TLR4-mediated activation of macrophages by the polysaccharide fraction from *Polyporus umbellatus*(pers.) Fries

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**A B S T R A C T**

**Aim of the Study:** Zhu Ling (*Polyporus umbellatus*) is well-known to reduce the risk of a variety of diseases. In this study, we explored the molecular mechanism of its immunostimulatory potency in immune responses of macrophages, using polysaccharides prepared from *Polyporus umbellatus* (PPS).

**Materials and methods:** Splenocyte proliferation was analyzed with \(^3\)H-TdR incorporation method. Nitric oxide (NO) was measured by Griess method and cytokines of culture supernatants was detected by enzyme linked immunosorbent assay (ELISA). The fluorescence-labeled PPS (Flu-PPS) and dextran (Flu-dextran) were prepared by the cyanogen bromide activation method. The cell-binding activity of Flu-PPS was analyzed with FACS and confocal microscopy. NF-κB activity was measured by ELISA assay.

**Results:** We found that PPS is able to strongly upregulate the functions of macrophages such as Nitric oxide (NO) production and cytokine expression. Compared with C3H/HeN group, PPS significantly stimulated the proliferation of splenocytes and the production of TNF-α, IL-1β and NO of peritoneal macrophages from C3H/HeJ mice. The function blocking antibodies to TLR-4, but not TLR-2 and CR3, markedly suppressed PPS-mediated TNF-α and IL-1β production. Flow cytometric and confocal laser-scan microscopic analysis showed that fluorescence-labeled PPS (Flu-PPS) can bind specifically to the target cells, and the binding can blocked by unlabeled PPS and anti-TLR4, but not anti-TLR2 and CR3 monoclonal antibodies.

**Conclusions:** Therefore, our data suggest that PPS may exert its immunostimulating potency via TLR-4 activation of signaling pathway.

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1. Introduction

Monocytes/macrophages provide an important bridge between innate and adaptive immunity (Ma et al., 2008). When the body is stimulated by pathologic stimuli or injury, macrophages release numerous proinflammatory cytokines, such as TNF-α and IL-1, and cytotoxic and inflammatory molecules, such as nitric oxide (NO) and reactive oxygen species (ROS) (Marcato et al., 2008; Wang et al., 2009). For these cellular events to occur, an intracellular signaling cascade is required that is triggered through a connection from cell surface receptors, including pattern-recognition receptors (PRRs) (Marcato et al., 2008). So far, toll-like receptor (TLR)-2, TLR-4, CR3 and dectin-1, as well as transcription factors such as nuclear factor (NF)-κB and activator protein (AP)-1, have been reported to be the major components of the signaling machinery (Lee et al., 2008; Marcato et al., 2008; Chan et al., 2009).

Zhu Ling, the sclerotium of *Polyporus umbellatus*(pers.) Fries, has been widely used in China for more than 2000 years. Some of the most promising modern research on Zhu Ling has been on its effect against parasites, boosting of the immune system (Zhang et al., 1991; Yang et al., 2004) and for the treatment of certain cancers (Wu et al., 1997), including liver cancer (You et al., 1994) and leukemia (Ohsawa et al., 1992). Several studies have also shown that the use of Zhu Ling in the treatment of hepatitis B is effective (Lin and Wu, 1988). Zhu Ling also demonstrates a protective effect in cases of toxic hepatitis (Lin and Wu, 1988).

A Chinese study found that *Polyporus umbellatus* polysaccharides (PPS) can offset the immunosuppression of the supernatant from S180 cell culture, possibly by down-regulating the synthesis and/or secretion of immunosuppressive substance by S180 cells (Yang et al., 2004). PPS were also found to improve the cellular immunity of normal mice and the mice with liver lesions (Zhang et al., 1991) and had superoxide and hydroxyl radical scavenging activities (Liu et al., 1997).

In spite of numerous studies, the exact biochemical and immunopharmacological properties of the active polysaccharides have remained largely elusive.

In this study, we investigated the activating roles and mechanisms of action of PPS on macrophage-mediated innate immune responses. To do this, functional events mediated by activated...
macrophages, such as the production of cytokines and the release of toxic molecules (NO), and PPS-binding receptors and signaling pathways were investigated.

2. Materials and methods

2.1. Mice

Female C3H/HeN and C3H/HeJ mice (6–8 weeks old) were purchased from the Shanghai Laboratory Animal Center, China. All animals were maintained and used in strict accordance with the guidelines issued by the Beijing Government on Animal Care.

2.2. Reagents

All cells were cultured in RPMI-1640 supplemented with 10% FCS (Hyclone), 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM l-glutamine and 5 × 10⁻³ M 2-ME. LPS from Escherichia coli, L8274 and dextran (70 kDa) were purchased from Sigma. Anti-mouse TLR-2, TLR-4 and CD11b (CR3) mAb were purchased from ebiosciences and dialyzed extensively against PBS to remove NaN₃ before use in cell culture.

Mouse IL-1β and TNF-α ELISA kits were purchased from R&D Systems. The Limulus amebocyte lysate (LAL) assay kit was purchased from Sigma. The NF-κB ELISA kit was obtained from Active Motif.

2.3. Preparation of PPS

PPS used in the present study was prepared and purified by a method reported previously (Xie et al., 2008; Tayama et al., 1985). Briefly, Zhu Ling was boiled in water and PPS was isolated from this water using ethanol precipitation, dialysis, and protein depletion via the Sevag method. The polysaccharide solution was dialyzed against distilled water for 7 days and then lyophilized. The percentage of PPS polysaccharide was approximately 95% according to the phenol-sulfuric acid colorimetric method.

The average molecular weight (Mw) was determined using size exclusion chromatography (SEC) combined with static light scattering (SLS). Wyatt Technology Astra software was used for data collection and analysis. The PPS molecules had a Mw of approximately 1.6 × 10⁶ and its molecular weight distribution (MM/Mn) is 2.914. Using gas chromatography (GC) analysis, the monosaccharide fractions were mixed, adjusted to 1 mg/ml, and stored at −20°C for future use. The concentration of fluoresceinamine in the P-PPS and dextran preparations was approximately 5 µg/ml.

2.4. Preparation of fluorescein-labeled PPS and dextran

Fluorescein-labeled PPS and dextran were generated as described previously (Glabe et al., 1983; Harty et al., 1983). Briefly, 20 mg/ml PPS or dextran solution was mixed with 200 µl 10 mg/ml CNBr in 1 ml water and maintained at pH 11 for 15 min by the addition of 0.2 M NaOH. After dialysis against Na₂B₄O₇ buffer at pH 8.0 for 20 h, the CNBr-activated PPS or dextran was mixed with 2 mg fluoresceinamine (Sigma) for 10 h in the dark at room temperature. Labeled polysaccharide was separated by gel filtration on a Sephadex G-25 column and the bright yellow fraction was collected. Carbohydrate concentration using the phenol–sulfuric acid method with dextran as the standards and the labeling density of the polysaccharides (the molar ratio of fluorophore and polysaccharide) was determined by measuring absorbency at 490 nm against a series of fluoresceinamine standards. Fluorescent-labeled polysaccharide fractions were mixed, adjusted to 1 ml, and stored at −20°C for future use. The concentration of fluoresceinamine in the F-PPS and f-dextran preparations was 5 µg/ml.

2.5. Preparation of mouse splenocytes and proliferation assays

Erythrocyte-depleted splenocytes were prepared as previously described (Shao et al., 2004). In brief, spleens from C3H/HeN mice were gently mashed by pressing with the flat surface of a syringe plunger against a stainless steel sieve (200 mesh). Red blood cells were lysed by brief treatment with distilled water. The splenocytes were washed twice and then resuspended in complete RPMI-1640. Splenocytes (4 × 10⁵) were cultured in round-bottomed 96-well plates (Nunc) in a volume of 200 µl/well in the presence LPS (1 µg/ml), PPS (50 µg/ml), f-PPS (50 µg/ml) or dextran (50 µg/ml). The cultures were incubated at 37°C and 5% CO₂ for 3 days. In the last 8 h of incubation, 0.2 µCi ³H-thymidine was added into each well. Thereafter, cells were harvested into scintillation filters and radioactivity measured using a scintillation counter (EG&G Wallac, USA).

2.6. Peritoneal macrophages (pMφs) preparation

pMφs were prepared as reported previously (Da Silva et al., 2009), with some modifications. Briefly, mice were killed by cervical dislocation, and 10 ml of ice-cold complete RPMI-1640 was injected intraperitoneally. Medium containing peritoneal exudate cells was recollected and kept on ice. The suspended cells were centrifuged at 800 × g for 5 min and resuspended with complete RPMI-1640. Peritoneal exudate cells were then seeded onto culture plates and allowed to adhere for 2 h at 37°C and 5% CO₂. After nonadherent cells were removed the remaining adherent cells were designated as pMφ. Cell viability was ≥90% in all experiments.

2.7. Fluorescence staining, FACS analysis and confocal laser-scanning microscopy

pMφs were harvested, centrifuged, and the cell pellets (1 × 10⁶/tube) were resuspended and incubated with 50 µl f-dextran or f-PPS for 30 min at 4°C. The cells were washed three times in PBS and resuspended in 1 ml of staining buffer (PBS supplemented with 0.1% NaN₃ and 5% FCS) for flow cytometric analysis on a FACS Calibur (Becton–Dickinson).

To perform inhibition assays, pMφs were treated with unlabeled PPS at 100 µg/ml, TLR4, TLR2 and CR3 mAb or the isotype control (50 µg/ml) or dextran (50 µg/ml) or dextran (50 µg/ml) or dextran (50 µg/ml). For confocal laser-scanning microscopy, the stained pMφs were spread on a glass slide, fixed and observed using a Leica TCS SP2 microscope (Germany).

2.8. Nitric oxide assay

pMφs from C3H/HeN mice were incubated with various concentrations of PPS for 24 h. The nitrite in the culture supernatants was determined using Griess reagent (Sigma).

2.9. Cytokine ELISA

pMφs were incubated with PPS at concentrations of 12.5, 25, 50 and 100 µg/ml for 24 h. Control cells were treated with dex-
3. Results

3.1. PPS-induced splenocyte activation

To determine the immunostimulatory activity of PPS, splenocytes from C3H/HeN and C3H/HeJ mice were exposed to PPS and proliferation measured. C3H/HeN mice have a point mutation in their TLR-4 molecule that their macrophages and B cells responding to LPS stimulation. LPS and PPS induced vigorous proliferation of splenocytes from C3H/HeN mice (Fig. 1A). In contrast, the responses of splenocytes from C3H/HeJ mice to PPS were decreased by about 60% (Fig. 1B). These results suggested that PPS has strong biological activity and that its action is at least partly mediated by TLR4-mediated signaling.

In addition, f-PPS was equally effective in stimulating C3H/HeN splenocytes in the same experiments, suggesting that conjugation of PPS with fluoresceinamine did not significantly alter its immunobiological activity (Fig. 1A).

2.10. Antibody inhibition experiments

pMφs were pre-treated with 20 μg/ml of TLR4, TLR2 and CR3 mAb for 30 min, and then 50 μg/ml PPS was added for 24 h. IL-1β and TNF-α levels in the culture supernatants were measured by ELISA.

2.11. NF-κB activity

pMφs from C3H/HeN mice were incubated with PPS (50 μg/ml), dextran (50 μg/ml) or LPS (1 μg/ml) at 37 °C in 5% CO2 for 24 h. Nuclear extracts were prepared using the TransAM nuclear extract kit (Active Motif) and used to measure the level of activated NF-κB using the ELISA-based transcription factor NF-κB p65 assay kit according to the manufacturer’s instructions.

2.12. Statistical analysis

All data are presented as the mean ± standard error (SEM). Student’s t-test was used to analyze the results. A P-value of <0.05 was used to determine significant differences between groups.

Fig. 1. PPS-induced proliferation of mouse splenic cells. (A) C3H/HeN mouse splenocytes were stimulated with various concentrations of PPS, f-PPS or dextran for 72 h. Thymidine was added to the cultures for the last 8 h of incubation and thymidine incorporation was measured (CPM). (B) Splenocytes from C3H/HeN (open bars) or C3H/HeJ (filled bars) mice were stimulated with 50 μg/ml dextran, 50 μg/ml PPS or 5 μg/ml LPS. C3H/HeJ mouse splenocytes did not respond to LPS stimulation but responded well to PPS activation. **P < 0.01.

Fig. 2. PPS induces NO production in C3H/HeN mouse pMφs. pMφs were cultured for 24 h in the presence of 1 μg/ml LPS, 50 μg/ml dextran or various concentrations of PPS. Cell culture supernatants were collected from the same triplicate wells after 24 h and tested for nitrite using the Griess reagent. *P < 0.05, comparing unstimulated (dextran) versus stimulated pMφ.

Fig. 3. PPS induces IL-1β and TNF-α production in C3H/HeN mouse pMφs. pMφs were cultured for 24 h in the presence of 1 μg/ml LPS, 50 μg/ml dextran or various concentrations of PPS. At the end of the incubation time, the culture medium was collected for cytokine analysis by ELISA. *P < 0.05, comparing unstimulated (dextran) versus stimulated pMφ.
**3.3. PPS induces IL-1β and TNF-α synthesis through TLR4**

In order to verify if TLR4 was required for PPS activation of pMφs, cells C3H/HeN and C3H/HeJ mice were incubated with PPS, LPS, dextran or IFN-γ for 24 h before measurement of IL-1β and TNF-α in their culture supernatants. C3H/HeJ pMφs did not respond to PPS stimulation in terms of IL-1β and TNF-α production (Fig. 4). Given that PPS was able to activate macrophages from C3H/HeN mice (Fig. 4), it is likely that TLR4 plays an important role in PPS-mediated macrophage activation. Furthermore, we demonstrated that the addition of a mAb against mouse TLR4 to C3H/HeN pMφs blocked PPS-induced IL-1β production by approximately 45%, but the addition of an anti-TLR2 or CR3 mAb did not (Fig. 5). These results suggest that TLR4 is involved in PPS activation of murine pMφs.

**3.4. Specific binding of PPS to pMφs**

In order to identify pMφs expressing specific receptors for PPS, f-PPS and f-dextran were used to stain C3H/HeN pMφs and expression was measured by flow cytometry (Fig. 6A) and confocal

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**Fig. 4.** PPS stimulated IL-1β and TNF-α production in C3H/HeN but not C3H/HeJ pMφs to produce IL-1β and TNF-α. C3H/HeN (open bars) and C3H/HeJ (filled bars) mouse pMφs were treated with PPS (50 μg/ml), dextran (50 μg/ml), LPS (1 μg/ml) or 10 U/ml IFN-γ for 24 h. IL-1β (A) or TNF-α (B) concentration (pg/ml) in the culture supernatant was determined by ELISA. *P < 0.05.

**Fig. 5.** Neutralization with TLR-4 mAb inhibits the synthesis of IL-1β in PPS-treated pMφs. C3H/HeN mouse pMφs were pre-treated with 20 μg/ml TLR2, TLR4, CR3 and IgG1 antibodies separately for 1 h before adding PPS (50 μg/ml). After 24 h, IL-1β in the culture supernatants was measured by ELISA. Significant differences between untreated (no Ab) and antibody treated pMφs is indicated by *P < 0.05.

**Fig. 6.** Specific binding of f-PPS to pMφs. (A) pMφs were stained with f-dextran or f-PPS for 30 min for flow cytometric analysis. (B) pMφs stained with f-PPS (a) or f-dextran (b) were also observed using a confocal laser-scanning microscope.

**Fig. 7.** TLR4-dependent staining of pMφs with f-PPS. f-PPS staining of pMφs from normal (C3H/HeN) mice was blocked by 100 μg/ml unlabeled PPS (A) and 200 μg/ml anti-TLR4 (B) but not by anti-TLR2 and CR3 (C).
laser-scanning microscopy (Fig. 6B). Positive staining of pMøs was observed using both methods.

Furthermore, the staining of 1 µg/ml F-PPS could be blocked by the addition of 100-fold molar excess (100 µg/ml) of unlabeled PPS (Fig. 7A). Anti-TLR4, but not anti-TLR2 and CR3, also significantly blocked pMø staining by f-PPS (Fig. 7B and C). In contrast, dextran did not exhibit any significant inhibitory effect in similar experiments (data not shown).

3.5. Stimulation of pMøs with PPS leads to NF-κB activation

Cell recognition of extracellular stimuli can lead to the activation of various intracellular signaling pathways, including NF-κB (Ahmed and Mehlhorn, 1999). Therefore, we next examined whether PPS induced the activation of NF-κB in pMøs. Stimulation of pMøs with 50 µg/ml PPS led to an increase in the DNA binding activity of NF-κB as measured by an ELISA-based assay (Fig. 8).

4. Discussion

Polysaccharides from *Polyporus umbellatus* (PPS) have potent anti-tumor and anti-viral activities (Xiong, 1993; Yang et al., 2004). However, the molecular mechanisms behind these activities remain largely unresolved. In this study we investigated the functional activation of macrophages by PPS and explored the molecular mechanisms involved.

We found that PPS can induce wild-type mouse pMøs to produce NO, IL-1β and TNF-α. However, production of IL-1β and TNF-α in PPS-stimulated pMøs from TLR4-deficient mice was significantly reduced. Moreover, an anti-TLR4 mAb blocked F-PPS binding to pMøs and inhibited the production of IL-1β. PPS can also upregulate transcription of NF-κB in pMøs. These results suggest that PPS can activate macrophages via pathways that involve TLR4 and NF-κB activation. A role for endotoxin/LPS in this effect was ruled out via the LAL assay.

Once activated, macrophages produce lots of bioactive compounds, including reactive oxygen/nitrogen species (ROS/RNS), such as superoxide (O$_2^-$) and NO, and several cytokines, such as IL-1β and TNF-α. These compounds are critical for successful defense against invading pathogens. NO can not only enhance the activity of macrophages, but also acts as an important signaling molecule in many biological functions, such as immunomodulating and cytotoxic action against tumor cells (Berdeaux, 1993; Arko-Mensah et al., 2007). IL-1β and TNF-α play important roles in defense against intracellular pathogens (Adler et al., 2008; Nichols et al., 2008). When incubated with PPS, pMøs increased their production of NO, IL-1β and TNF-α. Therefore, PPS-mediated activation of macrophages and the production of NO and cytokines contribute to immunostimulating, anti-viral and anti-tumor activities.

Macrophages recognize pathogens via pattern-recognition receptors (PRRs) including TLRs, CR3 and dectin-1. These PRRs can bind to pathogen associated molecular patterns (PAMPs), preserved motifs expressed on pathogens. The toll protein was first discovered in Drosophila, where it is essential for embryo development and acts as a receptor (Moncrieffe et al., 2008). Recent studies found members of the TLR family probably play a fundamental role in innate immunity, for example, by enhancing anti-tumor and anti-infection activities through the promotion of NO production (Zhou et al., 2009). It can also regulate adaptive immunity by inducing the production of IL-1, IL-6, TNF-α and IL-12 (Hsu et al., 2004; Rivas-Santiago et al., 2006).

Several natural polysaccharides, such as polysaccharides from *Carthamus tinctorius*, *Acanthopanax senticosus* and *Ganoderma lucidum* activate the transcription factor NF-κB via TLR4 and induce cytokine production by macrophages. *Ganoderma lucidum* polysaccharides can promote activation and maturation of dendritic cells (DC) via TLR4-modulated NF-κB activation. Therefore, in this study, the role of TLR4 in PPS-induced macrophage activation was investigated. Our results demonstrated that PPS activated pMøs from wild-type but not TLR4-deficient mice to produce TNF-α and IL-1β, indicating that TLR4 is involved in mediating the activities of PPS.

Besides TLR4, several PPRs such as TLR-2, CR3 (CD11b) and dectin-1 are also reported to be involved in mediating the in vivo immunomodulatory effects of fungi polysaccharides. Therefore, we investigated whether these molecules were capable of acting as receptors for PPS and if they could modulate macrophage activation using cytokine neutralizing experiments, flow cytometry and confocal microscopy analysis. As illustrated in Fig. 1A, F-PPS and PPS was equally effective in stimulating splenocytes in the same experiments, suggesting that conjugation of PPS with fluoresceinamine did not significantly alter its immunobiological activity. These results suggested that fluorescent-labeled PPS was suitable for binding studies. Flow cytometry and confocal laser-scanning microscope analysis showed that F-PPS can specifically bind to pMøs (Fig. 6), a process that can be inhibited by a mAb to TLR4, but not CR3 or TLR2 (Fig. 7). In contrast dextran and laminarin did not exhibit any significant inhibitory effect in similar experiments (data not shown).

These results indicate that TLR4 is a PPR of PPS. However, the existence of a receptor on macrophages other than TLR4 that recognizes PPS is likely, because TLR4 mAb only showed partial inhibition of IL-1β production (Fig. 4) and it did not completely inhibit F-PPS binding to pMøs. Ongoing experiments, including polysaccharide-membrane protein co-precipitation and proteomics, in our laboratory will thoroughly address this question.

The activation of macrophages is associated with transcriptional upregulation of numerous genes related to immune defense functions. Transcription is known to be regulated by lots of transcription factors, including NF-κB (Chen et al., 2007; Jacques et al., 2009). Since PSK (a polysaccharide from *Ganoderma lucidum*) and other natural polysaccharides have been reported to induce nuclear translocation of NF-κB, we tried to address whether PPS was capable of controlling the functional role of NF-κB. Our results demonstrated that NF-κB nuclear translocation and DNA binding activity were significantly increased after PPS stimulation, suggesting that NF-κB may play a central role in macrophage-mediated cellular activation by PPS. Detailed intracellular signal pathways will be further explored in the future.

In summary, our study demonstrated that PPS simulates pMøs to produce inflammatory mediators (NO) and cytokines (IL-1β and TNF-α) via pathways that involve TLR4 and NF-κB activation. These results indicate TLR4 is a receptor for PPS-mediated macrophage
activation and will contribute to our understanding of Zhu Ling-mediated immunomodulatory activities.

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References


Glossary

PPS: polysaccharides extract from Zhu Ling (Polyporus umbellatus(pers.) Fries)

fluorescein-labeled PPS

fluorescein-labeled dextran

nitric oxide