J Appl Biomed
DOI: 10.32725/jab.2024.005
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

# *Dictyophora indusiata* polysaccharide mediates priming of the NLRP3 inflammasome activation via TLR4/ NF-κB signaling pathway to exert immunostimulatory effects

Youyi Liu [1 a], Huanxiao Zhang [1 a], Yuxuan Li [1], Hanqian Zha [1], Yujie Gao [1], Hui Chen [2], Yalin Wang [1], Tongxin Zhou [2 *], Chao Deng [1 *]

[1] *Jiangnan University, Wuxi School of Medicine, Wuxi, Jiangsu 214122, P.R. China*
[2] *Yixing Hospital of Traditional Chinese Medicine, Wuxi, Jiangsu 214200, P.R. China*

**Abstract**
*Dictyophora indusiata*, commonly known as bamboo fungus, is a type of edible mushroom that is highly popular worldwide for its rich flavor and nutritional value. It is also recognized for its pharmaceutical efficacy, with medicinal benefits attributed to its consumption. One of the most important components of *Dictyophora indusiata* is polysaccharide, which has been acknowledged as a promising regulator of biological response due to its immunostimulatory and anti-inflammatory properties. However, the specific roles of polysaccharide in modulating the NOD-like receptor protein 3 (NLRP3) inflammasome activation within macrophages remain relatively under-researched. To investigate this further, the mechanism by which *Dictyophora indusiata* polysaccharide (DIP) exerts its immunostimulatory activity in RAW 264.7 macrophages was analyzed. Results indicated that DIP has the potential to facilitate the priming of NLRP3 inflammasome activation by enhancing TLR4 expression, phosphorylation of IκB-α, and nuclear translocation of NF-κB p65 subunit. It was noted that DIP was unable to mediate the second step of NLRP3 inflammasome activation. The findings of this study provide compelling evidence that DIP has immunomodulatory effects by modulating the NLRP3 inflammasome in RAW264.7 macrophages.

**Keywords:** *Dictyophora indusiata* polysaccharide; Immunostimulatory activity; Macrophage; NLRP3 inflammasome

**Highlights:**
- *Dictyophora indusiata* polysaccharide (DIP) is a promising immunomodulator.
- DIP activates NLRP3 inflammasome via TLR4/NF-κB signal pathway.
- The application of DIP is potentially important for immunomodulatory therapies.

**Abbreviations:**
ASC, Apoptosis-associated speck-like protein; DAMPs, Danger-associated molecular patterns; DIP, *Dictyophora indusiata* polysaccharide; FBS, Fetal bovine serum; IL, Interleukin; LPS, Lipopolysaccharide; NF-κB, Nuclear factor kappa B; NLRP3, NOD-like receptor protein 3; PAMPs, Pathogen-associated molecular pattern; P2X7R, P2X purinoceptor 7 channel; ROS, Reactive oxygen species; TLR4, Toll-like receptor-4

## Introduction

The immune system plays a critical role in recognizing and responding to various microbial pathogens, including Gram-negative bacteria (Yu et al., 2014). One of the key mechanisms by which the immune system recognizes Gram-negative bacteria is through the activation of Toll-like receptor 4 (TLR4), which recognizes bacterial lipopolysaccharide (LPS) in the outer membrane (Martinez-Micaelo et al., 2015). Upon activation, TLR4 triggers a cascade of inflammatory responses that lead to the activation of NF-κB and the subsequent induction of various inflammatory mediators, such as IL-6 (Guo et al., 2018; Tak and Firestein, 2001).

Inflammation is an essential process of the innate immune system that plays a crucial role in both the activation of adaptive immunity and the maintenance of homeostasis (Freudenberg et al., 2015). However, dysregulated immune responses

* **Corresponding authors:** Chao Deng, Wuxi School of Medicine, Jiangnan University, Wuxi 214122, China; e-mail: dcao@jiangnan.edu.cn. Tongxin Zhou, Pulmonary Medicine Department, Yixing Hospital of Traditional Chinese Medicine, Wuxi 214200, China; e-mail: 1920304422@qq.com
http://doi.org/10.32725/jab.2024.005
[a] These authors contributed equally to the study.

Submitted: 2023-10-09 • Accepted: 2024-03-13 • Prepublished online: 2024-03-13
J Appl Biomed 22/1: 23–32 • EISSN 1214-0287 • ISSN 1214-021X

and persistent inflammation can lead to severe outcomes, such as immune dysfunction, sepsis, tissue damage, organ failure, and even fatality (Xin et al., 2017).

The NOD-like receptor protein 3 (NLRP3) inflammasome is a component of the innate immune system that recognizes cellular danger signals and promotes the maturation of pro-inflammatory cytokines such as IL-1β and IL-18 (Schroder and Tschopp, 2010). The inflammasome consists of three key components: NLRP3, the (apoptosis-associated speck-like protein) ASC, and caspase-1. Upon recognizing cellular stress, NLRP3 recruits ASC and pro-caspase-1, leading to inflammasome maturation and the release of pro-inflammatory cytokines (Liu et al., 2018). Numerous studies have shown that the secretion of IL-18 is associated with various auto-inflammatory diseases, including Alzheimer's disease. Dysregulated inflammasome activity has been strongly linked to several human heritable and acquired disorders, further underscoring the importance of NLRP3 inflammasome-mediated immune responses (Mangan et al., 2018).

Activation of the NLRP3 inflammasome in certain cells like macrophages or neutrophils, follows a two-step mechanism. The first signal involves TLR-mediated activation of NF-κB, which transcribes pro-IL-1β and NLRP3 (Dowling and O'Neill, 2012; Lamkanfi and Dixit, 2014; Sharma and Kanneganti, 2016; Zeng et al., 2017). The second signal is initiated by the detection of molecules with functions similar to pathogen-associated molecular patterns (PAMPs), or danger-associated molecular patterns (DAMPs) such as Reactive Oxygen Species (ROS) and Extracellular ATP. This results in caspase-1-dependent cleavage and activation of the NLRP3 inflammasome (Janeway and Medzhitov, 2002; Sutterwala et al., 2014).

Polysaccharides are biopolymer compounds known to play fundamental roles in diverse physiological cellular processes. Research in the field of immunology and virology has explored the potential of certain polysaccharides in the regulation of immune response. These polysaccharides are often investigated for their ability to modulate the host immune system and potentially exhibit antiviral effects (Chen and Huang, 2018). *Dictyophora indusiata* is among the world's most renowned edible mushrooms, with the *Dictyophora indusiata* polysaccharide (DIP) considered a promising regulator of biological response due to its immunostimulatory and anti-inflammatory properties (Jin and Flavell, 2010). However, there is little knowledge regarding the immunostimulatory roles of DIP in the regulation of NLRP3 inflammasome activation in macrophages. Accordingly, this study aims to investigate the mechanisms underlying modulation of the NF-κB signal transduction pathway and NLRP3 inflammasome-mediated production of IL-18 and IL-1β in RAW 264.7 macrophages by DIP. The findings of this study could have important implications for the development of potential immunomodulatory therapies using DIP.

## Materials and methods

### *Chemicals and reagents*

To perform their experiments, the researchers in this study obtained LPS extracted from *Escherichia coli* 0111: B4 from Sigma-Aldrich (St. Louis, MO, USA) as well as ATP from InvivoGen (San Diego, CA, USA). The cell culture media and supplements utilized consisted of fetal bovine serum (FBS), penicillin G, streptomycin, and Dulbecco's modified eagle medium (DMEM) which were all purchased from Gibco (Carlsbad, CA, USA).

### *DIP preparation and characterization*

In this study, the fruiting bodies of *D. indusiata* were collected from Changning, China, and purified to isolate DIP via a series of methods as described previously (Wang et al., 2019). Initially, the fungal bodies were ground into powder, and lipids were extracted. Then, the powder was extracted with distilled water at 90 °C, and the supernatant was obtained by centrifugation to collect the polysaccharide fractions. The polysaccharides were then precipitated with ethanol to increase the yield, and protein was removed using the Sevage method. To further ensure purity, the polysaccharide was characterized via gel filtration chromatography, dialysis, and lyophilization. Subsequently, DIP was dissolved in DMEM, filtered with a 0.22 μm filter, and preserved at −20 °C in readiness for further analyses. The DIP was found to have a total sugar content of 95.67%, and in the DIP solution at a concentration of 25 μg/ml, the endotoxin content is below 0.01 ng/ml (Wang et al., 2019).

As shown in Fig. 1A, the molecular structure and chain of DIP have been previously established and reported (Deng et al., 2012). In this study, Fourier transform infrared (FTIR) spectroscopy, gel permeation chromatography (GPC), and nuclear magnetic resonance (NMR) were employed to validate the consistency of DIP with earlier findings. The FTIR analysis displayed characteristic absorption peaks of DIP distributed at 3313 $cm^{-1}$, 2927 $cm^{-1}$, and 1621 $cm^{-1}$ corresponding to the hydroxyl group stretching vibration, C-H band, and bound water, respectively (Fig. 1B). Moreover, the characteristic absorption at 891 $cm^{-1}$ confirmed the existence of a β-type glycosidic bond in DIP. The $^{13}$C-NMR spectrum exhibited six peaks relating to the carbons in [β-Glc(1→3)-]n (Fig. 1C). Based on Table 1, the molecular weight of DIP was calculated to be 651 kDa.

### *Cell culture and stimulation*

The RAW264.7 macrophage-like cell line was procured from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM supplemented with high glucose (4.5 g/l), 10% FBS, streptomycin (100 μg/ml), and penicillin (100 U/ml) at 37 °C in a 5% $CO_2$ humidified atmosphere.

To evaluate the potential of DIP to induce an inflammatory response by modulating NLRP3 inflammasome activation, RAW264.7 cells were divided into distinct groups, namely: (i) blank control: cells cultured with only DMEM medium, (ii) LPS group: cells stimulated with LPS (1 μg/ml) for 3 h, followed by another hour of stimulation with 5 mM ATP, and (iii–v) DIP groups: cells treated with DIP at varying concentrations of 12.5 μg/ml, 25 μg/ml, or 50 μg/ml for 4 h each. The DIP solution was diluted and prepared using DMEM.

### *Effect of DIP on TLR4 expression*

After treating the cells in each group with PE anti-CD284 (TLR4) antibody (BioLegend, USA) for 2 h, flow cytometry was performed to measure TLR4 expression.

### *RNA isolation and quantitative Real-Time PCR (qPCR)*

Total RNA was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcribed using a RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA). QPCR was conducted with a Roche LightCycler 96 Real-Time System, utilizing UltraSYBR mixture (CWBIO, China). The reaction mixture comprised of 95 °C for 10 min, followed by 40 cycles, and then 95 °C for 15 s and 60 °C for 1 min. The primer sequences (5'-3') for IL-6, IL-1β, and GAPDH were as follows: IL-6, ACAACCACGGCCTTCCCTACTT, and CACGATTTCCCAGAGAACATGTG; IL-1β, GCAACTGTTCCTGAACT-

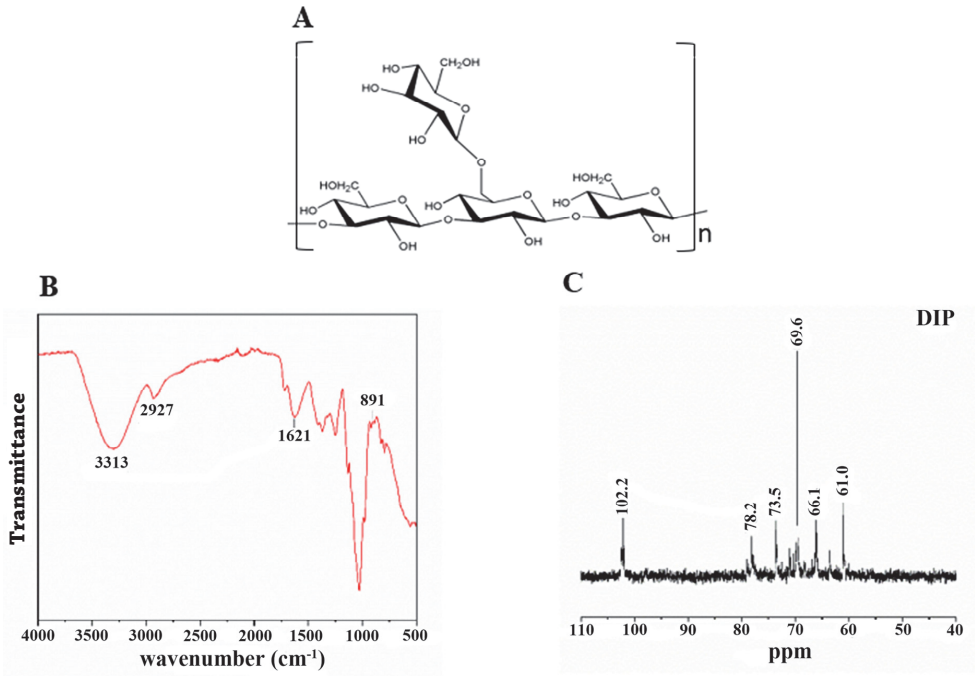


**Fig. 1.** The structure and chain of DIP (**A**) and its FTIR spectra (**B**), $^{13}$C-NMR spectra (**C**)

**Table 1. The molecular weight of DIP**

| Molecular weight | DIP |
|---|---|
| Mw (g/mol) | $6.512 \times 10^5$ |
| Mn (g/mol) | $3.181 \times 10^5$ |
| Polydispersity index (Mw/Mn) | 2.05 |

CAACT, and ATCTTTTGGGGTCCGTCAACT; GAPDH, GAGCCAAACGGGTCATCATCT and GAGGGGCCATCCACAGTCTT. The relative expression levels of the inflammatory cytokines IL-6 and IL-1β were normalized to GAPDH using the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

### *Western blot analysis*

Supernatant and cellular fractions were collected to assess NLRP3 inflammasome activation by Western blot analysis. Denatured proteins were separated on a 10% SDS-PAGE gel and transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 h at room temperature and then exposed to primary antibodies for 1 h at 37 °C. Primary antibodies utilized were rabbit anti-phospho-IκBα (Abcam, UK), anti-IκBα (Abcam, UK), anti-NLRP3 (Abcam, UK), anti-pro-caspase-1 (Abcam, UK), anti-GAPDH (Beyotime Biotechnology, China), or goat anti-IL-1β (R&D system, China). Subsequently, the membrane was washed and incubated with a corresponding HRP-conjugated secondary antibody. Semi-quantitative protein quantification was performed using ImageJ software (Bio-Rad, CA, USA).

### *Effects of DIP on NF-κB nuclear translocation*

To determine the impact of DIP on nuclear translocation of NF-κB, immunofluorescence analysis was conducted. RAW264.7 cells ($1 \times 10^5$) were seeded in a confocal plate and then treated with various concentrations of DIP. The cells were subjected to immunostaining blocking buffer (Beyotime, Shanghai, China), followed by overnight incubation with rabbit monoclonal antibody NF-κB p65 (1 : 200, Beyotime, Shanghai, China) at 4 °C and then with Cy3-labeled goat anti-rabbit IgG (Beyotime, Shanghai, China) for another hour in the dark. DAPI solution was used to stain the nuclei for 3 min before the images were viewed using confocal laser scanning microscopy (CLSM, Nikon A1, Japan).

### *Measurement of secretion of IL-6, IL-1β, and IL-18*

Mature forms of IL-1β, IL-18, and IL-6 cytokines were detected using commercial ELISA kits (Invitrogen, USA). The tests were conducted according to the manufacturer's guidelines, and all experiments were repeated at least three times.

### *Determination of caspase-1 activation*

To determine the activity of caspase-1 enzyme, a commercial kit (Beyotime, Shanghai, China) was employed following the manufacturer's instructions. The cellular extract was used after incubation with Ac-YVAD-pNA, a substrate of caspase-1, for 3 h at 37 °C in a 96-well microtiter plate. The absorbance values of pNA were then measured using a microplate reader at a wavelength of 405 nm (SpectraMax M5, Molecular Devices, USA).

### *Detection of ROS generation*

The level of ROS in the RAW264.7 cells was determined using a commercial kit obtained from Beyotime Biotechnology (Shanghai, China). This assay was conducted to assess the oxidative stress status of cells treated with DIP. The assay was performed in triplicate to ensure the reliability of the results.

### *Statistical analysis*

The data obtained from this study are presented as means ± standard deviation (SD), which is a standard format for reporting experimental results. Statistical analysis was performed using SPSS 23.0 software, which is commonly used for statistical analysis in biomedical research. To compare the data between different groups, ANOVA was employed, followed by a post-hoc test to determine significant differences. A *P*-value of less than 0.05 was regarded as statistically significant.

## Results

### *DIP enhanced TLR4 expression on the surface of macrophages*

Fig. 2 demonstrates the expression of TLR4 on macrophage surfaces in each experimental group. The fluorescent intensity was measured to assess TLR4 expression levels in the presence of DIP at concentrations of 12.5, 25, and 50 μg/ml. The results indicated a significant increase in TLR4 expression levels compared to the blank control group ($P < 0.05$). Macrophages treated with 25 μg/ml DIP showed the highest fluorescence intensity among the cells treated with DIP. Moreover, there was a statistically significant difference in the fluorescence intensity between the DIP group and the LPS + ATP group ($P < 0.05$). These findings suggested that DIP potentially stimulated the expression of TLR4 on macrophage surfaces, which is an essential receptor involved in the recognition of pathogen-associated molecular patterns and initiation of the immune system response.

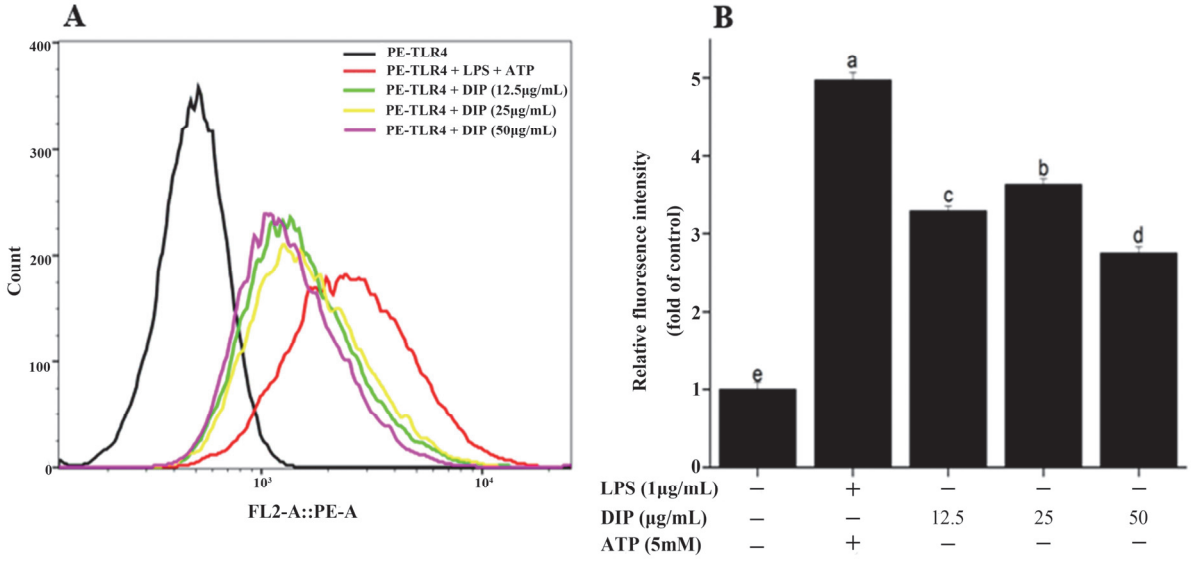


**Fig. 2.** The effects of DIP on expression of TLR4 in RAW 264.7 macrophages. (**A**) The expression of TLR4 in the cell surface was detected by flow cytometry. (**B**) Relative fluoresence intensity. Different lowercase letters indicated statistical differences ($P < 0.05$).

### *DIP up-regulates pro-inflammatory cytokine gene expression*

Fig. 3 presents the results of qRT-PCR analysis, which measured the expression levels of target genes in each experimental group. The mRNA expression levels of IL-1β and IL-6 were significantly higher in the DIP-treated groups compared to the blank control group ($P < 0.05$). Among the DIP-treated groups, macrophages exposed to 25 μg/ml DIP exhibited the highest expression levels of both IL-1β and IL-6. These findings suggested that DIP was capable of up-regulating the expression of pro-inflammatory cytokines IL-1β and IL-6 in macrophages.

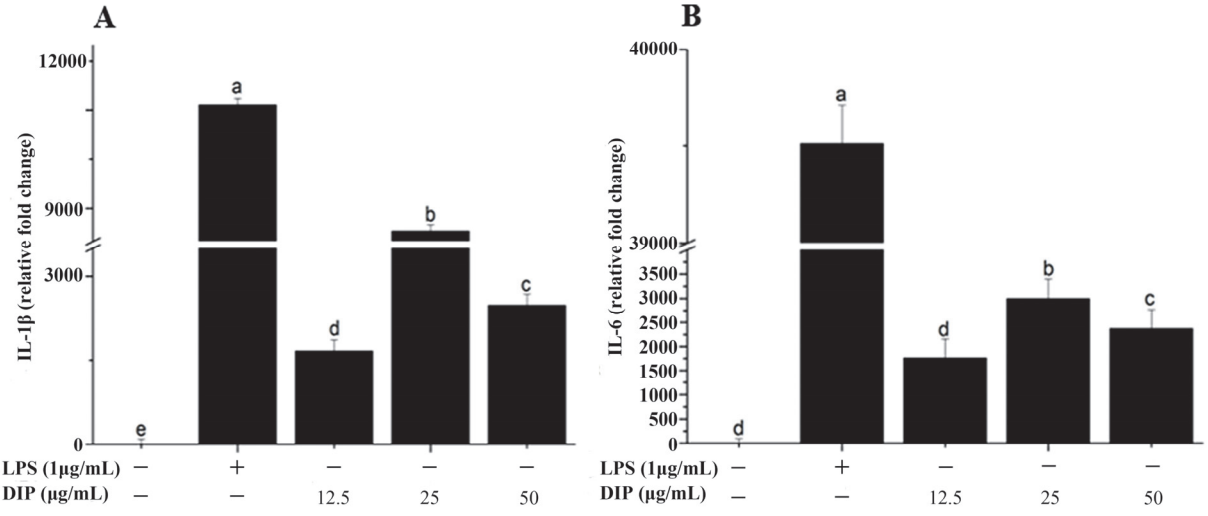


**Fig. 3.** The relative mRNA expression of IL-1β and IL-6 in the presence of DIP in RAW 264.7 cells detected by qRT-PCR

### *DIP promotes transcriptional activation of NF-κB*

The NF-κB signaling pathway plays a prominent role in the regulation of immune responses, inflammation, and physiological processes. Fig. 4 depicts the effects of DIP on the NF-κB signaling pathway in macrophages. In unstimulated cells, NF-κB remained in an inactive state by binding to its inhibitor protein IκBα and staying in the cytoplasm. However, DIP activation of TLR4 potentially led to phosphorylation of IκBα, resulting in the degradation of IκBα and subsequent nuclear translocation of NF-κB. Fig. 4A and 4B illustrate that DIP treatment significantly raised the level of phosphorylated IκBα in the intracellular of macrophage compared to the blank control group, and the maximum effect was observed at 25 μg/ml DIP concentration.

In Fig. 4C, macrophages treated with DIP displayed a remarkably enhanced nuclear NF-κB (p65) localization in a dose-dependent manner, particularly at a concentration of 25 μg/ml. Furthermore, Fig. 4D shows that DIP treated macrophages demonstrated higher IL-6 gene expression, which was also regulated by NF-κB.

### *DIP induces priming step of NLRP3 inflammasome activation*

Fig. 5 reports the effects of DIP on the expression of NLRP3 and pro-caspase-1 in macrophages. Fig. 5A and 5B demonstrate that the expression of NLRP3 was significantly up-regulated in the intracellular of macrophages stimulated with DIP (12.5, 25, and 50 μg/ml), particularly at a concentration of 25 μg/ml, similar to observations in the positive control group, where LPS induced a significant increase in the expression of NLRP3 compared to the blank control group. Nonetheless, DIP did not affect the expression of pro-caspase-1 in the supernatant (Fig. 5A and 5D), which suggested that DIP might be adequate to modulate the first step of NLRP3 inflammasome activation, *i.e.*, priming.

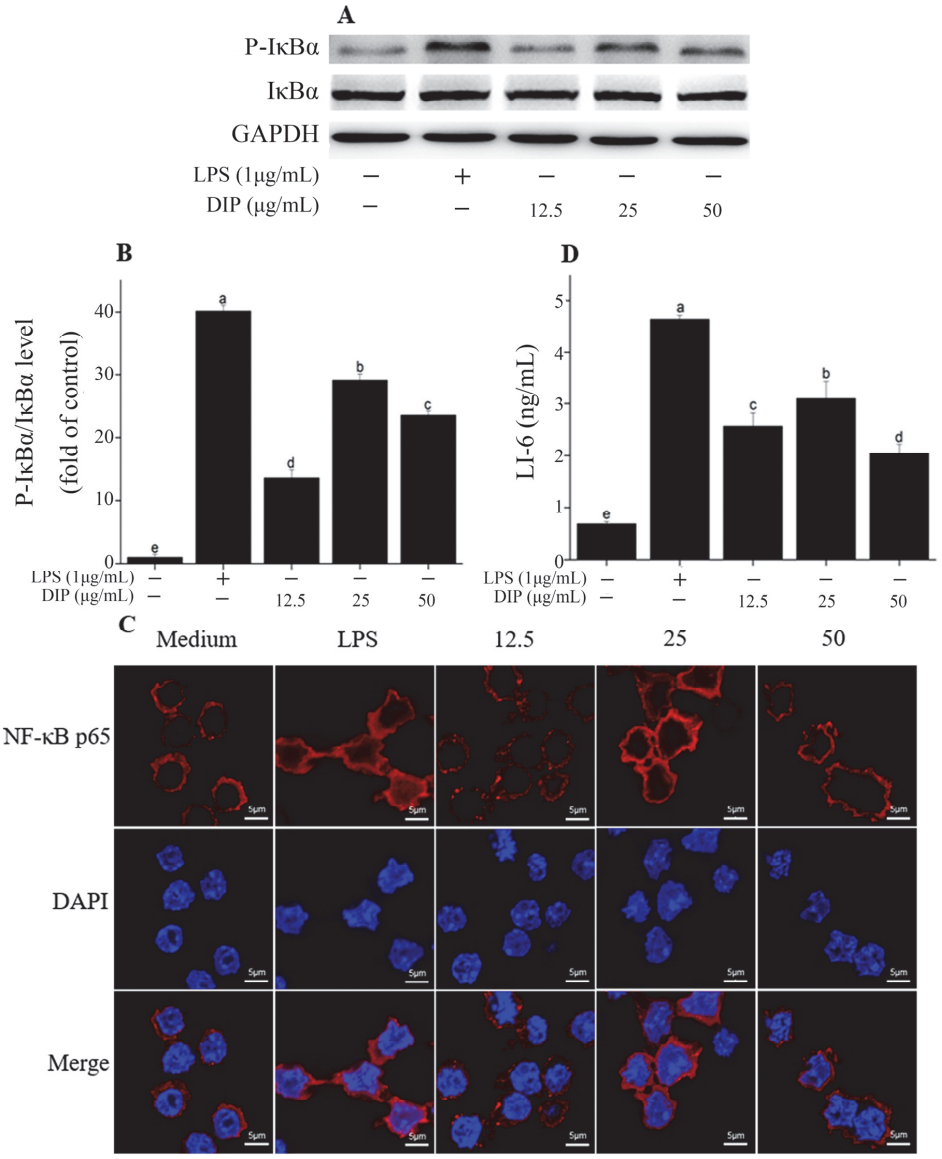


**Fig. 4.** The inducing effect of DIP on the activation of NF-κB. (**A**) Expression of P-IκBα and IκBα protein in the intracellular of macrophages determined using Western blot analysis. (**B**) Protein semi-quantification for P-IκBα and IκBα. (**C**) Nuclear translocation of NF-κB p65 was observed under a CLSM. (**D**) Secretion of IL-6 detected by ELISA. Bars with different lowercase letters indicated a significant difference between the groups ($P < 0.05$).

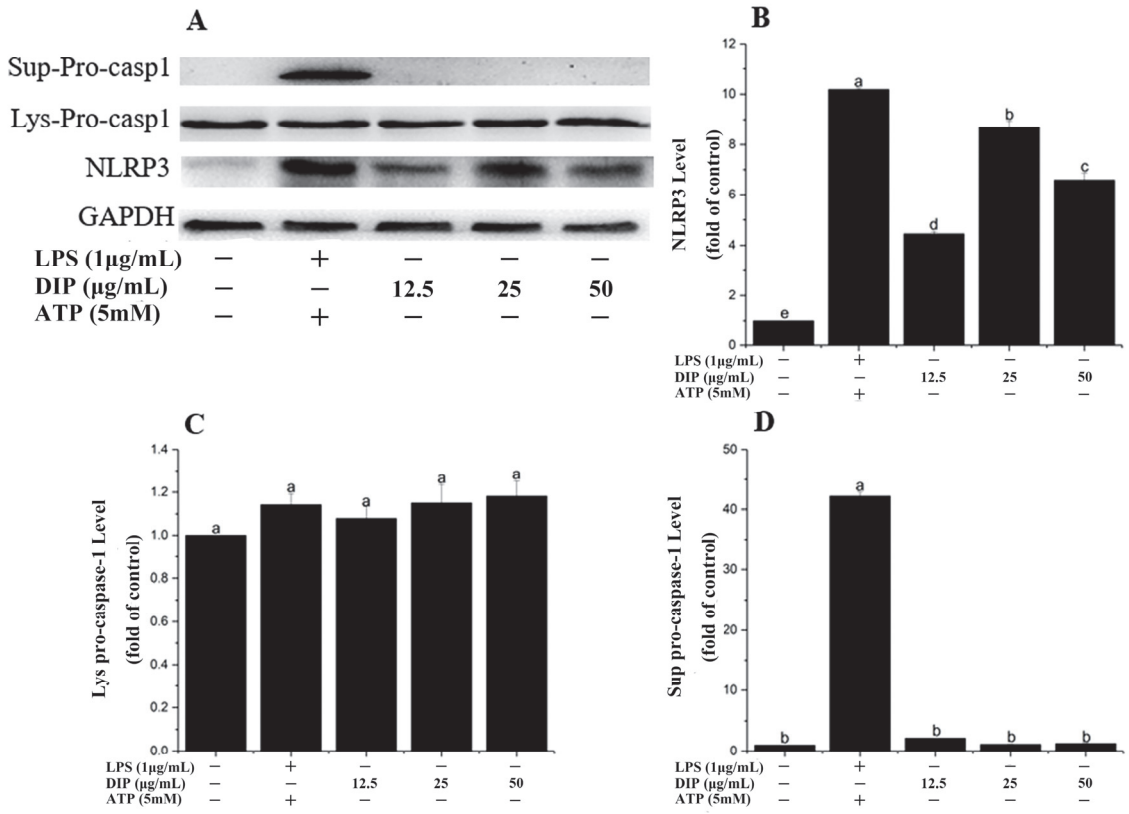


**Fig. 5.** Effects of DIP on expression of inflammasome components in priming of the NLRP3 inflammasome activation. (**A**) Western blot analysis of NLRP3 level in the intracellular of macrophages, and pro-caspase-1 level in intracellular and supernatant fractions. (**B–D**) Protein semi-quantifications were shown for NLRP3 and pro-caspase-1. Different lowercase letters indicated statistical differences ($P < 0.05$).

### *DIP has no effect on the second step of NLRP3 inflammasome activation*

The NLRP3 inflammasome and its downstream inflammatory mediators, including caspase-1, IL-1β, and IL-18, play crucial roles in the innate immune responses and inflammation (Elliott and Sutterwala, 2015). Fig. 6 illustrates the effects of DIP on the secretion of caspase-1, IL-1β, and IL-18 in macrophages. The level of IL-1β in the supernatant was detected using western blot and ELISA, while sup-IL-18 level was detected using ELISA. Despite DIP activating the NLRP3 inflammasome priming step, the results suggested that DIP had no significant impact on the secretion of caspase-1 (Fig. 6C), IL-1β (Fig. 6B and 6D), and IL-18 (Fig. 6E), indicating that the second step of NLRP3 inflammasome activation was unaffected by DIP.

### *DIP has no effects on ROS production*

The second step of NLRP3 inflammasome activation involves the recruitment and activation of precursor caspase-1 by the active NLRP3 inflammasome, leading to the generation of mature cytokines and exacerbating the inflammatory response. ROS have been implicated in NLRP3 inflammasome activation. Fig. 7 shows the effect of DIP on ROS production in macrophages. The results suggested that DIP had no significant effect on ROS production compared to the blank control group.

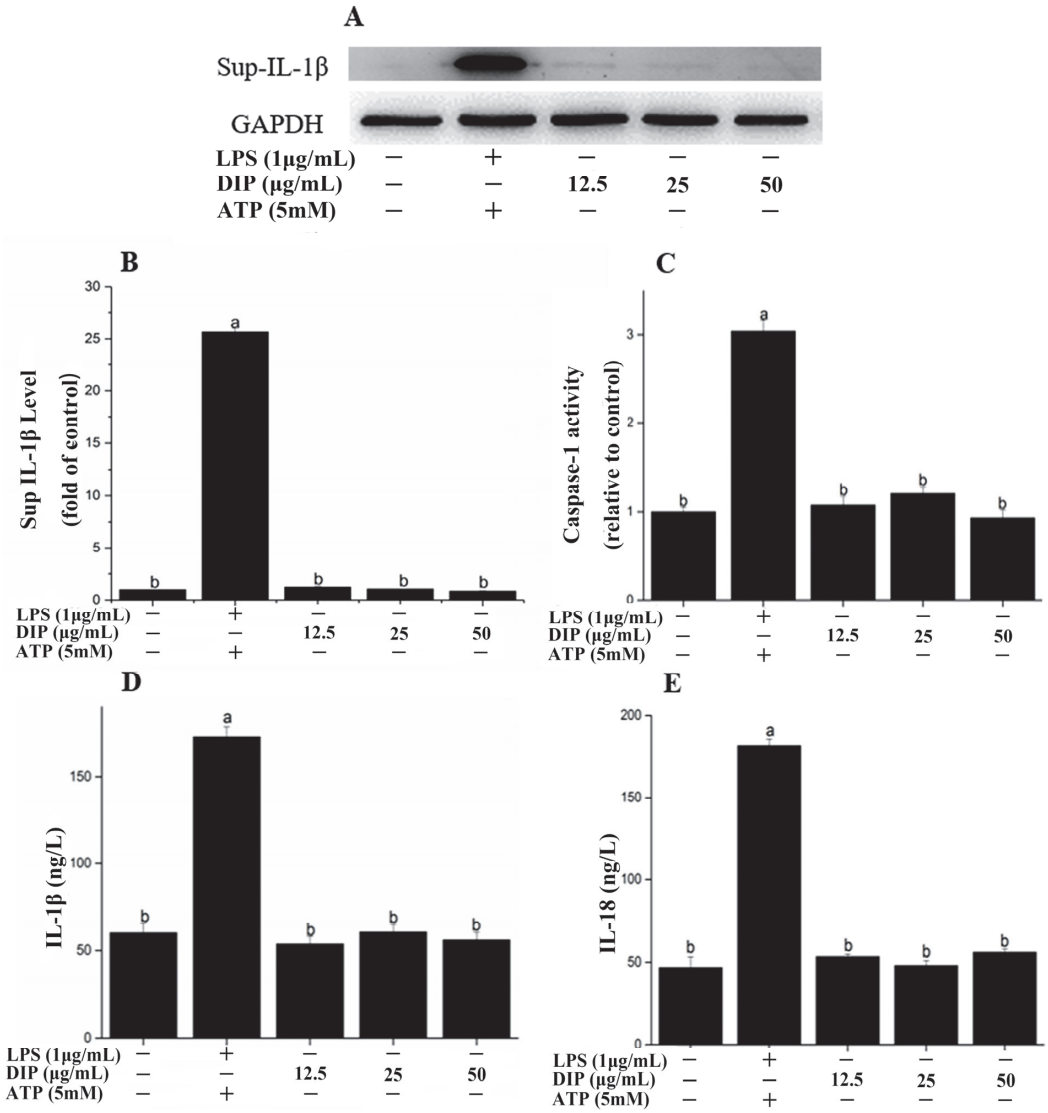


**Fig. 6.** Effects of DIP on the second step of activation of the NLRP3 inflammasome. (**A**) IL-1β protein level in supernatant. (**B**) Protein semi-quantifications were shown for supernatant IL-1β. (**C**) Analysis of caspase-1 activity using Ac-YVAD-*p*NA. (**D, E**) Secretion of IL-1β and IL-18 was detected by ELISA. Different lowercase letters indicated statistical differences ($P < 0.05$).

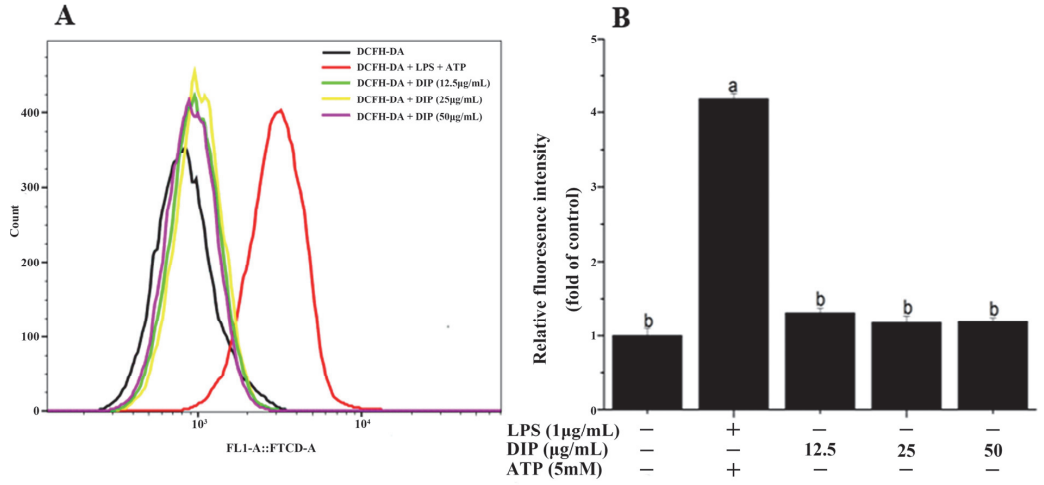


**Fig. 7.** Effects of DIP on ROS generation. (**A**) ROS generation in different DIP dose groups detected by flow cytometry. (**B**) Relative fluoresence intensity. Different lowercase letters indicated statistical differences ($P < 0.05$).

## Discussion

Inflammation is a complex process that is involved in the development of many human diseases (Martinez-Micaelo et al., 2015). DIP has gained attention due to its promising antioxidant, anti-inflammatory, and anti-cancer properties (Deng et al., 2013; Fu et al., 2015; Han et al., 2017). However, the mechanisms underlying the ability of DIP to modulate the immune response and its effects on the NLRP3 inflammasome are poorly understood. The present study aimed to investigate the impact of DIP on the NLRP3 inflammasome activation process.

The NLRP3 inflammasome is a crucial component of innate immunity, which senses pathogen invasion signals and cellular damage (Schroder and Tschopp, 2010). Upon activation, the NLRP3 inflammasome mediates the processing of precursors of IL-1β and IL-18 into mature forms, leading to an increase in inflammatory responses. The NLRP3 inflammasome is composed of the NLRP3 protein, ASC protein, and pro-caspase-1 zymogen. NLRP3, functioning as a receptor protein, can sense various stimuli both inside and outside the cell, such as bacteria, viruses, oxidative stress, and decreased intracellular potassium ion concentration. Upon activation, it recruits downstream adaptor proteins like ASC, subsequently regulating the activity of caspase-1, promoting the maturation of cytokines, triggering inflammatory responses, and mediating cellular pyroptosis (Janeway and Medzhitov, 2002; Sutterwala et al., 2014). However, the mechanisms underlying NLRP3 inflammasome activation remain largely unknown. One proposed mechanism is that NLRP3 inflammasome is activated by a common pathway of ROS, which is closely linked to mitochondria. A separate avenue of activation is seen through P2X7R channels, which permit external flow of K ions and require agonist ATP to activate NLRP3 inflammasome (Kanneganti et al., 2007; Liu et al., 2014; Pelegrin and Surprenant, 2006).

In our previous study, each experimental group was treated with LPS, providing an inflammatory stimulus, and ATP was added to each group, fully activating the macrophage inflammasomes, resulting in an inflammatory response. In this context, the investigation focused on the anti-inflammatory effects of DIP (Wang et al., 2019). In this study, LPS served as a positive control group, while in the experimental group, DIP was exclusively added for comparative analysis of its impact on inflammasome activation. The results demonstrated that DIP activated NLRP3 inflammasome priming, which involved enhancing TLR4 expression, phosphorylating IκB-α, and translocating the NF-κB p65 subunit into the nucleus. Additionally, DIP increased IL-6 secretion, a biomarker of inflammation that is regulated by the NF-κB pathway. However, DIP did not significantly affect the second step of NLRP3 inflammasome activation in RAW264.7 macrophages. This was primarily manifested by the inability of DIP stimulation to promote the secretion of pro-caspase-1, IL-1β, and IL-18. These findings suggested that DIP might enhance the immune response through the modulation of the NF-κB pathway and NLRP3 inflammasome priming without causing excessive inflammation or cellular damage. Further studies are required to determine the precise molecular mechanisms involved in the immunostimulatory effects of DIP on the NLRP3 inflammasome.

Interestingly, our results indicated that the immunostimulatory effect of DIP was better at a concentration of 25 ug/ml compared to a concentration of 50 ug/ml. This may be associated with the complexity of the interaction between polysaccharides and the immune system. The immune activity of polysaccharides is influenced by a variety of factors, and their mechanisms may involve complex networks of cellular and molecular interactions, such as receptor saturation effect, biphasic effect, and changes in bioavailability. Immune receptors may reach saturation at a certain concentration, meaning that further increases in polysaccharide concentration may not result in additional immune stimulation. Once receptors are saturated, higher concentrations may not induce a proportional immune response (Pizzuto et al., 2019). Some polysaccharides exhibit immune-stimulating effects at low concentrations but may demonstrate immune-suppressive effects at higher concentrations, creating a biphasic response. This dual effect could arise from diverse interactions between polysaccharides and different types of immune cells or molecules (Ruan et al., 2022; Zanoli and Zavatti, 2008). Furthermore, as the concentration increases, the bioavailability of polysaccharides may change, altering their accessibility or interaction within cells (Du et al., 2016). This complexity underscores the need for a nuanced understanding of the relationships between DIP and the immune system for the development of effective immunomodulatory strategies.

There are some limitations in this work. We focused on the detection of NLRP3 and caspase-1 due to their pivotal roles within the inflammasome, where ASC primarily functions as a bridging element. Given its more intermediary role, we chose to de-emphasize the detection of ASC in this investigation. Future studies and analyses could delve into the involvement of ASC to provide a more comprehensive understanding of the activation of inflammasome by DIP.

## Conclusion

Through our investigation, the data we have collected distinctly demonstrate that DIP can facilitate the priming of NLRP3 inflammasome activation by enhancing TLR4 expression, phosphorylating IκB-α, and translocating nuclear factor kappa B (NF-κB) p65 subunit. On the other hand, it has been observed that DIP is incapable of mediating the second step of NLRP3 inflammasome activation. These findings, for the first time, provide compelling evidence of the immunomodulatory effects of DIP through the regulation of NLRP3 inflammasome in RAW264.7 macrophages (Fig. 8).

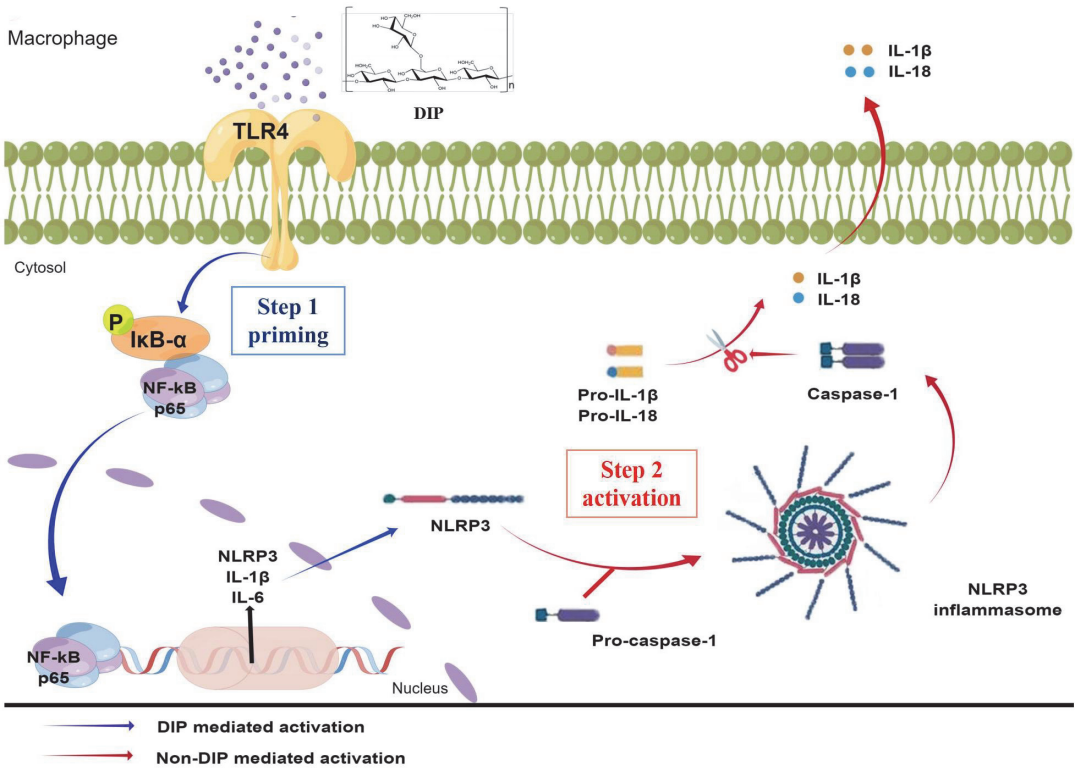


**Fig. 8.** DIP can facilitate the priming of NLRP3 inflammasome activation by enhancing TLR4 expression, phosphorylating IκB-α, and translocating nuclear factor kappa B (NF-κB) p65 subunit, but not mediating the second step of NLRP3 inflammasome activation

### *Author contributions*

*Youyi Liu:* Conceptualization, Methodology. *Huanxiao Zhang:* Methodology. *Yuxuan Li:* Writing – Original Draft. *Hanqian Zha:* Validation. *Yujie Gao:* Investigation; *Hui Chen:* Creation of models. *Yalin Wang:* Visualization. *Tongxin Zhou:* Resources, Review; *Chao Deng:* Project administration, Writing – Review and Editing. All authors approved the final manuscript.

### *Funding*

This work was supported by grants from the Postdoctoral Science Foundation of China (2021M691278), and the Research Project Plan of the Wuxi Municipal Health Commission (Q202243). We would like to thank Prof. Minchen Wu at the Wuxi School of Medicine for the technical assistance.

### *Ethical aspects and conflict of interest*

The authors have no conflict of interest to declare.

## References

Chen L, Huang G (2018). The antiviral activity of polysaccharides and their derivatives. Int J Biol Macromol 115: 77–82. DOI: 10.1016/j.ijbiomac.2018.04.056.

Deng C, Fu H, Teng L, Hu Z, Xu X, Chen J, Ren T (2013). Antitumor activity of the regenerated triple-helical polysaccharide from *Dictyophora indusiata*. Int J Biol Macromol 61: 453–458. DOI: 10.1016/j.ijbiomac.2013.08.007.

Deng C, Hu Z, Fu H, Hu M, Xu X, Chen J (2012). Chemical analysis and antioxidant activity *in vitro* of a β-D-glucan isolated from *Dictyophora indusiata*. Int J Biol Macromol 51: 70–75. DOI: 10.1016/j.ijbiomac.2012.05.001.

Dowling JK, O'Neill LAJ (2012). Biochemical regulation of the inflammasome. Crit Rev Biochem Mol Biol 47: 424–443. DOI: 10.3109/10409238.2012.694844.

Du LL, Fu QY, Xiang LP, Zheng XQ, Lu JL, Ye JH, et al. (2016). Tea Polysaccharides and Their Bioactivities. Molecules 21: 1449. DOI: 10.3390/molecules21111449.

Elliott EI, Sutterwala FS (2015). Initiation and perpetuation of NLRP3 inflammasome activation and assembly. Immunol Rev 265: 35–52. DOI: 10.1111/imr.12286.

Freudenberg U, Zieris A, Chwalek K, Tsurkan MV, Maitz MF, Atallah P, et al. (2015). Heparin desulfation modulates VEGF release and angiogenesis in diabetic wounds. J Control Release 220: 79–88. DOI: 10.1016/j.jconrel.2015.10.028.

Fu H, Deng C, Teng L, Yu L, Su T, Xu X, et al. (2015). Immunomodulatory Activities on RAW 264.7 Macrophages of a Polysaccharide from Veiled Lady Mushroom, *Dictyophora indusiata* (Higher Basidiomycetes). Int J Med Mushrooms 17: 151–160. DOI: 10.1615/intjmedmushrooms.v17.i2.60.

Guo J, Chen J, Lu X, Guo Z, Huang Z, Zeng S, et al. (2018). Proteomic Analysis Reveals Inflammation Modulation of κ/τ-Carrageenan Hexaoses in Lipopolysaccharide-Induced RAW264.7 Macrophages. J Agric Food Chem 66: 4758–4767. DOI: 10.1021/acs.jafc.8b01144.

Han S, Ma C, Hu M, Wang Y, Ma F, Tao N, Qin Z (2017). A polysaccharide from *Dictyophora indusiata* inhibits the immunosuppressive function of cancer-associated fibroblasts. Cell Biochem Funct 35: 414–419. DOI: 10.1002/cbf.3290.

Janeway CA, Medzhitov R (2002). Innate immune recognition. Annu Rev Immunol 20: 197–216. DOI: 10.1146/annurev.immunol.20.083001.084359.

Jin C, Flavell RA (2010). Molecular mechanism of NLRP3 inflammasome activation. J Clin Immunol 30: 628–631. DOI: 10.1007/s10875-010-9440-3.

Kanneganti TD, Lamkanfi M, Núñez G (2007). Intracellular NOD-like receptors in host defense and disease. Immunity 27: 549–559. DOI: 10.1016/j.immuni.2007.10.002.

Lamkanfi M, Dixit VM (2014). Mechanisms and functions of inflammasomes. Cell 157: 1013–1022. DOI: 10.1016/j.cell.2014.04.007.

Liu J, Wang Y, Ouyang X (2014). Beyond toll-like receptors: *Porphyromonas gingivalis* induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-κB and ERK signaling pathways in periodontal fibroblasts. Inflammation 37: 522–533. DOI: 10.1007/s10753-013-9766-0.

Liu Q, Zhang D, Hu D, Zhou X, Zhou Y (2018). The role of mitochondria in NLRP3 inflammasome activation. Mol Immunol 103: 115–124. DOI: 10.1016/j.molimm.2018.09.010.

Livak KJ, Schmittgen TD (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25: 402–408. DOI: 10.1006/meth.2001.1262.

Mangan MSJ, Olhava EJ, Roush WR, Seidel HM, Glick GD, Latz E (2018). Targeting the NLRP3 inflammasome in inflammatory diseases. Nat Rev Drug Discov 17: 688. DOI: 10.1038/nrd.2018.149.

Martinez-Micaelo N, González-Abuín N, Pinent M, Ardévol A, Blay M (2015). Procyanidin B2 inhibits inflammasome-mediated IL-1β production in lipopolysaccharide-stimulated macrophages. Mol Nutr Food Res 59: 262–269. DOI: 10.1002/mnfr.201400370.

Pelegrin P, Surprenant A (2006). Pannexin-1 mediates large pore formation and interleukin-1beta release by the ATP-gated P2X7 receptor. EMBO J 25: 5071–5082. DOI: 10.1038/sj.emboj.7601378.

Pizzuto M, Lonez C, Baroja-Mazo A, Martínez-Banaclocha H, Tourlomousis P, Gangloff M, et al. (2019). Saturation of acyl chains converts cardiolipin from an antagonist to an activator of Toll-like receptor-4. Cell Mol Life Sci 76: 3667–3678. DOI: 10.1007/s00018-019-03113-5.

Ruan S, Yang Y, Li W (2022). *Antrodia camphorata* Polysaccharide activates autophagy and regulates NLRP3 degradation to improve liver injury-related inflammatory response. Aging (Albany NY) 14: 8970–8981. DOI: 10.18632/aging.204330.

Schroder K, Tschopp J (2010). The inflammasomes. Cell 140: 821–832. DOI: 10.1016/j.cell.2010.01.040.

Sharma D, Kanneganti TD (2016). The cell biology of inflammasomes: Mechanisms of inflammasome activation and regulation. J Cell Biol 213: 617–629. DOI: 10.1083/jcb.201602089.

Sutterwala FS, Haasken S, Cassel SL (2014). Mechanism of NLRP3 inflammasome activation. Ann N Y Acad Sci 1319: 82–95. DOI: 10.1111/nyas.12458.

Tak PP, Firestein GS (2001). NF-kappaB: a key role in inflammatory diseases. J Clin Invest 107: 7–11. DOI: 10.1172/JCI11830.

Wang Y, Lai L, Teng L, Li Y, Cheng J, Chen J, Deng C (2019). Mechanism of the anti-inflammatory activity by a polysaccharide from *Dictyophora indusiata* in lipopolysaccharide-stimulated macrophages. Int J Biol Macromol 126: 1158–1166. DOI: 10.1016/j.ijbiomac.2019.01.022.

Xin W, Wang Q, Zhang D, Wang C (2017). A new mechanism of inhibition of IL-1β secretion by celastrol through the NLRP3 inflammasome pathway. Eur J Pharmacol 814: 240–247. DOI: 10.1016/j.ejphar.2017.08.036.

Yu Q, Nie SP, Wang JQ, Yin PF, Huang DF, Li WJ, Xie MY (2014). Toll-like receptor 4-mediated ROS signaling pathway involved in *Ganoderma atrum* polysaccharide-induced tumor necrosis factor-α secretion during macrophage activation. Food Chem Toxicol 66: 14–22. DOI: 10.1016/j.fct.2014.01.018.

Zanoli P, Zavatti M (2008). Pharmacognostic and pharmacological profile of *Humulus lupulus* L. J Ethnopharmacol 116: 383–396. DOI: 10.1016/j.jep.2008.01.011.

Zeng J, Chen Y, Ding R, Feng L, Fu Z, Yang S, et al. (2017). Isoliquiritigenin alleviates early brain injury after experimental intracerebral hemorrhage via suppressing ROS- and/or NF-κB-mediated NLRP3 inflammasome activation by promoting Nrf2 antioxidant pathway. J Neuroinflammation 14: 119. DOI: 10.1186/s12974-017-0895-5.