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Signaling pathway underlying splenocytes activation by polysaccharides from *Atractylodis macrocephalae Koidz.*

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ABSTRACT

Previous study demonstrated that total polysaccharides isolated from *Atractylodis macrocephalae Koidz*. (*RAMPtp*) were effective to eliminate intramammary infection in cows. The present study was designed to investigate the immunomodulatory activity of *RAMPtp* in mouse splenocytes. Splenocyte proliferation, natural killer (NK) cytotoxicity, productions of NO and cytokines, transcription factor activity as well as the signal pathways and receptor were examined. The results showed that *RAMPtp* significantly promoted splenocyte proliferation and made the cells enter S and G2/M phases, increased ratios of T/B cells, boosted NK cytotoxicity, enhanced transcriptional activities of nuclear factor of activated T cells (NFAT), nuclear factor κB (NF-κB) and activator protein 1 (AP-1), and stimulated secretions of NO, immunoglobulin G (IgG) and multiple cytokine families (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IFN-γ, TNF-α, G-CSF, GM-CSF, KC, MIP-1α, MIP-1β, RANTES and Eotaxin). In addition, all the specific inhibitors against the mitogen-activated protein kinases (MAPKs) and NF-κB significantly suppressed the IL-6 production induced by *RAMPtp*. Moreover, splenocytes from Toll-like receptor 4 (TLR4) deficient mouse responded equally to *RAMPtp* stimulation as the wild-type. Therefore, *RAMPtp* might induce splenocytes activation at least in part via the TLR4-independent MAPKs and NF-κB signaling pathways. The present results would be useful to further understand the immunomodulatory mechanisms of *RAMPtp* in elimination of intramammary infection in cows.

1. Introduction

Atractylodis macrocephalae Koidz. is a plant in the family of *Compositae* and has a plenty of natural resources in Zhejiang Province in China. Its rhizome (*RAM*) has been utilized for more than 2000 years as a traditional Chinese medicine as it is believed to enhance the body resistance and stimulate digestion and recorded in *Chinese Pharmacopoei* (Xie et al., 2013). Oral administration of decoction made from *RAM* has been reported to enhance the immune response to vaccination (Li et al., 2009). *RAM* contains various components, such as volatile oil, lactones and polysaccharides (Duan et al., 2008). After comparison of the constituents for their immunomodulation, Xie et al. (2012) reported oral administration of the polysaccharide fractions significantly increased the immune responses to immunization against foot-and-mouth disease (FMD). Recently, Xu et al. (2015) observed that *RAMPtp* injected in the area of supramammary lymph node (SMLN) had therapeutic effect on bovine subclinical mastitis and somatic cell count in milk and intramammary infections were significantly reduced. Xu et al. (2017) also observed the immunostimulatory effect of *RAMPtp* on lymphocytes isolated from SMLN in cows. Therefore, the present study was designed to investigate the immunomodulatory effects of *RAMP* on mouse splenocytes by determination of cell proliferation, natural killer (NK) cytotoxicity, productions of NO and cytokines, and also to explore the signal pathways.

2. Materials and methods

2.1. Materials and chemicals

Concanavalin A (Con A, L7647), lipopolysaccharide (LPS, L2630) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, M2128) were purchased from Sigma Chemical Co., USA; FuGENE HD transfection reagent was the product of Promega, Madison, WI, USA; SB203580, PD98059, SP600125, BAY117082, NO detection kit and Firefly Luciferase Reporter Gene Assay Kit were the products of Beyotime Biotechnology, Jiangsu, China; Hank's balanced salt solution (HBSS) was purchased from Solarbio Science and Technology Co., Ltd, Beijing, China; RPMI-1640 was obtained from GENOM Inc., Hangzhou,

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China; fetal bovine serum (FBS) was purchased from Gibco, Grand Island, New York, USA; Cell Cycle and Apoptosis Analysis Kit was obtained from 7sea Pharmatech Co., Ltd, Shanghai, China; allophycocyanin (APC)-conjugated anti-mouse CD3e (Clone: 145-2C11) and phycoerythrin (PE)-conjugated anti-mouse CD19 (Clone: 1D3) monoclonal antibodies were procured from eBioscience, INC., San Diego, CA, USA; Bio-plex Pro mouse Cytokine 23-plex array kit was purchased from Bio-Rad Laboratories, Inc., Hercules, CA, USA; the PathDetect pNFAT-luc, pNF-κB-luc and pAP-1-luc plasmids were purchased from Stratagene, Santa Clara, CA, USA; Mouse IL-2, IL-6, TNF-α and IgG Enzyme-linked immunosorbent assay (ELISA) kits were procured from MultiSciences Biotech Co., Ltd, Hangzhou, China; all the other chemicals used were of analytical grade.

RAMPtp with purity of 95.66% was prepared and characterized in our previous study (Xu et al., 2018, 2017). Stock solutions of *RAMPtp* with concentration of 10 mg/ml were prepared by dissolving in PBS and sterilized by passing through a 0.22 μm Millipore filter. Then the polysaccharide solutions were subjected to a Detoxi-Gel Column (Thermo Scientific) and confirmed free of endotoxin with Limulus amebocyte lysate (LAL) test.

2.2. Animals and cells

Female ICR and C3H/HeJ mice (18–20 g, 6–8 weeks old) were purchased from Shanghai Laboratory Animal Center (SLAC) Co. Ltd. (Shanghai, China), and were maintained under a clear air condition. All the animals were treated following the Institutional Animal Care and Use Committee at Zhejiang University and the National Institutes of Health guide for the care and use of Laboratory animals. YAC-1 lymphoma cell line was procured from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China) and cultured in a humidified incubator at 37 °C with 5% CO_2 in RPMI 1640 supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FBS.

2.3. Splenocyte proliferation

Spleen was aseptically collected from sacrificed ICR mice with scissors, minced in cold HBSS and passed through nylon mesh filters to obtain a homogenous cell suspension. The erythrocytes were removed by the addition of Tris-NH_4Cl lysis buffer and kept on mice for 3 min. After washing twice with PBS, the isolated splenocytes were counted by trypan blue exclusion with viability above 95%. The assay for splenocytes proliferation was conducted by MTT method as described previously (Su et al., 2012). Briefly, splenocytes were added in 96-well microplates at 5×10^5 cells/well in a complete medium (RPMI 1640 supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin and 10% heat inactivated FBS) with *RAMPtp* at the concentrations of 12.5–200 μg/ml. Cells treated with RPMI 1640 medium, Con A (final concentration of 2.5 μg/ml) or LPS (final concentration of 2.5 μg/ml) only served as control groups. All tests were carried out in triplicate. The plates were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 44 h, and then 20 μl of MTT solution (5 mg/ml) was added to each well followed by further incubation for another 4 h. After removal of untransformed MTT by centrifugation at 1400 × g for 5 min, 150 μl of a DMSO solution was added to each well and the optical density (OD) at 570 nm (A570 value) was determined using a microplate reader (Multiskan FC, Thermo, USA). After incubation with *RAMPtp* (100 μg/ml) for 48 h, splenocytes were observed under light microscope to investigate morphological change of the cells.

2.4. Test of NO production

Splenocytes were added in a 96-well plate at 5×10^5 cells/well in a complete RPMI 1640 medium without or with *RAMPtp* at concentrations of 25, 50 and 100 μg/ml. All tests were carried out in triplicate. After incubation for 48 h at 37 °C in 5% CO_2 atmosphere, the plate was centrifuged at 1400 × *g* for 5 min. Then the supernatant was used to evaluate NO production based on the Griess method and the absorbance was measured at 540 nm using a microplate reader (Multiskan FC, Thermo, USA).

2.5. NK cell cytotoxicity assay

NK cell activity assay was carried out as per the method described previously (Jing et al., 2014). Briefly, splenocytes from ICR mice were prepared as effector cells, whereas YAC-1 cells were used as target cells. The effector cells (10^7/ml) were treated without or with *RAMPtp* at the dose of 25, 50 and 100 μg/ml and incubated for 48 h at 37 °C in 5% CO_2 atmosphere. Then the same volume of YAC-1 cells was added at the ratio of 25: 1 (effector cells: target cells). Besides, the wells with effector cells or target cells added alone were taken as effector and target cells control, respectively. Each group was repeated in three wells. After 4 h of incubation at 37 °C in 5% CO_2 atmosphere, the MTT method described above was employed to detect NK cell cytotoxicity. The A570 value for each well was measured using a microplate reader (Multiskan FC, Thermo, USA). The percentages of NK cell cytotoxicity were calculated based on the following formula:

$$\text{NK cell cytotoxicity} = [1\text{-}(OD_S\text{-}OD_E)/OD_T] \times 100\%$$

where OD_S, OD_E and OD_T represented the OD value of test sample, effector cell control and target cell control, respectively (Ling et al., 2017).

2.6. Analysis of cell cycle profiles

Splenocytes were added in a 24-well plate at 3×10^6 cells/well in a complete RPMI 1640 medium without or with *RAMPtp* at the concentration of 100 μg/ml. Con A (2.5 μg/ml) was used as a positive control. After incubation for 48 h at 37 °C in 5% CO_2 atmosphere, the cells were harvested, washed twice with PBS and fixed in 1 ml of 70% pre-cold ethanol at 4 °C overnight. After washing twice with PBS, the cells were labeled with PI in the presence of RNaseA at 37 °C for 30 min and analyzed by FCM (BD FACSCalibur). The percentages of cells in each phase were determined using ModFit LT 5.0 cell cycle analysis software (Becton Dickinson, NJ, USA). Proliferation index was calculated as SPF and PI values according to the following equations:

$SPF = S/(G0/G1 + S + G2/M) \times 100\%$; $PI = (S + G2/M)/(G0/G1 + S + G2/M) \times 100\%$ (Li et al., 2004).

2.7. Determination of cytokines and IgG

Splenocytes were added in a 96-well microplate at 5×10^5 cells/well in a complete RPMI 1640 medium without or with *RAMPtp* at the concentration of 25 and 100 μg/ml. Each test was performed in triplicate. After incubation for 48 h at 37 °C in 5% CO_2 atmosphere, the plate was centrifuged at 10 000 rpm for 10 min and then the supernatant was transferred into new tubes for analysis of IgG and multiple cytokines using the Bio-Plex Pro™ Mouse Cytokine 23-Plex Panel and the Bio-Plex Cytokine assay system (Bio-Rad Laboratories, Inc.) according to the manufacturer's instructions.

2.8. Inhibition of MAPKs and NF-κB using specific inhibitors

Splenocytes were treated with 100 μg/ml of *RAMPtp* supplied without or with p38 MAPK inhibitor (SB203580), extracellular regulated protein kinase (ERK) inhibitor (PD98059), c-Jun N-terminal kinase (JNK) inhibitor (SP600125) or NF-κB inhibitor α (IκBα) inhibitor (BAY117082), respectively. After incubation for 24 h at 37 °C in 5% CO_2 atmosphere, IL-6 was measured in the culture supernatants using an ELISA kit.

2.9. Transient transfection and luciferase reporter assay

Splenocytes were transiently transfected with pNFAT-TA-luc, pAP-1-luc or pNF-κB-luc plasmid using FuGENE HD transfection reagent in accordance with the manufacturer's instructions. The transfected cells were incubated at 37 °C in 5% CO_2 atmosphere for 48 h. After that, the cells were placed into a 96-well plate at 10^5 cells/well and treated without or with 100 μg/ml of *RAMPtp*. Each group was repeated in three wells. After another 6 h of incubation, the cells were harvested, lysed and measured for the luciferase activity. The specific transactivation was obtained by subtracting the background of lysis buffer in each experimental value and expressed as the relative luciferase activity.

2.10. Measurement of subpopulations of lymphocytes

Splenocytes were placed into a 24-well plate at 1.5×10^6 cells/well in a complete RPMI 1640 medium in the absence or presence of *RAMPtp* at the concentration of 25, 50 and 100 μg/ml. After incubation for 48 h at 37 °C in 5% CO_2 atmosphere, the cells were collected and washed twice with PBS, followed by incubation with 2 μl of APC-CD3 and PE-CD19 monoclonal antibodies at 4 °C for 1 h. After washing twice with PBS, the cells were then analyzed by flow cytometry (FCM, BD FACSCalibur).

2.11. Fluorescence staining, FCM analysis and the relevance of TLR4

Fluorescently labeled dextran (*fldextran*) and *RAMPtp* (*flRAMPtp*) with 5–10 μg/ml of fluoresceinamine were prepared in our previous study using the cyanogen bromide (CNBr)-activation method (Shao et al., 2004; Xu et al., 2017). To explore the binding of *RAMPtp* to splenocytes from ICR mouse, the cells (10^6/tube) were incubated with *fldextran* or *flRAMPtp* (100 μg/ml) in the absence or presence of *RAMPtp* (500 μg/ml) for 1 h at 4 °C, respectively. Then the cells were washed twice with PBS and subjected to FCM analysis. To address the question whether TLR4 was related to the immunostimulatory effect of *RAMPtp*, splenocytes from C3H/HeJ mouse were tested for their ability to respond to *RAMPtp* stimulation based on the assays of proliferation, receptor binding and productions of cytokines (IL-2 and TNF-α) as described above.

2.12. Statistical analysis

Results were expressed as mean ± SE of data obtained from triplicate experiments. Data analysis was performed with SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). Student's *t*-test was conducted between the experimental and the control group, and $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Effect of RAMPtp on splenocytes proliferation and morphological change

The spleen plays a critical role in regulating both the innate and the adaptive immune responses. Thus, it has always been an ideal organ to be used to study the immune response (Mebius and Kraal, 2005). Considering that the immunologic action of polysaccharides may begin with activating effector cells such as lymphocytes and macrophages, we investigated the immunostimulatory effect of *RAMPtp* on mouse splenocyte proliferation using MTT method in the present study. As presented in Fig. 1A, *RAMPtp* (12.5–200 μg/ml) significantly promoted the splenocytes proliferation in a concentration-dependent manner as reflected by the increased A570 values. After observation of splenocytes incubated with *RAMPtp* (100 μg/ml) for 48 h under a microscope, no morphological changes were found.

3.2. Effect of RAMPtp on NO released by splenocytes

NO has been found to be effective in the control of infectious pathogens, T and B lymphocytes differentiation as well as the antitumor defense (Bogdan, 2015). Previous study revealed that exopolysaccharides produced by *Lactococcus lactis* subsp. *lactis* could induced a significant increase in NO production in mouse spleen lymphocytes (Liu et al., 2013). Similar results shown in Fig. 1B indicated that *RAMPtp* triggered release of NO by splenocytes. This might be a reason for induction of splenocytes proliferation by *RAMPtp*.

3.3. Effect of RAMPtp on NK cell cytotoxicity

NK cells are innate lymphocytes that act as a defense line against viral infections and also play a pivotal role in antitumor immune responses (Bar et al., 2014). They are capable of lysing infected cells, mediating antibody-dependent cellular cytotoxicity (ADCC) of opsonized cells, and secreting pro-inflammatory cytokines such as IFN-γ, and activation of phagocytes (Nielsen et al., 2016). It has been reported that polysaccharides from a various sources such as *Litchi Pulp*, *Panax ginseng* C.A. Meyer and *Pleurotus eryngii* exhibited the capacity to improve the cytotoxicity of NK cells (Huang et al., 2014; Liu et al., 2015; Shin et al., 2017). Similar results were found in our present study. As presented in Fig. 1C, *RAMPtp* augmented NK cell activity in a dose-dependent manner as evidenced by significantly increased lysing of YAC-1 cells.

3.4. Effect of RAMPtp on splenocyte cycle

Splenocyte proliferation is closely associated with cell cycle distribution resulting from DNA synthesis and mitosis. The division of immune cells is essential for an effective immune response. Polysaccharide from *Litchi chinensis* has been found to stimulate splenic lymphocyte proliferation by promoting cells to enter S phase (Jing et al., 2014). Since *RAMPtp* is capable of stimulating splenocytes proliferation, it is implied to promote cells to enter S and G2/M phases of the cell cycle. To test this hypothesis, splenocytes were stained with PI after *RAMPtp* stimulation and the cell cycle profile was analyzed by FCM. As shown in Fig. 2A, *RAMPtp* significantly reduced the percentage of splenocytes in G0/G1 phase and increased that in G2/M and S phases. Accordingly, the calculated SPF and PI values in *RAMPtp*-treated splenocytes were also significantly higher than those of the medium control (Fig. 2B).

3.5. Effect of RAMPtp on the ratios of T and B lymphocytes

Splenocytes mainly contain T and B lymphocytes. CD3 and CD19 molecules are specific surface markers for T and B lymphocytes, respectively (Hotchkiss et al., 2001). Proteoglycan isolated from *Phellinus linteus* has been reported to significantly increase the percentage of $CD19^+$ cells while slightly decreased the population of $CD3^+$ cells, indicating its selective activation on B lymphocytes (Kim et al., 2003). To examine the effect of *RAMPtp* on lymphocyte subpopulation, FCM was employed to determine the ratios of $CD3^+$ and $CD19^+$ lymphocytes in mouse splenocytes stimulated with *RAMPtp*. As shown in Fig. 3, *RAMPtp* dose-dependently upregulated the ratio of $CD3^+$ (T) while downregulated that of $CD19^+$ (B) lymphocytes in mouse splenocytes. Correspondingly, *RAMPtp*-treated splenocytes were 1.33, 2.55 and 2.88 times the $CD3^+$/$CD19^+$ (T/B) ratios of the control, respectively.

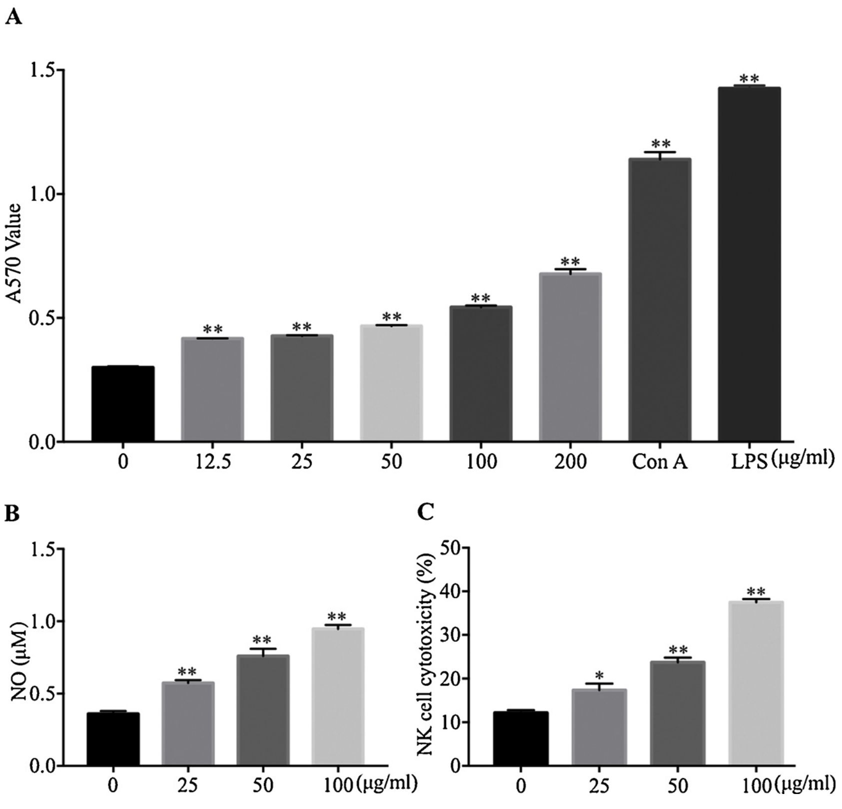

Fig. 1. Effect of *RAMPtp* on splenocyte proliferation, NO secretion and NK cell cytotoxicity. (A) Splenocytes were incubated with *RAMPtp*, Con A or LPS for 44 h, and then were subjected to MTT method to measure splenocyte proliferation. (B) Splenocytes were incubated with *RAMPtp* for 48 h and then measured for the NO level by Griess method. (C) Splenocytes as effectors were incubated with *RAMPtp*, YAC-1 cells as targets was added at the ratio of 25: 1 (effectors vs. targets), after 4 h of incubation, MTT method was performed to determine NK cell cytotoxicity. *$P < 0.05$ and **$P < 0.01$ versus the control group.

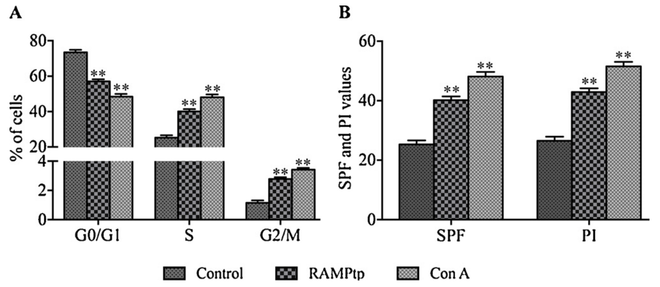

Fig. 2. Effect of *RAMPtp* on splenocyte cycle. (A) Percentages of splenocytes in G0/G1, S and G2/M phases; (B) SPF and PI values of mouse splenocytes. After stimulation with *RAMPtp* for 48 h, splenocytes were stained with PI and the cell cycle profile was determined using FCM followed by analysis with ModFit LT 5.0 software. **$P < 0.01$ versus the control group.

3.6. Effect of RAMPtp on secretions of interleukins, IFN-γ, TNF-α, CSF, chemokines and IgG

Cytokines are cell-secreted small peptides or proteins that play a fundamental role in cell-cell communication and regulation in the immune system (Stow et al., 2009). Most commonly cytokines have been classified into families of interleukins, colony-stimulating factors, interferons, tumor necrosis factors and chemokines (O'Brien et al., 2004). Granulocytes and macrophages release cytokines as soluble messengers to activate and recruit other cells during inflammation, or to kill targets directly (Hume, 2006).

Plenty of researchers have demonstrated that polysaccharides could induce secretions of various cytokines from many kinds of cell types. Jin et al. (2003) reported that polysaccharides from *Lentinus lepideuss* stimulated release of TNF-α, IL-1β, IL-10, IL-12, GM-CSF and IL-18 by PBMCs. Meng et al. (2014) found that polysaccharides from a novel *Cordyceps sinensis* fungus UM01 promoted diversified cytokines (IL-1α, IL-6, IL-10, TNF-α, MCP-1, MIP-1α, IP-10 and KC) released from RAW264.7 macrophages. Splenocytes consist of a variety of cell populations, including T and B lymphocytes, monocytes, dendritic cells and macrophages (PináChen, 2014). Feng et al. (2016) observed increased production of IL-4 and IFN-γ in splenocytes treated with *Eucommia ulmoides* polysaccharides. Wang et al. (2018) demonstrated the increased production of IL-2, TNF-α and IFN-γ in splenocytes stimulated by polysaccharides fractioned from marine clam *Meretrix meretrix*. To determine the effect of *RAMPtp* on secretion of cytokine, multiplex-bead ELISA was used to measure the cytokine concentrations in splenocytes after stimulation with 25 or 100 μg/ml of *RAMPtp*. As indicated in Fig. 4, *RAMPtp* promoted releases of interleukins (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-10, IL-12p40, IL-12p70 and IL-13), IFN-γ, TNF-α, colony-stimulating factors (G-CSF and GM-CSF) and chemokines (KC, MIP-1α, MIP-1β, RANTES and Eotaxin), indicating that *RAMPtp* could significantly stimulate splenocyte functions via regulating the secretion of multiple cytokines. In addition, the incereased IgG production was also found in *RAMPtp*-stimulated splenocytes, implying the activation of B lymphocytes.

3.7. Effect of specific pathway inhibitors on IL-6 production induced by RAMPtp

NF-κB plays a vital role in immune responses as well as transcriptional regulator of inflammatory cytokine genes. MAPKs family, composed of ERK, JNK, and p38 kinase, is also responsible for the signal transduction of the activation and cytokine secretion of lymphocytes. Polysaccharides from *Ganoderma atrum* and *Coriolus versicolor* have been reported to activate T and B lymphocytes via both MAPKs and NF-κB signaling pathways, respectively (Xiang et al., 2017; Yang et al., 2015). To determine the possible involvement of NF-κB and MAPK cascades in the immunomodulatory effect of *RAMPtp* on splenocytes, IL-6 was detected in splenocytes after 24 h exposure to *RAMPtp* in the presence or absence of specific inhibitors. As indicated Fig. 5A, all the inhibitors markedly reduced the production of IL-6, suggesting that NF-κB and MAPKs pathways were involved in the immunomodulatory activity of *RAMPtp* in mouse splenocytes.

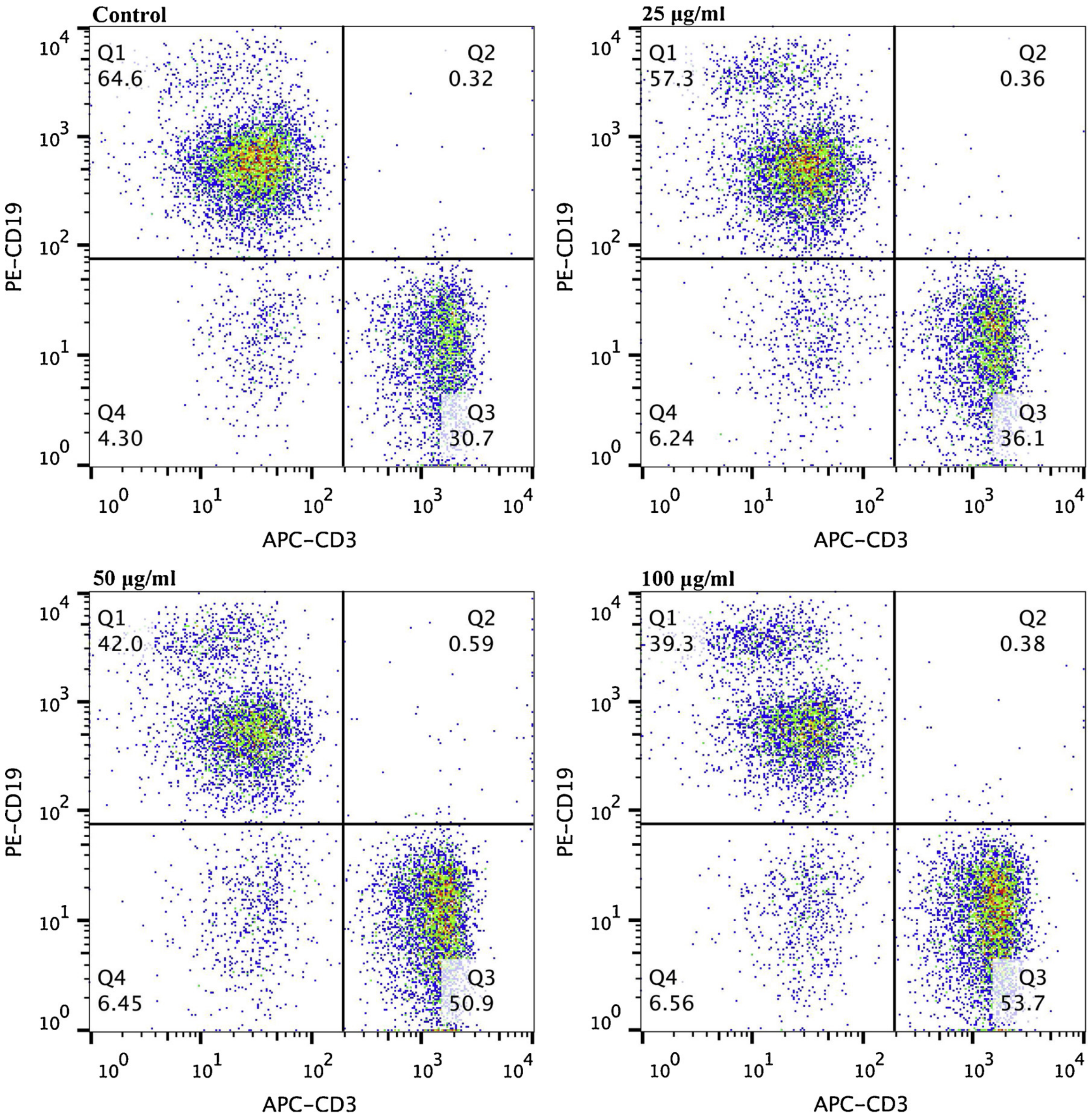

Fig. 3. Effect of *RAMPtp* on the ratios of CD3$^+$ and CD19$^+$ lymphocytes in miouse splenocytes. Splenocytes were incubated in absence or presence of *RAMPtp* for 48 h. The cells were then stained with APC-CD3 and PE-CD19 monoclonal antibodies at 4 °C for 1 h and analyzed by FCM.

3.8. Effect of RAMPtp on NFAT, NF-κB and AP-1 transcriptional activities

Transcription factors are proteins that bind specifically to defined DNA sequences to promote gene expression and therefore control cell function. Several families of transcription factors, including NFAT, NF-κB and AP-1, have already been proven to enter the nucleus and bind the promoter or enhancer of cytokine and chemokine genes to trigger the mRNA transcription when activated (Russell and Garrett-Sinha, 2010). Polysaccharide-protein complex from *Lycium barbarum* L. has been reported to induce secretions of IL-2 and IFN-γ in T lymphocytes via promoting the transcriptional activities of NFAT and AP-1 (Chen et al., 2008). Conversely, both gossypol and pseudoephedrine were found to suppresses T lymphocytes activation by inhibiting NF-κB, NFAT and AP-1 signaling pathways (Fiebich et al., 2012; Song et al., 2013). Additionally, artepillin C was found to inhibit the productions of IL-1β, IL-3, IL-4, IL-5, IL-9, IL-12p40, IL-13, IL-17, TNF-α, G-CSF, GM-CSF, MCP-1, MIP-1α, MIP-1β, RANTES and KC via blocking NF-κB in activated macrophages (Szliszka et al., 2013). To reveal the signaling pathways involved in the *RAMPtp*-mediated splenocytes activation, three transcription factors (NFAT, NF-κB and AP-1) were tested for their transcriptional activities. As shown in Fig. 5B, *RAMPtp* significantly activated NFAT, NF-κB and AP-1 compared to the medium control, suggesting that *RAMPtp* might induce splenocytes activation at least partly by targeting NFAT, NF-κB and AP-1 signaling pathways.

3.9. Role of TLR4 in RAMPtp-mediated activation of mice splenocytes

Mounting evidence suggested that the effective functions of polysaccharides were initiated from the recognition by pattern recognition receptors (PRRs), of which TLR4 was the most frequently studied polysaccharide receptors (Xie et al., 2016; Zhang et al., 2016). To demonstrate the binding of *RAMPtp* to target cells, freshly prepared splenocytes from ICR mouse were stained with either *fldextran* or *flRAMPtp* and then subjected to FCM analysis. Consistence with the functional study results, splenocytes were stained by *flRAMPtp*. In addition, the staining of *flRAMPtp* could be markedly inhibited by co-treatment with 500 μg/ml of *RAMPtp*, implying *RAMPtp* itself could bind to splenocytes (Fig. 6A). The observed broad distribution of fluorescence intensity was in line with an earlier work, which might be due to the polyclonal nature of B lymphocytes (Shao et al., 2004).

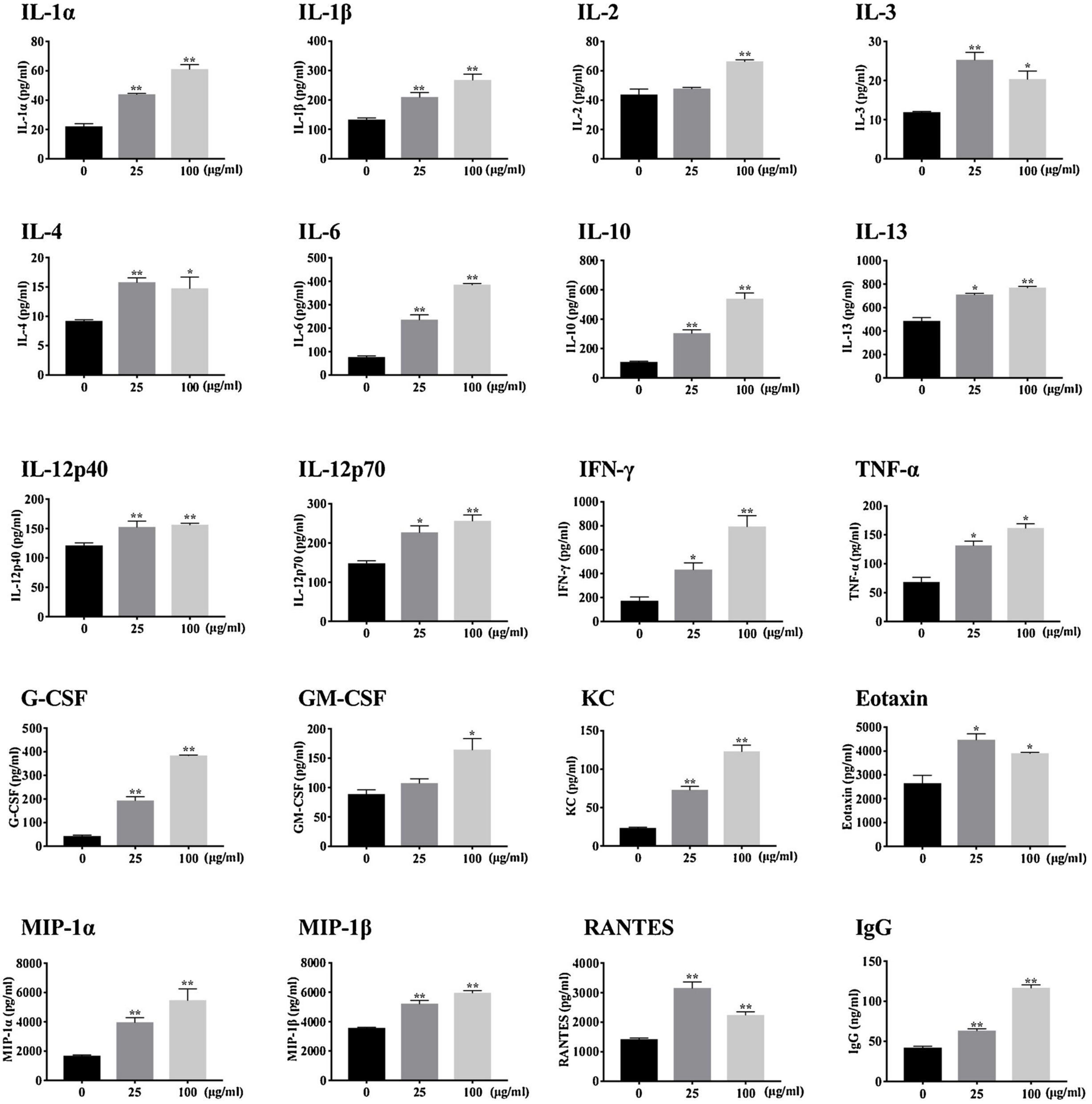

Fig. 4. Effect of *RAMPtp* on the multiple cytokines and IgG secreted from mouse splenocytes. Splenocytes were incubated in absence or presence of *RAMPtp* for 48 h, cytokines and IgG in the supernatant were then measured by ELISA. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus the control group.

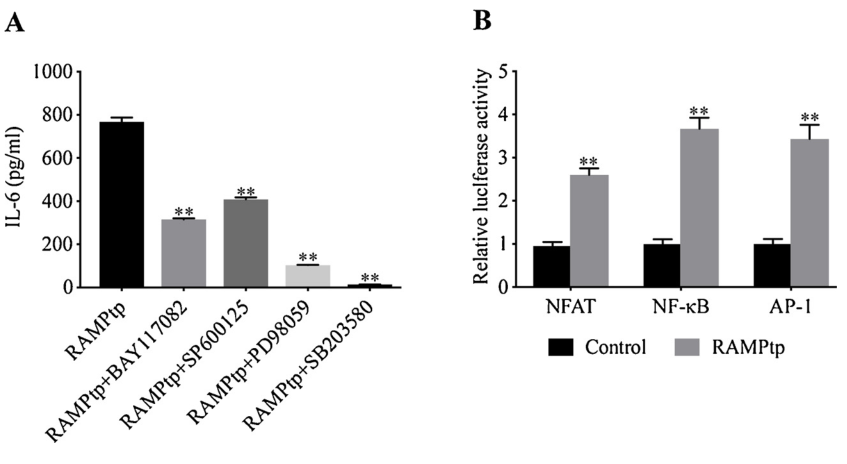

Fig. 5. Effect of *RAMPtp* on IL-6 secretion in the presence of specific inhibitors and transactivational activities of NFAT, NF-κB and AP-1 in mice splenocytes. (A) After incubation with *RAMPtp* in presence or absence of the indicated specific inhibitors for 24 h, IL-6 was measured in the supernatants using an ELISA kit. (B) Splenocytes transfected with NFAT, NFκB or AP-1 reporter vector were incubated for 48 h and then were stimulated with *RAMPtp* for another 6 h. After that, the cells were harvested, lysed and measured for the luciferase activity. $^{**}P < 0.01$ versus the control group.

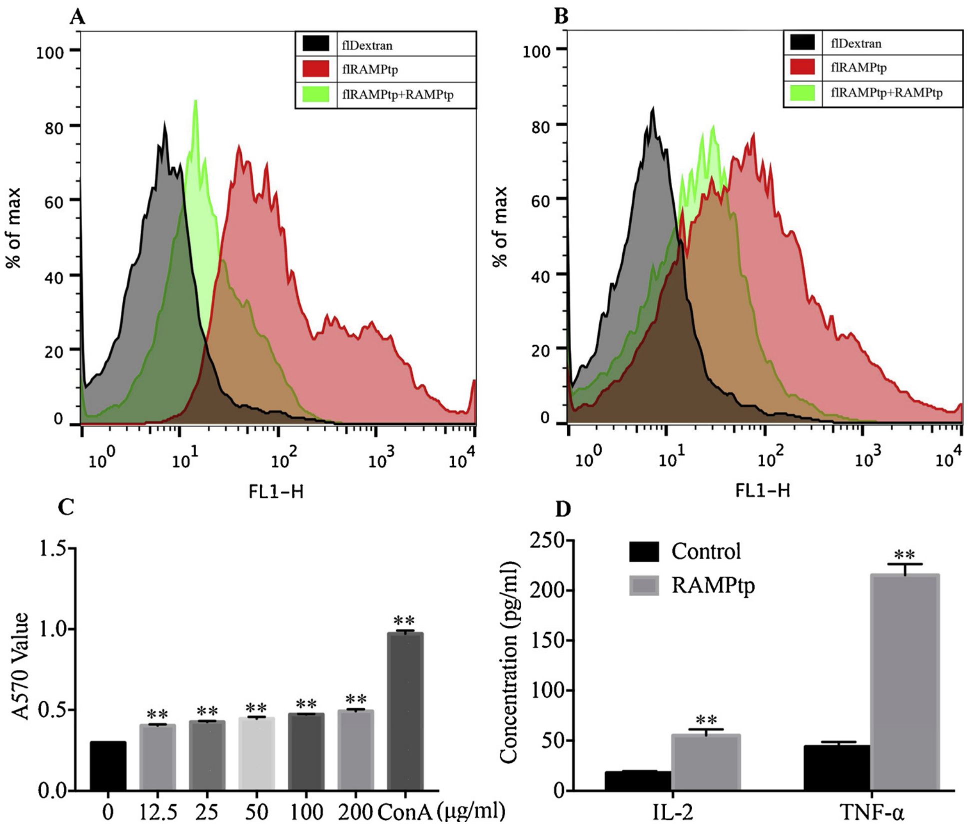

Fig. 6. Analysis of receptor binding, splenocyte proliferation and cytokine (IL-2 and TNF-α) production. (A and B) Splenocytes from ICR or C3H/HeJ mouse were incubated with *fldextran* or *flRAMPtp* in absence or presence of *RAMPtp* for 1 h at 4 °C and then were subjected to FCM analysis. (C) Splenocytes from C3H/HeJ mouse were incubated with *RAMPtp* for 44 h and then were subjected to proliferation assay by using MTT method. (D) Splenocytes from C3H/HeJ mouse were incubated without or with *RAMPtp* for 48 h, IL-2 and TNF-α concentrations in the supernatant were measured by ELISA. $^{**}P < 0.01$ versus the control group.

To verify whether TLR4 was the target receptor of *RAMPtp*, splenocytes from TLR4-deficient C3H/HeJ mouse were subjected to staining and inhibitory tests as described above. Similar results were obtained, in which *RAMPtp* was also found to bind to splenocytes from C3H/HeJ mouse (Fig. 6B). As shown in Fig. 6C/D, splenocytes derived from C3H/HeJ mouse responded equally to *RAMPtp* stimulation in terms of splenocytes proliferation and cytokine (IL-2 and TNF-α) production. Therefore, TLR4 might not be involved in *RAMPtp*-mediated splenocytes activation. However, given that splenocytes consist of various cell types, many issues with the immunomodulatory activity and the receptor of *RAMPtp* need to be further elucidated. Next, we will explore whether *RAMPtp* has direct impacts on purified T, B or monocytes from splenocytes and clarify its target receptor.

4. Conclusion

In summary, *RAMPtp* was investigated for its immunomodulatory activity in mouse splenocytes in vitro. The results showed that *RAMPtp* promoted splenocyte proliferation, increased T/B ratio, accelerated cell cycle progression, enhanced NK cytotoxicity and stimulated secretions of NO, IgG and various cytokines. The data from assays of specific inhibitors, transcriptional activity and receptor binding indicated that *RAMPtp* might induce splenocyte activation via the TLR4-independent MAPKs and NF-κB signaling pathways. However, considering that splenocytes consist of several types of cells, there are still many issues needed to be further clarified. Nevertheless, since it is the first time that the profile of *RAMPtp*-induced cytokine production in splenocytes was determined, our findings might offer theoretical evidence for *RAMPtp* used as a new-type immunotherapeutic agent and provide new mechanistic insights into the potential immunomodulatory activities of polysaccharides from medicinal herbs.

Conflict of interest

The authors declare that they have no competing interests.

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References

Bar, E., Whitney, P.G., Moor, K., Sousa, C.R.E., LeibundGut-Landmann, S., 2014. IL-17 regulates systemic fungal immunity by controlling the functional competence of NK cells. Immunity 40, 117–127.

Bogdan, C., 2015. Nitric oxide synthase in innate and adaptive immunity: an update. Trends Immunol. 36, 161–178.

Chen, Z., Tan, B.K.H., Chan, S.H., 2008. Activation of T lymphocytes by polysaccharide-protein complex from *Lycium barbarum* L. Int. Immunopharmacol. 8, 1663–1671.

Duan, Q., Xu, D., Liu, C., Li, C., 2008. Advances of the rhizome of *Atractylodis macrocephalae Koidz*. Chin. Tradit. Herb. Drugs. 39, 4–6.

Feng, H.B., Fan, J., Song, Z.H., Du, X.G., Chen, Y., Wang, J.S., Song, G.D., 2016. Characterization and immunoenhancement activities of *Eucommia ulmoides* polysaccharides. Carbohydr. Polym. 136, 803–811.

Fiebich, B.L., Collado, J.A., Stratz, C., Valina, C., Hochholzer, W., Muñoz, E., Bellido, L.M., 2012. Pseudoephedrine inhibits T-cell activation by targeting NF-κB, NFAT and AP-1 signaling pathways. Immunopharmacol. Immunotoxicol. 34, 98–106.

Hotchkiss, R.S., Tinsley, K.W., Swanson, P.E., Schmieg, R.E., Hui, J.J., Chang, K.C., Osborne, D.F., Freeman, B.D., Cobb, J.P., Buchman, T.G., 2001. Sepsis-induced apoptosis causes progressive profound depletion of B and $CD4^+$ T lymphocytes in humans. J. Immunol. 166, 6952–6963.

Huang, F., Zhang, R.F., Yi, Y., Tang, X.J., Zhang, M.W., Su, D.X., Deng, Y.Y., Wei, Z.C., 2014. Comparison of physicochemical properties and immunomodulatory activity of polysaccharides from fresh and dried Litchi pulp. Molecules 19, 3909–3925.

Hume, D.A., 2006. The mononuclear phagocyte system. Curr. Opin. Immunol. 18, 49–53.

Jin, M., Jung, H.J., Choi, J.J., Jeon, H., Oh, J.H., Kim, B., Shin, S.S., Lee, J.K., Yoon, K., Kim, S., 2003. Activation of selective transcription factors and cytokines by water-soluble extract from *Lentinus lepideus*. Exp. Biol. Med. 228, 749–758.

Jing, Y., Huang, L., Lv, W., Tong, H., Song, L., Hu, X., Yu, R., 2014. Structural characterization of a novel polysaccharide from pulp tissues of *Litchi chinensis* and its immunomodulatory activity. J. Agric. Food Chem. 62, 902–911.

Kim, G.Y., Park, S.K., Lee, M.K., Lee, S.H., Oh, Y.H., Kwak, J.Y., Yoon, S., Lee, J.D., Park, Y.M., 2003. Proteoglycan isolated from *Phellinus linteus* activates murine B lymphocytes via protein kinase C and protein tyrosine kinase. Int. Immunopharmacol. 3, 1281–1292.

Li, Z., Hu, D.Y., Chu, Q., Wu, J.H., Gao, C., Zhang, Y.Q., Huang, Y.R., 2004. Cell apoptosis and regeneration of hepatocellular carcinoma after transarterial chemoembolization. World J. Gastroenterol. 10, 1876.

Li, R.L., Sakwiwatkul, K., Li, Y.T., Hu, S.H., 2009. Enhancement of the immune responses to vaccination against foot-and-mouth disease in mice by oral administration of an extract made from *Rhizoma Atractylodis Macrocephalae* (RAM). Vaccine 27, 2094–2098.

Ling, N., Zhou, X., Ji, Y., Li, W., Ji, C., Qi, Z., 2017. Immunomodulatory and cellular antioxidant activities of κ-selenocarrageenan in combination with *Epirubicin* in H22 hepatoma-bearing mice. Biomed. Pharmacother. 91, 132–137.

Liu, L., Pan, D.D., Zeng, X.Q., Li, H., 2013. Effect of selenium-enriched exopolysaccharide produced by *Lactococcus lactis subsp lactis* on signaling molecules in mouse spleen lymphocytes. Food Funct. 4, 1489–1495.

Liu, X.K., Wang, L., Zhang, C.M., Wang, H.M., Zhang, X.H., Li, Y.X., 2015. Structure characterization and antitumor activity of a polysaccharide from the alkaline extract of king oyster mushroom. Carbohydr. Polym. 118, 101–106.

Mebius, R.E., Kraal, G., 2005. Structure and function of the spleen. Nat. Rev. Immunol. 5, 606–616.

Meng, L.Z., Feng, K., Wang, L.Y., Cheong, K.L., Nie, H., Zhao, J., Li, S.P., 2014. Activation of mouse macrophages and dendritic cells induced by polysaccharides from a novel *Cordyceps sinensis* fungus UM01. J. Funct. Food. 9, 242–253.

Nielsen, C.M., Wolf, A.S., Goodier, M.R., Riley, E.M., 2016. Synergy between common γ chain family cytokines and IL-18 potentiates innate and adaptive pathways of NK cell activation. Front. Immunol. 7, 101.

O'Brien, S.M., Scott, L.V., Dinan, T.G., 2004. Cytokines: abnormalities in major depression and implications for pharmacological treatment. Hum. Psychopharmacol. Clin. Exp. 19, 397–403.

PináChen, Y., 2014. Bioinspired resin acid-derived materials as anti-bacterial resistance agents with unexpected activities. Chem. Sci. 5, 2011–2016.

Russell, L., Garrett-Sinha, L.A., 2010. Transcription factor Ets-1 in cytokine and chemokine gene regulation. Cytokine 51, 217–226.

Shao, B.M., Xu, W., Dai, H., Tu, P., Li, Z., Gao, X.M., 2004. A study on the immune receptors for polysaccharides from the roots of Astragalus membranaceus, a Chinese medicinal herb. Biochem. Biophys. Res. Commun. 320, 1103–1111.

Shin, M.S., Hwang, S.H., Yoon, T.J., Shin, K.S., 2017. Polysaccharides from ginseng leaves inhibit tumor metastasis via macrophage and NK cell activation. Int. J. Biol. Macromol. 103, 1327–1333.

Song, B., Huang, G., Tong, C., Li, G., Wang, Z., Xiong, Y., Zhang, S., Lu, J., Guan, S., 2013. Gossypol suppresses mouse T lymphocytes via inhibition of NF-κB, NFAT and AP-1 pathways. Immunopharmacol. Immunotoxicol. 35, 615–621.

Stow, J.L., Low, P.C., Offenhauser, C., Sangermani, D., 2009. Cytokine secretion in macrophages and other cells: pathways and mediators. Immunobiology 214, 601–612.

Su, F., Yuan, L., Zhang, L., Hu, S., 2012. Ginsenosides Rg1 and Re act as adjuvant via TLR4 signaling pathway. Vaccine 30, 4106–4112.

Szliszka, E., Mertas, A., Czuba, Z.P., Król, W., 2013. Inhibition of inflammatory response by Artepillin C in activated RAW264. 7 macrophages. Evid. Based Complement. Altern. Med. 2013.

Wang, L., Chen, L., Li, J., Di, L., Wu, H., 2018. Structural elucidation and immune-enhancing activity of peculiar polysaccharides fractioned from marine clam *Meretrix meretrix* (Linnaeus). Carbohydr. Polym. 201, 500–513.

Xiang, Q.D., Yu, Q., Wang, H., Zhao, M.M., Liu, S.Y., Nie, S.P., Xie, M.Y., 2017. Immunomodulatory activity of *Ganoderma atrum* polysaccharide on purified T lymphocytes through $Ca2^{+}$/CaN and mitogen-activated protein kinase pathway based on RNA sequencing. J. Agric. Food Chem. 65, 5306–5315.

Xie, F., Li, Y.T., Su, F., Hu, S.H., 2012. Adjuvant effect of *Atractylodis macrocephalae Koidz.* polysaccharides on the immune response to foot-and-mouth disease vaccine. Carbohydr. Polym. 87, 1713–1719.

Xie, F., Sakwiwatkul, K., Zhang, C., Wang, Y., Zhai, L., Hu, S., 2013. *Atractylodis macrocephalae Koidz.* polysaccharides enhance both serum IgG response and gut mucosal immunity. Carbohydr. Polym. 91, 68–73.

Xie, S.Z., Hao, R., Zha, X.Q., Pan, L.H., Liu, J., Luo, J.-P., 2016. Polysaccharide of *Dendrobium huoshanense* activates macrophages via toll-like receptor 4-mediated signaling pathways. Carbohydr. Polym. 146, 292–300.

Xu, W., Guan, R., Lu, Y., Su, X., Xu, Y., Du, A., Hu, S., 2015. Therapeutic effect of polysaccharide fraction of *Atractylodis macrocephalae Koidz.* in bovine subclinical mastitis. BMC Vet. Res. 11, 165.

Xu, W., Guan, R., Shi, F., Du, A., Hu, S., 2017. Structural analysis and immunomodulatory effect of polysaccharide from *Atractylodis macrocephalae Koidz.* on bovine lymphocytes. Carbohydr. Polym. 174, 1213–1223.

Xu, W., Du, A., Hu, S., 2018. Transcriptome analysis of bovine lymphocytes stimulated by *Atractylodis macrocephalae Koidz.* polysaccharides *in vitro*. Vet. Immunol. Immunopathol. 196, 30–34.

Yang, S.F., Zhuang, T.F., Si, Y.M., Qi, K.Y., Zhao, J., 2015. *Coriolus versicolor* mushroom polysaccharides exert immunoregulatory effects on mouse B cells via membrane Ig and TLR4 to activate the MAPK and NF-κB signaling pathways. Mol. Immunol. 64, 144–151.

Zhang, X., Qi, C., Guo, Y., Zhou, W., Zhang, Y., 2016. Toll-like receptor 4-related immunostimulatory polysaccharides: primary structure, activity relationships, and possible interaction models. Carbohydr. Polym. 149, 186–206.