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Sargassum fusiforme polysaccharide activates nuclear factor kappa-B (NF-κB) and induces cytokine production via Toll-like receptors

Xiaoming Chen[a,b,*,1], Guoqing Yu[a,b,1,2], Sairong Fan[a,1], Manman Bian[a,b], Huijun Ma[a,b], Jianxin Lu[a,b], Liqin Jin[a,b,*]

[a] *Institute of Glycobiological Engineering/School of Laboratory Medicine & Life Science, Wenzhou Medical University, Wenzhou 325035, China*
[b] *Key Laboratory of Laboratory Medicine, Ministry of Education of China, School of Laboratory Medicine & Life Science, Wenzhou Medical University, Wenzhou 325035, China*

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ABSTRACT

This study was designed to investigate the mechanism of macrophage activation by the *Sargassum fusiforme* polysaccharide (SFPS). As a result, SFPS significantly enhanced cytokines and nitric oxide (NO) productions in peritoneal macrophages, and stimulated macrophages to produce the cytokines and NO through the induction of their genes expression. The pretreatment of peritoneal macrophages with special antibodies [Toll-like receptors (TLRs) antibody] significantly blocked SFPS-induced tumor necrosis factor alpha (TNF-α) and NO production. Furthermore, pyrrolidine dithiocarbamate (PDTC), a specific inhibitor of NF-κB, effectively suppressed SFPS-induced TNF-α and interleukin 1β (IL-1β) secretion in peritoneal macrophages, indicating that SFPS stimulated macrophages to produce cytokines through the NF-κB pathway and the result was further confirmed by the experiment of Western blotting (WB) and confocal laser scanning microscope (CLSM). Taken together, these results suggest that SFPS-mediated induction of cytokines and NO production in macrophages is mediated, at least in part, by TLRs/NF-κB signaling pathway.

1. Introduction

Polysaccharides isolated from natural sources are known to affect a variety of biological response, especially the immune response (Yang, Zhao, Wang, & Mei, 2007). Therefore, they have the potential as immunomodulators with wide applications (Tzianabos, 2000). The mechanisms of action of these polysaccharides are unclear, however, polysaccharides have been found to be the primary factor for macrophage stimulation through induction of the immune system of Toll-like receptors (Hsu et al., 2004; Schepetkin & Quinn, 2006).

Toll-like receptors (TLRs) are known to play a critical role in the early innate immune response (Lee & Kim, 2007; Takeda & Akira, 2004; Werling & Jungi, 2003). Upon stimulation, TLRs bind to the adaptor protein MyD88, and MyD88, in turn, associates with the serine threonine kinase IRAK (Aderem & Ulevitch, 2000; Hatada, Krappmann, & Scheidereit, 2000; Imler & Hoffmann, 2001). This is followed by the activation of tumor necrosis factor-associated factor 6 (TRAF6) and TRAF6 in turn activates inhibitor of kappa B (IκB) kinase (IKK) complex. The activation of IKK complex leads to the degradation of IκB and the activation of NF-κB, which regulates a wide spectrum of target genes (Anderson, 2000), including the pro-inflammatory cytokines such as TNF-α, IL-1β and interleukin 6 (IL-6) (Imler & Hoffmann, 2001; Lin et al., 2011; Shishido et al., 2006; Yang et al., 2011).

In our previous report, a polysaccharide (SFPS) isolated from *Sargassum fusiforme* (Harv.) Setchel, a kind of brown algae widely distributed in China, Korea, and Japan, has been shown to have anti-tumor and immunomodulatory activities (Chen, Nie, Fan, et al., 2012). SFPS also stimulated the production of inflammatory cytokines (interleukin 2 (IL-2), IL-6 and TNF-α) by peritoneal macrophages (Chen, Nie, Yu, et al., 2012). However, the molecular mechanism responsible for immunostimulatory activity of this polysaccharide is not fully understood, and it is not clear which receptors are stimulated by this polysaccharides to transmit intracellular signals.

Thus the present study was designed to investigate the molecular mechanism responsible for the activation of macrophages by SFPS. To do this, functional events mediated by activated macrophages, such as the production of cytokines (TNF-α, IL-1β

* Corresponding authors at: Institute of Glycobiological Engineering/School of Laboratory Medicine & Life Science, Wenzhou Medical University, Wenzhou 325035, China. Tel.: +86 577 86699652; fax: +86 577 86689717.
E-mail address: xmchen01@163.com (X. Chen).
[1] These authors contributed equally to this work.
[2] Present address: North Hospital of Huainan Xinhua Medical Group, China.

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and IL-6) and the release of toxic molecules (NO), and SFPS-binding receptors and signaling pathways were investigated. The current data suggest that the activation of macrophages by SFPS is mediated, at least partially, by activation of a member of the TLRs.

2. Materials and methods

2.1. Materials and chemicals

S. fusiforme was collected in Dongtou, Zhejiang province (China). Sephadex G-200, DEAE-52 cellulose, PDTC, propidium iodide (PI), lipopolysaccharide (LPS), fluorescein isothiocyanate (FITC) were purchased from Sigma Chemical Co. Fetal bovine serum (FBS) and RPMI1640 medium were the products of Gibco. Cytokine detecting ELISA kits were purchased from Changfeng Biotechnology Co. Ltd. Toll-like receptor 4 (TLR-4) antibody, Toll-like receptor 2 (TLR-2) antibody and cluster of differentiation 14 (CD14) antibody were the products of Beijing Biosynthesis Biotechnology Co. Ltd. NF-κB antibody was the product of Santa Cruz Biotechnology Inc. The assay kit for NO was purchased from Jiancheng Biologic Project Company. TRIZOL Reagent, DNA ladder marker and cDNA reverse transcription kits were obtained from TaKaRa Biotechnology Co. Ltd. Sprague-Dawley rats (body weight 250 ± 20 g) used for experiments were purchased from Experimental Center, Wenzhou Medical University, Zhejiang, China. All the other reagents were analytical grade.

2.2. Preparation of the polysaccharide

The polysaccharide (SFPS) was prepared as described previously (Chen, Nie, Yu, et al., 2012). Briefly, the air dried *S. fusiforme* was soaked with 95% ethanol to remove the pigments and small lipophilic molecules. The residue was then extracted with 10-time volume of distilled water at 90 °C for 3 h thrice. All water-extracts were combined, filtrated, concentrated, and precipitated with 95% ethanol (1:4, v/v) at 4 °C for overnight. The precipitate was collected by centrifugation and deproteinated by Sevag method (Staub, 1965). Finally the deproteinated supernatant was lyophilized to give crude polysaccharides. The crude polysaccharides were purified by DEAE-52 cellulose and Sephadex G-200, and the main polysaccharide fraction (SFPS) was collected and lyophilized. SFPS was used for further study.

The average molecular weight of SFPS was determined as 299 kDa by high performance gel permeation chromatography (HPGPC), which is mainly comprise of D-fucose, L-xylose, D-mannose and D-galactose with a relative molar ratio of 5.9:1.0:2.3:2.2 by gas chromatography (GC) analysis. The uronic acid content of SFPS was 6.48%. The ester sulfate content of SFPS was found to be 10.74%.

2.3. Cell isolation and culture

Peritoneal macrophages were prepared from Sprague-Dawley rats as described previously (Chen et al., 2008). Briefly, peritoneal macrophages were harvested from 2 to 3 Sprague-Dawley rats, which had been injected intraperitoneally with 2 mL of thioglycollate three days before sterile peritoneal lavage with 10 mL of Hank's balanced salt solution. The collected cells were seeded and cultured in RPMI1640 containing 10% FBS at a density of 1×10^6 cells/well in the 24-well cultures plate at 37 °C in a humidified atmosphere of 5% CO_2 incubator. Then cultures were washed with RPMI1640 to remove nonadherent cells prior to the addition of fresh RPMI1640 containing 10% FBS.

2.4. Cytokine assay

Adhered peritoneal macrophages at 1×10^6 cells/well incubated with either various concentrations of SFPS (or 10 μg/mL LPS) at 37 °C for 24 h, or incubated with 500 μg/mL SFPS (or 10 μg/mL LPS) at 37 °C for 1–48 h. After incubation, conditioned supernatants were collected for assaying cytokines using commercial ELISA kits. The assay was carried out according to the procedures recommended in the ELISA kit manual, the absorbance was read at 450 nm on an automatic ELISA plate reader.

2.5. NO assay

NO production was determined indirectly by assaying the culture supernatant for accumulated nitrite, the stable end product of NO reacted with molecular oxygen as previously described (Green et al., 1982). One hundred μL of culture supernatants was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% N-1-naphthylethylenediamine dihydrochloride and 2.5% phosphoric acid) and incubated at room temperature for 10 min. Nitrite products were determined by measuring absorbance at 550 nm versus a $NaNO_2$ standard curve.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIZOL Reagent, followed by cDNA synthesis using a commercially available cDNA reverse transcription kit in a Bio-Rad thermocycler. The final volume of 20 μL consisted of 1 μL dNTPs, 1 μL oligo dT primer, 5 μL total RNA, 4 μL 5 × buffer, 1 μL retroviridase and 8 μL DEPC-H_2O. The reverse transcription conditions were as follows: 42 °C for 60 min, 70 °C for 15 min and 4 °C for 14 min. Then the obtained cDNA was amplified by PCR. PCR was performed in a final volume of 25 μL as follows: 2.0 μL cDNA, 2.5 μL 10 × PCR buffer, 1 μL dNTPs, 0.5 μL forward primer, 0.5 μL reverse primer, 0.25 μL Taq DNA polymerase and 18.25 μL DEPC-H_2O. The sequences used were as follows: IL-1β, forward primer: 5′-TGATGACGACCTGCTAGTGT-3′, reverse primer: 5′-CTTCTTTGGGTATTGTTTGG-3′, IL-6, forward primer: 5′-TGCCTTCTTGGGACTGATGT-3′, reverse primer: 5′-GCTCTGAATGACTCTGGCTTTG-3′, TNF-α: forward primer: 5′-CCACGCTCTTCTGTCTACTGAACT-3′, reverse primer: 5′-GAGGCTGACTTTCTCCTGGTATG-3′, Inducible Nitric Oxide Synthase (iNOS), forward primer: 5′-GCAGCACTTGGATCAATAACCT-3, reverse primer: 5′-ACATCGCCACAAACATAAAGG-3′, β-actin, forward primer: 5′-CACCCGCGAGTACAACCTTC-3′, reverse primer: 5′-CCCATACCCACCATCACACC-3′. The PCR ampliformed as follows: an initial denaturing at 94 °C for 4 min, followed by 30 cycles of denaturing at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, with a final extension at 72 °C for 10 min. The products of amplification were confirmed using electrophoresis in 1.0% agarose gels and visualized by staining with ethidium bromide (EB).

2.7. TLR antibody blocking

Peritoneal macrophage cells (1×10^6 cells/mL) were treated with 20 μg/mL anti-TLR2, anti-TLR4, anti-CD14 antibody for 1 h before SFPS (200 μg/mL) or LPS (10 μg/mL) treatment. Cytokine production and nitric oxide production in the culture medium were measured 24 h later using ELISA and Griess reagent, respectively.

2.8. Assay of cytokines in SFPS-treated cells with or without NF-κB inhibitor

Peritoneal macrophage cells (1×10^6 cells/mL) were treated with or without 100 μmol/L PDTC for 2 h before SFPS (50, 200, 500

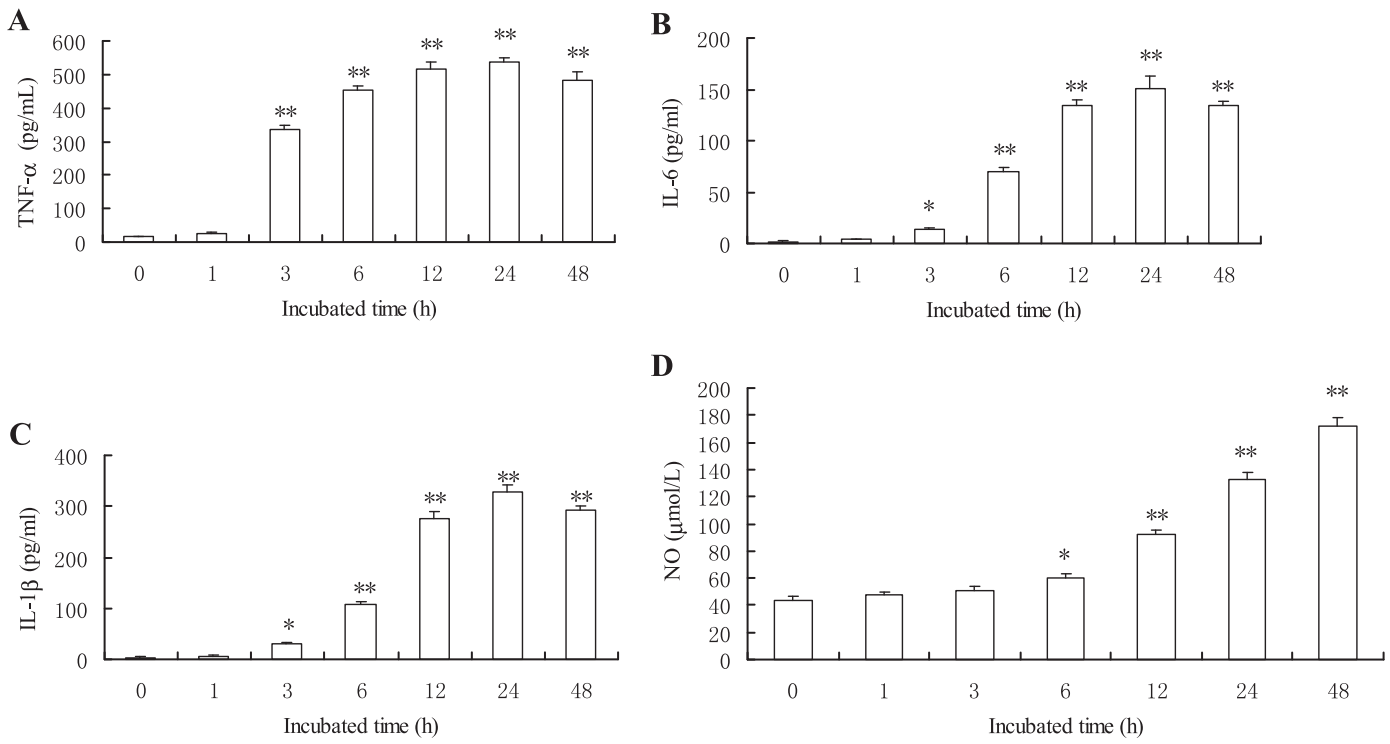

Fig. 1. Time-dependent induction of cytokines and NO production by SFPS (500 μg/mL) in peritoneal macrophages (PMφ). The macrophages (1×10^6 cells/well) were incubated with 500 μg/mL of SFPS for the indicated time and the productions were determined as described in Section 2. (A) TNF-α production in the PMφ; (B) IL-6 production in the PMφ; (C) IL-1β production in the PMφ; (D) NO production in the PMφ. The values are presented as means ± S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ (compared with control group).

and 1000 μg/mL) or LPS (0.1, 0.3, 1.0, 3.0 and 10 μg/mL) treatment. Cytokine productions in the culture medium were measured 24 h later using ELISA.

2.9. Western blot

Cells (1×10^6 cells/mL) were initially incubated for 4 h at 37 °C and then stimulated with SFPS (500 μg/mL) for 1–10 h, or stimulated with either various concentrations of SFPS (or 10 μg/mL LPS) for 10 h, after which cells were washed with ice-cold PBS and lysed in 200 μL RIPA lysis buffer (50 mmol Tris, pH 7.4, 150 mmol NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 0.05 mmol EDTA). After centrifugation (16,000 × *g*, 10 min), supernatants were collected. Nuclear lysates (50 μg) were separated by 10% SDS-PAGE, and transferred onto polyvinylidene fluoride membranes. The membranes were pre-incubated for 1 h in PBS, 5% skim milk, 0.1% Tween 20. Then, the membranes were incubated with NF-κB specific antibodies overnight at 4 °C. Immunoreactive bands were detected by incubation with conjugates of anti-rabbit IgG with horseradish peroxidase and enhanced chemiluminescence reagents (Amersham Biosciences).

2.10. Immunofluorescence assay

Cells (1×10^6 cells/mL) were initially incubated for 4 h at 37 °C and then stimulated with SFPS (100 μg/mL) for 6 h, after which cells were washed with ice-cold PBS. Cells were fixed for 10 min in 3% paraformaldehyde solution and permeabilized by treating with 0.1% Triton X-100 in PBS for 10 min. After incubated with NF-κB specific antibodies for 2 h, cells were incubated with conjugates of anti-rabbit IgG with FITC for 30 min. Then cells were incubated with 10 μg/mL PI for 30 min at 37 °C and observed using a TCS4D confocal microscope based on a DM microscope interfaced with a mixed-gas argon-krypton laser (OLYMPUS FV1000 + SIMS).

2.11. Statistical analysis

All results were presented as mean ± S.D. Data were analyzed by one-way ANOVA using SPSS and Dunnett's test. *P*-values of less than 0.05 were considered to be statistically significant.

3. Results

3.1. SFPS induce the production of various cytokines and NO by peritoneal macrophages

To understand the mechanism of the immunoregulatory activity of SFPS, we first examined the ability of SFPS to stimulate peritoneal macrophages to secrete cytokines. As illustrated in Figs. 1 and 2, treatment of the peritoneal macrophages with SFPS resulted in a marked increase in TNF-α (Fig. 1A), IL-6 (Fig. 1B) and IL-1β (Fig. 1C) production in a time-related manner, and the maximal effect was observed in 24 h. When the incubated time was longer than 24 h, a slight decreased trend was observed. It was also found that SFPS at concentrations of 50, 200, 500 and 1000 μg/mL significantly increased the production of TNF-α (Fig. 2A), IL-6 (Fig. 2B) and IL-1β (Fig. 2C) in a dose-dependent manner.

Because such induced immunological events accompany oxidative bursts when cells are exposed to foreign substances, we test whether SFPS stimulates macrophages to release NO. Indeed, SFPS induced peritoneal macrophages to release NO in time- and dose-dependent manner (Figs. 1D and 2D).

3.2. Effects of SFPS on cytokines and iNOS mRNA expression in peritoneal macrophages

Our results indicated that SFPS induced peritoneal macrophages to produced cytokines and NO, and it was concluded that SFPS possessed the potential of enhancing innate immune response.

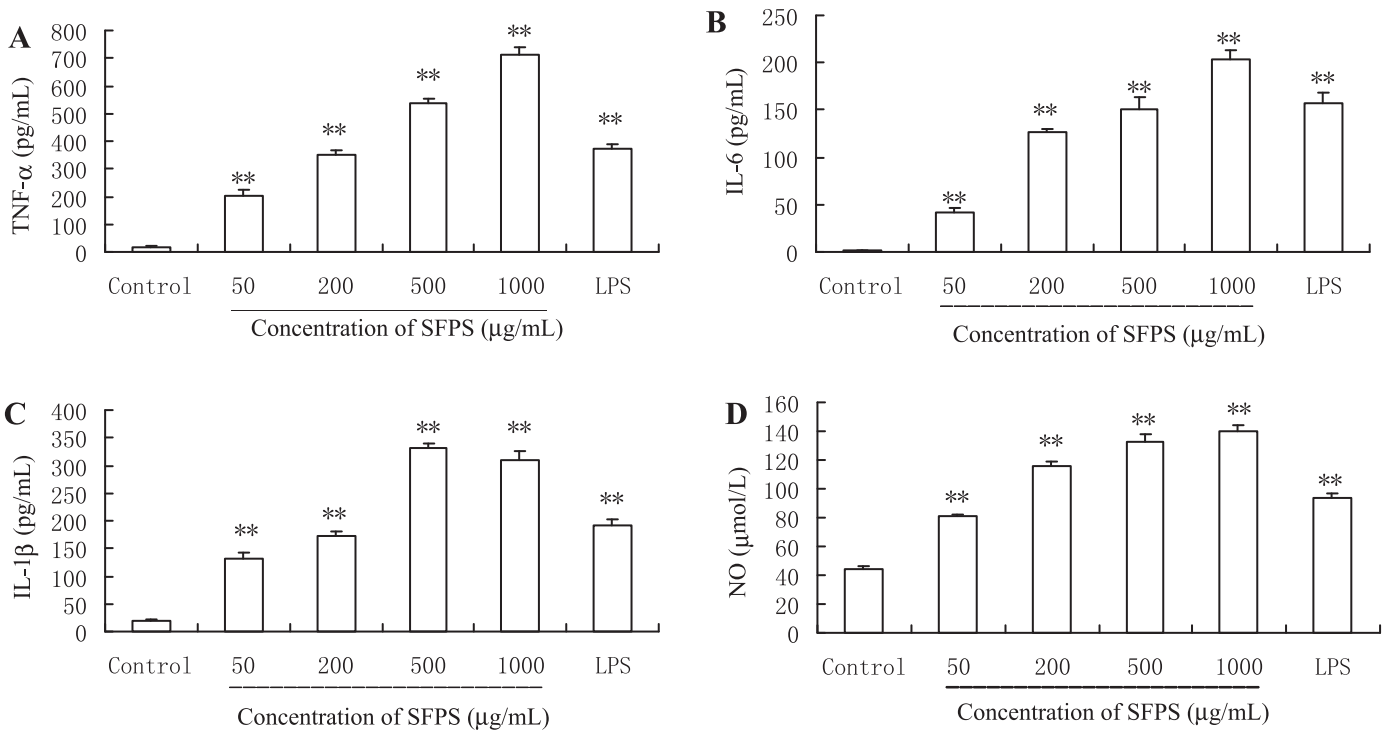

Fig. 2. Effects of SFPS on cytokines and NO production in the peritoneal macrophages (PMφ). Culture macrophages were treated with stimulus (10 μg/mL LPS or SFPS at the concentration of 50, 200, 500 and 1000 μg/mL, respectively) for 24 h. Culture supernatants were collected, and the production of (A) TNF-α, (B) IL-6, (C) IL-1β and (D) NO was measured. The values are presented as means ± S.D. $^{**}P < 0.01$ (compared with control group).

However, it was unclear whether the stimulatory effect of SFPS on TNF-α, IL-6, IL-1β and NO production was attributable to its influence on the expression of their genes expression. To elucidate the underlying mechanisms of the immunoregulatory activity of SFPS, the TNF-α, IL-6, IL-1β and iNOS mRNA expression levels in macrophages were investigated by RT-PCR under the stimulation of SFPS at 0–500 μg/mL (10 μg/mL of LPS as positive control). As shown in Fig. 3, treatment of macrophages with either SFPS or LPS resulted in a drastic increase in the expression of TNF-α, IL-6, IL-1β and iNOS mRNA, and the transcription of TNF-α and iNOS mRNA was concentration-dependently induced by SFPS. The mRNA expression of β-actin, an internal control, was not affected by SFPS treatment. This experiment showed that the increase of cytokines (TNF-α, IL-6 and IL-1β) and NO in peritoneal macrophages were mediated by the expression of the genes.

3.3. *TLRs-dependent activation of macrophages by SFPS*

TLRs have been reported to mediate cytokine expression by macrophages in response to several kinds of polysaccharides (Cohen et al., 2008). However, there are no reports about the role of TLRs in the activation of macrophages by SFPS. To further insight into the activation mechanism and investigate the membrane receptor involved in the activation of cytokines and NO productions, we examined the effect of SFPS on the production of TNF-α and NO in the pretreatment of peritoneal macrophages with specific antibodies (anti-TLR4, anti-TLR2 and anti-CD14). Fig. 4A and B shows the effects of the treatment of macrophages with anti-TLR4, anti-TLR2 and anti-CD14 antibody on SFPS or LPS-induced TNF-α and NO productions by macrophages, respectively. As shown in Fig. 4A, the pretreatment of macrophages with anti-TLR4, anti-TLR2 and anti-CD14 antibody remarkably inhibited the TNF-α secretion from cells. Similarly, LPS-induced TNF-α secretion was also significantly suppressed by the pretreatment. Anti-TLR4, anti-TLR2 and anti-CD14 antibody also significantly blocked SFPS-mediated production of NO in peritoneal macrophages, and LPS-induced NO production was also suppressed by pretreatment of peritoneal macrophages with these antibodies (Fig. 4B). The antibodies have similar effects on the block TNF-α and NO production induced by SFPS as well as LPS. The results suggested that SFPS induces cytokines and NO production mediated through TLRs

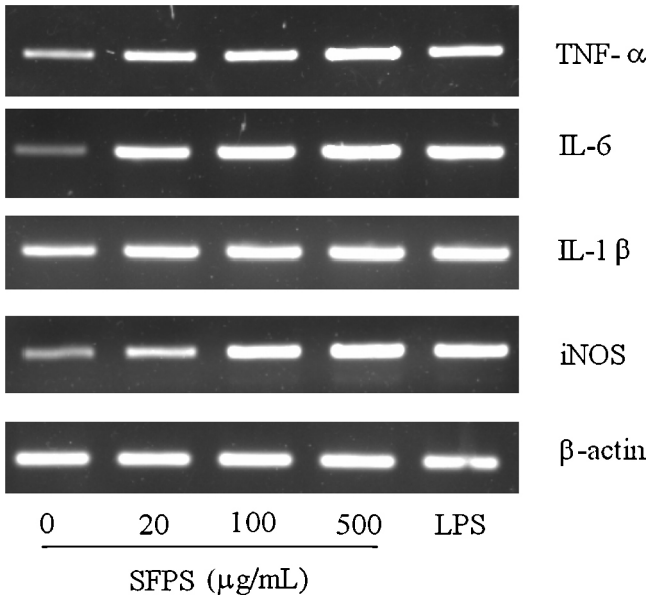

Fig. 3. Effects of SFPS on the gene expression of TNF-α, IL-6, IL-1β and iNOS mRNA in the peritoneal macrophages (PMφ). Culture macrophages were treated with stimulus (5 μg/mL LPS or SFPS at the concentration of 20, 100 and 500 μg/mL, respectively) for 24 h. Total RNA was isolated and analyzed for magnitude of the mRNA expression of TNF-α, IL-6, IL-1β and iNOS using RT-PCR as described in Section 2.

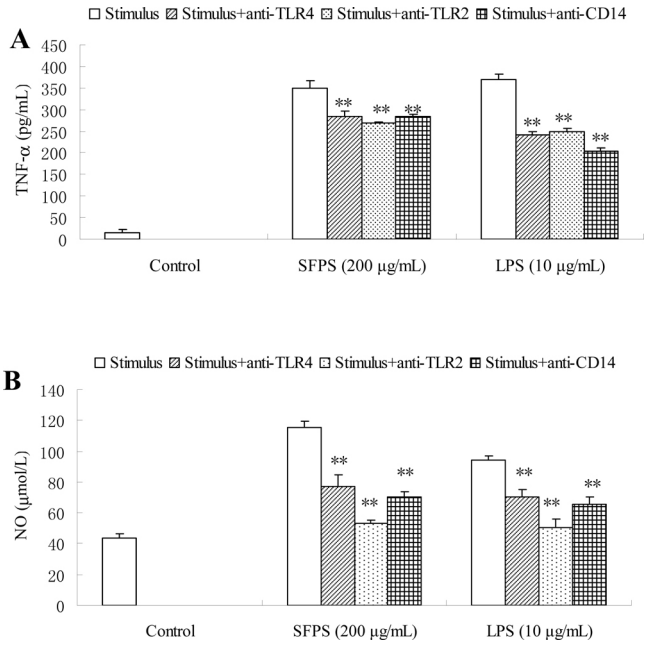

Fig. 4. Effect of the pretreatment of macrophages with anti-TLRs and anti-CD14 antibody on TNF-α and NO production in the peritoneal macrophages (PMφ). The cells were pre-incubated with 20 μg/mL of anti-TLRs and anti-CD14 antibody for 1 h, and then treated with SFPS (200 μg/mL) or LPS (10 μg/mL) for 24 h. Culture supernatants were collected, and the production of (A) TNF-α and (B) NO was measured. The values are presented as means ± S.D. $^{**}P < 0.01$ (compared to the values obtained in only stimulus-treated cells).

in macrophages and may shares common membrane receptors with LPS.

3.4. Effect of PDTC (an inhibitor of NF-κB) on SFPS-induced TNF-α and IL-1β secretion in macrophages

As described previously, signals originated from TLRs are known to active NF-κB. The transcription factor NF-κB signaling pathway is activated by LPS and essential for the induction of cytokines such as TNF-α (Hatada et al., 2000). Once activated, NF-κB transcriptionally regulates many cellular genes implicated in early immune, acute phase, and inflammatory responses, including TNF-α, IL-1β, IL-2, IL-6, IL-12 (Jobin & Sartor, 2000). To explore SFPS-mediated signal transduction pathways, the effects of PDTC, a specific inhibitor of NF-κB, was investigated. As illustrated in Fig. 5, Both TNF-α (Fig. 5A) and IL-1β (Fig. 5B) production induced by SFPS, at concentrations of 50, 200, 500 and 1000 μg/mL, significantly decreased when cells were the pretreatment with PDTC, except the TNF-α production induced by 50 μg/mL of SFPS. Similarly, LPS-induced TNF-α (Fig. 5C) and IL-1β (Fig. 5D) secretion was also significantly suppressed by the pretreatment. PDTC also inhibited the TNF-α mRNA expression induced by SFPS, as shown in Fig. 5E. The mRNA expression of β-actin, an internal control, was not affected by the PDTC pretreatment. These results indicate that activation of macrophages by SFPS is mediated by the TLRs-dependent activation of transcription factor NF-κB, an important downstream regulator of TLR4.

3.5. Induction of NF-κB by SFPS in macrophages

To convincingly demonstrate the specificity of NF-κB activation by SFPS, WB and CLSM was used to examine the activation of NF-κB in macrophages stimulated by SFPS. The protein bands stand for nuclear NF-κB were detected on nitrocellulose. As shown in Fig. 6, it was found that SFPS at concentrations of 62.5, 125, 250 and 500 μg/mL significantly increased the production of NF-κB in a dose-related manner, and the maximal effect was observed at a concentration of 500 μg/mL. When the concentration was higher than 500 μg/mL, a decreased trend was observed. The production of β-actin, an internal control, was not affected by the SFPS. To further conform, the activation of NF-κB in macrophages stimulated by SFPS with FITC-tagged antibody and its presence in the nucleus and cytoplasm was monitored by measuring the green fluorescence integrated over the nucleus, which was counterstained with PI, respectively. As shown in Fig. 7, NF-κB associated fluorescence can be observed in the nuclei of SFPS stimulated macrophages, no such fluorescence were observed in the nuclei of control cells.

4. Discussion

SFPS, a polysaccharide isolated from *S. fusiforme*, is claimed to have a variety of biological activities, including immunostimulation and antitumor effects. However, the molecular mechanism responsible for immuostimulatory activity of this polysaccharide is not full understood. In the present study, we attempted to characterize membrane receptors and subsequent intracellular signaling pathways involved in the activation of macrophages by SFPS.

In the present study, we first investigated that SFPS induces the activation of macrophages in vitro, as manifested by the production of cytokines (TNF-α, IL-1β and IL-6) and NO. It is well known that the activation of macrophages produce a variety of cytokines, including TNF-α, IL-1β and IL-6. In turn, TNF-α, IL-1β and IL-6 induce the proliferation of other cells and the expression of various genes via the autocrine and paracrine system. TNF-α also plays a pivotal role in host defense and can induce the expression of a number of other immunoregulatory and inflammatory mediators (Baugh & Bucala, 2001). IL-6 is one of the most important immune and inflammatory mediators that regulate diverse cell functions including proliferation and differentiation of B-cells and T-cells (Sobota et al., 2008). Activated macrophages also results in the expression of iNOS, which catalyzes the production of a large amount of NO from L-arginine and molecular oxygen (Alderton, Cooper, & Knowles, 2001), which contributes to the killing of microorganisms and mediating a variety of biological functions as an intracellular messenger molecule (Palmer, Ashton, & Moncada, 1988). In our study, TNF-α, IL-1β, IL-6 and NO production by the macrophages in response to SFPS was found to be time and dose dependent. Moreover, the induction of TNF-α, IL-1β, IL-6 and iNOS gene expression was also significantly increased by SFPS treatment. These results indicated that SFPS stimulated macrophages to produce cytokines and NO through the induction of the genes expression.

TLRs are a family of proteins that play an essential role in the recognition of invaded pathogens and the activation of cytokine production by macrophages. There have been reports demonstrating TLR-dependent activation of macrophages by several kinds of polysaccharides, such as *Spirulina* (Balachandran, Pugh, Ma, & Pasco, 2006), *safflower* (Ando et al., 2002), *Acanthopanax koreanum* (Han et al., 2003) and *Platycodon grandiflorum* (Yoon et al., 2003). These reports also suggest that TLR has a relatively broad specificity to polysaccharides isolated from a variety of sources, and various membrane receptors other than TLR4, including TLR2, CD19 and CD79b were also involved. CD14, another membrane receptor of macrophages, acts as a pattern recognition receptor and enhances signaling via TLRs by immobilizing the ligands at high local concentration (Medzhitov, 2001). This prompted us to study TLR4, TLR2 and CD14 as the candidate receptor/binding site for SFPS. Our data showed that anti-TLR4, anti-TLR2 and anti-CD14 antibody reduced SFPS- and LPS-induced TNF-α secretion and NO production (Fig. 4A and B). The blocking of antibodies on SFPS-induced TNF-α secretion and NO production was quite similar to that on the action of LPS. The

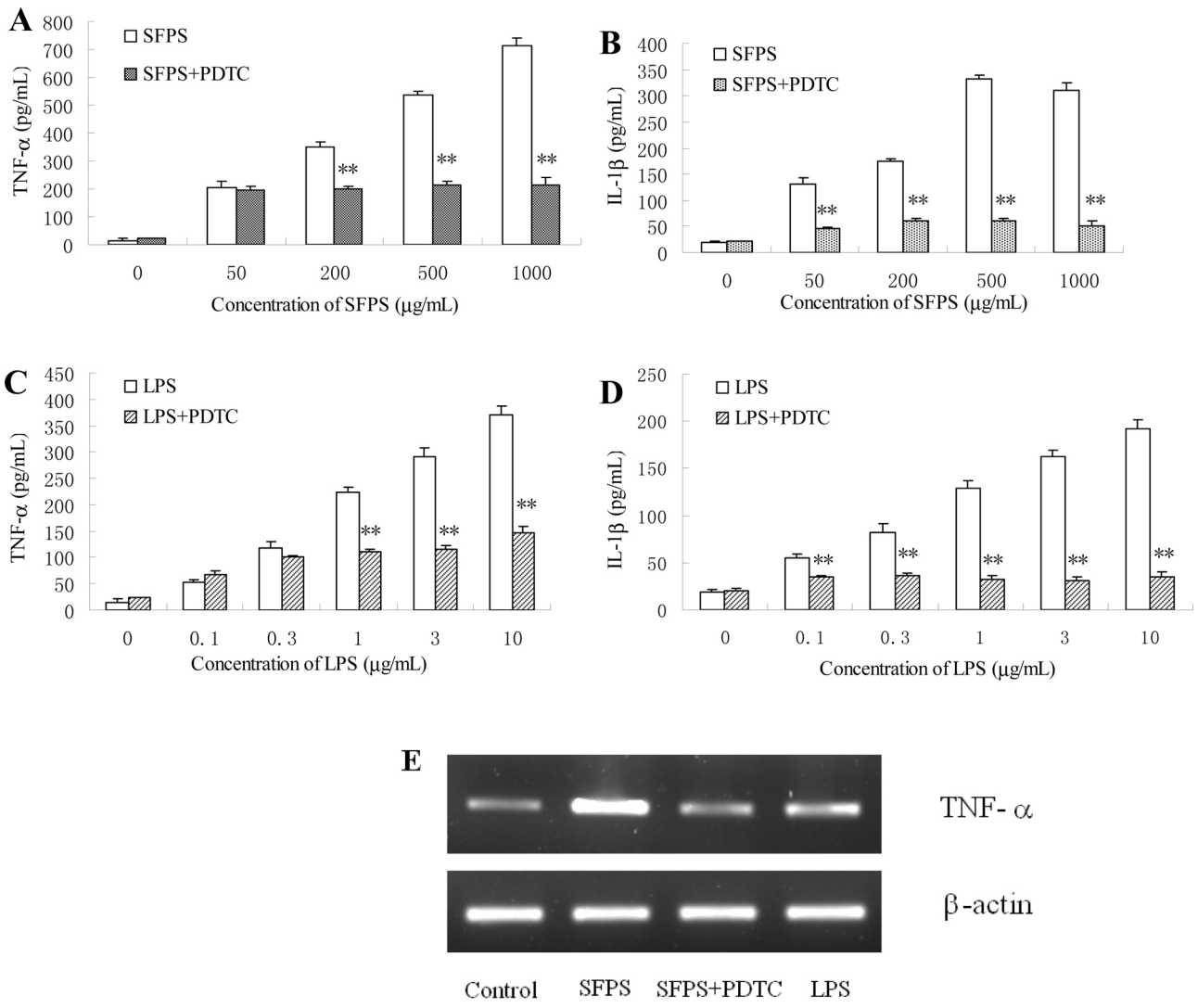

Fig. 5. Effect of the pretreatment of macrophages with NF-κB inhibitor (PDTC) on SFPS- or LPS-induced TNF-α and IL-1β secretion. Macrophages were pre-incubated with PDTC (100 μmol/L) for 2 h, and then culture with indicated concentration of SFPS (A and B) or LPS (C and D) for 24 h. Cells were treated with SFPS (500 μg/mL) or LPS (10 μg/mL) for 24 h as described in Section 2, then total RNA was isolated an analyzed for magnitude of the mRNA expression of TNF-α using RT-PCR (E). The values are presented as means ± S.D. Significance was determined using the Student's *t*-test versus the corresponding group without PDTC treatment ($^{**}P < 0.01$).

result indicated that binding (interaction) of SFPS to TLRs triggers the downstream signaling and related immunomodulatory activities, including expression of the cytokine TNF-α and NO. The result also showed that CD14 is involved in the activation of macrophages by SFPS.

TLRs trigger immune responses primarily through the activation of the transcription factor NF-κB (Imler & Hoffmann, 2001). In unstimulated cells, NF-κB forms a complex with IκB and is sequestered in the cytosol. Immediately after phosphorylation, IκB is ubiquitinated and degraded by 26S proteasome, which allows the translocation of NF-κB to the nucleus where it activates the transcription of a variety of target genes (Hatada et al., 2000), including IL-1β, TNF-α, IL-6, IL-8, IL-12, inducible nitric oxide synthase (iNOS). For investigated the role of NF-κB in the SFPS induced cytokines and NO production in peritoneal macrophages, PDTC (an NF-κB inhibitor) was used. It was observed that SFPS induced TNF-α and IL-1β productions were sensitive to PDTC. This suggested that NF-κB pathways is involved in SFPS induced signal transduction of macrophage activation. To further confirm our observation we checked the direct involvement of NF-κB by WB and CLSM. The results showed that SFPS significantly increased the production of NF-κB in a dose-related manner and translocation of NF-κB to the nucleus. These results indicated that SFPS-mediated induction of cytokines and NO production in macrophages is mediated, at least in part, by TLRs/NF-κB signaling pathway.

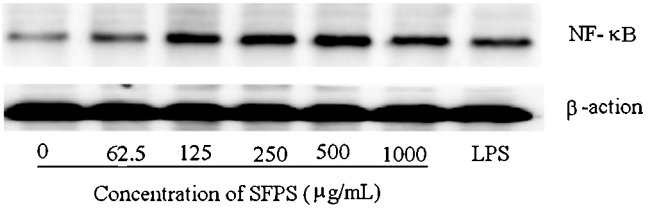

Fig. 6. Effect of SFPS on NF-κB production in the peritoneal macrophages (PMφ). Culture macrophages were treated with indicated concentration of SFPS or 10 μg/mL LPS for 10 h. Nuclear extracts were then prepared and subjected to Western immunoblotting using antibody specific for NF-κB. Results are representative of three independent experiments.

In summary, we have used a macrophage model to study in vitro the immunomodulatory effect of SFPS and investigated the signaling pathways in association with immune response. Taken together, the results presented in this report suggest that SFPS induces macrophage activation through TLRs/NF-κB signaling

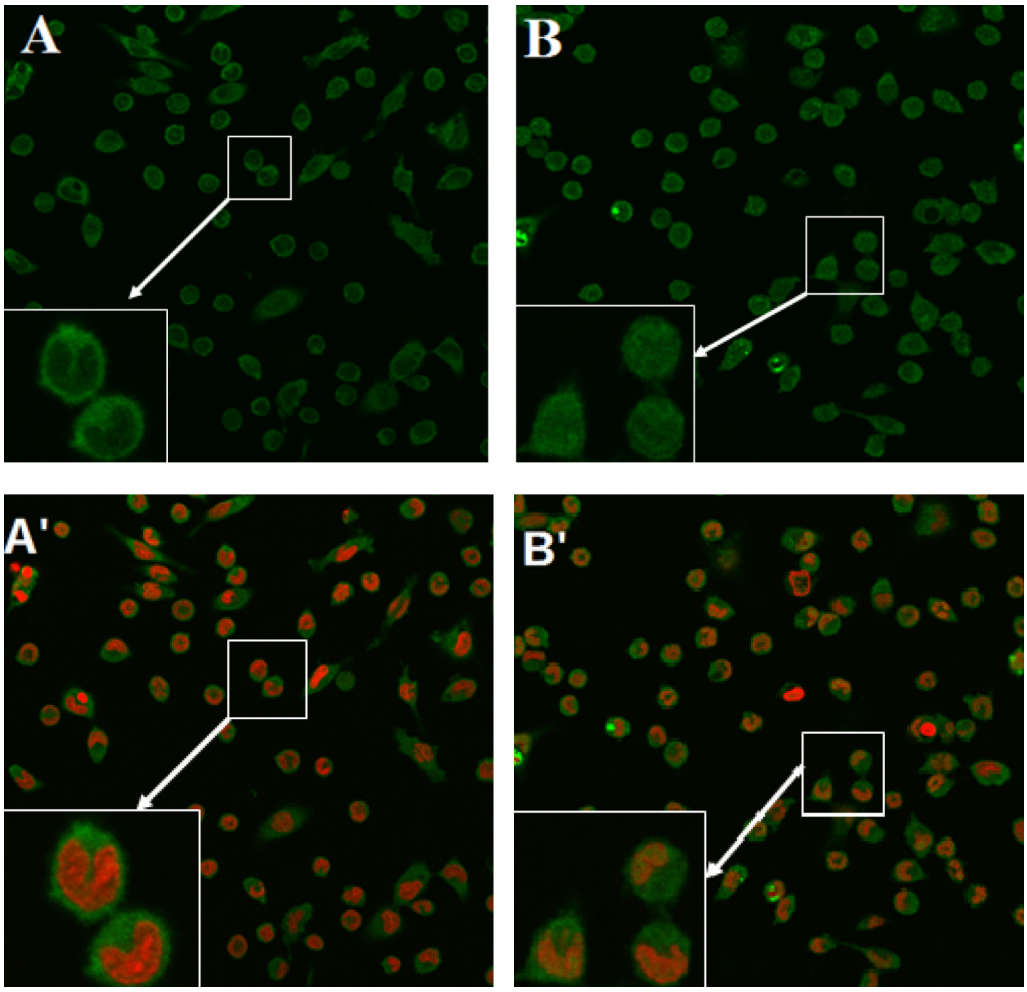

Fig. 7. Effect of SFPS on NF-κB activation in the peritoneal macrophages (PMφ) by confocal laser scanning microscope. Cells stained with FITC-tagged antibody (A: control; B: 100 μg/mL SFPS) or propidium iodide (PI). Micrographs in (A′: control; B′: 100 μg/mL SFPS) represent merged images obtained through the red and green fluorescence channels. The activation of NF-κB in peritoneal macrophages stimulus by SFPS with FITC-tagged antibody and its present in the nucleus was monitored by measuring the green fluorescence integrated over the nucleus, which was counterstained with propidium iodide (PI). Results are representative of three independent experiments. Magnification, 600×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

pathway. However, further studies should be undertaken to fully understand overall intracellular process related to the macrophage activation by SFPS. The immunostimulatory effect of SFPS also suggests a possible application of SFPS as an immunostimulant or a supportive agent in anticancer therapy.

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