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Research Paper

Immunomodulating activity of the polysaccharide TLH-3 from Tricholomalobayense in RAW264.7 macrophages

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ABSTRACT

Polysaccharide TLH-3 extracted from Tricholoma lobayense possessed unique antioxidant and anti-aging activities, whereas its immunomodulatory properties remain unexplored. Herein in order to explore TLH-3 biological activities, the immunomodulatory effects on RAW264.7 macrophages and its molecular mechanisms were investigated. It was showed that TLH-3 could significantly enhance the phagocytic activity, releasing toxic molecules NO (nitricoxide), secretion of the cytokine TNF-?(tumor necrosis factor-?, IL-6 (interleukin-6). Further, TNF-?and IL-6 were blocked by the inhibitor of TLR4 (Toll-like receptor4), suggesting TLR4 was a receptor of TLH-3, and immunomodulatory activity of TLH-3 was mediated by TLR4. Moreover, immunofluorescence indicated that TLH-3 lead to the nuclear translocation of NF-?B (nuclear factor-?B) subunit p65. Western blotting demonstrated that NF-?B levels in nucleuses increased and cytoplasmic I?B-?(inhibitor of NF-?B) degraded after TLH-3 treatment, suggesting that TLH-3 probably stimulated macrophage by activating the IκB-α-NF-κB pathway via TLR-4. This study demonstrated that TLH-3 could be potentially used as immunomodulatory agent for healthcare and disease control.

1. Introduction

Macrophages, which are derived from blood monocytes, occupy a uniquerole in immune system, inthat they can not only initiate innate immune responses, but also be effector cells that contribute to fight against infection and inflammation [1]. Macrophages could kill causative agent directly by phagocytosis or indirectly via secrete pro-inflammatory factors including TNF-α, IL-6 and NO, which can confer bactericidal and antitumor effects [2,3]. Therefore, macrophages served as an important target of some antitumor and immunomodulatory agent [4]. Immunomodulatory effect of polysaccharides has been widely reported in recent years, especially from edible fungi polysaccharide [5–7]. The important mechanism of polysaccharides in the immunostimulatory activity is their ability to enhance macrophage efficiency [8,9]. Thereinto these cellular events, cell surface pattern-recognition receptors (PRRs), particularly TLR-4 could be bound by natural polysaccharides [10,11], and then triggered through NF-κB signaling pathways. Subsquently NF-κB as a transcription factor was translocated from cytoplasm to nucleus, accumulating in the nucleus to active the immunomodulation of macrophages through the promotion of cytokines production and phagocytic uptake.

Tricholoma lobayense Heim is kind of rare and valuable edible fungus due to its pharmacological potential [12,13]. The immune regulation and anti-tumor activities of the proteoglycans from Tricholoma lobayense Heim were reported elsewhere [14], in additional, the structural elucidations of three anti-oxidative polysaccharides from Tricholoma lobayense Heim have be expound in our previous study [15]. It is believed that TLH-3 possesses a low molecular weight, high branch degree, versatile linkage types and complex conformation among three polysaccharides (Fig.S1). Interestingly, TLH-3 manifested admirable antioxidant activity in vitro, which was close to that of Vitamin C with IC50 value of 126 μg/ml [13]. TLH-3 also had the ability to reduce t-BHP induced oxidative damage on HELF (Human embryonic lungfiberblast) cells and successfully alleviated oxidative damage in serum and liver tissue of D-gal-induced aging [12]. Apart from this, the changellge of isolation and purification for activity fraction TLH-3 polysaccharide from T.lobayense has been resolved [16]. Beyond of these, the sulfated derivativeSTLH-3, was markedly improve its anti-oxidative activity and cytotoxicity activity for cancer cell [17].

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However, the antioxidant and immunostimulatory-effects are-natural activities of polysaccharides [18–22], and previously our laboratory focused on the TLH-3 of antioxidant and anti-aging function, thus the immunostimulatory effect of TLH-3 has been rarely investigated up to now. Therefore, in the present study, we investigated the immunstimulatory effect of TLH-3 on RAW264.7 cells, which is commonly accepted as a tool to investigate the molecular mechanisms of macrophages about regulating immunity [23]. Our studies indicated the immunostimulatory of TLH-3, which activating the IκB-α-NF-κB pathway via TLR-4 in RAW264.7 cells, then inducing phagocytes is and secreting pro-inflammatory cytokines including TNF-α, IL-6 and NO. This study suggested that TLH-3 potentially could be used as immunomodulatory agent.

2. Materials and methods

2.1. Materials

TLH-3 polysaccharide, which is made up of 1,3-linked-β-D-glucopyranosyl branched at C-6 and 1,3-linked-β-D-galactopyranosyl [15], was separated and purified according to our research group previous report [16]. DMEM (Dulbecco?s modified eagle medium) high glucose medium was acquired from Wisten Biotechnology (WISTEN Co. Ltd., Nanjing), fetal bovine serum(FBS), penicillin and streptomycin were purchased from GIBCO (life technology USA).3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide(MTT),Lipopolysaccharides (LPS) and neutral red were purchased from Sigma Chemical Co. (SaintLouis, USA).Mouse IL-6 ELISA kit and TNF-α ELISA kit were purchased from eBioscience (San Diego, CA, USA), NO assay kit, cellular NF-κB translocation kit were supplied by Beyotime Institute of Biotechnology (Shanghai, China), lipopolysaccharide (LPS, Escherichia coli 0111:B4), Polymyxin B(PMB) (Sigma?Aldrich, St. Luis, MO, USA), anti-TLR4-antibody (ab13556), anti-IκB-α antibody (ab32518), anti-NF-κB –antibody (ab32536), anti-β-actin antibody (ab59381) were purchased from Abcam (Cambridge, UK), HRP-labeled goat anti-rabbit IgG and HRP-labeled goat anti-Mouse IgG antibody were purchased from Protein techBiotechnology (Proteintech Group, Inc, Wuhan). All other reagents were of the highest grade commercially available.

2.2. Cell culture

RAW264.7 was purchased from Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). The cells were maintained in DMEM medium supplemented with 100 U/ml penicillin, 100 g/ml streptomycin, and 10% heat-inactivated FBS at 37?C in an incubator with a humidified atmosphere and 5% CO2.

2.3. Assay for cell viability

The effect of TLH-3 on the viability of RAW264.7 cells was determined by MTT method. Adherent RAW 264.7 cells were treated with increasing concentrations of TLH-3 (25–800 μg/ml) in the growth medium at 37 °C in 96-well plates. After 24 h incubation, MTT (5 mg/ml) was added to each well and incubated for additional 4 h. The supernatant was then removed and 100 μl of DMSO was added. The cell culture plate was shaken for 10 min until no particulate matter was visible. Absorbance in each well was measured at 570 nm using a micro-plate reader (Biotek, USA).

2.4. Cell proliferation assay

The proliferation of RAW264.7 was measured using xCELLigence RTCA TP (ACEA Biosciences, Inc). RAW264.7 cells (5 × 104 cells/well) were pre-incubated in E-Plate8 at 37 °C in 5% CO2 incubators on RTCATP Analyzer. After 24 h, the various concentrations of TLH-3 were added into each well according to the procedure and settings in RTCA Control Unit before the experiment started. After 24 h incubated, the assay of RAW264.7 proliferation was acquired and processed using RTCA Control Unit and Graph Pad Prism 6 software.

2.5. Measurement of NO production

NO production was monitored by assessment of nitrite accumulation as previous study [24,25]. RAW264.7 cells (1 × 105 cells/well) were pre-incubated in 12-well plates for 24 h at 37 °C in 5% CO2incubator and then were incubated with TLH-3 (25–200 μg/ml) or LPS (1 μg/ml) for 24 h. One hundred microliter of culture supernatants was mixed with an equal volume of 10% Griess-reagent Sodium nitrite (NaNO2) was used to generate a standard curve, and nitrite production was determined by measuring absorbance at 540 nm.

2.6. Assay for phagocytic activity

The phagocytic ability of macrophage was measured by neutral red uptake. After cells (1 × 105 cells/well) cultured in 12-well with TLH-3 (25–200 μg/ml) or LPS (1 μg/ml)(Sigma) for 24 h, 100 μl neutral red solutions (dissolved in PBS with the concentration of 0.075%) was added and incubated for 30 min. The supernatant was discarded and the cells in 12-well plates were washed with PBS thrice to remove the neutral red that was not phagocytized by RAW264.7 cells. Then cell lysate (50% ethanol and 1% acetic acid at the ratio of 1:1, 100 μl/well) was added to lyse cells at 4 °C for 2 h. After cells were incubated overnight at room temperature, the optical density was measured by a micro-plate reader at 540 nm (Biotek, USA).

2.7. ELISA for quantitative analysis of cytokines and antibody inhibition experiments

RAW 264.7 cells (1 × 105 cells/well) in 12-well were pre-treated with 20 μg/ml of TLR4mAb for 30 min, and then incubated with TLH-3 (25–200 μg/ml) or LPS (1 μg/ml) was used as positive control for 24 h. The supernatant TNF-α and IL-6 levels were determined using ELISA kit. The specific method was carried out according to the ELISA kit manual. The TNF-α and IL-6 concentration was estimated from a reference to a standard curve of murine recombinant TNF-α and IL-6.

2.8. Morphologic observations and immunofluorescence of nuclear translocation of NF-κB

RAW 264.7 cells were grown on glass bottom cell culture dishes and treated with TLH-3 (200 μg/ml) or LPS (1 μg/ml) for 24 h. The morphological change was observed under a phase contrast microscope (Olympus, Japan). For analysis of nuclear translocation of NF-κB, cells were washed with PBS and stained with cellular NF-κB translocation kit according to the manufacturer's instructions after removing supernatant. Briefly, cells were fixed and incubated with a blocking buffer for 1 h to suppress non-specific binding. Cells were then incubated with the primary NF-κB p65 antibody at 4?C overnight, followed by incubation with a Cy3-conjugated secondary antibody at room temperature for another 1 h. DAPI as nuclear staining was used after 5 min incubation with cells. Samples were visualized under laser-scanning confocal microscopy (Olympus FV1000, Japan). Cells were luminescently imaged on Olympus FV1000 upright confocal-laser scanning microscope with 60X and 100X oil-immersion lenses. For DAPI (500 nM), excitation

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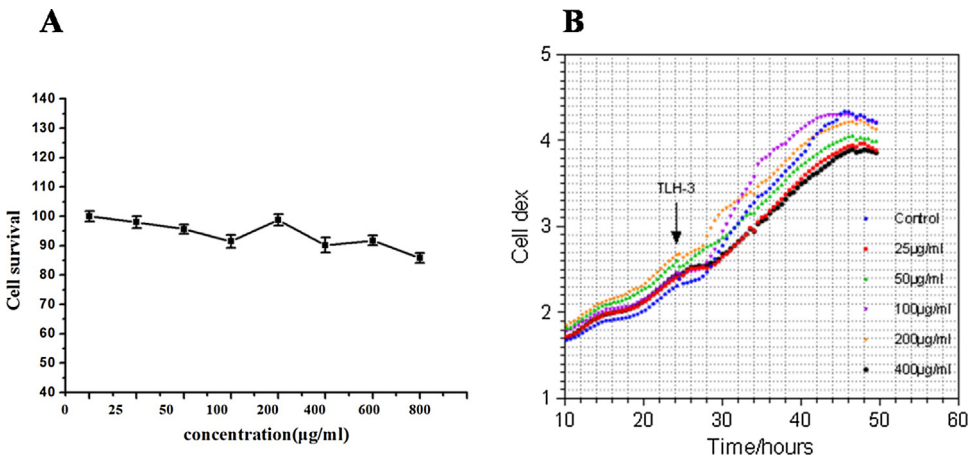

Fig 1. Effect of different concentrations of TLH-3 on the viability (A) and prolifration (B) of RAW264.7 cells analyzed by MTT and RTCATP Analyzer respectively.The results were expressed as means ±SD (n = 8) for MTT assay. Significant difference from the control group was designated as **P < 0.001.

energy 405 nm was used and fluorescence emission was measured at 420–450 nm. For Cy3 secondary antibody, excitation energy 545 nm was used and fluorescence emission was measured at 560–600 nm.

2.9. Western blot analysis

After various treatments, cells were washed with PBS and the IκB-α and NF-κB protein expression of RAW264.7 was performed cytoplasm and nuclei according to Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime, Shanghai, China). And the protein BCA quantitative method was performed to determine total protein content in the sample stored at −80 °C. Each 30 μg cytoplasmic or nuclear protein samples were boiled with loading buffer for 5 min followed being put through 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidenedifluoride membranes (Immun-Blot PVDF membrane, 0.2 m; BIOSHARP), The membrane was blocked with 5% skim milk with 1*Tris-buffered saline(TBS) containing 0.1% Tween-20 for 2 h and then incubated with primary antibodies at 4 °C overnight. After washing twice with TBST (15 min/time), the membrane was incubated with HRP conjugated goat anti rabbit-IgG or HRP conjugated goat anti rabbit-IgG for 2 h, and the antibody-specific protein was visualized by enhanced chemiluminesece detection system with ECL kit (Beyotime, Shanghai, China).

2.10. Statistical analysis

Statistical analyses were carried out with Graph Pad Prism 6software, and data were presented as mean ± SD. Statistical comparisons between multiple groups were performed using one-way ANOVA, followed by student *t*-test. P < 0.001 was considered statistically significant.

3. Results and discussion

3.1. Effect of TLH-3 on the viability and proliferation of RAW 264.7 cells

Cytotoxicity and proliferation of TLH-3 on RAW264.7 cells were firstly evaluated. After treated with TLH-3 (25–800 μg/ml) for 24 h, cells were detected for the viability using standard MTT assay. As shown in Fig. 1A, it is found that TLH-3 displayed little toxic againstRAW264.7 even up to 800 μg/ml. In addition, TLH-3 did not alter cell proliferation at a concentration of 25–400 μg/ml (Fig. 1B),

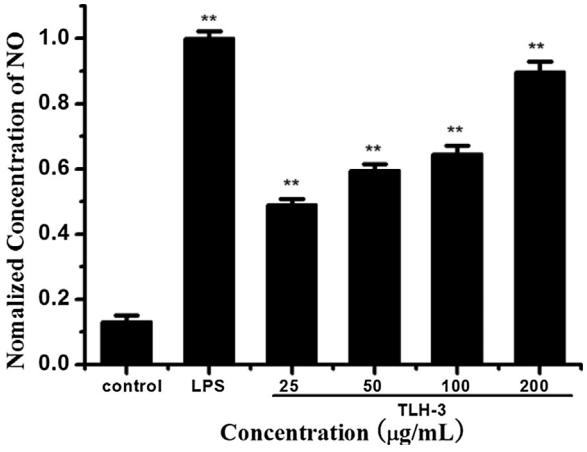

Fig. 2. Effect of TLH-3 on the production of NO in RAW 264.7 cells. RAW264.7 cells were treated with TLH-3 (25, 50, 100, and 200 μg/ml), positive control LPS (1 μg/ml) for 24 h. Production of NO was detected by Griess reagent, these results were expressed as ±SD (n = 3). Significant difference from the control group was designated as **P < 0.001.

which in agreement with MTT assay that indicated TLH-3 was non-toxic to RAW264.7 cells over time.

3.2. TLH-3 induced the production of NO from RAW264.7 cells

NO is an important inflammatory mediator, which is involved in the regulation of apoptosis and in host defenses against microorganisms and tumor cells [26]. Activated macrophages synthesize NO is an important cytotoxic/cytostatic mechanism of non-specific immunity [27]. As shown in Fig. 2, a minimum amount of NO was released when RAW264.7 cells were exposed to medium alone, whereas 25–200 μg/ml TLH-3couldsignificantly stimulate the production of NO in RAW 264.7 cells in a dose-dependent manner. It is noteworthy that the 200 μg/ml TLH-3 was caused equivalent NO production with 1 μg/ml LPS (positive control) and ∼6 fold higher compared to negative control group. It may be possible that these effects of TLH-3 were possibly LPS contamination of the polysaccharide. It is important to verify the absence or presence of LPS. Thus, in an attempt to eliminate the possibilities, both polysaccharides were pretreated with polymyxin B. Fig. 3 shows NO production by LPS was significantly suppressed by the pretreatment. In contrast, the polymyxin B-pretreated cells still possessed the activity for NO production in the presence of TLH-3. It means there have no LPS contamination of the polysaccharides. This reliably suggested that

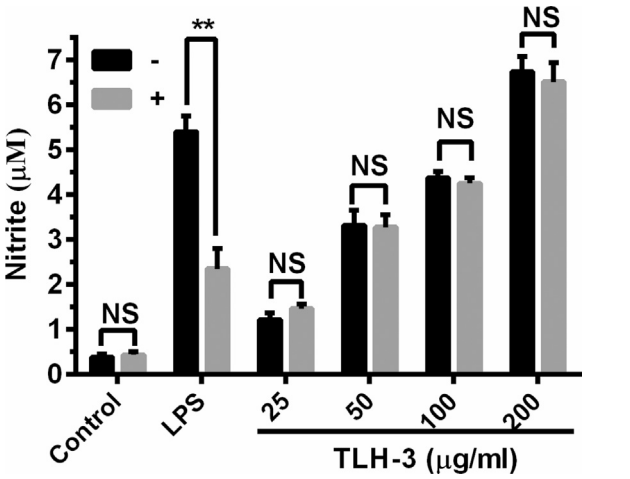

Fig. 3. Effect of polymyxin B on the NO production stimulated by polysaccharides. Positive control LPS (1 μg/ml) and TLH-3 (25, 50, 100, and 200 μg/ml) were pretreated with polymyxin B (100 μg/ml) at 37 °C for 1 h before treat RAW264.7 cells. These results were expressed as ±SD (n = 3). Significant difference from the control group was designated as **P < 0.001.

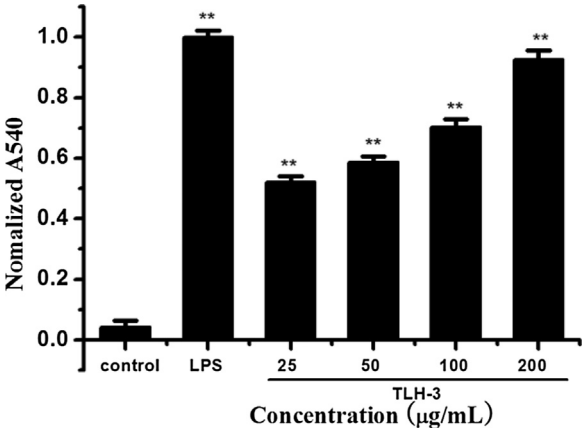

Fig. 4. The effect of TLH-3 on the phagocytic activity of RAW 264.7 cells. Cells were incubated with increasing concentrations of TLH-3 (25, 50, 100, and 200 μg/ml), positive control LPS (1 μg/ml) for 24 h. The phagocytic activity of RAW 264.7 cells was examined by the uptake of neutral red. These results were expressed as mean?SD (n = 3). Significant difference from the control group was designated as **P < 0.001.

TLH-3 might stimulate the NO production of RAW264.7 cells as an inhibitor to bacteria and tumor cells.

3.3. Determination of phagocytic uptake

Macrophages are monocyte-derived phagocytic cells that play crucial roles in adaptive and innate immunity [28]. The phagocytic function of macrophages is the primary and indispensable step the immune function to some extent [29,30]. The phagocytic activity of RAW 264.7 cells phagocytosis of neutral red assay treated by TLH-3 was examined. As shown in Fig. 4, compared to the control group, the phagocytic activity of macrophages treated withTLH-3 from 25 to 200 μg/ml for 24 h was enhanced significantly. The enhancement of the phagocytic activity was approximately 20 times higher at 200 μg/ml compared to the control group. This enhancement was about equivalent with LPS (1 μg/ml) treatment group. This result suggested that TLH-3 might be applied for therapy of microbial infection and tumor.

3.4. Quantitative analysis of cytokines and antibody inhibition experiments

Tumor necrosis factor-α (TNF-α) as the major proinflammatory cytokine produced by monocyticleukocytes in response to various stimuli and induce the expression of other inflammatory and immunoregulatory mediators [31]. Interleukin 6 (IL-6),a lymphocytes produced by activated T cells and fibroblasts, which can induce B cell precursors to become antibody secreting cell; and enhance the natural killer cell lysisfunction. Both of this are of great importance for immunoregulatory mediator. As show in Fig. 5, TLH-3significantly stimulated the secretion of TNF-α (Fig. 5A) and IL-6 (Fig. 5B) from 50?200 μg/ml. The enhancement of the TNF-α and IL-6 production was approximately 34 times and 4 times higher at 200 μg/ml compared with the control group and equivalent with LPS (1 μg/ml) treatment group. This result suggested that TLH-3 can facilitate the secretion of TNF-α and IL-6 in RAW 264.7 cells, which can also explain the NO production increase because TNF-α can induce the up-regulation of inducible nitric oxide synthase (iNOS) leading to release of NO [32].

Although Dectin-1 was known to be β-glucan specific receptor involved in the binding of polysaccharides to macrophages [33], several PRRs, especially TLR-4 also reported to participate in the regulation macrophage activation [10,11]. In order to verify if TLR4 was required for TLH-3 activation of RAW264.7 in cytokine production, in spite of western blot assays of TLR-4 shown no difference in various treatments(data no shown). TLR4 mAb inhibition experiments blocked TLH-3 induced TNF-α and IL-6 production was approximately 23% and 52% (Fig. 5) indicate that TLR4 plays an important role in TLH-3-mediated macrophage activation.

3.5. Effects of TLH-3 on the morphological change of RAW 264.7cells and nuclear translocation of NF-κB

Deformation of macrophages, especially the emergence of dendritic-like morphology, often reflects the activation of macrophages [34,35]. Herein macrophages showed a significant increase in morphologically active after LPS/TLH-3 stimulation RAW264.7 (Fig. 6). While he normal morphology of RAW 264.7 cells was round and form aggregates in the control group, after treated with LPS (1 μg/ml) for 24 h, nearly all of cells showed polygonal and dendritic-like structure. The RAW264.7 treated with different concentrations showed similar morphological changes as LPS-treat group in a dose-dependent manner.

The NF-κB family of transcription factors that ubiquitously controls the activity of genes involved in apoptosis, cell senescence, inflammation, and immunity. NF-κB as dimmers was constitutively present in the cytoplasm of RAW264.7 cells, sequestered by IκB-α protein. On stimulation, IκB-α protein are phosphorylated, ubiquitinated, and degraded, therefore freeing NF-κB dimers that become able to translocate to the nucleus and activate the transcription of their target genes [36]. Here NF-κB p65 antibodies and nuclear dye DAPI colocolization were conducted by NF-κB nuclear translocation. As shown in Fig. 7A, NF-κB p65 nuclear translocation using immunofluorescence staining measured by confocal laser-scanning microscopy. The fluorescence of proteinp65 was weak at nucleus in the control group, however in the LPS (1 μg/ml) and TLH-3 (200 μg/ml) treated group, the p65 fluorescence was clearly observed in nuclear region. This was further confirmed via the 3D viewer of TLH-3 treat group Fig. 7B, showed that p65 was translocated from cytoplasm to nucleus and accumulated in great extend within the nucleus to active the immunomodulation of RAW264.7 through the promotion of NO, IL-6, TNF-α production and phagocytic uptake.

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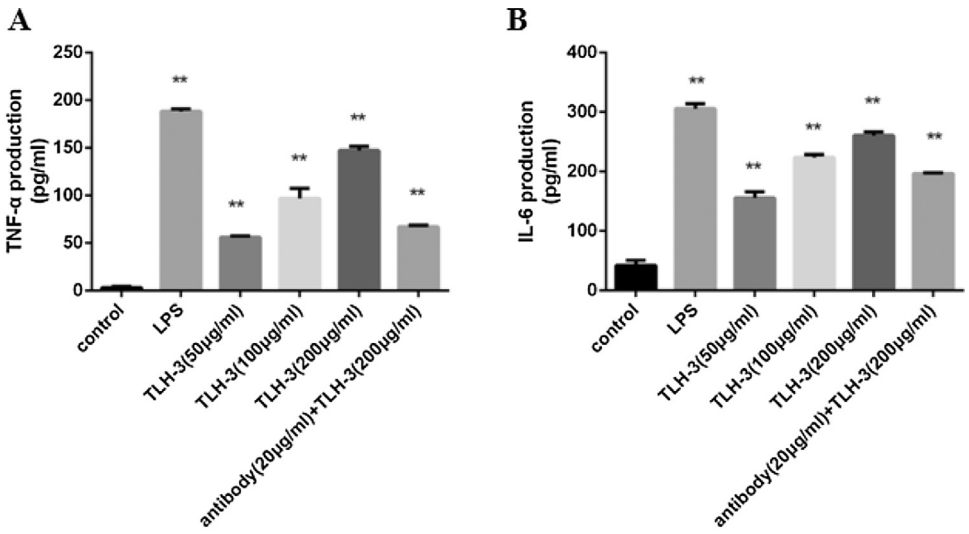

Fig. 5. Effects of TLH-3 on the production of TNF-α and IL-6 in RAW 264.7 cells, cells were incubated with increasing concentrations of TLH-3 (50, 100, and 200 μg/ml), positive control LPS (1 μg/ml) for 24 h; and the effects of TLR-4 mAb inhibits on the productions of TNF-?and IL-6 induced by TLH-3 in RAW 264.7 cells. RAW 264.7 cells were pre-incubated with TLR-4 mAb (20 μg/ml) for 1 h, followed by TLH-3 (200 μg/ml) stimulation for additional 24 hThese results were expressed as mean ±SD (n = 3). Significant difference from the control group was designated as *P < 0.05 and **P < 0.001.

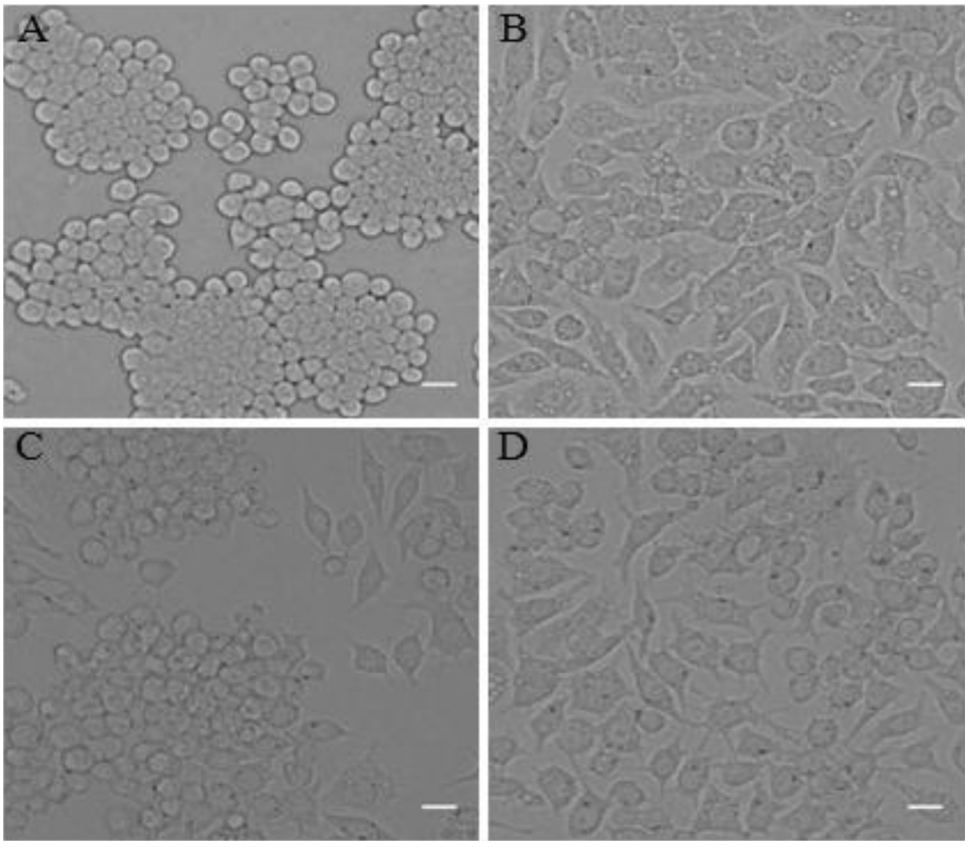

Fig. 6. The effect of TLH-3 on morphological changes of RAW264.7 cells. Cells were treated with TLH-3 (50, 200 μg/ml) or LPS (1 μg/ml) for 24 h. The morphological changes were observed under phase contrast microscope (Olympus, Japan). (A) showed the control group, (B) showed LPS (1 μg/ml) positive control group, (C) showed TLH-3 (50 μg/ml) treated group. (D) showed TLH-3 (200 μg/ml) treated group.

3.6. TLH-3 induced the IκB-α degradation and NF-κB activation in RAW264.7 cells

Degradation of cytoplasmic inhibitor IκB-α is a vital step for activating the NF- κB family of transcription factors. It was important to investigate the degradation of IκB-α in the cytoplasm and the translocation of NF-κB from the cytoplasm to the nucleus. The effect of TLH-3 on degradation of IκB-α was examined using western blotting analysis firstly. As shown in Fig. 8A, the protein in the cytoplasm content ratio between IκB-α and β-actin observably decreased from 0.864 ± 0.023 (control group) to 0.755 ± 0.018,0.674 ± 0.023,0.633 ± 0.016 (normalized intensity)at

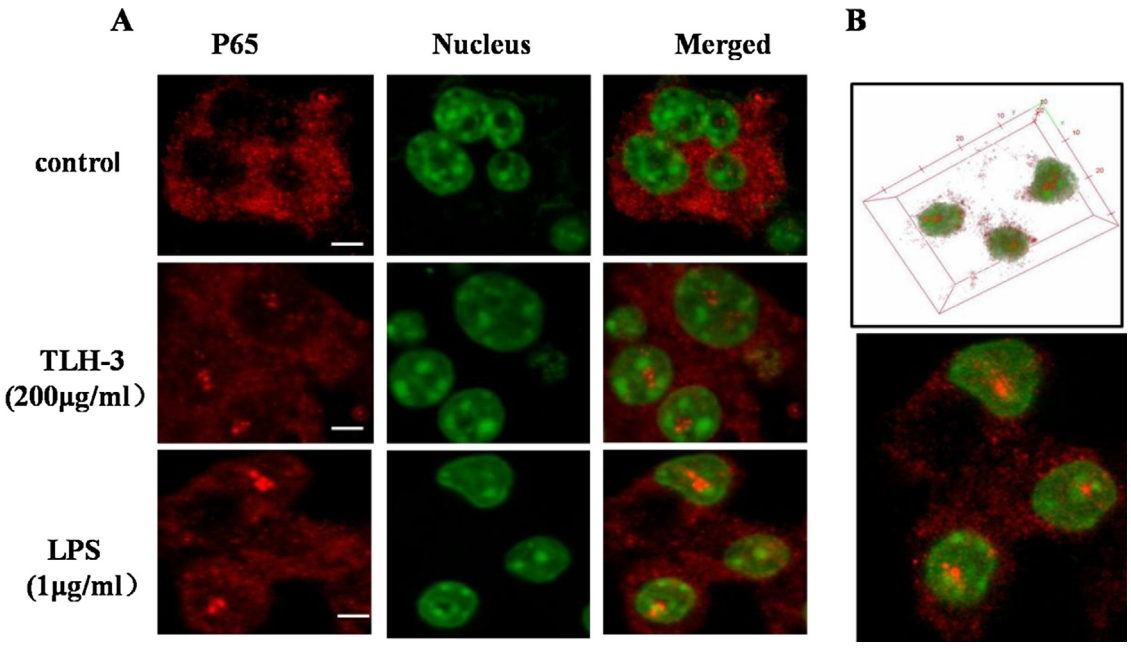

Fig. 7. Effect of TLH-3 on p65 nuclear translocation of RAW264.7 cells. (A)RAW 264.7 cells were treated with control medium, TLH-3 (200 μg/ml) and LPS (1 μg/ml) for 24 h, respectively. Then, cells were immuno-stained with p65 (red) mAb and DAPI (nucleus, green). The nuclear localization of p65 was determined using fluorescence microscopy after staining with DAPI, anti-p65, and Cy3 fluorescein-conjugated secondary antibody (p65 translocation into nucleus were indicated by white arrows).(B) 3D Z-stack confocal of TLH-3 (200 μg/ml) on p65 nuclear translocation of RAW264.7 cells.Images shown here are representatives of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

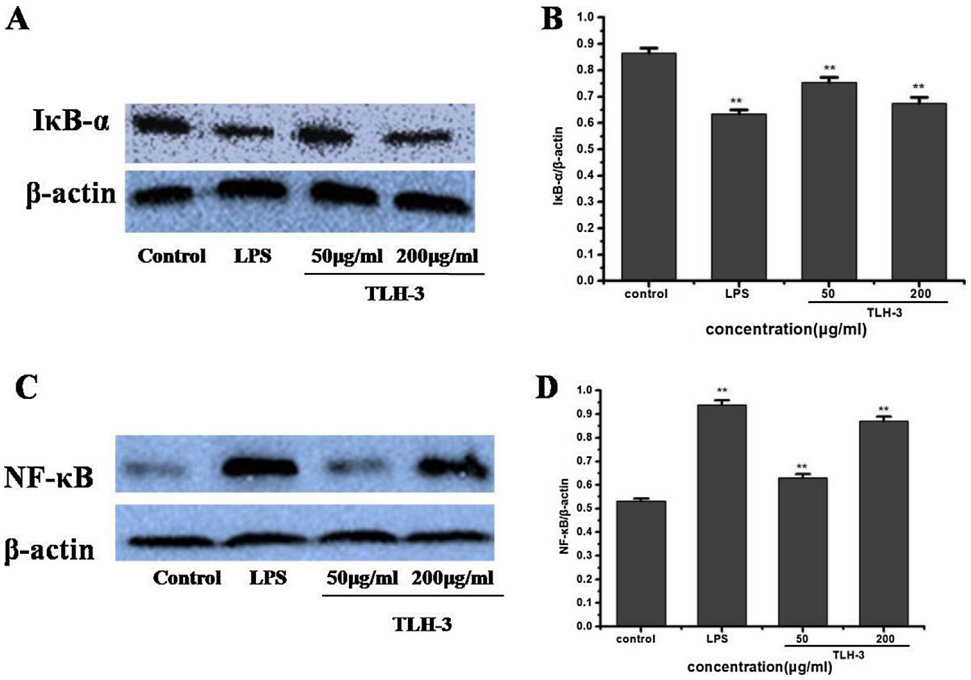

Fig. 8. TLH-3 induced degradation of IκB-α and NF- κB nuclear translocation in RAW 264.7 cells. RAW 264.7 cells were incubated with TLH-3 (200 μg/ml) and LPS (1 μg/ml) for 24 h. The degradation of IκB-α in cytoplasm and NF- κB in nuclear were determined by western blot. The anti-β-actin antibody was used as the control blots. These results were expressed as mean ±SD (n = 3). Significant difference from the control group was designated as $^{**}P < 0.001$.

the concentrations of 50,200 μg/ml of TLH-3 and LPS respectively. We next examined whether TLH-3 induced the translocation of NF- κB to nucleus in RAW 264.7 cells. As shown in Fig. 8C, stimulation of RAW264.7 with 200 μg/ml of TLH-3 led to an increase translocation of NF- κB to the nucleus as measured by western blotting assay. The ratio of protein content between NF- κB and β-actin was 0.630 ± 0.016, 0.870 ± 0.021, 0.939 ± 0.020, 0.531 ± 0.011 at the concentrations of 50, 200 μg/ml of TLH-3, 1 μg/ml of LPS and control group. This result further proved that TLH-3 could activate the nuclear transcription factor NF- κB in RAW 264.7 cells consistent with the above results.

4. Concluding

In this study, we found that the acidic polysaccharide TLH-3 obtained from Tricholoma lobayense Heim has potent immunos-

timulatory activity at cellular level. It is showed that TLH-3could increase NO production, IL-6, TNF-α secretion, as well as enhances the phagocytic uptake capacity in RAW264.7 macrophages through NF- κB signaling pathways via TLR-4.Therefore, this study provided new evidence that TLH-3 as a natural product could regard as a promising candidate as an immunomodulator. Future experiments are necessary to fully understand the detailed molecular mechanisms of TLH-3 activating macrophages and the overall immunostimulatory effect of TLH-3 in vivo.

Acknowledgments

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