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Acetylation of polysaccharide from *Morchella angusticeps* peck enhances its immune activation and anti-inflammatory activities in macrophage RAW264.7 cells

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ABSTRACT

Morchella angusticeps Peck has been recognized as a resource of nutraceuticals and drug discovery. Three acetylated polysaccharides (Ac-PMEP$_{1\text{-}3}$) with appropriate degree of substitution were obtained from *Morchella angusticeps* Peck, chemically characterized, and cultured with macrophage RAW264.7 cells to evaluate their immune activation and anti-inflammatory activities. Results of ultraviolet–visible spectroscopy and fourier-transform infrared showed these modifications were successful. Compared with the control group, PMEP and Ac-PMEP$_{1\text{-}3}$ enhanced cell proliferation and the production of nitric oxide and tumor necrosis factor-α of RAW264.7 macrophages (cultured without lipopolysaccharide). Compared with PMEP, Ac-PMEP$_3$ enhanced cell viability and NO production by inducing the degradation of cytoplasmic IκBα and nuclear translocation of NF-κB subunit p65 as well as the expression of iNOS and phosphorylated-p38. Moreover, in lipopolysaccharide-stimulated RAW264.7 macrophages, Ac-PMEP$_3$ showed a stronger ability to suppress the overproduction of nitric oxide and tumor necrosis factor-α by down-regulating the level of nuclear NF-κB p65, iNOS, and phosphorylated-p38 and inhibiting the degradation of cytoplasmic IκBα. Therefore, Ac-PMEP enhanced immune activation and anti-inflammatory activities via nuclear factor κB and p38/mitogen-activated protein kinase signaling pathways.

1. Introduction

Immune response is an important physiological process of the living organism, where macrophages play a significant role via its phagocytic, cytotoxic and intracellular killing activities (Ren et al., 2017). Once activated, macrophages can kill pathogens directly by phagocytosis and indirectly by producing biological factors including nitric oxide (NO) and tumor necrosis factor-α (TNF-α) (Ma et al., 2016). However, excessive macrophages activation results in the production of a large amount of pro-inflammatory chemicals which bring damages to the living organism (Sun et al., 2016). Inflammation is an essential response of the immune system that helps protect the body against infection and tissue injury, but chronic inflammation increases the risk of chronic diseases such as cancer, diabetics, rheumatoid arthritis, and cardiovascular diseases (Byun et al., 2014; Divate and Chung, 2017). Lipopolysaccharide (LPS) -stimulated RAW 264.7 murine macrophage is a well-established model to study inflammatory responses (Yang et al., 2018; Mendis et al., 2016). Recently, interest in developing polysaccharides as nutraceuticals and medicines to regulate immune system via controlling macrophages activation and cytokines production is increasing (Wang et al., 2017). It has been reported that polysaccharides have a dual effect on macrophage, namely immunological activation and anti-inflammatory immunosuppression (Sun et al., 2016). Polysaccharides isolated from *Pleurotus nebrodensis* can not only promote the production of TNF-α, interleukin-6 (IL-6), interleukin-10 (IL-10), and iNOS in normal RAW264.7 macrophages, but also inhibit these cytokines secretion in LPS-induced inflammatory macrophages (Wang et al., 2014a). Moreover, previous studies found that acetylated modification of pumpkin polysaccharides could significantly improve their antioxidant and cytoprotective activities (Song et al., 2013).

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Morchella angusticeps Peck, a precious edible mushroom belonging to *Morchella esculenta* (L.) Pers., has been used as a traditional Chinese medicine for thousands of years. Recent studies have revealed that polysaccharides from *Morchella esculenta* exhibit a wide range of pharmacological activities, including antitumor activity, anti-oxidative activity, and cholesterol-lowering activity (Liu et al., 2016; Fu et al., 2013; Li et al., 2017). Our previous study showed that the water-soluble polysaccharide from *Morchella angusticeps* Peck (PMEP) with the molecular weight of 43,600 Da had a hypolipidemic activity (Ming et al., 2009a, 2009b). To the best of our knowledge, the immunostimulatory and anti-inflammatory activities of acetylated PMEP have not been reported yet.

Therefore, this study was carried out to 1) prepare and characterize chemically acetylated PMEP (Ac-PMEP); and 2) study the immunostimulatory and anti-inflammatory activities of Ac-PMEP in RAW264.7 cells under different experimental models compared with that of PMEP, with focusing on the expression of p65, IκB-α, iNOS, p38, and p-p38.

2. Materials and methods

2.1. Materials

The water-soluble PMEP was obtained according to our previous method (Li et al., 2017). RAW264.7 cells were purchased from American Type Culture Collection (Manassas, VA, USA). William's Medium E and Dulbecco's modified eagle's medium (DMEM) were purchased from Sigma-Aldrich (St Louis, MO, USA). Fetal bovine serum (FBS) and Trypsin-EDTA 0.05% were obtained from Life-Technologies Corporation (Carlsbad, CA, USA). Neutral red was obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Antibodies of p65, IκB-α, iNOS, p38, p-p38 were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, Jiangsu, China). LPS was purchased from Sigma-Aldrich. All of the other reagents and chemicals were of analytical reagent grade.

2.2. Acetylation of PMEP

Acetylation of PMEP was performed by the reported method with modifications (Chen et al., 2014). Briefly, PMEP (0.5 g) was dispersed with 15 mL ultrapure water under stirring. The pH was adjusted to 9.0 with 10 M NaOH and then the homogeneous solution was kept at room temperature for 10 min. To obtain acetylated derivatives with variable degree of substitution, 1 mL, 4 mL, 6 mL of acetic anhydride were added, respectively. 10 M NaOH was subsequently added to each mixture to maintain the pH at 8.0–8.5. After 30 min, the reaction was stopped with 10 M HCl and the pH was adjusted to 7.0. The resultant solution was dialyzed against tap water for 48 h and then distilled water for another 48 h with a 8000 Da MW cut off membrane, concentrated, and precipitated by 95% alcohol. The precipitates were dissolved in distilled water and freeze-dried to obtain three types of acetylated polysaccharides, namely Ac-PMEP$_1$ (1 mL acetic anhydride), Ac-PMEP$_2$ (4 mL acetic anhydride), and Ac-PMEP$_3$ (6 mL acetic anhydride).

2.3. Total sugar content

The total sugar content of PMEP and Ac-PMEP$_{1\text{-}3}$ was determined using phenol-sulfuric acid colorimetric method with glucose as a standard at 620 nm (Dubois et al., 1956). Briefly, 1 mL sample solution and 5 mL phenol-sulfuric acid were added to the test tube, boiled, stirred for 10 min, and then cooled to room temperature for 15 min before measurement.

2.4. Degree of substitution of Ac-PMEP

The degree of substitution (DS) of Ac-PMEP$_{1\text{-}3}$ was determined as described by Sánchez-Rivera et al. (2010) with a slight modification. 20 mg Ac-PMEP$_{1\text{-}3}$ was dissolved in 10 mL NaOH (0.01 M) and agitated at 50 °C for 2 h. The excessive amount of NaOH was back-titrated with 0.01 M HCl with phenolphthalein as an indicator. The acetyl content (A, %) and the DS were calculated as the following equations with PMEP as a control group:

$$A\ (\%) = [(V_0C_0 - V_1C_1) \times 0.043 \times 100] / W$$

$$DS = 162A / \qquad (4300\text{-}42A)$$

Where V_0 is the volume of NaOH (mL), C_0 is the concentration of NaOH (mol/L), V_1 represents the volume of HCL (mL), C_1 represents the concentration HCl (mol/L), and W is the sample mass.

2.5. UV spectra analysis

UV Spectra of the PMEP and Ac-PMEP$_{1\text{-}3}$ were detected by a UV-2450 ultraviolet spectrograph (Shimadzu, Japan) as modified method of Feng et al. (2010). Briefly, 0.1 mg sample was dissolved in 1 mL distilled water and UV spectra were carried out at the range of 400–190 nm.

2.6. The fourier-transform infrared spectroscopy (FT-IR) analysis

PMEP and Ac-PMEP$_{1\text{-}3}$ were characterized by a 100 FT-IR spectrometer (PerkinElmer, Akron, OH, USA) as modified method of Chen et al. (2014). 5 mg sample and 100 mg KBr powder were dried, ground, and pressed into a pellet for scans over the spectral range of 4000–400 cm^{-1}.

2.7. Cell viability

Cell viability was measured according to the modified method reported by Mosmann (1983). Briefly, RAW264.7 cells diluted into 5×10^5 cell/well were inoculated in 96-well plates at 37 °C in an atmosphere of 5% CO_2. After 24 h, 100 μL fresh medium containing 10 μL distilled water or different concentrations of samples solutions was added to corresponding wells (the final concentration of the samples were 0, 3.125, 12.5, 50, 200, and 400 μg/mL, respectively) to culture for another 24 h. LPS (1 μg/mL) was used as a positive control. Then, the culture medium was removed and cells were washed with phosphate buffered saline (PBS) (0.01 M, pH 7.4). Cells were stained with methylene blue and the absorbance was measured at 570 nm using an ELISA reader (Molecular Devices co. USA). Cell viability (%) was calculated as follows: $A_2/A_1 \times 100$, where A_2 and A_1 represent the OD values of the stimulated and negative control wells, respectively. The percentage of cell viability in the negative control well was designated as 100%. Noncytotoxic doses of PMEP and Ac-PMEP$_{1\text{-}3}$ were selected to conduct immunization and anti-inflammatory assessments.

2.8. Phagocytic capacity

The phagocytic activity of macrophage was evaluated through neutral red uptake assay reported by Liu et al. (2017) with slight modifications. RAW264.7 cells were cultured with 100 μL fresh medium containing 10 μL distilled water or different concentrations of samples solutions for 24 h. LPS (1 μg/mL) was used as a positive control. Then, the culture medium in each well was replaced with 100 μL 0.01% of neutral red solution, incubated for another 3 h, and washed five times with PBS. Cells were then lysed with addition of 200 μL of cell lysis buffer (50% ethanol and 50% acetic acid, v/v) and shaken slightly for 30 min at room temperature. The absorbance was measured at 540 nm using an ELISA reader. Cell phagocytosis (%) was calculated as follows: $A_2/A_1 \times 100$, where A_2 and A_1 represent the OD values of the stimulated and negative control wells, respectively. The percentage of cell phagocytosis in the negative control well was designated as 100%.

2.9. RAW264.7 cells in the absence or presence of LPS under two experimental models to assess the immunostimulatory and anti-inflammatory effects of PMEP and Ac-PMEP$_{1-3}$

For experiment I, RAW264.7 cells with samples at different concentrations were cultured in 96-well plates and incubated for 24 h. The supernatants in the cell cultures were collected and stored at −80 °C for further analysis.

For experiment II, RAW264.7 cells were firstly incubated with samples at different concentrations for 12 h. Then, the supernatant was removed and the cultured cells were washed three times with PBS. LPS (1 μg/mL) was added to each well to evaluate the preventive effects of Ac-PMEP$_{1-3}$ on LPS-induced inflammation. The plate was then incubated for another 24 h. Distilled water and LPS (1 μg/mL) were used as negative and positive controls, respectively.

2.9.1. Release of NO and TNF-α

RAW264.7 cells culture supernatant in treatment I& II was collected to measure NO and TNF-α levels. The release of NO was measured according to instructions provided by NO Kit (Beijing Biyuntian Company, China). TNF-α level was measured according to instructions of TNF-α Kit (Shanghai Sino Best Biological Technology Co., Ltd., China).

2.9.2. Western blotting analysis

RAW264.7 cells were cultured according to experiment I & II in the absence or presence of samples at different concentrations for 24 h on a 6-well plate. The supernatant was removed and cells were washed twice with PBS. Cells were then lysed, ultrasonicated, and centrifuged at 14000 *g* for 15 min to isolate total cellular protein. Hypotonic buffer was added and centrifuged at 15000 rpm for another 10 min to obtain nuclear protein. Western blotting was carried out as reported by Burnette (1981). Membranes were cultured with 1:200 anti-iNOS, 1:200 anti-p65, 1:200 anti-IκBα, 1:200 anti-p38, 1:200 anti-p-p38, and 1:1000 β-actin antibodies, respectively, (Nanjing Jiancheng Bioengineering Institute) overnight at 4 °C. After that, membranes were washed with TBST solution and incubated with 1:4000 dilutions secondary antibody (Nanjing Jiancheng Bioengineering Institute) at 4 °C for another 1–2 h. Densitometry was analyzed using BioRad Quantity One® (BioRad Laboratories, Hercules, USA). Protein concentrations of iNOS, p65, IκBα, p38 and p-p38 were normalized with β-actin (Santa Cruz Biotechnology, Santa Cruz, Paso Robles, CA, USA).

2.10. Statistical analysis

Results were presented as mean ± standard deviation (SD), n = 3. All data were analyzed using one-way analysis of variance (ANOVA) followed by Dun-can's multiple-range test (SPSS Inc, PASW Statistics for Windows, Version 16.0, Chicago, IL, USA). Significance was considered as $p < 0.05$.

3. Results

3.1. Characterization of PMEP and Ac-PMEP$_{1-3}$

The total sugar content, yield, and DS of Ac-PMEP$_{1-3}$ are shown in Table 1. The total sugar content of Ac-PMEP$_{1-3}$ was about 73%. The yield was increased as the DS of Ac-PMEP increased as follows: Ac-PMEP$_3$ > Ac-PMEP$_2$ > Ac-PMEP$_1$. As shown in Fig. 1A, there was no significant difference among the four spectra of PMEP, Ac-PMEP$_1$, Ac-PMEP$_2$, and Ac-PMEP$_3$ between 190 and 400 nm. The FT-IR spectra of PMEP and Ac-PMEP$_{1-3}$ in 4000–400 cm^{-1} were similar (Fig. 1B). After acetylation, a new absorption occurred at around 1738.65 cm^{-1}. In addition, the absorption at around 1248.93 cm^{-1} remarkably increased (Liu et al., 2012). These results showed that PMEP was successfully acetylated with minor changes of the molecular structure.

Table 1
Total sugar content, degree of substitution (DS), and yield of acetylated polysaccharide from *Morchella angusticeps* Peck (Ac-PMEP).

Acetylated derivatives	Ac-PMEP$_1$	Ac-PMEP$_2$	Ac-PMEP$_3$
Total sugar content (%)	73.30 ± 2.79	73.61 ± 1.54	72.65 ± 1.58
Yield (%)	61.30 ± 0.88	65.18 ± 0.64	74.64 ± 1.05
DS	0.20 ± 0.02	0.29 ± 0.02	0.40 ± 0.07

Data are expressed as mean ± SD. Ac-PMEP$_1$, polysaccharide from *Morchella angusticeps* Peck acetylated with 1 mL acetic anhydride; Ac-PMEP$_2$, polysaccharide from *Morchella angusticeps* Peck acetylated with 4 mL acetic anhydride; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride.

3.2. Effects of PMEP and Ac-PMEP$_{1-3}$ on the viability and phagocytosis of RAW264.7 cells

Fig. 2 illustrates the viability of RAW264.7 cells treated with PMEP and Ac-PMEP$_{1-3}$ with different concentrations. Compared with the negative control group (cells without polysaccharides or LPS), Ac-PMEP$_3$ had the highest proliferation ratio of 112.73 ± 1.93% at the concentration of 50 μg/mL. However, the viability of RAW264.7 cells treated with PMEP and Ac-PMEP$_{1-3}$ decreased at the concentration of 400 μg/mL compared to the negative control group. Therefore, treatment of PMEP and Ac-PMEP$_{1-3}$ at concentrations of 3.125, 12.5, 50, and 200 μg/mL were selected to perform cytokine secretion assays.

Compared with the negative control group, PMEP at the concentration of 3.125–50 μg/mL increased the phagocytosis of RAW264.7 cells significantly while Ac-PMEP$_3$ enhanced phagocytic activity of RAW264.7 cells from 3.125 to 200 μg/mL ($p < 0.05$, Fig. 3).

3.3. Effects of PMEP and Ac-PMEP$_{1-3}$ treated alone on the production of NO and TNF-α in RAW264.7 cells

To examine the effect of the immunoregulatory activity of obtained polysaccharides, PMEP and Ac-PMEP$_{1-3}$ at the concentrations of 3.125–200 μg/mL were added to RAW264.7 cells cultures for 24 h (Fig. 4). In experiment I, compared with the negative control, levels of NO and TNF-α secreted by the macrophages were significantly increased by LPS ($p < 0.01$). Immunity signaling molecule secretions of TNF-α and NO were increased dramatically by PMEP and Ac-PMEP$_{1-3}$ ($p < 0.05$) (Fig. 4A, C). Compared with the negative control group, NO production of RAW264.7 cells treated with PMEP and Ac-PMEP$_{1-3}$ at the concentrations of 3.125 and 12.5 μg/mL were increased by 48–117% and 121–170%, respectively. Moreover, TNF-α secretion of RAW264.7 cells treated with PMEP and Ac-PMEP$_3$ at the concentrations of 12.5 and 200 μg/mL were increased by 13–23% and 40–43%, respectively. Thus, Ac-PMEP presented a higher immunoregulatory activity than PMEP. However, TNF-α secretion of RAW264.7 cells treated with PMEP, Ac-PMEP$_2$ and Ac-PMEP$_3$ at the concentration of 200 μg/mL were higher than that treated with LPS, indicating 200 μg/mL of PMEP or Ac-PMEP might exert pro-inflammatory effect.

3.4. Anti-inflammatory effects of PMEP and Ac-PMEP$_{1-3}$ on LPS-stimulated secretion levels of NO and TNF-α in RAW264.7 cells

To examine the preventive effect of PMEP and Ac-PMEP$_{1-3}$ on LPS-triggered inflammation, PMEP and Ac-PMEP$_{1-3}$ were added to RAW264.7 cells cultures for 12 h before LPS stimulation. In experiment II, compared with the RAW264.7 cells treated alone with LPS, NO and TNF-α secretions by LPS-stimulated RAW264.7 cells were markedly ($p < 0.01$) inhibited by PMEP and Ac-PMEP$_{1-3}$ at the concentrations of 50 and 200 μg/mL (Fig. 4B, D). Compared with the positive control group, NO production of RAW264.7 cells treated with PMEP + LPS and Ac-PMEP$_{1-3}$ + LPS at the concentrations of 50 and 200 μg/mL were decreased by 6–33% and 13–55%, respectively. Furthermore, TNF-α

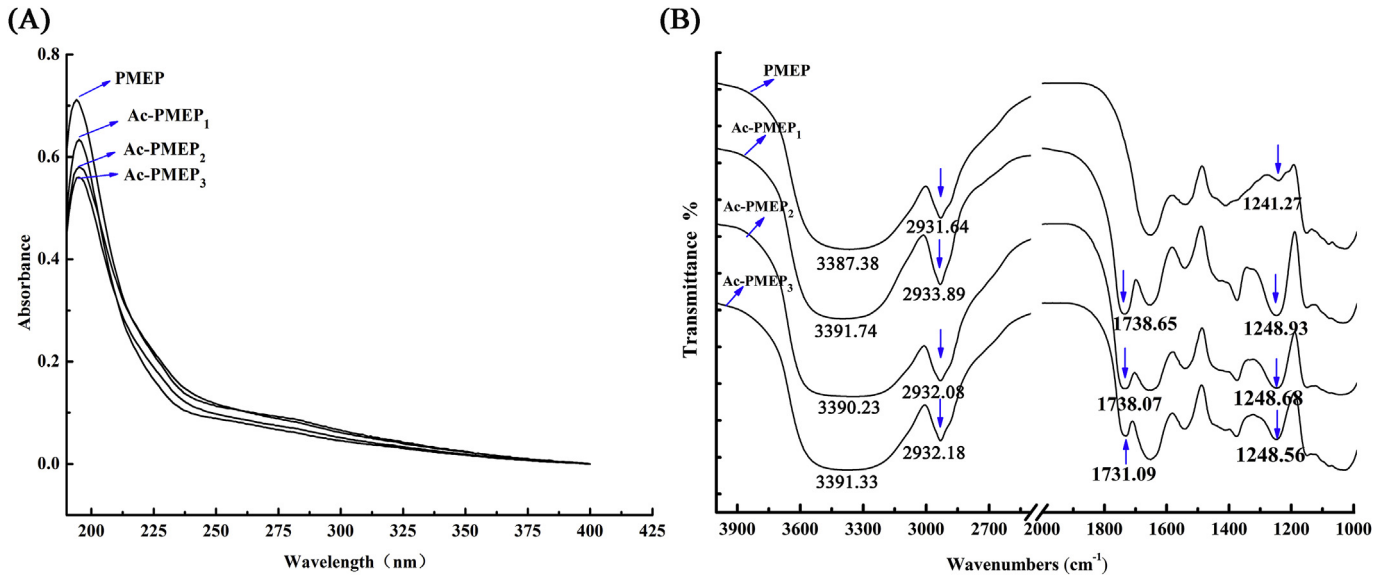

Fig. 1. Ultraviolet scanning spectra (A) and fourier transforms infrared spectra (B) of PMEP and Ac-PMEP$_{1\text{-}3}$. PMEP, polysaccharide from *Morchella angusticeps* Peck; Ac-PMEP$_1$, polysaccharide from *Morchella angusticeps* Peck acetylated with 1 mL acetic anhydride; Ac-PMEP$_2$, polysaccharide from *Morchella angusticeps* Peck acetylated with 4 mL acetic anhydride; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride.

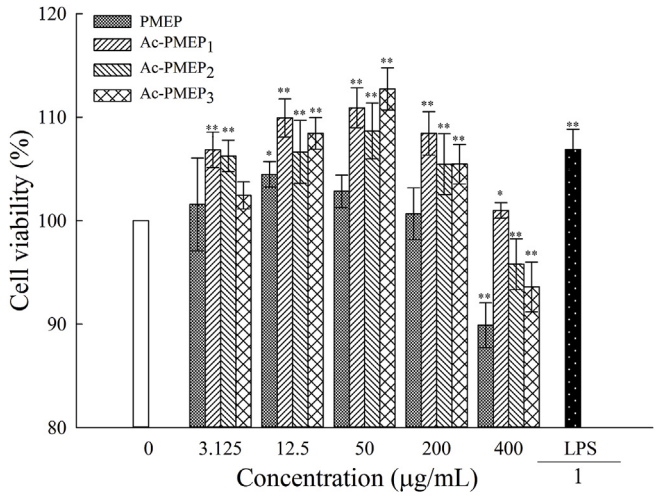

Fig. 2. Effects of PMEP and Ac-PMEP$_{1\text{-}3}$ on the viability of RAW264.7 cells. RAW264.7 cells were treated with different concentrations of PMEP and Ac-PMEP$_{1\text{-}3}$ or LPS (1 μg/mL). PMEP, polysaccharide from *Morchella angusticeps* Peck; Ac-PMEP$_1$, polysaccharide from *Morchella angusticeps* Peck acetylated with 1 mL acetic anhydride; Ac-PMEP$_2$, polysaccharide from *Morchella angusticeps* Peck acetylated with 4 mL acetic anhydride; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride; LPS, lipopolysaccharides. Significant differences with control cells are designated as $*p < 0.05$, $**p < 0.01$.

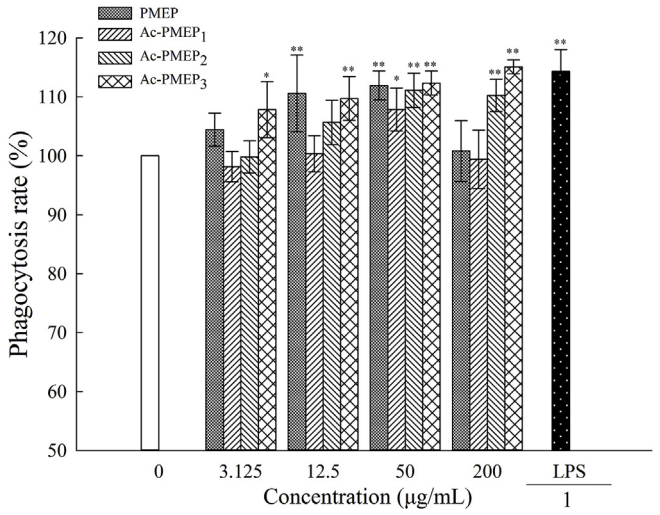

Fig. 3. Effects of PMEP and Ac-PMEP$_{1\text{-}3}$ on phagocytosis of RAW264.7 cells. RAW264.7 cells were treated with various concentrations of PMEP and Ac-PMEP$_{1\text{-}3}$ or LPS (1 μg/mL). PMEP, polysaccharide from *Morchella angusticeps* Peck; Ac-PMEP$_1$, polysaccharide from *Morchella angusticeps* Peck acetylated with 1 mL acetic anhydride; Ac-PMEP$_2$, polysaccharide from *Morchella angusticeps* Peck acetylated with 4 mL acetic anhydride; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride; LPS, lipopolysaccharides. Significant differences with control group are designated as $*p < 0.05$ and $**p < 0.01$.

production of RAW264.7 cells treated with PMEP + LPS and Ac-PMEP$_{1\text{-}3}$ + LPS at the concentrations of 50 and 200 μg/mL were decreased by 1–8% and 5–16%, respectively. Especially, Ac-PMEP$_3$ + LPS showed the strongest inhibitory effect on the production of TNF-α and NO. Therefore, Ac-PMEP showed a stronger anti-inflammatory activity than PMEP. Since 200 μg/mL of PMEP and Ac-PMEP$_3$ might exert pro-inflammatory effect (Fig. 4C), Ac-PMEP$_3$ at doses of 3.125 μg/mL (Ac-PMEP$_3$-L) and 50 μg/mL (Ac-PMEP$_3$-H) was selected for conducting the potential mechanism of immunomodulatory and anti-inflammatory activities in RAW264.7 cells.

3.5. PMEP and Ac-PMEP$_3$ promoted NF-κB p65 translocation into the nucleus

To certify whether NF-κB signaling pathway participated in immunostimulatory and anti-inflammatory effects of PMEP and Ac-PMEP in macrophages, the protein expression of p65 in nucleus and the degradation of cytoplasmic inhibitor-IκBα as well as the protein level of iNOS were detected by western blotting. The protein levels of p65, iNOS and IκBα in macrophages treated with PMEP and Ac-PMEP$_3$ at different concentrations were presented in Fig. 5A–C. Compared with the negative control group, PMEP and Ac-PMEP$_3$ significantly promoted the expression of p65 and iNOS while decreased the expression

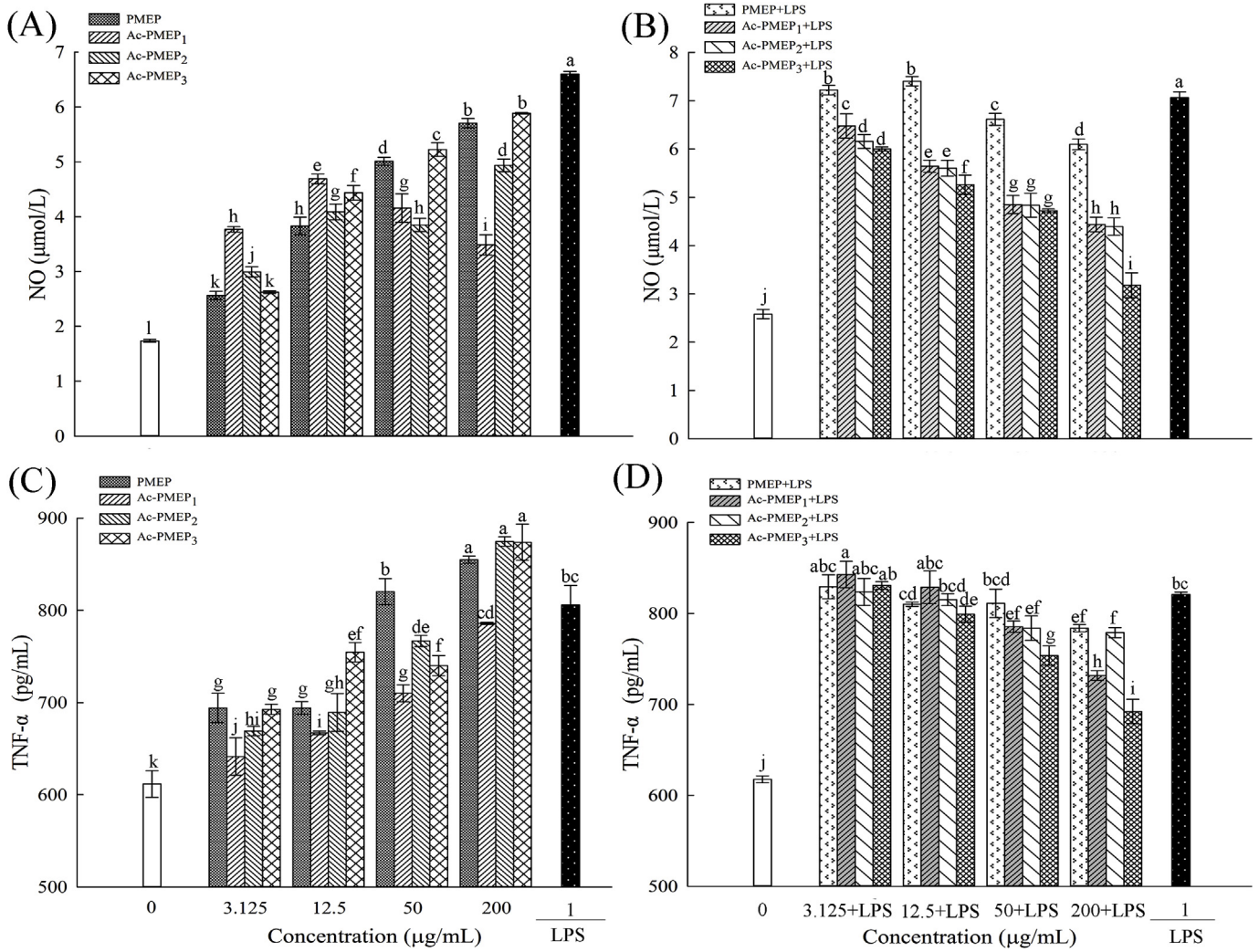

Fig. 4. The stimulating effects of PMEP and Ac-PMEP$_{1\text{-}3}$ on RAW264.7 cells. (A) NO production of RAW264.7 cells treated with various concentrations of PMEP and Ac-PMEP$_{1\text{-}3}$ or LPS (1 μg/mL); (B) NO production of RAW264.7 cells treated with different concentrations of PMEP with LPS (1 μg/mL), Ac-PMEP$_{1\text{-}3}$ with LPS (1 μg/mL) or LPS (1 μg/mL); (C) TNF-α production of RAW264.7 cells treated with various concentrations of PMEP and Ac-PMEP$_{1\text{-}3}$ or LPS (1 μg/mL); (D)TNF-α production of RAW264.7 cells different concentrations of PMEP with LPS (1 μg/mL), Ac-PMEP$_{1\text{-}3}$ with LPS (1 μg/mL) or LPS (1 μg/mL). PMEP, polysaccharide from *Morchella angusticeps* Peck; Ac-PMEP$_1$, polysaccharide from *Morchella angusticeps* Peck acetylated with 1 mL acetic anhydride; Ac-PMEP$_2$, polysaccharide from *Morchella angusticeps* Peck acetylated with 4 mL acetic anhydride; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride; LPS, lipopolysaccharides. Different letters of each group means significantly different ($p < 0.05$).

of IκBα ($p < 0.05$) (Fig. 5A, B, C). Compared with the positive control group, the protein levels of p65 and iNOS were significantly decreased whereas the expression of IκBα was increased by PMEP-H + LPS and Ac-PMEP$_3$-H + LPS, respectively. The anti-inflammatory activity of Ac-PMEP$_3$-H + LPS (p65, 132%; iNOS, 173%; IκBα, 78%) in RAW264.7 cells was stronger than that of PMEP-H + LPS (p65, 160%; iNOS, 187%; IκBα, 56%) ($p < 0.05$).

3.6. p38/MAPK signaling pathway participated in PMEP and Ac-PMEP$_3$-stimulated immunostimulatory and anti-inflammatory activities in macrophages

The protein expressions of p38 and phosphorylated p38 in macrophages treated with PMEP and Ac-PMEP$_3$ at different concentrations were presented in Fig. 5D and E. Compared with the negative control, the expression of p-p38 was significantly increased at a dose of 50 μg/mL ($p < 0.05$) (Fig. 5E). In LPS-stimulated cells, PMEP-H and Ac-PMEP$_3$-H inhibited the protein expression of p-p38 by 23.29% and 38.97%, respectively, compared to LPS group ($p < 0.05$) (Fig. 5D and E).

4. Discussion

In this study, we prepared three types of acetylated derivatives of PMEP. PMEP was characterized by gas chromatography-masss pectrometry (GC-MS), fourier transform infrared spectroscopy (FT-IR), and nuclear magnetic resonance (NMR) in our previous study (Ming et al., 2009a). Data showed PMEP contained D-arabinose, D-mannose, D-glucose, and D-galactose (molar ratio of 1:2.37:4.79:3.09). The main linkage types were found to be (1 → 4)-α-D-glucose, (1 → 6)-α-D-galactose, (1 → 2)-α-D-mannose, and (1 → 5)-α-D-arabinose; and the branches were found to be (1 → 2→6)-α-D-mannose, (1 → 2→6)-α-D-glucose, and (1 → 2→6)-β-D-galactose. Physicochemical analysis showed that PMEP did not contain free monosaccharide, uronic acid, protein, and polyphenols (Li et al., 2017). In our present study, PMEP and Ac-PMEP had a weak absorption peak at 260 nm and 280 nm, indicating that PMEP and its acetylated derivatives were substantially free of nucleic acids and proteins (Fig. 1A) (Feng et al., 2010). The intensity of an absorption at 194 nm, a characteristic symbol of a polysaccharide, decreased with the increasing of DS (Li et al., 2012). The broad -intense peaks in four polysaccharides at around 3390 cm^{-1}

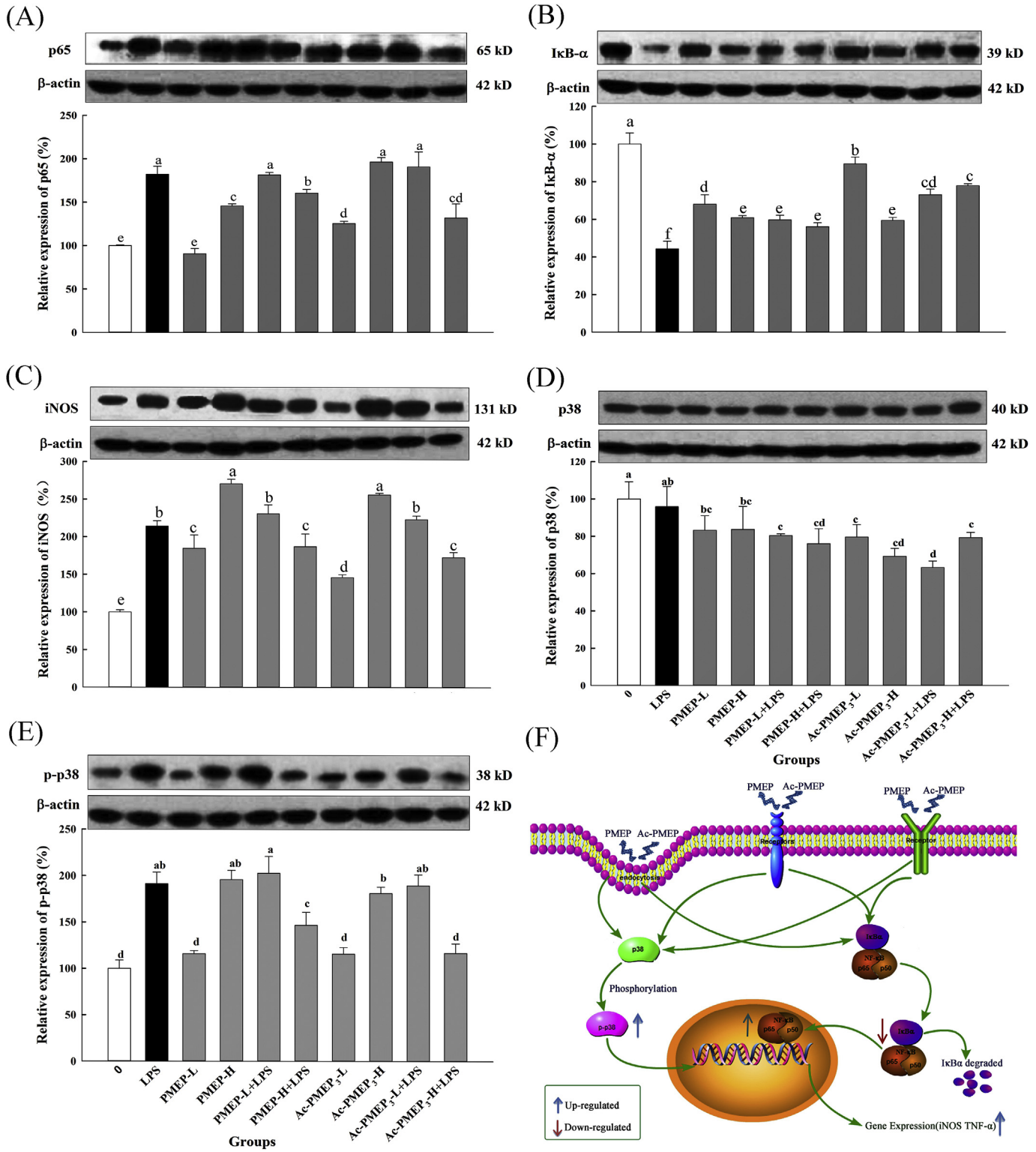

Fig. 5. Effects of PMEP and Ac-PMEP$_3$ on protein expression of p65 protein in the nucleus (A), IκB-α protein (B), iNOS protein (C), p38 protein (D), and p-p38 protein (E) at the doses of 3.125 μg/mL (PMEP-L and Ac-PMEP$_3$-L) and 50 μg/mL (PMEP-H and Ac-PMEP$_3$-H). The possible molecular mechanism of immunomodulatory activity of PMEP and Ac-PMEP$_3$ was proposed (F). RAW 264.7 cells (5×10^5 cell/mL) were incubated with PMEP, Ac-PMEP$_3$ or LPS (1 μg/mL). PMEP, polysaccharide from *Morchella angusticeps* Peck; Ac-PMEP$_3$, polysaccharide from *Morchella angusticeps* Peck acetylated with 6 mL acetic anhydride. Different letters of each group means significantly different ($p < 0.05$).

were assigned to inter- and intramolecular hydrogen bonds and −OH stretching (Kumar et al., 2004). The weak peaks at around 2932 cm^{-1} indicated stretching vibration and deformation vibration of C−H (Fang et al., 2015). After acetylation, a new absorption at around 1738.65 cm^{-1} was corresponded to C=O stretching vibrations of esters and the increased absorption intense at 1248.93 cm^{-1} assigned to C−O stretching vibration in carbonyl groups (Fig. 1B) (Ren et al., 2007). Our findings were in accordance with the results of acetylated

polysaccharides from *Cyclocarya paliurus* leaves (Xie et al., 2015). Therefore, the acetylation of PMEP with different amounts of acetic anhydride was successful.

It has been reported that polysaccharides possess multiple biological activities, moreover, chemical acetylation of polysaccharides could enhance their beneficial properties (Fan et al., 2017; Chen et al., 2014; Liu et al., 2017). In our study, PMEP and Ac-$PMEP_{1-3}$ were not cytotoxic to macrophages RAW264.7 and induced cell proliferation at the concentration of 3.125–200 μg/mL. Furthermore, Ac-$PMEP_3$ showed a stronger immunoregulatory activity than PMEP, with NO production of RAW264.7 cells treated with PMEP and Ac-$PMEP_3$ at the concentration of 50 μg/mL being increased by 188% and 200%, respectively. Moreover, we observed that Ac-$PMEP_3$ performed a stronger anti-inflammatory activity in LPS-stimulated macrophages, with NO production of RAW264.7 cells treated with PMEP + LPS and Ac-$PMEP_3$ + LPS at the concentration of 50 μg/mL being decreased by 6% and 33%, respectively. Thus, acetylation of PMEP enhanced its immunoregulatory and anti-inflammatory activities in macrophages. As far as we know, it was the first time that the underlying mechanisms by which PMEP and its acetylated derivatives had the immunostimulatory and anti-inflammatory effects on macrophages was investigated in our study.

Macrophages have a pleiotropic biological function including regulation of inflammation and induction of immunity (Kasimu et al., 2017). Once activated, macrophages exerted 1) direct cytotoxicity towards tumor cells, or 2) indirect cytotoxicity via secreting bioactive molecules, such as NO and TNF-α, which can further activate the anti-tumor functions of other cell types (Siveen and Kuttan, 2009; Ma et al., 2016). NO and TNF-α are two pivotal pleiotropic molecules, which can kill tumor cells by stimulating macrophages, inducing other immune cells to release cytokines, and participating in immune response (Siveen and Kuttan, 2009). NO and TNF-α have been extensively used both in vitro and in vivo to evaluate immune activation and immunotoxicity (Divate and Chung, 2017). In our present study, we found that Ac-PMEP with appropriate DS enhanced the NO production of RAW264.7 macrophages in the absence of LPS (Fig. 4A, C), thus improving its immunomodulatory activity (Fang et al., 2015). Evidence showed that excessive activation of macrophages and overproduction of NO and TNF-α could mediate the inflammatory response (Divate and Chung, 2017). LPS, a potent activator of macrophages, is considered as one of the major virulence factors of inflammatory response by inducing the release of a large number of inflammatory mediators (Gasparrini et al., 2017). From a toxicological perspective, an effect was considered immunotoxic if there were significant differences between the control and experimental groups when investigation of changes in physiological parameters (Galbiati et al., 2018). We further found that the up-regulation of NO and TNF-α by LPS was inhibited by Ac-PMEP under an excessive immune experimental model, indicating that Ac-PMEP had an anti-inflammatory effect (Fig. 4B, D). Therefore, Ac-PMEP regulated the secretion of NO and TNF-α of macrophages in a preventive manner to coordinate innate immunity and inflammatory responses (Wang et al., 2014a).

As a ligand, polysaccharide recognizes the receptors on the surface of macrophage through the TLR signal pathway and then activates various downstream signal molecules, such as NF-κB family (Kim et al., 2012). Previous studies have demonstrated that polysaccharides activated NF-κB via TLRs and promoted the secretion of NO and cytokines (Ma et al., 2016; Wang et al., 2014b). NF-κB, as a transcription factor, has a crucial effect on immune system. Non-activated NF-κB resides in the cytoplasm in the form of p50-p65-inhibitor κB (IκB) trimer. Under the activation signals, IκB is phosphorylated by IκB kinase and then dissociated from NF-κB, allowing the NF-κB p65 subunit transfer into nucleus (Ren et al., 2017). Thus, an increased content of p65 protein in the nucleus reflects the activation of NF-κB. iNOS is a key enzyme in NO synthesis and it can be up-regulated when macrophages were stimulated by upstream signals including NF-κB in immune reaction (Huang et al., 2012). In contrast, the suppressive effects of bioactive compounds on iNOS expression and NF-κB activation are considered as the possible therapeutic approach of inflammation (Kim et al., 2006). In this study, we found that Ac-$PMEP_3$ up-regulated the protein expression of p65 and iNOS as well as the degradation of IκBα in the immunity experimental model (Fig. 5A and B). However, the increased expression of p65 and iNOS while the decreased expression of IκBα were inhibited by Ac-$PMEP_3$ in the inflammatory experimental model. Therefore, acetylated PMEP could activate NF-κB to regulate immunity and inflammation of macrophages. Similar results were found in the polysaccharides from *Artemisia sphaerocephala* Krasch seed (Ren et al., 2017).

p38 kinase, a member of MAPKs family, is involved in immunomodulatory and inflammatory reactions leading to cytokine production, which plays an important role in iNOS expression and NO synthesis (Nakamura et al., 2006). Similarly, inhibitory effects of polysaccharides on secretion of cytokine and NO production were mediated by inhibiting the p38/MAPK signaling pathway in LPS-induced inflammation. *Poria cocos* polysaccharides stimulated macrophages by activating NF-κB and p38 kinase while fucoidan inhibited LPS-induced inflammation by the suppression of NF-κB and p38/MAPK (Nakamura et al., 2006; Lee et al., 2004). Therefore, we attempted to clarify whether p38/MAPK were involved in immunostimulatory and anti-inflammatory activities of Ac-PMEP in RAW264.7 cells. Our results demonstrated that Ac-$PMEP_3$ could increase the expression of phosphorylated p38 in normal macrophages (Fig. 5E). In addition, the enhancement of p-p38 induced by LPS was down-regulated by Ac-$PMEP_3$. Meanwhile, the concentration of non-phosphorylated p38 was obviously influenced by Ac-$PMEP_3$ (Fig. 5D). These results suggested that acetylated PMEP regulated the activation of macrophages mediated by p38/MAPK signal pathway. Our results were in agreement with previous results, which found that the polysaccharides from *Morchella conica* regulated NO production of LPS-treated macrophages by NF-κB and p38/MAPK signal pathway (Huang et al., 2012).

5. Conclusion

In summary, acetylated PMEP were successfully obtained and their underlying mechanism of immunomodulatory and anti-inflammatory activities in RAW264.7 macrophage cells were evaluated. Results showed that PMEP and Ac-PMEP presented obvious immunomodulatory and anti-inflammatory activities with promoting the cell viability, phagocytosis, NO production and TNF-α secretion in RAW264.7 macrophages. Ac-PMEP with appropriate DS enhanced proliferative activity and production of NO of RAW264.7 cells compared with the PMEP. More importantly, Ac-PMEP showed a stronger suppression in LPS-induced inflammation. The immunoregulatory and anti-inflammatory activities of PMEP and Ac-PMEP were mainly mediated via NF-κB and p38/MAPK signaling pathways.

Conflicts of interest

The authors declare there are no conflicts of interest.

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