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Chitosan oligosaccharides inhibit LPS-induced over-expression of IL-6 and TNF-α in RAW264.7 macrophage cells through blockade of mitogen-activated protein kinase (MAPK) and PI3K/Akt signaling pathways

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ABSTRACT

Chitosan oligomers show various biological activities. However, its molecular mechanisms remain unknown in LPS-stimulated macrophages. Here, we explored the inhibitive effects of chitosan oligomers on LPS-induced IL-6/TNF-α production in macrophages. The results indicated chitosan oligomers pre-treatment effectively inhibited LPS-induced over-expression of both inflammatory cytokines. Signal transduction studies show chitosan oligomers may repress not only the phosphorylation of p38, ERK1/2, JNK, phosphatidylinositol 3-kinase (PI3K) and Akt, but also the activation of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Furthermore, both the activation of NF-κB/AP-1 and the subsequent IL-6/TNF-α over-expression in LPS-induced macrophages are inhibited by specific p38 inhibitor (SB203580), ERK1/2 inhibitor (PD98059), JNK inhibitor (SP600125) and PI3K inhibitor (LY294002). In conclusion, our investigation suggests chitosan oligomers inhibited the elevated expression of IL-6/TNF-α in LPS-induced macrophages, regulated by MAPKs and PI3K/Akt pathways dependent on NF-κB/AP-1 activation.

1. Introduction

Inflammatory response is the primary mechanism of host defense against infection. By pro-inflammatory factor stimulation, the micro-organ-induced pathogenic injury can be effectively prevented in human body. However, high levels of inflammatory cytokines in body circulation or in local inflammatory sites will lead to the occurrence of severe diseases (Liu et al., 2005; Serhan & Savill, 2005). For example, the over-production of TNF-α will cause metabolic disorder, tissue injury and septic shock by the induced body fever or inflammatory reactions (Ettinger et al., 1998; Shohami, Ginis, & Hallenbeck, 1999). Like TNF-α, IL-6 is also one of the most important mediators of fever and acute-phase responses, which can be secreted by macrophages under specific microbial molecules (Yoon et al., 2010). Therefore, it may be of great pathological significance to inhibit the macrophage activation followed by a series of inflammation cascades (Lee, Lee, et al., 2008). Previous studies showed that inflammatory responses could be initiated by a variety of heterologous stimuli including pneumococcus, influenza virus and Gram-positive/negative bacterial (Kukavica-Ibrulj et al., 2009; Lapara & Kelly, 2010; Schwerbrock, Karlsson, Shi, Sheridan, & Beck, 2009). Among these inflammatory agents, lipopolysaccharide (LPS), an integral part of Gram-negative bacteria, has been shown to induce production of pro-inflammatory cytokines, chemokines, growth factors and many other factors in macrophages, fibroblasts and monocytes, etc. (Schumann et al., 1996). Further, LPS recognition and signaling have been proved to be the key events in host defense against Gram-negative bacteria. It was reported that LPS-induced inflammatory reaction was mainly regulated by mitogen-activated protein kinases (MAPKs) – and/or phosphatidylinositol 3-kinase (PI3K)-associated signaling pathways in macrophage cells (Ku, Huang, Huang, & Chiou, 2008; Mendes Sdos et al., 2009).

Chitosan oligomers have been shown various biological effects including anti-microbial, anti-oxidative, anti-tumor and immuno-promoting activities (Dou et al., 2007; Huang, Mendis, Rajapakse, & Kim, 2006; Park, Je, & Kim, 2003; Yin, Du, & Zhang, 2009). Recently, more attention was paid to the effects of chitosan oligomers on inflammatory responses in macrophages. For example, chitosan oligomers (~10 kDa) have been found to inhibit NO and cytokine expression in LPS-induced macrophages (Yoon, Moon, Park, Im, & Kim, 2007). Chitosan with a molecular weight of 20 kDa displayed inhibitive effect on NO production in resting macrophages, but chitooligosaccharide mixture remarkably increased NO production (Wu & Tsai, 2007). Also, the investigation by Han et al., sug-

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gests that chitosan oligomers (~1 kDa) can activate macrophages directly (Han, Zhao, Yu, Feng, & Yu., 2005). Based on these above-mentioned, the anti-inflammatory activity of chitosan oligomers may directly depend on its molecular weight and polymerization degree composition. Hitherto, there is limited knowledge about the anti-inflammatory effect of chitosan oligomers with low molecular weight in macrophages. Further, the molecular mechanisms through which chitosan oligomers exert their anti-inflammatory effect remain to be known.

RAW264.7 macrophage cells, derived from BALB/c mice ascites, are commonly accepted as a tool to investigate the molecular mechanisms of macrophage involved in regulating immunity (Hartley et al., 2008). In present study, we aimed to investigate the inhibitory effects of chitosan oligomers on LPS-induced over-expression of IL-6 and TNF-α in macrophages at both transcription and translation levels. To explore the underlying action mechanisms of chitosan oligomers, the involvement of MAPKs and PI3K/Akt signaling pathway was studied, and the roles played by nuclear factor-κB (NF-κB) and activator protein-1 (AP-1) were also elucidated.

2. Materials and methods

2.1. Chemicals and regents

Chitosan oligomers were prepared by our laboratory (the degree of deacetylation was above 95%) (Zhang, Du, Yu, Mitsutomi, & Aiba, 1999). The weight percentages of chitosan oligomers with DP (degree of polymerization) 2–6 in oligomixture were 3.7%, 16.1%, 28.8%, 37.2% and 14.2%, respectively. RAW264.7 cell line was purchased from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. Lipopolysaccharide (LPS) from *Escherichia coli*, MTT, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE), JNK/SAPK inhibitor (SP600125) and PI3K inhibitor (LY294002) were obtained from Sigma. ERK1/2 inhibitor (PD98059) and p38 MAPK inhibitor (SB203580) were purchased from Invitrogen Corporation. Polyclonal antibody against NF-κB was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). The other antibodies were purchased from Santa Cruz Biotechnology. RPMI 1640 medium and fetal bovine serum (FBS) were from Gibco.

2.2. Cell culture and drug treatment

Macrophages were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 units/ml penicillin and 100 units/ml streptomycin at 37 °C under 5% CO_2 and 95% air.

For most experiments, after growing to sub-confluence, cells were pretreated with vehicle or various concentrations of chitosan oligomers (50–200 μg/ml) in RPMI 1640 with 10% FBS for indicated time. After that, the culture medium was removed as followed by washing twice with phosphate buffered saline (PBS, pH 7.4), and then the cells were exposed to LPS (100 ng/ml) diluted in culture medium for different time intervals at 37 °C until further analysis.

2.3. Determination of cell viability

The viability of macrophages was measured using the MTT assay. The absorbance was measured at 570 nm using a Sunrise Remote Microplate Reader (Grödig, Austria). The viability of macrophages in each well was presented as percentage of control group (untreated).

2.4. Cell proliferation activity analysis

The proliferation activity of macrophages was evaluated by CFSE assay (Parish, 1999). The cells (3×10^5 cells/ml) were sub-cultured into 6-well culture plates for 24 h. Subsequently, the supernatant was removed and the cells were incubated with CFSE (40 μM) in culture medium at 37 °C for 5 h. After the PBS washing, cells were pre-treated with chitosan oligomers (50–200 μg/ml) for 24 h and then exposed to 100 ng/ml LPS for another 12 h. After that, the cells were washed, harvested and re-suspended in culture medium. The fluorescence in cells was quantitatively analyzed at an emission wavelength of 530 nm and an excitation wavelength of 480 nm using a Vantage SE flow cytometer with fluorescence activated cell sorting (FACS) system (Becton Dickinson, San Jose, CA, USA).

2.5. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from macrophages using TRIZOL reagent (TaKaRa, Dalian, China) according to the protocol. RT-PCR assay was performed to examine the alteration in target gene expression. The primers for RT-PCR were used: 5′-CTT CTT GGG ACT GAT GCT GGT G-3′ (sense), 5′-CGC TGG CTT TGT CTT TCT TGT TA-3′ (anti-sense) for IL-6 (383 bp); 5′-GGC GGT GCC TAT GTC TCA-3′ (sense), 5′-GGC AGC CTT GTC CCT TGA-3′ (anti-sense) for TNF-α (363 bp); 5′-CGG TTG GCC TTA GGG TTC AGG GGG G-3′ (sense), 5'-GTG GGC CGC TCT AGG CAC CA-3' (anti-sense) for β-actin (246 bp). The PCR products were detected by electrophoresis in 1.5% agarose gel containing 1% Goldview™. The band intensity was analyzed with ImageJ system (NIH, USA) and presented as a fold of the control group.

2.6. ELISA assay for detecting IL-6 and TNF-α production

Macrophages were pretreated with chitosan oligomers for 24 h and then exposed to LPS (100 ng/ml) for 12 h. The culture media were measured using commercially available ELISA kits. All procedures were performed as the protocol instructions strictly. The samples were analyzed in triplicate.

2.7. Cell lysate preparation

For isolation of total cell extracts, the macrophages were lysed in 200 μl of cell lysis buffer (20 mM Tris–HCl with pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4 and 1 μg/ml leupeptin), to which 1 mM PMSF was added before use. For cytoplasmic and nuclear extract isolation, the Nuclear and Cytoplasmic Protein Extraction kit was used according to the protocol (Beyotime Institute of Biotechnology, Jiangsu, China). Concentration of protein samples was determined with the use of Bicinchoninic Acid Protein Assay kit (Biomed Biotech Co., Ltd., Beijing, China) and all samples were kept at −80 °C until further analysis.

2.8. Western blot analysis

For Western blot analysis, an aliquot of cell lysates containing 60 μg of protein was separated on 8–12% sodium dodecyl sulphate-polyacrylamide gels and transferred to 0.45 μm polyvinylidene fluoride membranes. The densitometric analysis was performed with the use of PDI Imageware System (Bio-Rad, Hercules, CA, USA).

2.9. Statistical analysis

Statistical evaluation was performed using SPSS 10.0 package (SPSS Inc., Chicago, IL, USA). Data were expressed as mean ± SD of 3–5 independent experiments. One-way ANOVA and Student's *T*-test followed by a Bonferroni correction were used to analyze

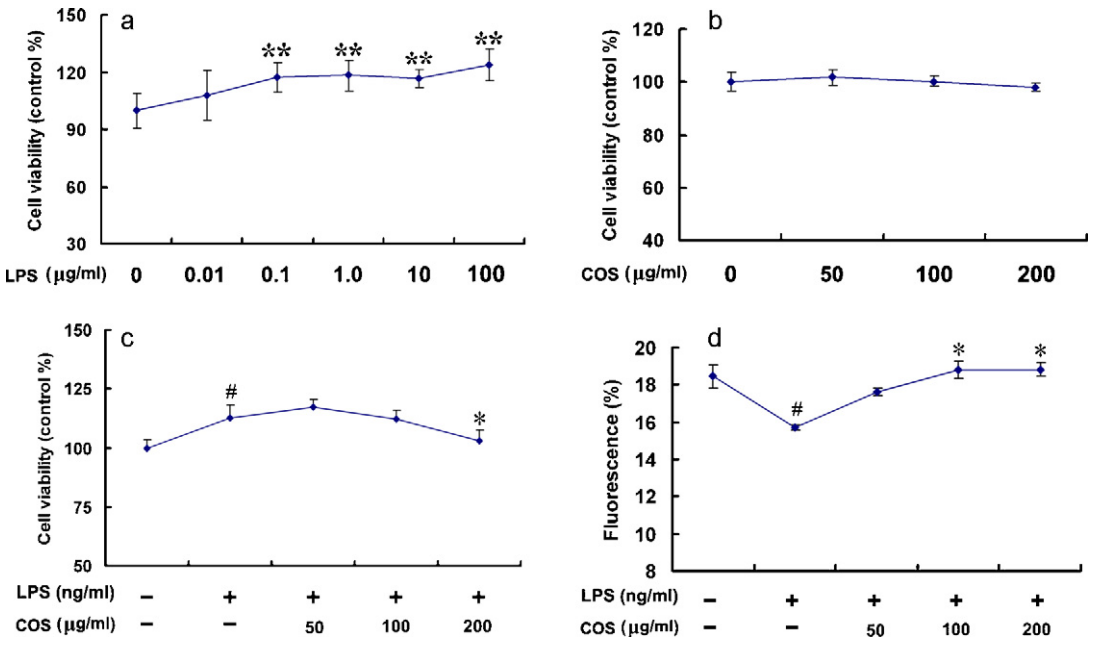

Fig. 1. Effects of chitosan oligomers and/or LPS on cell viability of macrophages. (A) Cells were treated with LPS (0.01–100 μg/ml) alone for 12 h. (B) Cells were treated with chitosan oligomers (50–200 μg/ml) alone for 24 h. (C) and (D) Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h before exposure to LPS (100 ng/ml) for 12 h. Data are presented as means ± SD ($n = 5$). $^{\#}P < 0.05$ compared to the control group; $^{*}P < 0.05$ compared to the LPS-treated group, and $^{**}P < 0.01$ compared to the control group.

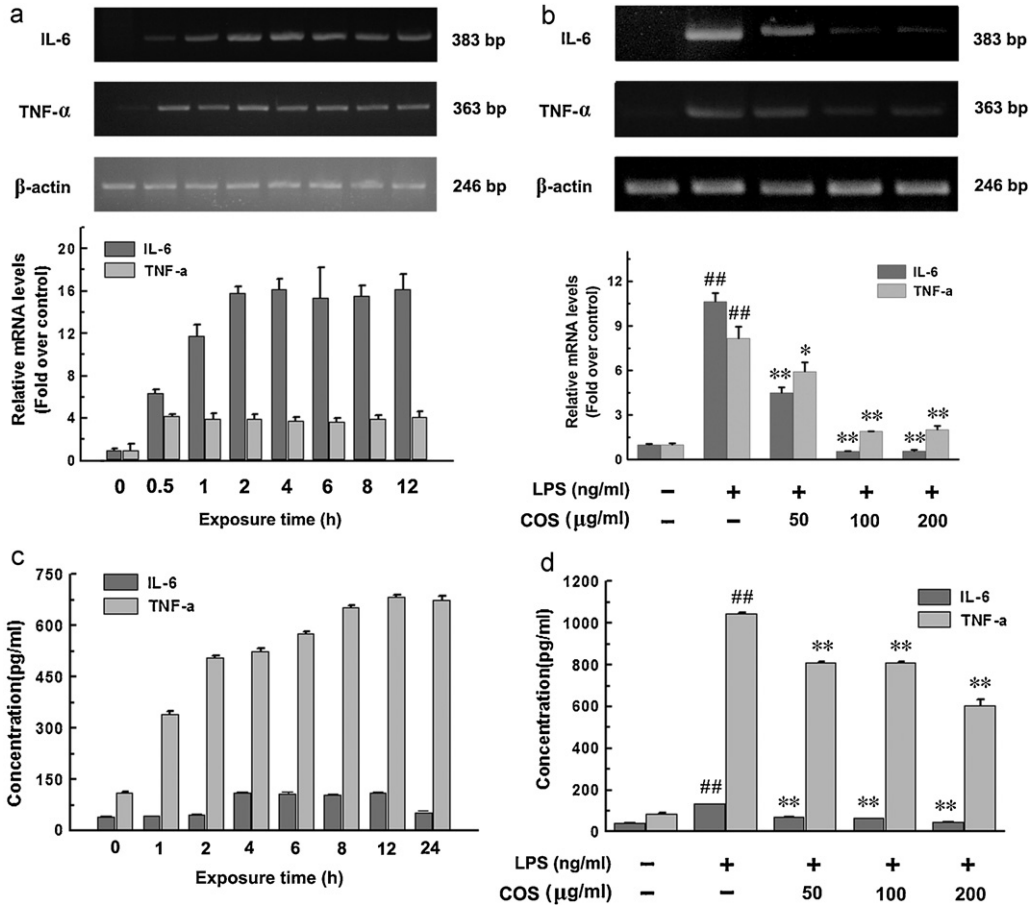

Fig. 2. Effects of chitosan oligomers on LPS-induced IL-6 and TNF-α expression in macrophages at mRNA and protein levels. (A) Cells were treated with LPS (100 ng/ml) for the indicated time. (B) Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h followed by LPS (100 ng/ml) exposure for 12 h. (C) Cells were induced by LPS (100 ng/ml) for the indicated time. (D) Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h and then exposed to LPS (100 ng/ml) for 12 h. Data are represented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

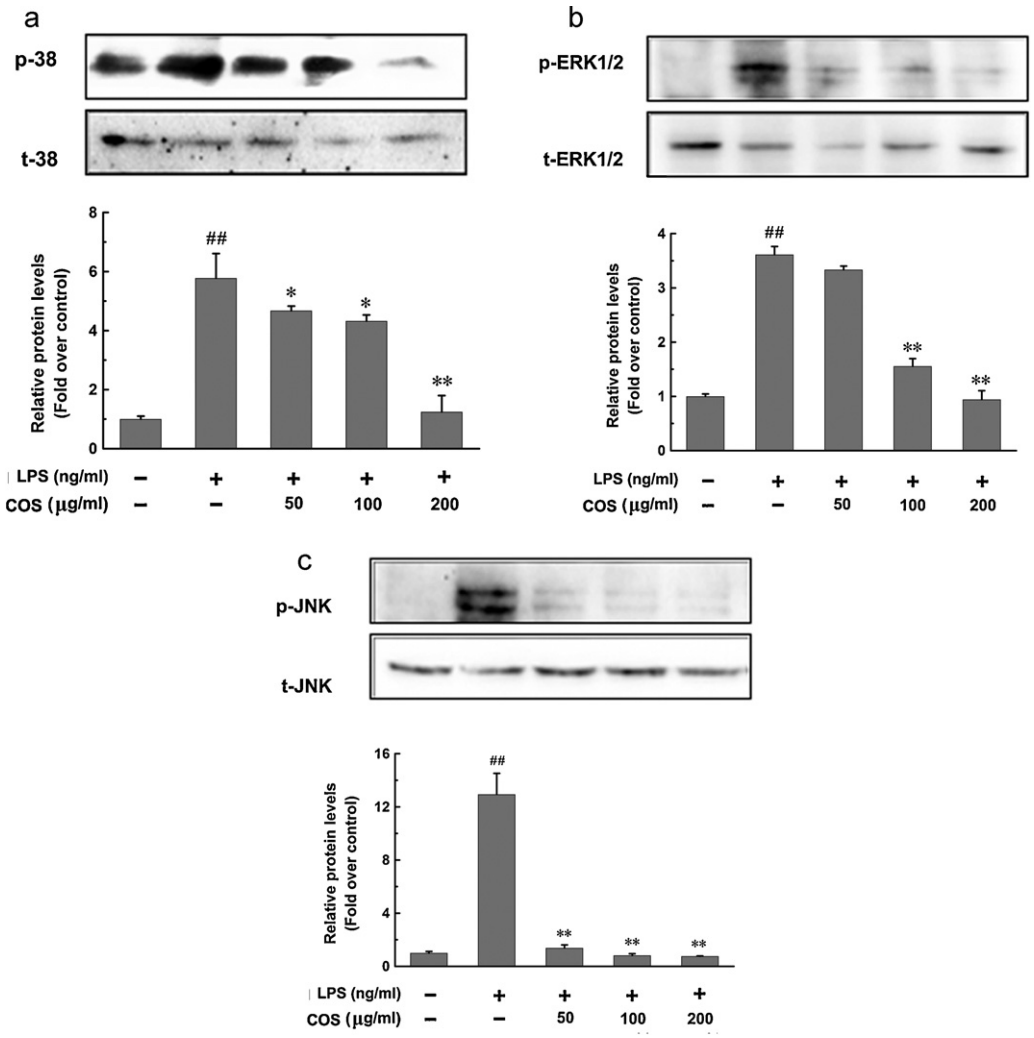

Fig. 3. Inhibitive effects of chitosan oligomers on LPS-induced p38 MAPK (A), ERK1/2 (B) and JNK (C) phosphorylation in macrophages. Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h before exposure to LPS (100 ng/ml) for 15 min. Data are expressed as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

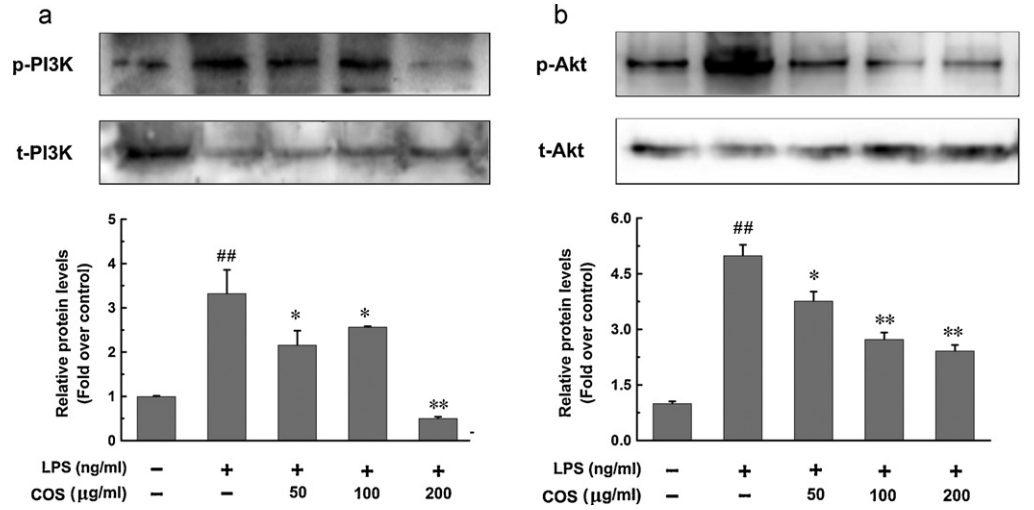

Fig. 4. Blocking effects of chitosan oligomers on LPS-stimulated PI3K (A) and Akt (B) phosphorylation in macrophages. Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h before exposure to LPS (100 ng/ml) for 15 min. Data are presented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

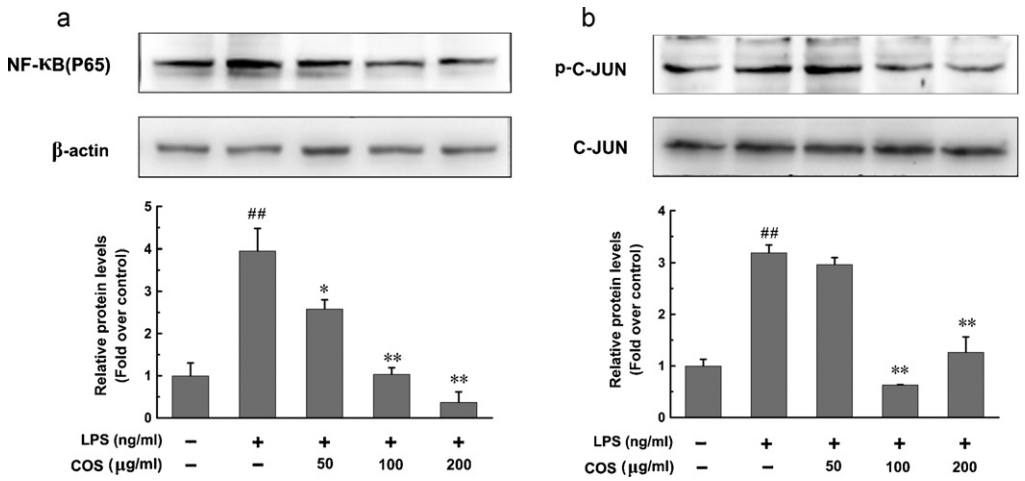

Fig. 5. Inhibitive effects of chitosan oligomers on LPS-induced NF-κB (A) and AP-1 (B) activation in macrophages. Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h and then exposed to LPS (100 ng/ml) for 30 min. Data were represented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

statistical significance. Values of $P < 0.05$ were considered to be statistically significant.

3. Results and discussions

3.1. Effect of chitosan oligomers pretreatment on cell viability of LPS-induced macrophages

The effect of chitosan oligomers pretreatment on cell viability of LPS-induced macrophages was evaluated by MTT assay. As shown in Fig. 1A, LPS treatment (0.1–100 μg/ml) for 12 h obviously enhanced the cell viability in a concentration-dependent manner, which was reversed by chitosan oligomers pretreatment (50–200 μg/ml) for 24 h (Fig. 1C). In parallel, the same results were obtained by CFSE proliferation assay (Fig. 1D). It should be noted that this inhibitory effect was not due to cytotoxic and cytostatic activities of chitosan oligomers, because the cell viability was not affected by chitosan oligomers treatment alone (Fig. 1B).

3.2. Inhibition of chitosan oligomers pretreatment on LPS-induced IL-6 and TNF-α production in macrophages

To investigate the inhibitory effect of chitosan oligomers on LPS-induced IL-6 and TNF-α expression in macrophages, we first performed the time–response studies of IL-6 and TNF-α mRNA levels by RT-PCR. As indicated in Fig. 2A, the expression of both IL-6 and TNF-α was sharply increased after LPS treatment (100 ng/ml), while chitosan oligomers pretreatment (50–200 μg/ml) for 24 h effectively down-regulated LPS-induced over-expression of both inflammatory cytokines (Fig. 2B).

Also, we further explored the effects of chitosan oligomers on LPS-induced production of IL-6 and TNF-α at translation level by ELISA analysis. Results suggested that chitosan oligomers exerted marked suppressive effect on LPS-induced IL-6 and TNF-α secretion in macrophages (Fig. 2C and D), and the result was consistent with that determined by RT-PCR assay.

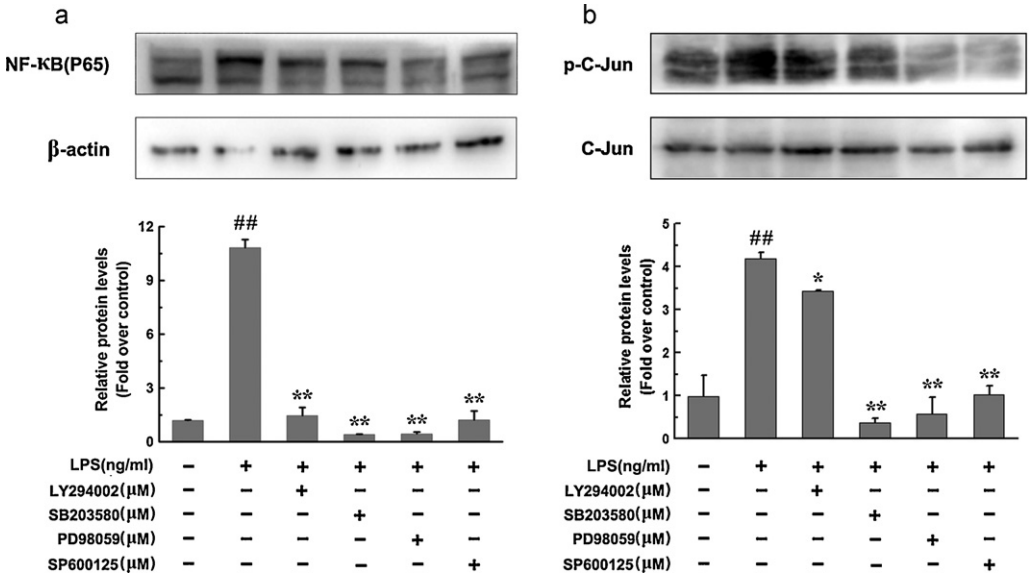

Fig. 6. Effects of MAPK and PI3K/Akt pathways on LPS-induced NF-κB and AP-1 activation in macrophages. Four specific inhibitors, i.e. SB203580 (10 μM), PD98059 (25 μM), and SP600125 (20 μM) and LY294002 (50 μM), pre-incubated cells for 1 h followed by LPS (100 ng/ml) exposure for 30 min. Data were presented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

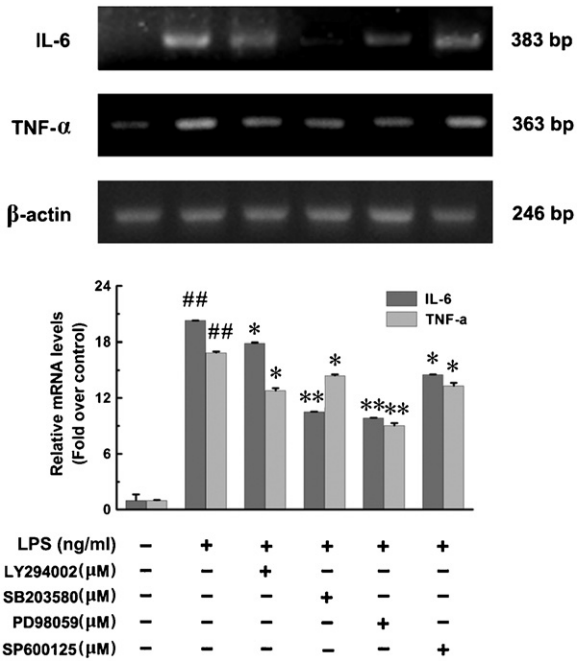

Fig. 7. Roles of MAPK and PI3K/Akt pathways on inhibiting LPS-induced over-production of IL-6 and TNF-α mRNA levels in macrophages. Four specific inhibitors, LY294002 (50 μM), SB203580 (10 μM), PD98059 (25 μM), and SP600125 (20 μM), pre-incubated macrophages for 1 h followed by LPS (100 ng/ml) exposure for12 h. Data were represented as means ± SD ($n = 3$). $^{\#\#}P < 0.01$ compared to the control group; $^{*}P < 0.05$, and $^{**}P < 0.01$ compared to the LPS-treated group.

3.3. Inhibitory effects of chitosan oligomers pretreatment on LPS-induced MAPK phosphorylation in macrophages

MAPKs, mainly composed of three sub-families including p38 MAPK, ERK1/2 and JNK, are serine/threonine-specific protein kinases, which can be triggered by various extracellular stimuli such as mitogens or pro-inflammatory cytokines (Pearson et al., 2001). To determine the inhibitory effect of chitosan oligomers on MAPK activation in macrophages induced by LPS, cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h before exposure to LPS (100 ng/ml) for 15 min. As shown in Fig. 3, LPS induced a sharp increase in the phosphorylated levels of p38 MAPK, ERK1/2 and JNK, and all of which were reverted by chitosan oligomers pretreatment to certain extent. Noticeably, the LPS-induced JNK phosphorylation was entirely suppressed by chitosan oligomers. Based on the above results, it can be suggested that chitosan oligomers prevented macrophages from LPS-induced MAPK activation.

3.4. Suppression of chitosan oligomers pretreatment on LPS-induced PI3K/AKT phosphorylation in macrophages

PI3K is a family of enzymes involved in various cellular functions, and Akt is the effector of PI3K. It was reported that the PI3K/Akt signaling pathway could be activated in response to LPS (Kim et al., 2008). So far, the role of PI3K/Akt signals in regulation of inflammatory response still remains to be controversial. For example, Luyendyk et al. and Tsukamoto et al. demonstrated that PI3K/Akt pathway negatively regulated LPS-induced inflammatory response (Luyendyk et al., 2008; Tsukamoto et al., 2008), while the studies by other groups indicate that LPS led to inflammation in macrophages by activating the PI3K and Akt signals (Lee, Vinodhkumar, et al., 2008; Martins, Ferracini, Ravanelli, Landgraf, & Jancar, 2008; Pan et al., 2008). Since PI3K/Akt pathway played an important role in regulating the inflammation responses in macrophage cells, we next examined the effects of chitosan oligomers on PI3K/Akt signaling pathway in LPS-induced macrophages. Cells were pretreated with chitosan oligomers (50–200 μg/ml) for 24 h before LPS (100 ng/ml) exposure for 15 min. As shown in Fig. 4, LPS induced rapid increase in the levels of both p-PI3K and p-Akt in macrophage cells. On the contrary, chitosan oligomers displayed marked inhibitory effects on the phosphorylation of both kinases in a concentration-dependent manner.

3.5. Inhibition of LPS-induced NF-κB and AP-1 activation of macrophages, by chitosan oligomers pretreatment

NF-κB is an important transcription factor in mediating the pro-inflammatory responses. In quiescent cells, NF-κB is located in the cytoplasm as an inactive complex bound to the inhibitor of κB (I-κB). After the activation by phosphorylation or ubiquitination, NF-κB will disassociate with I-κB and translocate into nucleus to initiate the transcription of target genes (Liu et al., 2010). Also, AP-1 is another major transcription factor, mainly consisting of homodimers or heterodimers of jun, fos or activating transcription factor (ATF) proteins. Once exposure to stimuli, both c-jun and ATF2 will be rapidly phosphorylated followed by AP-1 activation. Although the jun protein forms very stable heterodimers with the other members of the AP-1 family, they can also homodimerize among themselves to function as a transcription factor (Karin, 1995; Karin, Liu, & Zandi, 1997). It has been widely confirmed that both NF-κB and AP-1 will be activated and induce inflammation reaction in response to exogenous stimuli (Ortiz-Lazareno et al., 2008). Particularly, Studies show that both NF-κB and AP-1 played key roles in LPS-induced over-production of IL-6 and TNF-α in macrophage cells (Kim et al., 2007; Wu, Chen, Ueng, & Chen, 2008). Thus, the inhibition of two transcription factors might be a critical step in suppressing LPS-induced inflammatory responses in macrophages. We, here, investigated whether chitosan oligomers pretreatment could suppress LPS-induced NF-κB and AP-1 activation in macrophages. Results showed that LPS stimulation (100 ng/ml) for 30 min markedly promoted the levels of p-c-Jun in the nucleus and the translocation of NF-κB into nucleus of macrophages (Fig. 5). However, chitosan oligomers pretreatment (50–200 μg/ml) for 24 h led to a significant down-regulation of both proteins in the nucleus. In addition, we explored the involvement of c-fos, another member of the AP-1 family, which can be induced transcription rapidly and transiently by many different stimuli, such as LPS, followed by combination with c-jun proteins to form AP-1 dimers (Lee et al., in press). Moreover, the result seemed to be consistent with the above ones, chitosan oligomers could effectively suppress LPS-induced c-fos expression in macrophages in a concentration-dependent manner (unpublished data). Therefore, chitosan oligomers may be a potential inhibitor against NF-κB- and AP-1-mediated inflammation responses in macrophages.

3.6. Roles of MAPK and PI3K/Akt signaling pathways in LPS-induced NF-κB and AP-1 activation in macrophages

In the present study, chitosan oligomers displayed remarkable inhibitive effects on LPS-induced MAPKs and PI3K/Akt phosphorylation in macrophages. It was proved that MAPKs played a critical role in LPS-induced NF-κB/AP-1 activation and subsequent pro-inflammatory cytokine over-expression (Lee & Lim, 2009; Ock, Kim, & Suk, 2009). In parallel, PI3K/Akt pathway has been documented to positively regulate NF-κB or AP-1 activation in LPS-stimulated monocyte/macrophage cells (Guha & Mackman, 2002; Lai et al., 2009). Therefore, to confirm the roles of MAPK and PI3K/Akt signaling pathways played in the NF-κB and AP-1 activation induced by LPS in macrophages, the specific kinase inhibitors were used in

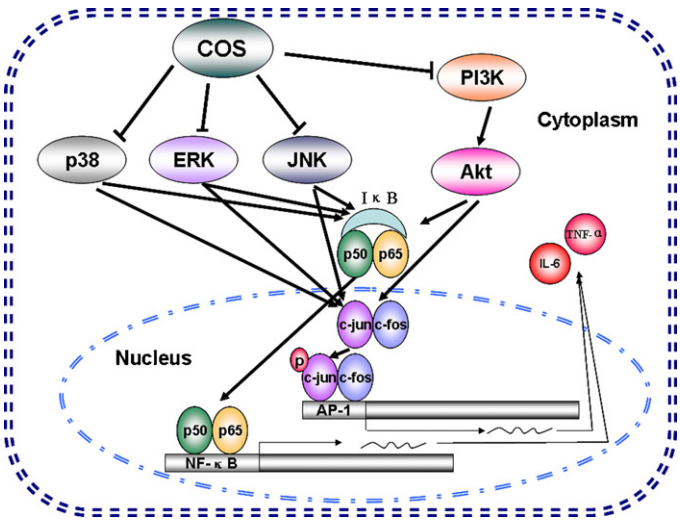

Fig. 8. Diagram of the underlying molecular mechanisms about the effects of chitosan oligomers on anti-inflammation in LPS-induced macrophages.

present study. As shown in Fig. 6, after the cells were pretreated for 1 h with p38 MAPK inhibitor (SB203580, 10 μM), ERK1/2 inhibitor (PD98059, 25 μM), JNK inhibitor (SP600125, 20 μM) and PI3K inhibitor LY294002 (50 μM) separately, the LPS-induced NF-κB and AP-1 activation in macrophage cells were effectively inhibited to certain extents, which implies that MAPK and PI3K/Akt pathways may act upstream of NF-κB and AP-1, and be involved in the LPS-induced activation of both transcription factors in macrophage cells.

3.7. Roles of MAPKs and PI3K/Akt signaling pathways in LPS-induced over-expression of IL-6 and TNF-α in macrophages

To clarify whether MAPK and PI3K/Akt signaling pathways were involved in LPS-induced IL-6 and TNF-α production, the macrophages were pretreated with four inhibitors as described before and then exposed to LPS (100 ng/ml) for 12 h. The results suggested that all the four inhibitors could suppress LPS-induced over-expression of IL-6 and TNF-α at transcription level (Fig. 7), indicating that MAPK and PI3K/Akt signaling pathways may be responsible for the suppressive effects of chitosan oligomers on LPS-induced over-production of both pro-inflammatory cytokines in macrophage cells.

4. Conclusions

In this study, our findings indicated that chitosan oligomers suppressed LPS-induced over-expression of IL-6 and TNF-α in macrophage cells at both transcription and translation levels. As shown in Fig. 8, the underlying molecular mechanisms may be that, chitosan oligomers inhibited the LPS-induced IL-6 and TNF-α production in macrophages by down-regulating the phosphorylated levels of MAPK and PI3K/Akt signaling pathways and subsequent NF-κB and AP-1 activation.

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References

Dou, J. L., Tan, C. Y., Du, Y. G., Bai, X. F., Wang, K. Y., & Ma, X. J. (2007). Effects of chitooligosaccharides on rabbit neutrophils in vitro. *Carbohydrate Polymers*, *69*, 209–213.

Ettinger, R., Mebius, R., Browning, J. L., Michie, S. A., van Tuijl, S., Kraal, G., et al. (1998). Effects of tumor necrosis factor and lymphotoxin on peripheral lymphoid tissue development. *International Immunology*, *10*, 727–741.

Guha, M., & Mackman, N. (2002). The phosphatidylinositol 3-kinase-Akt pathway limits lipopolysaccharide activation of signaling pathways and expression of inflammatory mediators in human monocytic cells. *The Journal of Biological Chemistry*, *277*(*35*), 32124–32132.

Han, Y., Zhao, L., Yu, Z., Feng, J., & Yu., Q. (2005). Role of mannose receptor in oligochitosan-mediated stimulation. *International Immunopharmacology*, *5*, 1533–1542.

Huang, R., Mendis, E., Rajapakse, N., & Kim, S. K. (2006). Strong electronic charge as an important factor for anticancer activity of chitooligosaccharides (COS). *Life Sciences*, *78*, 2399–2408.

Hartley, J. W., Evans, L. H., Green, K. Y., Naghashfar, Z., Macias, A. R., Zerfas, P. M., et al. (2008). Expression of infectious murine leukemia viruses by RAW264.7 cells, a potential complication for studies with a widely used mouse macrophage cell line. *Retrovirology*, *5*, 1–6.

Karin, M. (1995). The regulation of AP-1 activity by mitogen-activated protein kinases. *The Journal of Biological Chemistry*, *270*(*28*), 16483–16486.

Karin, M., Liu, Z., & Zandi, E. (1997). AP-1 function and regulation. *Current Opinion in Cell Biology*, *9*, 240–246.

Kim, J. B., Han, A. R., Park, E. Y., Kim, J. Y., Cho, W., Lee, J., et al. (2007). Inhibition of LPS-induced iNOS, COX-2 and cytokines expression by poncirin through the NF-κB inactivation in RAW264.7 macrophage cells. *Biological Pharmaceutical Bulletin*, *30*(12), 2345–2351.

Kim, J. H., Na, H. J., Kim, C. K., Kim, J. Y., Ha, K. S., Lee, H., et al. (2008). The non-provitamin A carotenoid, lutein, inhibits NF-κB-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF-κB-inducing kinase pathways: Role of H_2O_2 in NF-κB activation. *Free Radical Biology & Medicine*, *45*, 885–896.

Ku, K. T., Huang, Y. L., Huang, Y. J., & Chiou, W. F. (2008). Miyabenol A inhibits LPS-induced NO production via IKK/IkappaB inactivation in RAW 264.7 macrophages: Possible involvement of the p38 and PI3K pathways. *Journal of agricultural and Food Chemistry*, *56*(*19*), 8911–8918.

Kukavica-Ibrulj, I., Hamelin, M. E., Prince, G. A., Gagnon, C., Bergeron, Y., Bergeron, M. G., et al. (2009). Infection with human metapneumovirus predisposes mice to severe pneumococcal puneumonia. *Journal of Virology*, *83*, 1341–1349.

Liu, J., Li, X., Yue, Y., Li, J., He, T., & He, Y. (2005). The inhibitory effect of quercetin on IL-6 production by LPS-stimulated neutrophils. *Cellular & Molecular Immunology*, *2*(6), 455–460.

Lee, Y. G., Lee, W. M., Kim, J. Y., Lee, J. Y., Lee, I. K., Yun, B. S., et al. (2008). Src kinase-targeted anti-inflammatory activity of davallialactone from Inonotus xeranticus in lipopolysaccharide-activated RAW264.7 cells. *British Journal of Pharmacology*, *154*, 852–863.

Lee, H. C., Vinodhkumar, R., Yoon, J. W., Park, S. K., Lee, C. W., & Kim, H. Y. (2008). Enhanced inhibitory effect of ultra-fine granules of red ginseng on LPS-induced cytokine expression in the monocyte-derived macrophage THP-1 cells. *International Journal of Molecular Sciences*, *9*, 1379–1392.

Luyendyk, J. P., Schabbauer, G. A., Tencati, M., Holscher, T., Pawlinski, R., & Mackman, N. (2008). Genetic analysis of the role of the PI3K-Akt pathway in lipopolysaccharide-induced cytokine and tissue factor gene expression in monocytes/macrophages. *The Journal of Immunology*, *180*, 4218–4226.

Lai, C. S., Lee, J. H., Ho, C. T., Liu, C. B., Wang, J. M., Wang, Y. J., et al. (2009). Rosmanol potently inhibits lipopolysaccharide-induced iNOS and COX-2 expression through downregulating MAPK, NF-κB, STAT3 and C/EBP signaling pathways. *Journal of Agricultural and Food Chemistry*, *57*, 10990–10998.

Lee, S. J., & Lim, K. T. (2009). Inhibitory effect of ZPDC glycoprotein on the expression of inflammation-related cytokines through p38 MAP kinase and JNK in lipopolysaccharide-stimulated RAW264.7 cells. *Inflammation Research*, *58*, 184–191.

Lapara, N. J., & Kelly, B. L. (2010). Suppression of LPS-induced inflammatory responses in macrophages infected with Leishmania. *Journal of Inflammation*, *7*, 1–9.

Lee, J. J., Liu, C. L., Tsai, P. S., Yang, C. L., Lao, H. C., & Huang, C. J. Platonin inhibits endotoxin-induced MAPK and AP-1 up-regulation. *Journal of Surgical Research*, in press, doi:10.1016/j.jss.2009.11.738.

Liu, H. T., Li, W. M., Huang, P., Chen, W. J., Liu, Q. S., Bai, X. F., et al. (2010). Chitosan oligosaccharides inhibit TNF-α-induced VCAM-1 and ICAM-1 expression in human umbilical vein endothelial cells by blocking p38 and ERK1/2 signaling pathways. *Carbohydrate Polymers*, *81*, 49–56.

Martins, J. O., Ferracini, M., Ravanelli, N., Landgraf, R. G., & Jancar, S. (2008). Insulin inhibits LPS-induced signaling pathways in alveolar macrophages. *Cellular Physiology and Biochemistry*, *21*, 297–304.

Mendes Sdos, S., Candi, A., Vansteenbrugge, M., Pignon, M. R., Bult, H., Boudjeltia, K. Z., et al. (2009). Microarray analyses of the effects of NF-kappaB or PI3K pathway inhibitors on the LPS-induced gene expression profile in RAW264.7 cells: Synergistic effects of rapamycin on LPS-induced MMP9-overexpression. *Cellular Signalling*, *21*(7), 1109–1122.

Ortiz-Lazareno, P. C., Hernandez-Flores, G., Dominguez-Rodriguez, J. R., Lerma-Diaz, J. M., Jave-Suarez, L. F., Aguilar-Lemarroy, A., et al. (2008). MG132 proteasome

inhibitor modulates pro-inflammatory cytokines production and expression of their receptors in U937 cells: Involvement of nuclear factor-κB and activator protein-1. *Immunology*, *124*, 534–541.

Ock, J., Kim, S., & Suk, K. (2009). Anti-inflammatory effects of a fluorovinyloxyacetamide compound KT-15087 in microglia cells. *Pharmacological Research*, *59*, 414–422.

Parish, C. R. (1999). Fluorescent dyes for lymphocyte migration and proliferation studies. *Immunology and Cell Biology*, *77*(6), 499–508.

Pearson, G., Robinson, F., Beers Gibson, T., Xu, B., Karandikar, M., Berman, K., et al. (2001). Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocrine Reviews*, *22*(*2*), 153–183.

Park, P. J., Je, J. Y., & Kim, S. K. (2003). Free radical scavenging activity of chitooligosaccharides by electron spin resonance spectrometry. *Journal of agricultural and Food Chemistry*, *51*, 4624–4627.

Pan, M. H., Hsieh, M. C., Hsu, P. C., Ho, S. Y., Lai, C. S., Wu, H., et al. (2008). 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Molecular Nutrition & Food Research*, *52*, 1467–1477.

Schumann, R. R., Pfeil, D., Lamping, N., Kirschning, C., Scherzinger, G., Schlag, P., et al. (1996). Lipopolysaccharide induces the rapid tyrosine phosphorylation of the mitogen-activated protein kinases erk-1 and p38 in cultured human vascular endothelial cells requiring the presence of soluble CD14. *Blood*, *87*(7), 2805–2814.

Shohami, E., Ginis, I., & Hallenbeck, J. M. (1999). Dual role of tumor necrosis factor alpha in brain injury. *Cytokine & Growth Factor Reviews*, *10*, 119–130.

Serhan, C. N., & Savill, J. (2005). Resolution of inflammation: The beginning programs the end. *Nature Immunology*, *6*(*12*), 1191–1197.

Schwerbrock, N. M. J., Karlsson, E. A., Shi, Q., Sheridan, P. A., & Beck, M. A. (2009). Fish oil-fed mice have impaired resistance to influenza infection. *The Journal of Nutrition*, *139*, 1588–1594.

Tsukamoto, K., Hazeki, K., Hoshi, M., Nigorikawa, K., Inoue, N., Sasaki, T., et al. (2008). Critical roles of the p110β subtype of phosphoinositide 3-kinase in lipopolysaccharide-induced Akt activation and negative regulation of nitrite production in RAW264.7 cells. *The Journal of Immunology*, *180*, 2054–2061.

Wu, G. J., & Tsai, G. J. (2007). Chitooligosaccharides in combination with interferon-γ increase nitric oxide production via nuclear factor-κB activation in murine RAW264.7 macrophages. *Food and Chemical Toxicology*, *45*, 250–258.

Wu, G. J., Chen, T. L., Ueng, Y. F., & Chen, R. M. (2008). Ketamine inhibits tumor necrosis factor-α and interleukin-6 gene expressions in lipopolysaccharide-stimulated macrophages through suppression of toll-like receptor 4-mediated c-Jun N-terminal kinase phosphorylation and activator protein-1 activation. *Toxicology and Applied Pharmacology*, *228*, 105–113.

Yoon, H. J., Moon, M. E., Park, H. S., Im, S. Y., & Kim, Y. H. (2007). Chitosan oligosaccharide (COS) inhibits LPS-induced inflammatory effects in RAW 264.7 macrophage cells. *Biochemical and Biophysical Research Communications*, *358*, 954–959.

Yin, H., Du, Y. G., & Zhang, J. Z. (2009). Low molecular weight and oligomeric chitosans and their bioactivities. *Current Topics Medicinal Chemistry*, *9*, 1546–1559.

Yoon, W. J., Moon, J. Y., Song, G., Lee, Y. K., Han, M. S., Lee, J. S., et al. (2010). Artemisia fukudo essential oil attenuates LPS-induced inflammation by suppressing NF-κB and MAPK activation in RAW264.7 macrophages. *Food and Chemical Toxicology*, *48*, 1222–1229.

Zhang, H., Du, Y. G., Yu, X., Mitsutomi, M., & Aiba, S. (1999). Preparation of chitooligosaccharides from chitosan by a complex enzyme. *Carbohydrate Research*, *320*, 257–260.