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Study on the immunomodulatory activity of a novel polysaccharide from the lichen *Umbilicaria Esculenta*

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ABSTRACT

Umbilicaria esculenta possesses nutritional properties, such as antioxidant, antithrombotic and lipid peroxidation inhibitory activity. The immune effects of its polysaccharide (*Umbilicaria esculenta* polysaccharide, UEP) on murine macrophages RAW264.7 were investigated for the first time. UEP promoted their proliferation and phagocytic activity obviously. At the concentration of 600 μg mL^{-1}, UEP stimulated their proliferation and phagocytosis to 1.4 and 2.5 times, respectively, as compared with the negative group. Moreover, UEP induced their release of nitric oxide (NO), NO synthase (NOS), tumor necrosis factor-α (TNF-α), interferon-γ (IFN-γ) and interleukin factors (IL-1β, IL-6 and IL-10) in a concentration-dependent manner. The production of NO and NOS was increased to 25.2 and 2.7 times, respectively, while the secretion of those cytokines was enhanced to multiples from 1.9 to 2.6 times. At the same time, TAK242, an inhibitor of TLR4 (Toll-like receptor 4), inhibited UEP influences on these factors with inhibition rates up to 50%, which indicated that UEP acted on murine macrophages mainly via TLR4. UEP has positive immunomodulatory functions in murine macrophages, and can be developed as a potential novel immunomodulator.

1. Introduction

Macrophages are derived from blood monocytes and occupy a unique niche in the immune system against microbial infections and tumors [1]. They are the first line of defense in innate immunity. They engulf microorganisms and present antigens for triggering adaptive immune responses [2]. Macrophages can kill pathogens directly by phagocytosis and indirectly via the secretion of pro-inflammatory cytokines and inflammatory molecules including tumor necrosis factor-α (TNF-α), interleukin factors (IL-1α, IL-1β, IL-6 and IL-10) and reactive oxygen species (ROS) [3–5]. During phagocytosis, macrophages secrete preformed granule constituents and newly synthesized products that play a critical role in inflammation and tissue repair [6]. Phagocytic activity and macrophage reactivity are crucial in the assessment of macrophage function. At the same time, they also secret inflammation mediators like nitric oxide (NO) produced by the action of inducible NO synthetase (iNOS), and cyclooxygenase-2 (COX-2) [7].

Various biological substances can activate macrophages, such as polysaccharides, proteins and polyphenols. Recently, many polysaccharides have been isolated from natural sources, such as mushroom, algae and other edible food [8–10]. Natural polysaccharides have long been appreciated for their potent immunomodulatory activity [11–13]. These polysaccharides can bind to pattern recognition receptors on the surface of macrophages, such as Toll-like receptors (TLRs) and complement receptor type 3 (CR3), and then trigger several signaling pathways to activate macrophages [14–16]. Activated macrophages have several functions such as processing and presentation of antigens to lymphocytes, killing pathogenic microorganisms, removal of cell debris as well as releasing proinflammatory cytokines and cytotoxic molecules, so these polysaccharides enhance macrophage function [17]. More importantly, most polysaccharides from food are relatively nontoxic and do not cause significant side effects [6].

Umbilicaria esculenta, a kind of lichen, is popular as a traditional food in China. Lichen is regarded as a special simple plant or a specific fungus, which comes from symbiotic growth of algae and epiphyte or fungi, with 13,500 distinct species. Among them, only about 100 lichens have been studied [18,19]. The polysaccharides from *Umbilicaria esculenta* (UEP) may have particular molecular structures and different bioactivities, if *Umbilicaria esculenta* is from various areas of China. In our previous study, our research concentrated on the *Umbilicaria esculenta* from Huangshan Mountain (30°N, 1864 m altitude) in Anhui province, China. Its UEP was purified and characterized, and its in vitro and in vivo antithrombotic activity was reported for the first time in our laboratory [20]. Its UEP consist of two polysaccharides. One is composed of mannose, rhamnose, glucose and galactose with 124.7 kD, while the other composed of mannose and glucose with

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249.4 kD. Its UEP had significant preventive effect on thrombosis, and oral administration of UEP indicated a significant dose dependent preventive effect against thrombotic death or paralysis [20].

However, up to now, no investigations of its immunomodulatory activity have been conducted. Based on our previous study of UEP, the principal objective of this study was to evaluate the immunostimulatory effect of UEP on murine macrophages RAW 264.7 and the possible molecular mechanism was revealed.

2. Materials and methods

2.1. Materials and chemicals

Umbilicaria esculenta was obtained from Huangshan Mountain (30°N, 1864 m altitude) in Anhui province, China. They were identified according to their morphological characteristics [20]. RPMI-1640 was from Ruji Biotechnology Co., Ltd. (Nanjing, China). Fetal Bovine Serum (FBS) was from Cobioer Biotechnology Co., Ltd. (Nanjing, China). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), LPS (lipopolysaccharide), DMSO (dimethyl sulfoxide) and TMB (tetramethyl benzidine) were purchased from Sigma-Aldrich. TAK242 (TLR4 signaling inhibitor) was from Medchemexpress Co.,Ltd. (Shanghai, China). A (sulphanilamide) and B (N-1-naphtylethylenediamine dihydrochloride) in Griess reagent were obtained from Sbjbio Co., Ltd. (Nanjing, China). ABC (avidin–biotin–peroxidase complex) was from Luwen Biotechnology Co., Ltd. (Shanghai, China). Murine NOS, TNF-α, L-1β, IL-6 and IL-10 assay ELISA (enzyme-linked immune sorbent assay) kits were from Jiancheng Bioengineering Institute (Nanjing, China). Unless stated otherwise, other chemicals and reagents were of analytical grade and obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Deionized water was used throughout the experiments and purified by a Mill-Q water purification system from Millipore (Bedford, MA, USA).

2.2. Preparation of polysaccharide from Umbilicaria esculenta

The thalli of *Umbilicaria esculenta* (100.0 g) was cleaned, lyophilized (a Model 2K-XL Lyophilizer, Virtis Corporation, American), and ground with a Model ZN-100 Grinder (Zhongnan Pharmaceutical Machinery Factory, Shanghai, China). Its powders (70.8 g) were obtained and successively extracted with deionized water (800 mL) at 100 °C for 3 h (×3) to isolate the residue. Each aqueous extract was added to excess absolute ethyl alcohol (ratio of 3:1; v/v) to form a precipitate (14.5 g). The precipitate was isolated through centrifugation (Model 5804R, Eppendorf Corporation, Germany) at 4680 g for 20 min at 25 °C and then lyophilized. Hence, crude UEP (10.2 g) was obtained. Crude UEP was then dissolved in 0.1 M NaCl (10 mg mL^{-1}) and 10 mL of solution were applied to a column of DEAE Cellulose-52 (2.5× 60 cm), followed by stepwise elution with 0.1, 0.3, 0.5 and 0.7 M sodium chloride solutions at a flow rate of 0.24 BV h^{-1}. Eluate was collected, dialyzed with water, and lyophilized to obtain purified UEP.

2.3. Cell culture

The murine macrophages RAW264.7 were obtained from BeNa Culture Collection (Beijing, China) and routinely cultured in RPMI-1640 medium supplemented with 10% inactivated FBS, 2 mM glutamine, 100 U mL^{-1} penicillin and 100 μg/ml streptomycin. Cultures were maintained at 37 °C in an incubator with humidified air containing 5% CO_2. Cells were used when confluence reached 75%. They were harvested by gentle scraping, washed by PBS (phosphate buffered solution) and centrifuged at 1000 ×*g* for 5 min to obtain their sediments. Cells were resuspended into 5.0×10^5 cells mL^{-1} with the culture medium for the following experiments.

2.4. Macrophage viability assay

Cell viability was determined by a modified MTT method [21–23]. RAW264.7 cells were seeded into 96-well plates at a density of 5.0×10^5 cells mL^{-1}, 200 μL per well. The plates were incubated in the incubator under similar conditions for 24 h, and then their culture medium was removed. With the culture medium as their solvent, UEP at series of concentrations and a mixture of UEP and TAK242, were added into each well, 200 μL per well. At the same time, a negative control group of the culture medium and a positive control group of LPS (1 μg mL) were set up. The plates were incubated under similar condition again. After 24 h, their culture supernatants were removed and RAW264.7 cells were washed with PBS. 20 μL of MTT (5 mg/ml in PBS) was then added to each well and incubated for additional 4 h. Their supernatant was then discarded carefully. 150 μL of DMSO was added to each well, and the plates were shaken for 10 min until no particulate matter was visible. The absorbance values at 570 nm were measured using a Synergy TM HT-Mode ELISA microplate reader (Bio-Tek Instruments Inc., USA).

2.5. Phagocytic activity assay

The phagocytic activity of RAW264.7 cells was measured by the neutral red uptake method [24]. Briefly, as before, the plates were incubated with UEP, a mixture of UEP and TAK242 (UEP + T for short), RPMI-1640 medium or LPS (1 μg mL) for 24 h. Their culture supernatants were removed and cells were washed with PBS. 100 μL of 0.1% neutral red solution (dissolved in 10 mM PBS, 0.075%) was added to each well and incubated for further 4 h. The supernatants were discarded and the cells were washed with PBS three times to remove the neutral red that was not phagocytized. 100 μL of the cell lysis buffer (ethanol: glacial acetic acid = 1:1, (v/v)) was then added to each well and incubated at 4 °C for 2 h to extract the dye phagocytized by macrophages. Their absorbance values at 540 nm were detected by the ELISA microplate reader.

2.6. NO assay

NO released by cells is unstable and easily converted into nitrite, so the amount of nitrite was used as an indicator determined by the Griess reaction method [25,26]. The plates were also incubated with samples, RPMI-1640 medium or LPS (1 μg mL^{-1}) for 24 h. 100 μL of each supernatant were distributed in new 96-well plates and then equal volumes of the Griess reagent were added. It was worth mentioning that the Griess reagent was a mixture of solution A (1% sulphanilamide in 5% phosphoric acid) and equal volume of solution B (0.1% N-(1-naphtyl)-ethylenediamine dihydrochloride in distilled water). The reaction was allowed to proceed at room temperature in the dark place for 10 min. Their absorbance values at 550 nm were measured using the ELISA microplate reader.

2.7. NOS assay

The secretion amounts of NOS in RAW264.7 cells were detected using commercial ELISA kits [24]. Cell culture and experimental groups were operated according to the above process in analyzing NO amounts. After incubation for 24 h, the cells were collected for NOS assay, while the supernatant was used for the above NO assay. PBS (300 μL) was added to each well after the macrophages were washed twice with PBS. A JY92-IIN Sonicator (Ningbo Biotechnology Co., Ltd., China) was used to break the cells for releasing NOS. The suspension was centrifuged at 1000 ×*g* for 10 min, and 100 μL of supernatant was then fetched out for measuring NOS with NOS ELISA assay kid by the ELISA microplate reader, according to the manufacturer's instruction.

2.8. Cytokines assay

Secretions of cytokines including TNF-α, IFN-γ and IL-1β, IL-6 and IL-10 in the culture supernatants were detected using commercial ELISA kits [27]. The RAW264.7 cells were incubated in the presence of varying concentrations of samples as above. After 24 h, some cell culture supernatants were collected for NO assay described above, while others were for analyzing cytokines using commercial ELISA kits according to the manufacturer's protocols. In general, the culture supernatants or cytokine standards were added to new 96-well plates coated with coating antibody, and the plates then incubated at 37 °C for 2 h. The plates were washed and a detecting antibody was added to each well. The plates were incubated at 37 °C for 1 h before addition of avidin–biotin–peroxidase complex (ABC). After incubation for 30 min, the plates were washed twice with PBS and mixed with TMB at 37 °C for 15 min. The reaction was ceased with100 μL of stop solution. Their absorbance values were measured in the ELISA microplate reader, and cytokine concentrations were calculated by an extrapolation from the standard curve of the recombinant cytokines in the ELISA kits [28].

2.9. Statistical analysis

All experiments were repeated at least three times, each with three replicates. The data were represented as means ± SD (standard deviations). One-way ANOVA and Duncan's multiple range tests were carried out to assess a significance of differences between the various experimental groups using Statistical Product and Service Solutions software17.0. The significance of the level was considered at $p < 0.05$.

3. Results and discussion

3.1. Effect of UEP on the proliferation of murine RAW264.7

Macrophages play a critical role in nonspecific defense and help initiate specific defense mechanisms by recruiting other immune cells. The effect of UEP on the viability of murine macrophages was revealed in Fig. 1. UEP was nontoxic to the RAW264.7 cells within tested concentrations, which ranged from 0 to 1000 μg mL^{-1} ($p < 0.05$). In general, UEP induced and promoted the proliferation of macrophages in a dose-dependent manner. With the increasing UEP concentration from 0 to 600 μg mL^{-1}, their viability (OD) was increased from 0.537 ± 0.014 to 0.673 ± 0.029. At 600 μg mL^{-1}, UEP promoted the proliferation of macrophages to 1.4 times, as compared with the negative control. With the further increase of UEP concentrations, their viability even fell lower ($p < 0.05$). At the tested concentrations, UEP was effective and nontoxic, and the value of 600 μg mL^{-1} was chosen.

3.2. Effect of UEP on the phagocytosis of murine RAW264.7

Macrophage activation signifies an up-regulation of an innate immune response. One of the most distinguished features of macrophage activation is an increase in phagocytosis, which has nonspecific immune responses to organisms and is the first defense mechanism against foreign substances [29,30]. After activation, macrophages initiate phagocytosis to scavenge foreign substances through the innate immune response. Phagocytosis of pathogens by macrophages, which plays an important role in immune regulation, involves the migration and adhesion of macrophages. The phagocytic activity of macrophages triggered by UEP was evaluated by measuring the uptake of neutral red, an effective cationic dye for staining vesicle walls or vesicles contents. As presented in Fig. 2, UEP had abilities to enhance the phagocytic activity of macrophages in a dose-dependent manner. The effects of UEP at the concentrations from 300 to 800 μg mL^{-1} were always higher than that of the native control group ($p < 0.05$). Their phagocytic activity (OD) was increased significantly from 0.556 ± 0.015 to 0.825 ± 0.017 ($p < 0.05$) with the increasing UEP concentration from 300 to 600 μg mL^{-1}, which was consistent with the results in their above-mentioned proliferation assay. At 600 μg mL^{-1}, UEP increased the phagocytic activity of macrophages to 2.5 times, as compared with the negative control. However, with a further increase in its concentration, there was no significant difference between 600 and 700 μg mL^{-1} ($p > 0.05$), and the phagocytic activity even fell at 800 μg mL^{-1} ($p < 0.05$). The stimulatory effects of UEP at concentrations from 400 to 600 μg mL^{-1} on the phagocytic activity of RAW264.7 were significantly high than that of LPS (1 μg mL^{-1}, $p < 0.05$). UEP could prime macrophages for an enhanced phagocytic capacity, which was comparable to or greater than that observed for LPS.

As compared with UEP at 600 μg mL^{-1}, the phagocytic activity of RAW264.7 simulated by UEP + T, a mixture of UEP (600 μg mL^{-1}) and TAK242 (TLR4 signaling inhibitor), dropped to 53%, which indicated that TAK242 had an adverse effect on UEP, but it was not suppressed entirely. That is, TLR4 was essential for UEP, and UEP activated the phagocytic activity of murine macrophages mainly via TLR4. Because the adverse effect was incomplete, UEP could also simulate the cells via other receptors [14,31].

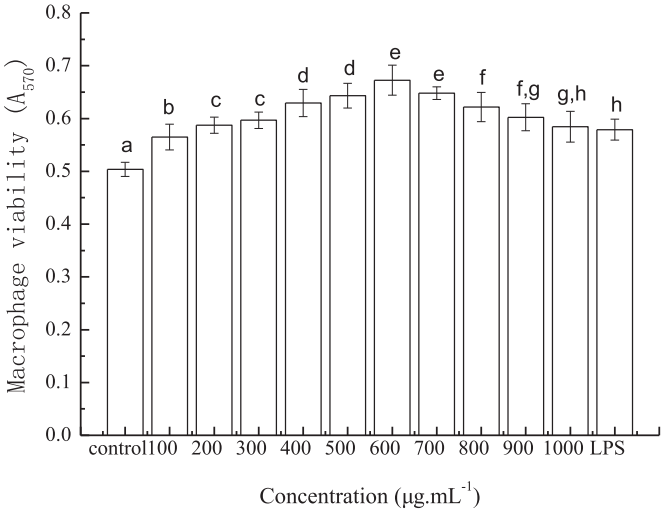

Fig. 1. The effect of UEP on the viability of murine RAW264.7. Data are expressed as the means ± SD (n = 3). The error bar represents the standard deviation. Values marked with different letters are significantly different ($p < 0.05$).

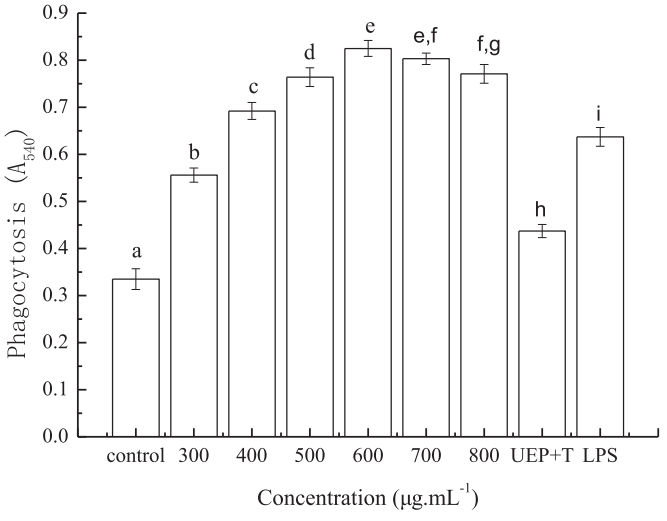

Fig. 2. The effect of UEP on the phagocytic activity of murine RAW264.7. Data are expressed as the means ± SD (n = 3). The error bar represents the standard deviation. Values marked with different letters are significantly different ($p < 0.05$).

3.3. Effects of UEP on the NO and NOS production in murine RAW264.7

To evaluate NO and NOS in RAW264.7, Griess reaction method and ELISA kits assay were adopted to determine the nitrite and NOS accumulation. NO is an important mediator of the non-specific host defense against invading tumor cells and pathogenic microorganisms [32]. As reported in Fig. 3A, UEP simulated the NO production in a dose-dependent manner, and its results approached to that of LSP (the positive control). The amount of NO in the cells treated with UEP at 600 μg mL^{-1} reached 20.736 ± 1.122 μmol L^{-1}, which was 25.2 times higher than that of the negative control group (0.823 ± 0.217 μmol L^{-1}). At the same time, the results of UEP at the concentrations from 600 to 800 μg mL^{-1} were not significantly different ($p > 0.05$).

Correspondingly, at 600, 700 and 800 μg mL^{-1}, the amounts of NOS in UEP group were 1.752 ± 0.047, 1.750 ± 0.116, 1.757 ± 0.124 U mL^{-1}, respectively, and they were not significantly different ($p > 0.05$). They reached the level of 2.7 times higher than that of the negative control group (Fig. 3B), which revealed that UEP improved the NO amount via increasing the NOS secretion. The increase of NO and NOS implied that UEP might promote a bactericidal and tumoricidal activity of murine RAW264.7.

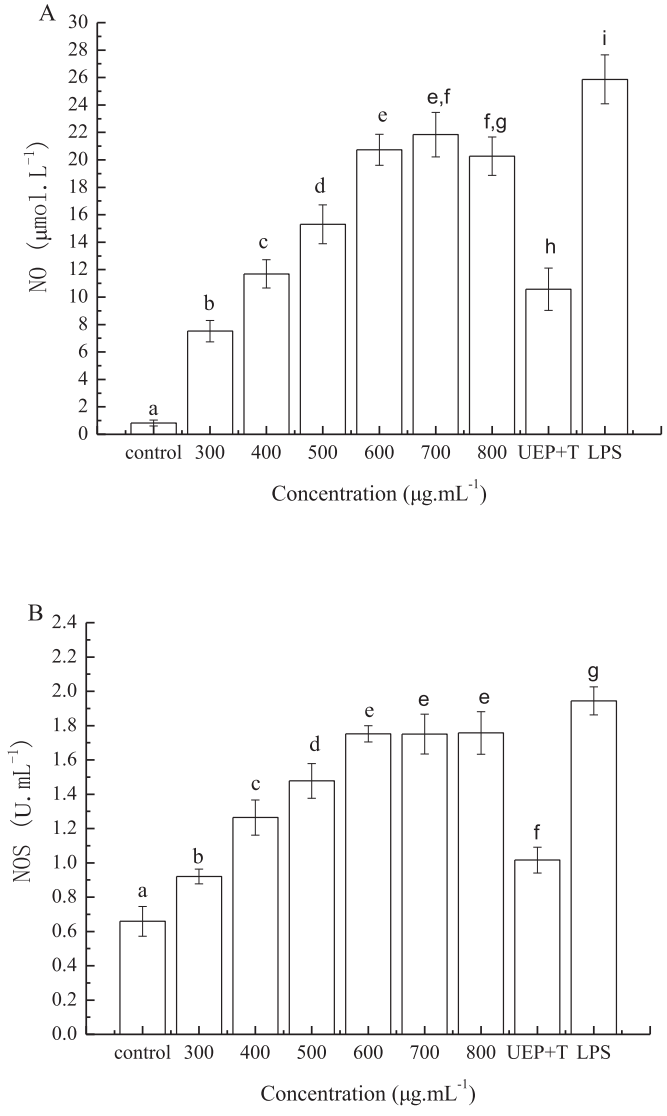

Fig. 3. The effect of UEP on NO and NOS in murine RAW264.7. (A) NO production; (B) NOS secretion. Data are expressed as the means ± SD (n = 3). The error bar represents the standard deviation. Values marked with different letters are significantly different ($p < 0.05$).

As also can be seen in Fig. 3, TAK242 (TLR4 signaling inhibitor) significantly inhibited the effects of UEP on NO and NOS production in macrophages, and their inhibition rates were about 51% and 58%, respectively, but not 100%. Although UEP definitely activated macrophages through TLR4, other receptors were also involved [14,31], which was consistent with the above phagocytosis result.

3.4. Effects of UEP on the cytokines secretion in murine RAW264.7

To investigate the immunological activity of UEP deeply, secretions of TNF-α, IFN-γ and IL-1β, IL-6 and IL-10 were evaluated in murine RAW264.7. As can be seen in Fig. 4, in general, UEP significantly induced murine RAW264.7 to release TNF-α, IFN-γ and IL-1β, IL-6 and IL-10 all in a dose-dependent manner. As compared with the negative control group, the secretion amounts of TNF-α, IL-1β and IL-10 reached the level of 1.9 (Fig. 4A), 2.5 (Fig. 4C) and 2.6 times (Fig. 4E), respectively, when the cells were incubated with UEP at 600 μg mL^{-1}. Moreover, TAK242 significantly inhibited the effects of UEP on TNF-α, IL-1β and IL-10 releases in the cells, and their inhibition rate were about 53% (Fig. 4A), 51% (Fig. 4C) and 60% (Fig. 4E), respectively, which was consistent with the above experimental results of NO and NOS. Like TNF-α, IL-1β and IL-10, IFN-γ and IL-6 are also considered as the most important immune mediators involved in the initiation of immunologic cascade during the induction of acute phase protein response [33]. In comparison with the untreated cells, UEP at 700 μg mL^{-1} caused a 95% (Fig. 4B) and 256% (Fig. 4D) increase in the release of IFN-γ and IL-6, respectively. That is, the amounts of IFN-γ and IL-6 in the cells treated with UEP were 2.0 and 3.6 times, respectively, higher than that of the negative control group. TAK242 also influenced the effects of UEP on IFN-γ and IL-6, as well as TNF-α, IL-1β and IL-10, and their inhibition rates were about 61% (Fig. 4B) and 55% (Fig. 4D), respectively.

Diverse receptors on the surfaces of macrophages may bind polysaccharides and activate the cells to produce cytokines. The above results suggested that UEP activated the immune system by increasing the secretion of pro-inflammatory cytokines. Cytokines are mainly polypeptide molecules released by stimulated immune cells, which play a very important role in intercellular interactions, proliferation, and other functions. They are involved in the inflammatory response, so they can expand the inflammation, and further deepen the tissue injury. TNF-α and IL-1β act on macrophages in an autocrine manner, strengthens immune responses and induces the expression of other immunomodulatory factors [34,35]. IL-1β is also easily involved in fever during the induction of the acute phase protein response. In addition, it can cooperate with IFN-γ to induce tumor death by NK cells. IL-6 plays important roles in phagocytosis, antigen presenting, inflammatory regulation, and it can regulate the cellular immunity and humoral immunity by offering a third signal to T cells [36]. As for anti-inflammatory substances, IL-10 has been recognized as a master anti-inflammatory cytokine exhibiting immune suppressive effects in inflammation and disease [37].

TAK242, the TLR4 inhibitor, significantly inhibited UEP influences on these cytokines with inhibition rates up to 50% or even to 60%, which implied that UEP activated macrophages mainly via TLR4. TLR4 is the main glyco-receptor on the cell surface of macrophages. Some polysaccharides from food or plants have been confirmed to activate macrophages via TLR4 [4,38]. When these polysaccharides act primarily through TLR4, TLR4 interacts with different combinations of adapter proteins and activates various transcription factors such as NF-κB (nuclear factor), AP-1(activatingprotein-1) and interferon regulatory factors. Among them, the NF-κB pathway has long been considered as a prototypical pro-inflammatory signaling pathway [16,39].

Once activated by bacterial toxins such as LPS, the macrophages are easily evoked to proliferate, and moreover their phagocytosis gets enhanced. At the same time, they release NO, NOS and cytokines to inhibit the growth of extensive invasive substances as an immune response. Similar to LPS, UEP induced macrophages to secrete NO, NOS and

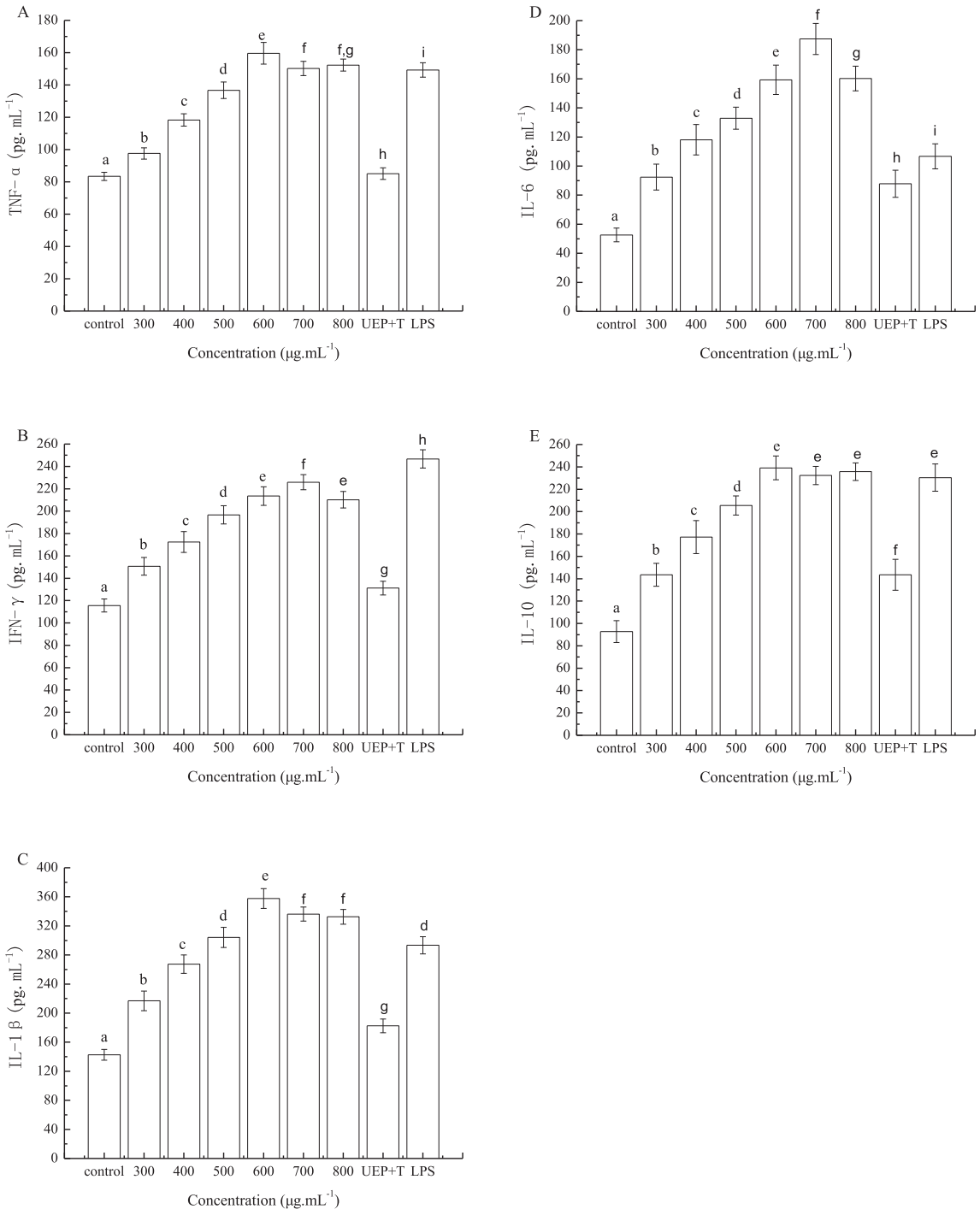

Fig. 4. The effects of UEP on cytokines in murine RAW264.7. (A) TNF-α secretion; (B) IFN-γ secretion; (C) IL-1β secretion: (D) IL-6 secretion: (E) IL-10 secretion. Data are expressed as the means ± SD (n = 3). The error bar represents the standard deviation. Values marked with different letters are significantly different ($p < 0.05$).

these pro-inflammatory cytokines. These physiological defensive reactions, in turn, would protect biological organisms against pathogens, damaged cells, or tumor cells [34,39]. These results were also consistent with the effects of some other polysaccharides on the cytokine production by macrophages [27,40,41].

4. Conclusions

In the present study, the polysaccharide from *Umbilicaria esculenta* (UEP) has positive immunomodulatory functions in murine macrophages RAW264.7. It can be used to trigger and increase the proliferation and phagocytosis of the cells. Moreover, it activates macrophages mainly via TLR4, and significantly promotes macrophages to secrete NO, NO synthase, TNF-α, IFN-γ, IL-1β, IL-6 and IL-10. UEP can be used as a novel potential immunomodulator for food purposes. Further studies on its specific signaling pathways and structure-activity relationship are still of great urgency and in progress in our laboratory.

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