg/ml respectively. Furthermore, we also investigated the effect of RAP and LPS on p65 nuclear translocation using immunofluorescence staining. Results clearly showed that p65 was translocated from cytoplasm to nucleus and accumulated in the nucleus after 30 min treatment with RAP (30 μg/ml) (Figure 6B). A similar trend was also observed in RAW264.7 cells treated with LPS (100 ng/ml) (Figure 6B).

4. Discussion

Macrophage is one of the most important antigen-presenting cells (APCs) of innate immunity. The first event of innate immune response in mammals is activation of macrophages; these cells engulf invading pathogens and secrete intercellular messengers, such as IL-6, TNF-α, and NO (Zong et al., 2012). Furthermore, macrophages may be the first line of tumor resistance, as they rapidly colonize and secrete cytokines that attack tumor cells and they activate dendritic cells and natural
killer cells (Mills et al., 2000). Our study found that RAP alone was not cytotoxic
towards tumor cells 4T1, but it significantly enhanced the cytotoxicity of supernatants
from RAW264.7 cells on 4T1 cells (Figure 1). Enhanced production of TNF-α, IL-6,
and NO in RAW264.7 macrophages cells may explain the anti-tumor effects of
supernatants from RAP-treated RAW264.7 cells because IL-6 and TNF-α are typical
examples of multifunctional cytokines involved in the regulation of the immune
response, hematopoiesis and inflammation (Akira et al., 1990). IL-6 functions as a
differentiation factor on B cells and an activation factor on T cells. TNF-α and NO
play important roles in macrophage cytotoxic function in certain tumors in vivo
(MacMicking et al., 1997).

TLRs play a key role in innate immune responses to enhance the body’s defense
system against bacterial and viral infections (Kawai and Akira, 2006). Among TLRs,
TLR4 is known to induce production of TNF-α and IL-6. Moreover, because many
studies have showed that TLR4 mediates polysaccharide-induced cytokine production
(Hsu et al., 2004; Li and Xu, 2011). Various natural polysaccharides, such as
polysaccharides from *Ganoderma lucidum* (Hsu et al., 2004; Shao et al., 2004a),
*Polyporus umbellatus* (Li and Xu, 2011), safflower (Ando et al., 2002), *Platycodon
grandiflorum* (Yoon et al., 2003), and *Acanthopanax senticosus* (Han et al., 2003)
induce activations of macrophages via TLR4 receptor. It was hypothesized that TLR4
might be a candidate receptor/binding site for RAP. A previous study suggested that
Astragalus polysaccharides may interact with TLR4 in affecting peritoneal macrophages (Shao et al., 2004). Our results confirm that TLR4 is involved in the signaling pathways of cytokine production (Figures 3 and 5). However, the existence of a receptor recognizing RAP on macrophages other than TLR4 is also possible, because TLR4 inhibitor did not completely inhibit of TNF-α, IL-6, and NO production (Figure 3). Further experiments will be conducted for detecting other membrane receptor and/or intracellular receptors.

TLR signaling is mostly mediated by MAP kinases, such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK) (Kawai and Akira, 2006), which regulate cell functions including proliferation, gene expression, differentiation, mitosis, and apoptosis (Pearson et al., 2001). The results obtained in this study indicate that incubation of RAW264.7 cells with RAP quickly activates MAPKs, including phosphorylated-ERK, phosphorylated-JNK and phosphorylated-p38 (Figure 4). In addition, the signaling of phosphorylated-ERK, phosphorylated-JNK and phosphorylated-p38 may be associated with stimulation of downstream IL-6, TNF-α, and iNOS gene expression.

TLR4 interacts with different combinations of adapter proteins and activates various transcription factors such as nuclear factor NF-κB, AP-1 (activating protein-1), and interferon regulatory factors. The NF-κB pathway has long been
considered as a prototypical pro-inflammatory signaling pathway, largely based on the
essential role of NF-κB in the expression of cytokines genes (Lawrence, 2009). The
phosphorylation of IκB-α leads to its degradation, the release of NF-κB, and the
activation of NF-κB dependent genes, such as TNF-α, IL-1, and IL-6. Some studies
have showed that *Astragalus membranaceus* polysaccharides increases the production
of TNF-α, GM-CSF, and NO; this possibly occurs through the activation of
NF-κB/Rel (Lee and Jeon, 2005; Zhao et al., 2011). We tried to address whether RAP
was capable of activating NF-κB protein. In accordance with this previous study,
IκB-α degradation and NF-κB translocation induced by RAP (Figure 6) also prove
that NF-κB is an important signaling pathway in RAP-treated RAW264.7 cells.

In conclusion, RAP showed significant immunomodulating activities, such as
inducing production of the inflammatory cytokines TNF-α, IL-6 and NO. Moreover,
these cytokines may be associated with the anti-tumor effects of supernatants of
RAW264.7 cells induced by RAP. Besides, RAP triggers TLR4-mediated signaling
pathways through the production of phosphorylated ERK, phosphorylated JNK and
phosphorylated p38 and translocation of NF-κB; these signaling pathways may be
responsible for the cytokine production. RAP demonstrated strong
immunoenhancement activity through MAPKs and NF-κB signaling pathways, which
provides us a better understanding of the molecular mechanisms of RAP in the
activation of murine macrophages. Further understanding the signaling pathways and
the activation effects of macrophage induced by RAP might provide novel insights
into the mechanisms of immunomodulation and new opportunities for rational
application of RAP.

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7 Conflict of Interest

The authors declare no conflict of interest.
References


binding cassette transporter A1 in THP-1 derived foam cells exposed to tumor necrosis factor-alpha. Phytotherapy Research 24, 393-398.


**Figure 1.** MTT assays of RAP’s effects on cell viability. (A) RAW264.7 cells were treated with different concentrations of RAP and LPS for 24 h; (B) 4T1 cells were
treated with RAP and LPS for 24 h; (C) 4T1 cells were cultured with the supernatants of RAW264.7 cells which were treated with RAP and LPS for 24 h. LPS was used as positive control. Data are presented as mean±SD.*p<0.05, ****p<0.0001 compared with the control group.

Figure 2. Effects of RAP on the production of NO (A1), IL-6 (B1), and TNF-α (C1) and on gene expression of iNOS (A2), IL-6 (B2), and TNF-α (C2) in RAW 264.7 cells. RAW264.7 cells were treated with RAP (30, 100, and 300 μg/ml), positive control LPS (100 ng/ml) or vehicle for 24 h. Production of NO was detected by Griess reagent; IL-6 and TNF-α were measured by ELISA assays. Gene expression levels of iNOS, IL-6, and TNF-α were measured by RT-PCR. Gene expression levels of iNOS, IL-6, and TNF-α were normalized to that of β-actin. Values are shown as the fold-change relative to the control group. Data are presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with the control group.

Figure 3. TLR4 inhibitor’s effect on RAP-induced production of NO (A1), IL-6 (B1), and TNF-α (C1), and gene expression of iNOS (A2), IL-6 (B2), and TNF-α (C2) in RAW264.7 cells. RAW264.7 cells were pre-incubated with TLR4 inhibitor or control peptide for 1 h, and then treated with RAP (30 μg/ml) or LPS (100 ng/ml) for 24 h. NO production was determined by Griess reagent and the production of IL-6 and TNF-α were measured by ELISA assays. Gene expression levels of iNOS, IL-6, and TNF-α were measured by real-time PCR and normalized to that of β-actin. Values are
shown as the fold-change relative to the control group. Data are presented as mean±SD. *  \( p<0.05 \), **  \( p<0.01 \), ***  \( p<0.001 \), ****  \( p<0.0001 \) compared with the control group.

**Figure 4.** Western blot analysis of p-p38 (A), p-ERK (C), p-JNK (E), p38 (B), ERK (D), and JNK (F) induced by RAP in RAW264.7 cells. Cells were treated with RAP (30, 100, and 300 µg/ml), positive control LPS (100 ng/ml) for 30 min and then were lysed. Expression levels of p-p38 (A), p38 (B), p-ERK(C), ERK (D), p-JNK (E), and JNK (F) were determined by Western blot. **  \( p<0.01 \), ***  \( p<0.001 \), ****  \( p<0.0001 \) compared with the control group.

**Figure 5.** Effect of PK inhibitors, including SB239063 (p38 inhibitor), SP600125 (JNK inhibitor), and PD184352 (ERK inhibitor) on RAP-induced NO, IL-6, and TNF-α production in RAW264.7 cells. Cells were pre-incubated with various concentrations of individual inhibitors for 1 h, and treated with RAP (100 µg/ml) for 24 h. The supernatants were subjected to Greiss reagent for detecting NO production and ELISA assays for determining production of IL-6 and TNF-α. Data are presented as mean±SD (n=3). **  \( p<0.01 \), ***  \( p<0.001 \), ****  \( p<0.0001 \) compared with the control group.

**Figure 6.** Effects of RAP on IκB-α degradation and p65 nuclear translocation of RAW264.7 cells. (A) Cells were treated with RAP (30, 100, and 300 µg/ml), positive
control LPS (100 ng/ml) or control medium for 30 min. Cells were lysed and IκB-α expression level was determined by Western blot. (B) RAW 264.7 cells were treated with control medium, RAP (100 µg/mL) and LPS (100 ng/mL) for 30 min, respectively. Then, cells were immuno-stained with p65 (red) mAb and DAPI (nucleus, blue). The nuclear localization of p65 was determined using fluorescence microscopy after staining with DAPI, anti-p65, and FITC-labeled anti-rabbit IgG antibody (p65 translocation into nucleus were indicated by green arrows). Results are presented as mean±SD (n=3). Images shown here are representatives of three independent experiments.

**Figure 7.** The proposed RAP-mediated signaling pathways in the regulation of TNF-α, IL-6, and NO secretion.

**Supplementary information Figure 1.** Effect of PK inhibitors, including SB239063 (p38 inhibitor), SP600125 (JNK inhibitor), and PD184352 (ERK inhibitor) on RAP-induced protein levels of iNOS, IL-6, and TNF-α in RAW264.7 cells. RAW264.7 cells were pre-incubated with various concentrations of inhibitor for 1 h, followed by RAP stimulation for 24 h. After incubation, samples were subjected to Western blotting for detecting protein volumes of iNOS, IL-6, and TNF-α.
**Figure 1.** MTT assays of RAP’s effects on cell viability. (A) RAW264.7 cells were treated with different concentrations of RAP and LPS for 24 h; (B) 4T1 cells were treated with RAP and LPS for 24 h; (C) 4T1 cells were cultured with the supernatants of RAW264.7 cells which were treated with RAP and LPS for 24 h. LPS was used as positive control. Data are presented as mean±SD.*p<0.05, ****p<0.0001 compared with the control group.
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Figure 3. TLR4 inhibitor’s effect on RAP-induced production of NO (A₁), IL-6 (B₁) and TNF-α (C₁), and gene expression of iNOS (A₂), IL-6 (B₂) and TNF-α (C₂) in RAW264.7 cells. RAW264.7 cells were pre-incubated with TLR4 inhibitor or control peptide for 1 h, and then treated with RAP (30 μg/ml) or LPS (100 ng/ml) for 24 h. NO production was determined by Griess reagent and the production of IL-6 and TNF-α were measured by ELISA assays. Gene expression levels of iNOS, IL-6, and TNF-α were measured by real-time PCR and normalized to that of β-actin. Values are shown as the fold-change relative to the control group. Data are presented as mean±SD. * p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared with the control group.
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