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Signaling pathways associated with macrophage-activating polysaccharide isolated from the fermentation liquor of *Rhizopus nigricans*

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ABSTRACT

Our previous reports showed that the structural features and immunologic enhancement of polysaccharide (EPS1-1) from *Rhizopus nigricans*. However, the molecular mechanism in cellular immunomodulatory of EPS1-1 remains unclear. Here the experiments for the molecular mechanisms of EPS1-1 on the peritoneal macrophages were performed. The results demonstrated that the expression of TLR4 was significantly improved by EPS1-1. Subsequently, the phosphorylation of p38MAPK, ERK1/2, JNK and IKKα/β were promoted. Moreover, EPS1-1 enhanced the expressions of IL-2, TNF-α and iNOS in EPS1-1-induced macrophages which were pretreated with MAPK signaling pathway inhibitors, and reduced the blocking effects of the inhibitors to the expressions of p-p38MAPK, p-ERK1/2 and p-IKKα/β. Therefore, these results illustrated that EPS1-1 could improve the immune functions of peritoneal macrophages by promoting the gene expressions of IL-2, TNF-α and iNOS via the MAPK and NF-κB signaling pathways.

Polysaccharides, as metabolic products from plants, animals and microorganisms, have been found with beneficial biological functions in human health, such as anti-tumour, antioxidant, and antiviral agents.[1,2] Moreover, it has been reported that polysaccharides with medical and nutritional value was confirmed to possess immunomodulatory functions without toxic or side effects.[3,4] Macrophage, one of the most important immune cells, is the target cell of polysaccharides which exert their immune function by promoting cytokine secretion and antibody production. It is known that many polysaccharides were reported to modulate the host immunity by activating immune cells, especially macrophages function.[5–7] The activation of macrophages is absolutely essential for immune defense by functioning as antigen-presenting cells, initiating the innate immune response and interacting with T lymphocytes to modulate the adaptive immune response.

In recent decades, the immunostimulatory mechanism of polysaccharides has been researched and attracted a great deal of attentions, especially the activation effect on macrophage function. These polysaccharides have been shown to increase macrophage activity that stimulated and modulated the host's immunity to against tumor cells and microorganisms via increasing the secretion of cytokines and chemokines, the production of reactive oxygen species (ROS) and nitric oxide (NO). The mechanism of polysaccharides in modulating the function of macrophages is being studied. Research has been shown that polysaccharides might activate or bind to some cell surface receptors on macrophages, such as Toll-like receptors (TLRs), thus triggering the intracellular signaling cascades to activate macrophages.[5] Subsequently, the activated macrophages could produce relative cytokines to enhance the immune response or directly kill pathogens or tumor cells by phagocytosis. Wu et al. identified a novel polysaccharide from *Dendrobium devonianum* which served as a TLR4 agonist for stimulating macrophages through activating MAPK and NF-κB signaling pathways.[8] Han Wool Kim et al. reported that polysaccharide (BF-I) enhanced macrophage activation properties by activating the JNK signaling pathway via several macrophage receptors (dectin-1, TLR4, SR, and CD14), resulting the increase of IL-6, IL-12, TNF-α and NO.[9] Consequently, it has been well-documented that polysaccharides can be considered as potential macrophage immunopotentiator used in medical and food industries.

Our previous studies have isolated and purified an extracellular polysaccharide (EPS1-1) from the fermentation liquor of *Rhizopus nigricans*, which was composed of glucose, mannose, galactose and

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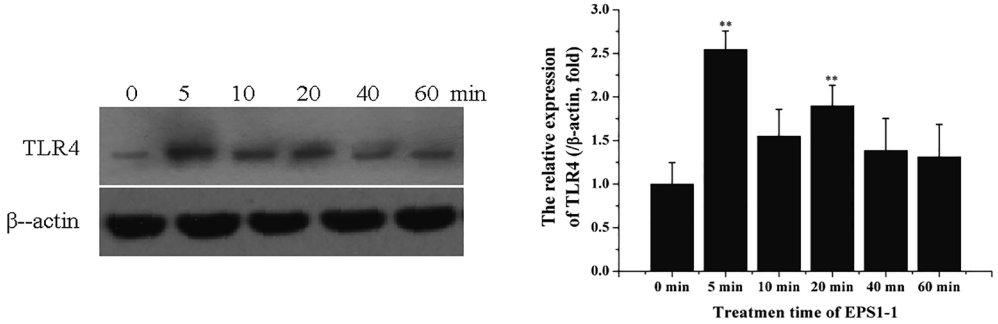

Fig. 1. Effect of EPS1-1 on the expression of macrophage cell surface receptor TLR4. The peritoneal macrophages were treated with EPS1-1 (0.2 mg/ml) for 0, 5, 10, 20, 40 and 60 min. The expression TLR4 was measured by western blotting. Data are presented as means ± SD. Significant differences from control group are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$.

fructose in the molar ration of 5.89:3.64:3.20:1.00 with average molecular weight of 9.7 × 10^3 g/mol with β-anomeric configuration.[10] Bioactive research showed that EPS1-1 possesses immunomodulatory activity *in vitro* and *in vivo*.[10–12] EPS1-1 stimulated the activities of macrophage to increase the phagocytosis, the secretion of NO and cytokines. However, the activation mechanism of EPS1-1 on macrophages remains unclear.

To determine whether TLR4 is required for EPS1-1 to stimulate the activation of macrophages, we determined the expression of TLR4 (PDB: 3VQ1) in macrophages after treatment with EPS1-1 (0.2 mg/ml) for 0, 5, 10, 20, 40 and 60 min, respectively. Western blot analysis showed that EPS1-1 increased the expression of TLR4 (Fig. 1), especially treatments for 5 min and 20 min with the significant difference compared with control group ($p < 0.01$). These results demonstrated that EPS1-1 might bind to the cell surface receptor TLR4 to activated macrophages via signaling cascades.

TLR4 mediates the activation of downstream events, such as mitogen-activated protein kinase (MAPK) and NF-κB pathway signaling, which are crucial elements for macrophages to induce the immune response. Western blotting was used to detect the phosphorylation and expression levels of proteins involved in MAPK and NF-κB signaling pathway, which showed that EPS1-1 increased the phosphorylation of p38MAPK (PDB: 3O17), JNK1/2 (PDB: 3O2M), ERK1/2 (PDB: 1VEU) and IKKα/β (PDB: 3BRV) (Fig. 2). Compared to the control group, EPS1-1 dramatically induced the phosphorylation of ERK1/2 after treatment for 5, 10 and 60 min (Fig. 2A and B). EPS1-1 also significantly increased the phosphorylation of p38 MAPK after treatment for 5, 10 and 20 min and phosphorylation of JNK for 60 min ($p < 0.01$) (Fig. 2A and C). Moreover, the phosphorylation of IKKα/β was significantly increased at 20, 40 and 60 min ($p < 0.01$) (Fig. 2D and E). These findings suggested that EPS1-1 might activate the MAPK and NF-κB signaling pathway to enhance the function of macrophages.

Since EPS1-1 could increase the expressions of IL-2, TNF-α and iNOS in macrophages reported in our previous study,[11] and might activated macrophages via MAPK signaling pathway. Here, further research confirmed whether MAPK signaling pathway involved in the gene expression in macrophages treated with EPS1-1. MAPK signaling pathways was specifically blocked by SB203580 (p38 inhibitor) and PD98059 (Erk1/2 inhibitor). The mRNA levels of iNOS, IL-2 and TNF-α were significantly elevated after EPS1-1 treatment and significantly decreased after treatment with inhibitors (Fig. 3). When the EPS1-1 was used to treat macrophages with the SB203580, the mRNA levels of iNOS, IL-2 and TNF-α were improved compared with the groups. However, the expression was not completely suppressed by the inhibitors and not fully recovered after treatment with EPS1-1, suggesting that other signaling pathways might participate in the process the activation of macrophage by EPS1-1.

In order to further indentify the signaling pathways involved in the activation of macrophages by EPS1-1, we determined the expression of TLR4 and the phosphorylation levels of ERK1/2, p38MAPK and IKKα/β in macrophages pretreated with the EPS1-1 and/or inhibitors (Fig. 4). Compared to the control, inhibitors dramatically suppressed the protein level of TLR4, while the block effect could be relived by EPS1-1. Furthermore, the similar tendency could be identified with the phosphorylation of ERK1/2, IKKα/β and p38MAPK. These findings demonstrated that TLR4 might be involved in macrophage activation mediated by EPS1-1 via p38MAPK and NF-κB signaling pathway.

In recent years, natural products from microorganisms, plants and animals, especially polysaccharides, have received increasing attention for their biomedical functions, such as immune enhanced activities that potentially improving the quality of life. EPS1-1 with β-anomeric configuration has been reported to have antitumor and immuoregulation effects.[10,11] As an potential adjuvant in cancer treatment, EPS1-1 can enhance the immunity of normal and immunosuppressed mice by contributing to the improvement of host immune response, such as boosting the activation of macrophages, promoting the proliferation of lymphocyte and increasing levels of serum antibody.[11,13] However, the mechanisms by which EPS1-1 activated the function of peritoneal macrophages remained unclear. In the present research, we investigated the underlying molecular mechanisms of EPS1-1 in activating macrophages.

Many polysaccharides modulate the immune functions mainly through macrophages which may be the first line in immune response and can be activated to release cytotoxic molecules.[3,14] Polysaccharides from *Poria cocos* sclerotium mediated the macrophage function through Ca^{2+}/PKC/p38/NF-κB signaling pathway.[15] Okra polysaccharides have been proven to be immune modulators for the macrophages stimulation.[16] It has been believed that polysaccharides could not directly enter cells and can be recognized by pattern recognition receptors (PRRs) of cell surface to activate macrophages.[17] TLRs, as a category of PRRs, play an essential role in the interaction of extracellular and intracellular signaling. Polysaccharides from microorganisms have been reported to possess considerable affinity to TLRs, especially TLR4.[3,18] We found EPS1-1 could significantly increase the expression of TLR4 to induce the expressions of IL-2, TNF-α and iNOS. Additionally, EPS1-1 weakened the effects of MAPK signaling inhibitors on the protein level of TLR4, indicating EPS1-1 might bind to the surface receptor TLR4 thus activating the intercellular signal cascade reaction.

The activation of MAPK and NF-κB signaling pathways in macrophages is associated with the cell surface receptor of TLR4, which activates various transcription factors to enhance the generation of nitric oxide (NO), inflammatory cytokines and chemokine.[19] Increasing evidence demonstrates that a number of polysaccharides could boost macrophages to produce cytotoxic molecules via MAPK signaling pathway, thus leading to the immune stimulatory. Polysaccharides isolated from *Nostoc commune* Vaucher suppress colorectal cancer growth *in vivo* via activating macrophages via NF-κB and AKT/JNK1/2.[20] Polysaccharides, isolated from the fruiting bodies of *Sarcodon aspratus*, could induce the activity of RAW264.7 via TLR4-mediated NF-κB and MAPK signaling pathways.[21] EPS1-1 could increase the acid phosphatase activity and the generation of NO and cytokines,[11] and it maybe worked through NF-κB and MAPK signaling pathways by increasing the phosphorylation of p38MAPK, JNK1/2, ERK1/2 and IKKα/β. Additionally, signaling pathway inhibitors were used to further

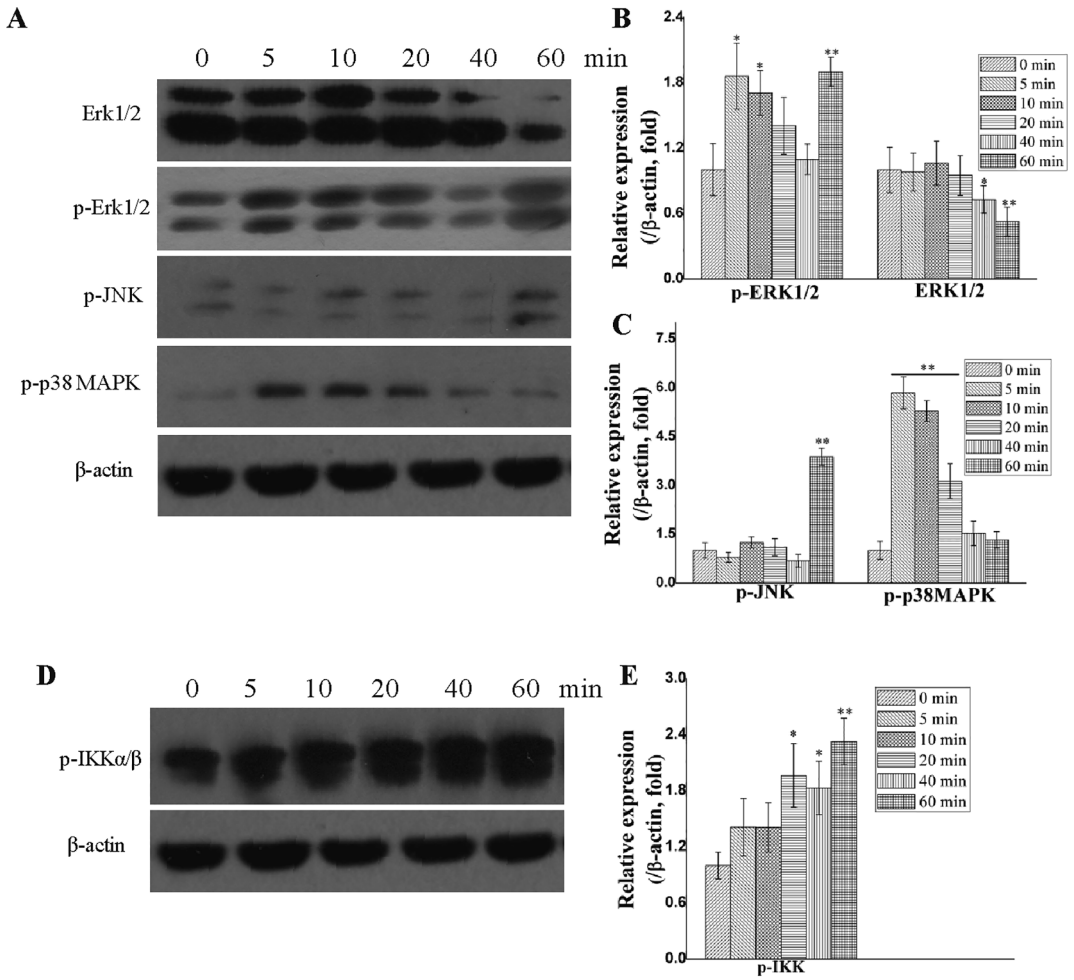

Fig. 2. Effect of EPS1-1 on MAPK and NF-κB signaling pathway. The peritoneal macrophages were treated EPS1-1 (0.2 mg/ml) for 0, 5, 10, 20, 40 and 60 min. The expressions of ERK1/2, p-ERK1/2, p-p38MAPK p-JNK, and p-IKKα/β involved in MAPK and NF-κB signaling were measured by western blotting. Data are presented as means ± SD. Significant differences from control group are indicated by $^{*}p < 0.05$, $^{**}p < 0.01$.

confirm which signaling pathway was involved in the activation effects of EPS1-1 on macrophages. We found that the mRNA levels of iNOS, IL-2 and TNF-α in MAPK signaling pathway inhibitor-treated macrophages were inhibited, and subsequently increased by the addition of EPS1-1. Moreover, the expressions of p-ERK1/2, p-IKKα/β and p-p38MAPK in EPS1-1-induced macrophages that pretreated with inhibitors were higher compared with that in cells only treated with inhibitor. These results indicated that both MAPK and NF-κB signaling pathways were the way that EPS1-1 activating macrophages, especially the MAPK signaling pathway. Therefore, EPS1-1, as a hetero-polysaccharide isolated from the fermentation liquor of *Rhizopus nigricans*, might be a good candidate for promoting immune functions, which possessed the properties similar to the traditional medical herbs polysaccharides.

In conclusion, the activation mechanism of EPS1-1on the macrophages might be to bind to TLR4, thus triggering the MAPK and NF-κB signaling pathways via enhancing the phosphorylation of p38MAPK, JNK1/2, ERK1/2 and IKKα/β. The activation of macrophages resulted the increasing expressions of IL-2, TNF-α and iNOS that mediated the immune response. These results demonstrated the possible molecular mechanisms of EPS1-1 in activating macrophages, which made it to be a potential immunomodulator used in adjuvant drug or functional food.

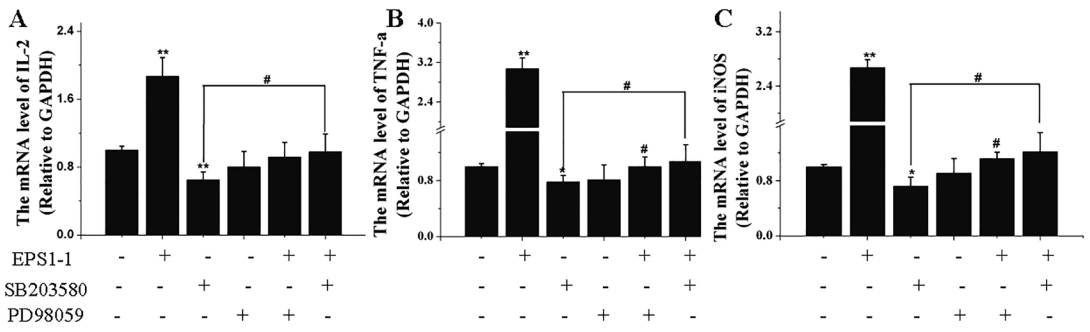

Fig. 3. The mRNA levels of IL-2, TNF-α and iNOS in the peritoneal macrophages with or without EPS1-1 and SB203580 or PD98059. Data are presented as means ± SD (n = 3), $^{*}p < 0.05$ vs control group, $^{\#}p < 0.05$ vs SB203580 only group.

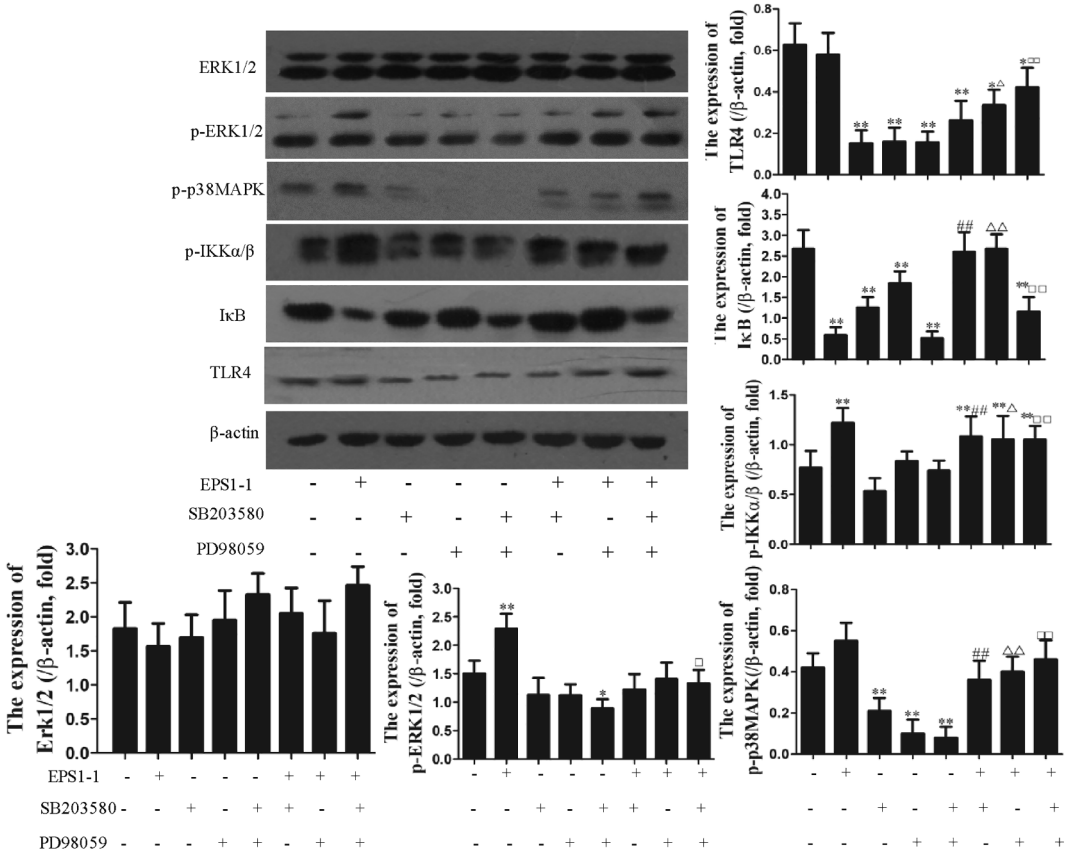

Fig. 4. Effects of EPS1-1 on the protein expression in the peritoneal macrophages with or without SB203580 and/or PD98059. Data are presented as means ± SD (n = 3), $^{*}p < 0.05$ and $^{**}p < 0.01$vs control group; $^{\#\#}p < 0.01$ vs SB203580 only group; $^{\triangle\triangle}p < 0.01$ vs PD98059 only group; $^{\square\square}p < 0.01$ vs PD98059 and PD98059 group.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bmcl.2020.127297.

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