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RESEARCH ARTICLE

Bioactive hyaluronic acid fragments inhibit lipopolysaccharide-induced inflammatory responses via the Toll-like receptor 4 signaling pathway

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Abstract **The high- and the low-molecular weight hyaluronic acids (HMW-HA and LMW-HA, respectively) showed different biological activities in inflammation. However, the role of LMW-HA in inflammatory response is controversial. In this study, we aimed to investigate the effect of bioactive hyaluronan (B-HA) on lipopolysaccharide (LPS)-induced inflammatory responses in human macrophages and mice. B-HA was produced from HA treated with glycosylated recombinant human hyaluronidase PH20. Human THP-1 cells were induced to differentiate into macrophages. THP-1-derived macrophages were treated with B-HA, LPS, or B-HA + LPS. The mRNA expression and the production of inflammatory cytokines were determined using quantitative real-time PCR and enzyme-linked immunosorbent assay. The phosphorylation levels of proteins in the nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and IRF-3 signaling pathways were measured using Western blot. The *in vivo* efficacy of B-HA was assessed in a mouse model of LPS-induced inflammation. Results showed that B-HA inhibited the expression of TNF-α, IL-6, IL-1, and IFN-β, and enhanced the expression of the anti-inflammatory cytokine IL-10 in LPS-induced inflammatory responses in THP-1-derived macrophages and *in vivo*. B-HA significantly suppressed the phosphorylation of the TLR4 signaling pathway proteins p65, IKKα/β, IκBα, JNK1/2, ERK1/2, p38, and IRF-3. In conclusion, our results demonstrated that the B-HA attenuated the LPS-stimulated inflammatory response by inhibiting the activation of the TLR4 signaling pathway. B-HA could be a potential anti-inflammatory drug in the treatment of inflammatory disease.**

Keywords bioactive hyaluronan; lipopolysaccharide; inflammatory cytokines; TLR4; human macrophages

Introduction

Hyaluronic acid (HA), a negatively charged polymer with repeated units of β-(1,4)-D-glucuronic acid–β-(1,3)-N-acetyl-D-glucosamine, is a nonprotein extracellular molecule secreted by many cell types [1,2]. HA is abundant in a variety of tissues, such as the vitreous of the eyes, umbilical cord, skeletal tissues, synovial fluid, heart valves, and skin [3] and initially thought to be principally structural. However, further investigation suggests that HA is also involved in regulating immune responses [4]. HA exists as a high-molecular weight polymer ($>$ 1000 kDa) under physiologic conditions and plays a critical role in tissue hydration, structure maintenance, and elasticity [5]. In tissue injury and inflammation, high-molecular weight HA (HMW-HA) is degraded by hyaluronidases into low-molecular weight HA (LMW-HA) fragments, which are inflammatory, angiogenic, and immunostimulatory [6]. The expression of HA fragments is actively increased by hyaluronan synthases and reactive oxygen species (ROS) during tissue injury, tissue repair, and wound healing [7]. Recent research has indicated that HA has differential functions that are partly dependent on its molecular weight [8–13]. The exogenous administration of HMW-HA can serve as a novel therapeutic strategy for lipopolysaccharide (LPS)-induced sepsis [14]. LMW-HA can activate intracellular inflammasomes [8], trigger sterile inflammation

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[15], and participate in cancer progression [16]. However, the role of LMW-HA in inflammation remains unclear.

Inflammation is related to the immune response of infection and the pathogenesis of diseases, such as type 2 diabetes, atherosclerosis, and cancer [17–19]. Macrophages as an important antigen-presenting cell play a crucial role in inflammatory response and modulate the pathogenesis of diseases [20,21]. The activated macrophages can secrete several proinflammatory cytokines, such as TNF-α, IL-1, and IL-6. TNF-α and IL-1 are early proinflammatory cytokines in the pathogenesis of sepsis [22]. The damage-associated molecular patterns (DAMPs) recognized by TLR-4 on innate immune cells can stimulate the expression of proinflammatory cytokines in macrophages [23]. Nadeem Akhter *et al.* have revealed that LPS induces CCL-2 via the Toll-like receptor 4 (TLR4)/myeloid differentiation factor 88 (MyD88) signaling, which leads to the activation of nuclear factor-κB (NF-κB)/AP-1 transcription factors on monocytes/macrophages in metabolic inflammation [24].

HA and its degraded products can combine with diverse HA-binding proteins, including CD44, TLR4, HA-binding protein 2, and receptor for HA-mediated motility, which are present in the blood, nucleus, extracellular matrix, cytosol, and cell plasma membrane [25]. HA can modulate the TLR4 signaling and has different biological effects *in vitro* and *in vivo* [26–28]. The activation of the TLR4 signaling pathway after LPS stimulation plays an important role in the secretion of inflammatory cytokines by its downstream signaling pathway, including mitogen-activated protein kinase (MAPK) and NF-κB pathways [29] via the MyD88-dependent pathway and the TIR domain-containing adaptor-inducing IFN (TRIF)-dependent pathway on macrophages [30,31]. Therefore, the inhibition of the TLR4 signal pathway can reduce the production of inflammatory cytokines [32]. At present, the inhibitory mechanism of HA on LPS activation is not well understood and may involve multiple receptors and partly depend on the cell type. In the present study, we investigated the effect of bioactive hyaluronan (B-HA), a HA fragment with an average molecular weight ranging from 10 kDa to 60 kDa, on LPS-induced inflammatory responses in THP-1 cell-derived macrophages and a mouse model of LPS-induced inflammation.

Materials and methods

Preparation of recombinant human hyaluronidase PH20 and bioactive HA

The Chinese hamster ovary (CHO) cells were used to produce the glycosylated recombinant human hyaluronidase PH20. A gene-encoding glycosylated recombinant human hyaluronidase PH20 was synthesized and inserted into GC-rich pMH3, pMH4, and pMH5 empty expression vectors to construct the pMH3–PH20, pMH4–PH20, and pMH5–PH20 expression vectors, respectively. The CHO cell line was transfected with the pMH3–PH20, pMH4–PH20, or pMH5–PH20 expression vector and screened for the PH20 high-expression cell clone. The glycosylated recombinant human hyaluronidase PH20 (130 000–160 000 activity units/mg) was prepared from the PH20 high-expression cells by using a series of separation and purification procedures. A patent for the preparation of glycosylated recombinant human hyaluronidase PH20 is in publication in China (Application number: CN 103468662 A).

B-HA with an average molecular weight ranging from 10 kDa to 60 kDa was prepared from HA treated with glycosylated recombinant human hyaluronidase PH20 in osmotic pressure of 280–300 mOsm/L at 37 °C for 6 h. B-HA was isolated and purified using affinity chromatography, precipitation, ultrafiltration, and dialysis. B-HA (5 mL) consisted of 100 mg HA and 4 mL saline supplemented with Mg^{2+}. The residual protein, RNA, and DNA of the prepared B-HA (HMW-HA and LMW-HA) were detected. A patent for the B-HA preparation is in publication in China (Application number: CN 107303271 A).

Cells and reagents

Human monocyte THP-1 cells were obtained from the cell bank of the Shanghai Institute of Biochemistry and Biology (Chinese Academy of Sciences, Shanghai, China). THP-1 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 10 mmol/L HEPES, 1% penicillin/streptomycin and 2.0 mmol/L L-glutamine in a humidified incubator maintained at 37 °C under 5% CO_2. Cells (3.5×10^5 cells/well) were cultured in 24-well plates and treated with 10 ng/mL phorbol 12-myristate 13-acetate (PMA) for 48 h to induce the differentiation into macrophages [33]. The LPS from *Escherichia coli* (O111:B4) and PMA were obtained from Sigma (St. Louis, MO, USA). FBS and RPMI-1640 medium were purchased from Gibco (Carlsbad, CA, USA). The GAPDH rabbit, p-SAPK/JNK mouse, p-p44/42 MAPK rabbit, p-p38 MAPK rabbit, p-IκBα mouse, p-IKKα/β rabbit, p-p65 rabbit, and p-IRF-3 rabbit monoclonal antibodies; goat antimouse IgG–HRP; and goat antirabbit IgG–HRP were obtained from Cell Signaling Technology (Danvers, MA, USA).

Quantitative real-time PCR (qRT-PCR)

The total RNA of THP-1 cell-derived macrophages treated with B-HA and LPS was extracted using a total RNA kit (Omega, Norcross, GA, USA). The RNA was reversely transcribed using the PrimeScript RT Master Mix kit

(TaKaRa, Shiga, Japan). The cDNA was diluted to 40 μL and used as template in qRT-PCR. The qRT-PCR was operated using the standard SYBR Green PCR kit (TaKaRa, Kofu, Japan) and a RT-PCR system (Lightcycler 2.0, Roche, Switzerland). The primer sequences for amplifying the genes encoding IL-1, IL-6, IL-10, TNF-α, and IFN-β have been previously described [34]. Relative RNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Enzyme-linked immunosorbent assay (ELISA)

Supernatants were collected from THP-1 cell-derived macrophages treated with B-HA and LPS. The levels of cytokines TNF-α, IL-1, IL-6, IL-10, and IFN-β were measured using the ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions.

Western blot

THP-1 cell-derived macrophages treated with B-HA and LPS were harvested and lysed in the RIPA lysis buffer to determine the phosphorylation levels of IKKα/β, IκBα, p65, JNK1/2, ERK1/2, p38, and IRF-3. The total protein was separated using 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The protein was then transferred onto a membrane and incubated with primary and secondary antibodies. The analysis of electrochemiluminescence was performed in accordance with the manufacturer's instructions (Tanon 5200, Shanghai, China).

In vivo study

Eight-week old C57BL/6 mice were maintained in a HEPA-filtered environment at a standard condition. Animal experiments were approved by the Ethical Committee of Jinling Hospital. A total of 24 animals were randomly divided into four groups ($n = 6$), namely, control, LPS, LPS + B-HA, and B-HA groups. The LPS group was treated with 1 mg/kg LPS intraperitoneally. The B-HA group was treated with B-HA intraperitoneally. The LPS + B-HA group was treated with 1 mg/kg LPS by intraperitoneal injection 2 h after B-HA injection. Each animal was observed for clinical signs. The blood sample was collected from each animal 90 min after LPS injection. The serum was prepared from the collected blood sample. The serum level of inflammatory cytokines TNF-α, IL-1, IL-6, and IL-10 were evaluated using ELISA.

Statistical analysis

Results were presented as mean ± standard deviation, and all assays were repeated thrice. The statistical significance of the data was analyzed using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA), and $P < 0.05$ was considered statistically significant.

Results

Effect of different B-HA concentrations on LPS-induced inflammatory response

THP-1 cell-derived macrophages were treated with different concentrations of B-HA (0.5%, 1%, and 2%) for 2 h and LPS (100 ng/mL) for 4 h. The mRNA expression levels of TNF-α, IL-1, IL-6, IFN-β, and IL-10 were detected using qRT-PCR to determine the effective concentration of B-HA for the inhibition of LPS-induced inflammatory response. As shown in Fig. 1, the B-HA treatment reduced the expression of TNF-α, IL-1, IL-6, and IFN-β in a concentration-dependent manner compared with the LPS treatment alone. The LPS-induced inflammatory response was significantly inhibited by 2% B-HA ($P < 0.05$). Moreover, the B-HA treatment significantly increased the IL-10 expression in a concentration-dependent manner compared with the LPS treatment alone ($P < 0.01$). Therefore, 2% B-HA was considered as the most effective concentration for the inhibition of LPS-induced inflammatory response and selected for subsequent experiments.

Effect of B-HA on the mRNA expression of inflammatory cytokines in LPS-induced inflammatory response

THP-1 cell-derived macrophages were treated with LPS (100 ng/mL), B-HA (2%), or LPS + B-HA. The mRNA expression levels of inflammatory cytokines TNF-α, IL-1, IL-6, IL-10, and IFN-β in B-HA- and LPS-treated cells were detected 2, 4, and 8 h after LPS treatment. The mRNA expression levels of TNF-α, IL-1, IL-6, and IFN-β in LPS-treated cells increased with a peak at 4 h after LPS treatment. The pretreatment with 2% B-HA reduced mRNA expression levels of TNF-α, IL-1, IL-6, and IFN-β at 2 and 4 h compared with the LPS treatment alone. However, the B-HA + LPS treated group was observed with increased IL-10 mRNA expression compared with the LPS treatment group (Fig. 2). Results indicated that the B-HA suppressed proinflammatory cytokines and increased anti-inflammatory cytokines in LPS-induced inflammatory response.

Effect of B-HA on the secretion of inflammatory cytokines in LPS-induced inflammatory response

The levels of inflammatory cytokines in the supernatants of THP-1 cell-derived macrophages treated with LPS (100 ng/mL), B-HA (2%), or LPS + B-HA were detected using ELISA. As shown in Fig. 3, the LPS treatment remarkably

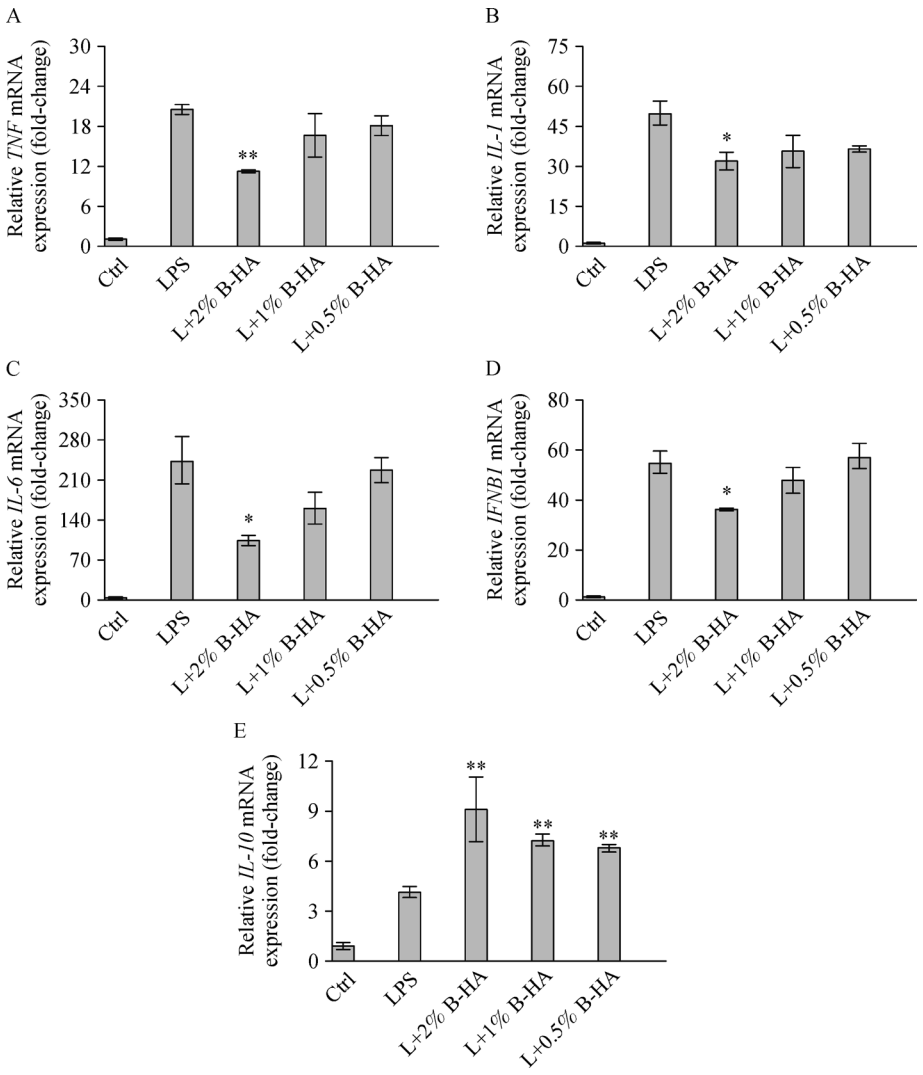

Fig. 1 Effect of B-HA concentration on LPS-induced inflammatory response. THP-1 cell-derived macrophages were pretreated with 0.5%, 1%, or 2% B-HA 2 h before LPS (100 ng/mL) treatment. The mRNA levels of *TNF* (A), *IL-1* (B), *IL-6* (C), *IFNB1* (D), and *IL-10* (E) were measured using qRT-PCR 4 h after LPS treatment. L, LPS; B-HA, bioactive hyaluronan. $*P < 0.05$, $**P < 0.01$ compared with LPS treatment alone.

increased the levels of TNF-α, IL-1, IL-6, IL10, and IFN-β compared with the control. The B-HA treatment considerably reduced the levels of TNF-α, IL-1, IL-6, and IFN-β compared with the LPS treatment alone. However, the B-HA treatment largely increased the IL-10 level compared with the LPS treatment alone. These results suggested that B-HA affected the secretion of inflammatory cytokines in LPS-induced inflammatory cells.

Downregulation of the TLR4 signaling pathway in LPS-induced inflammatory response by B-HA

The effect of B-HA on the TLR4 signaling pathway was investigated by measuring the phosphorylation levels of proteins in the NF-κB, MAPK, and IRF-3 signaling pathways. As shown in Figs. 4–6, the LPS treatment increased the phosphorylation levels of p65, IκBα, IKKα/β, ERK1/2, JNK1/2, p38, and IRF-3 in THP-1 cell-derived macrophages. However, the phosphorylation levels of these proteins were remarkably reduced when the cells were pretreated with 2% B-HA, indicating that B-HA could inhibit LPS-induced inflammatory response by downregulating the TLR4 signaling pathway.

Effect of B-HA on the production of inflammatory cytokines *in vivo*

The effect of B-HA on the production of inflammatory cytokines *in vivo* was assessed in a mouse model of LPS-induced inflammation. As shown in Fig. 7, the production of inflammatory cytokines TNF-α, IL-1, IL-6, and IL-10 was increased by LPS treatment. However, the B-HA

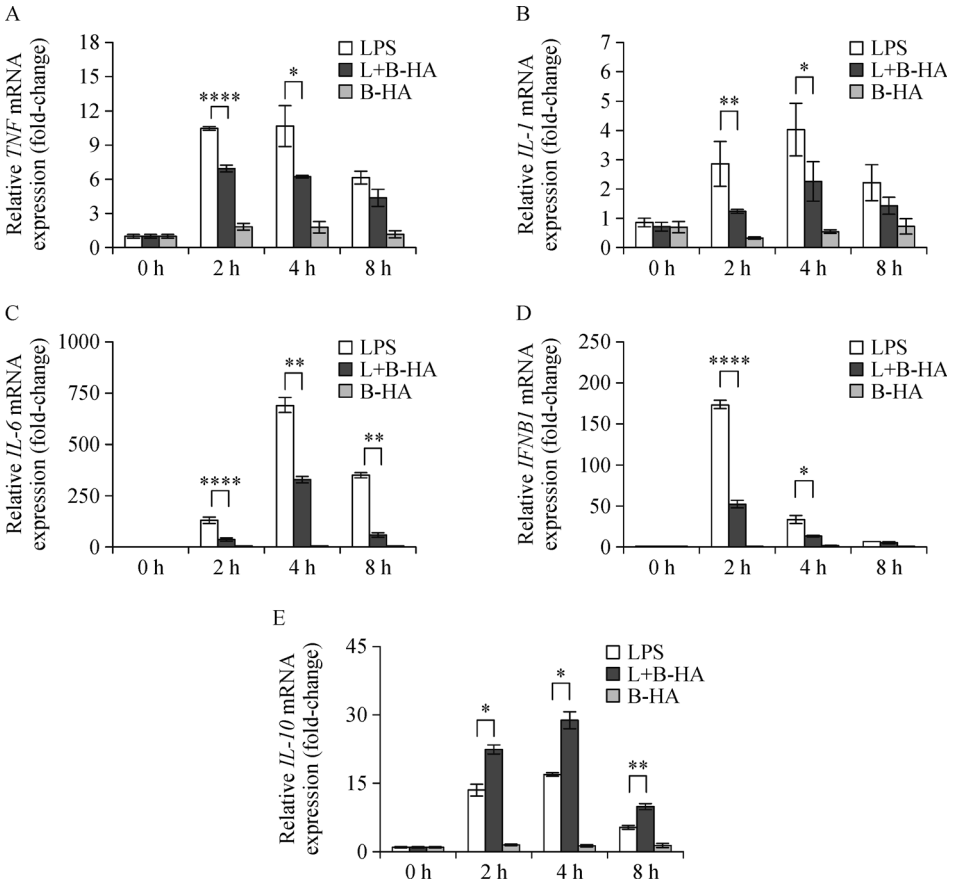

Fig. 2 Effect of B-HA on cytokine transcription in LPS-treated THP-1 cells. The B-HA pretreatment suppresses the transcription of *TNF* (A), *IL-1* (B), *IL-6* (C), and *IFNB1* (D) and enhances the transcription of *IL-10* (E) in LPS-treated THP-1 cells. Cells were pretreated with HA for 2 h and treated with 100 ng/mL LPS for 2, 4, or 8 h. Transcript levels were determined using qRT-PCR. L, LPS; B-HA, bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.001$ compared with the LPS group.

treatment considerably reduced the levels of proinflammatory cytokines TNF-α, IL-1, and IL-6 compared with the LPS treatment alone. The B-HA treatment largely increased the anti-inflammatory cytokine IL-10 compared with the LPS treatment alone.

Discussion

The endogenous or the exogenous HA can be degraded by hyaluronidases *in vivo,* and the mixture of degraded HA fragments may inhibit some inflammatory responses in cells and mice [12,35]. In this study, a small HA oligosaccharide (B-HA) shows an inhibitory effect on LPS-induced inflammatory response, suggesting a potential for the prevention and the treatment for LPS-induced sepsis or sepsis shock. Macrophages play an important role in tissue injury and infection and can produce inflammatory cytokines, such as TNF-α and IL-6, to regulate the inflammatory response [36]. However, the excessive production of inflammatory cytokines can result in multiple organ failure and even death in patients [37,38]. Therefore, the inhibition of excessive cytokine may be a therapeutic strategy for regulating the inflammatory response. Our results demonstrate that B-HA reduces the production of TNF-α, IL-1, IL-6, and IFN-β in human macrophages by LPS-induced inflammatory response in a concentration-dependent manner. Moreover, B-HA increases the production of the anti-inflammatory cytokine IL-10. The *in vivo* study also confirms the effect of B-HA on the production of inflammatory cytokines in an LPS-induced inflammation mouse model.

TLR4 recognizes pathogen-associated molecular patterns (PAMPs) or endogenously sourced DAMPs, and the TLR4 signaling pathway is the basis of innate and adaptive immune responses [39]. The innate recognition of PAMPs by TLRs triggers an inflammatory response characterized by the recruitment of immune cells and the production of inflammatory cytokines. The ROS-mediated oxidative stress induces TLR10 gene/protein expression in monocytes and PBMCs, which are associated with the increased expression of proinflammatory cytokines/chemokines [40]. The activation of the NF-κB, MAPK, and IRF-3

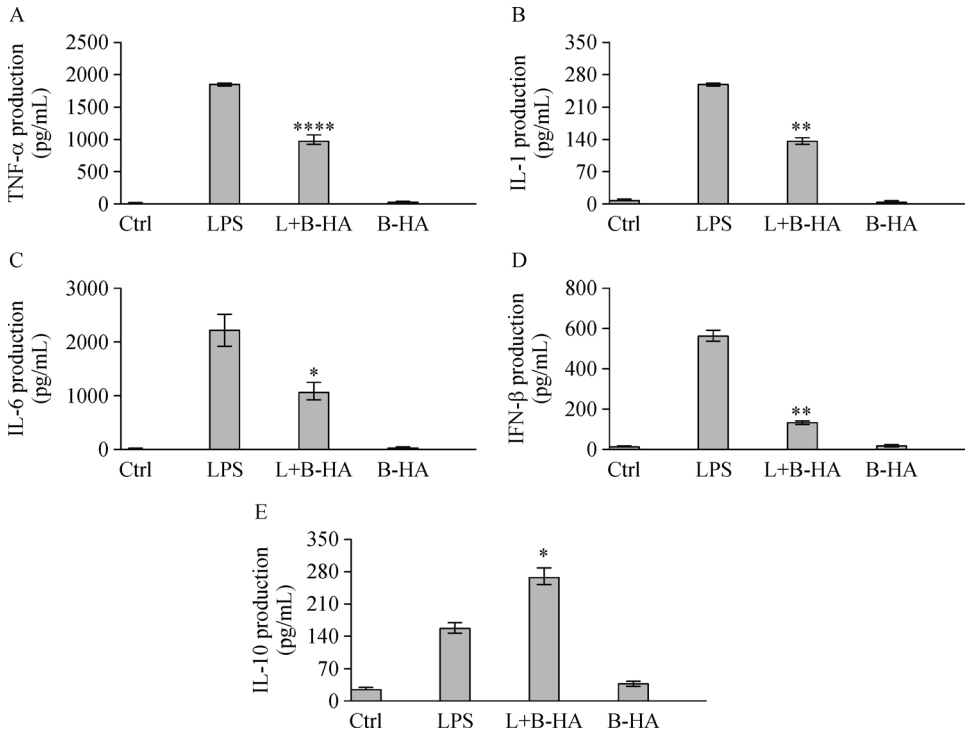

Fig. 3 Effect of B-HA on the secretion of inflammatory cytokines in LPS-induced inflammatory response. The levels of TNF-α (A), IL-1 (B), IL-6 (C), IFN-β (D), and IL-10 (E) in the supernatants of the treated cells were measured using ELISA. L, LPS; B-HA, 2% bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$, **** $P < 0.000$ compared with LPS group.

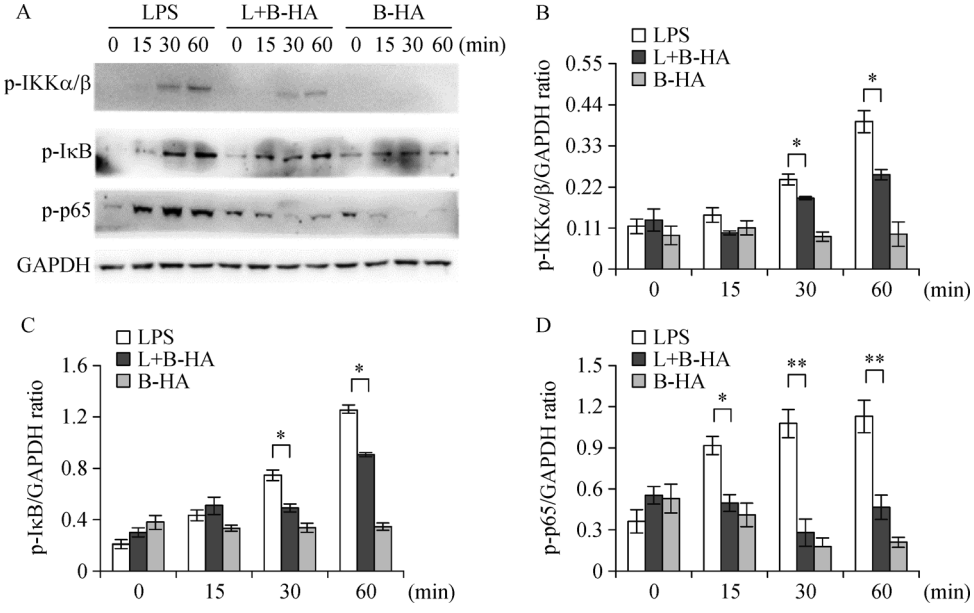

Fig. 4 Inhibition of NF-κB activation by B-HA in LPS-induced inflammatory response. The phosphorylation levels of IKK, IκB, and p65 proteins were detected in THP-1 cell-derived macrophages treated with B-HA and LPS by Western blot. L, LPS; B-HA, bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$ compared with the LPS group.

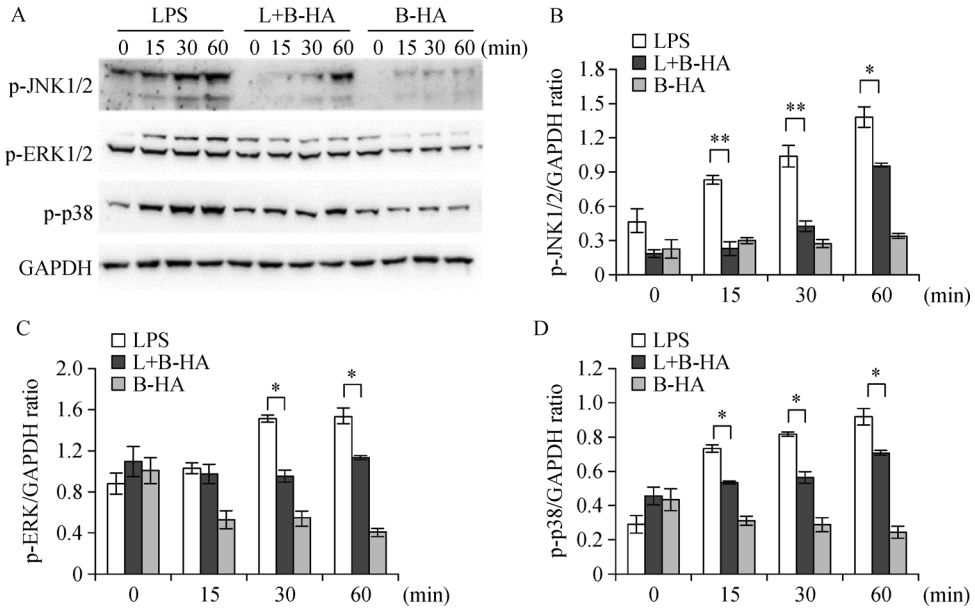

Fig. 5 Inhibition of MAPK activation by B-HA in LPS-induced inflammatory response. The phosphorylation levels of p-JNK1/2, p-ERK1/2, and p38 proteins were detected in THP-1 cell-derived macrophages treated with B-HA and LPS by Western blot. L, LPS; B-HA, bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$ compared with the LPS group.

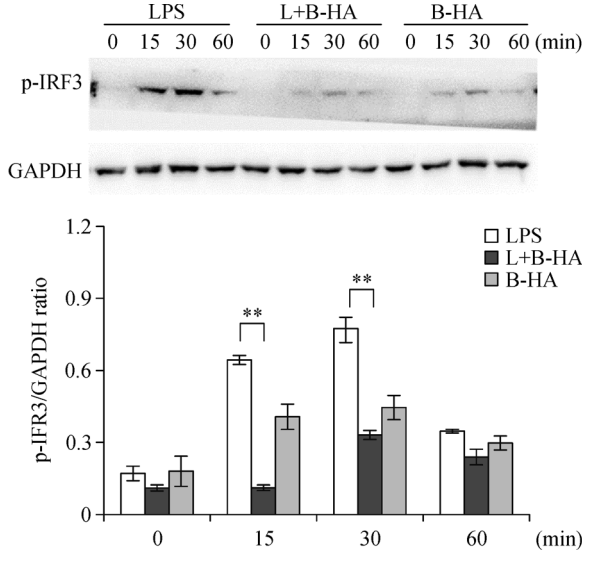

Fig. 6 Inhibition of IRF-3 activation by B-HA in LPS-induced inflammatory response. The phosphorylation level of the IRF-3 protein was detected in THP-1 cell-derived macrophages treated with B-HA and LPS by Western blot. L, LPS; B-HA, bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$ compared with the LPS group.

pathways in the TLR4 signaling in LPS-induced inflammatory responses is an important signal transduction in regulating inflammatory cytokines [41,42]. Obesity-associated chronic low-grade inflammation is also called metabolic inflammation [43]. Research shows that palmitate alone or in combination with TNF-α elevates CCL4 and MMP-9 expression via the TLR4/MyD88-dependent signaling pathway in human monocytic cells [44–46]. IL-8 and CCL2 production are enhanced by palmitate and TNF-α cooperatively, and this phenomenon is completely or partly dependent on the TRIF/IRF3 pathway [37,47]. Several intracellular proteins are considered as negative regulators of TLR signaling [48]. In the present study, we have demonstrated that B-HA inhibits the phosphorylation of p65, IKKα/β, IκBα, JNK1/2, ERK1/2, p38, and IRF-3 in human macrophages, thereby supporting our hypothesis that B-HA inhibits inflammation through the NF-κB, MAPK, and IRF-3 pathways [49].

LPS has a higher affinity for the TLR4 receptor than HA [50]. In Gram-negative bacteria, the lipid A domain of LPS, a phosphorylated glucosamine disaccharide with multiple fatty acids, is responsible for the activity of LPS. We hypothesize that B-HA is an antagonist of LPS because HA has repetitive glucosamine disaccharides similar to LPS, and this finding may explain the inhibitory effects of B-HA on LPS-stimulated macrophages.

IL-10 is a powerful anti-inflammatory factor produced by many immunocytes, such as macrophages [51]. IL-10 plays a vital role in regulating inflammation and the level of activation of adaptive immune responses [52]. In the present study, we have found that B-HA increases the IL-10 expression and the production in LPS-induced inflammatory cells and further demonstrates the anti-

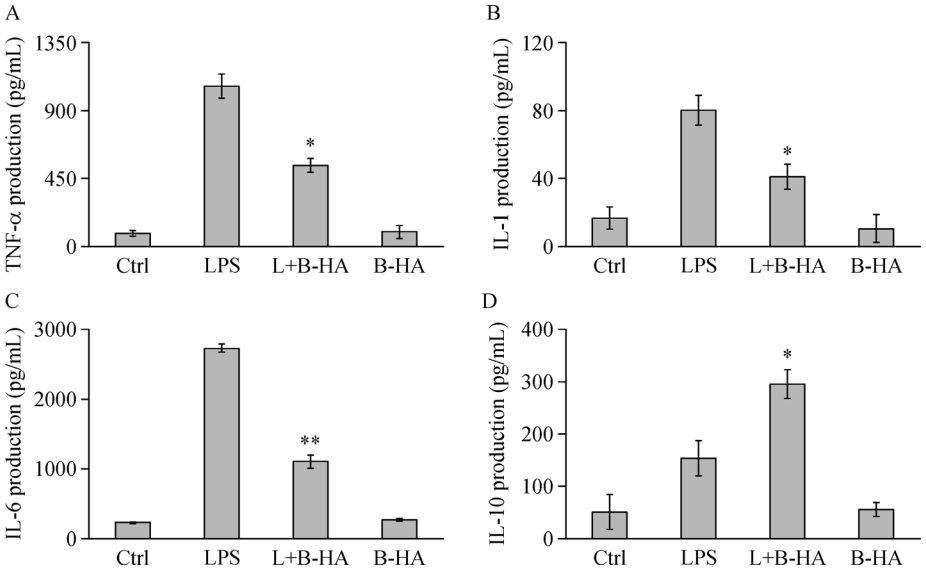

Fig. 7 Effect of B-HA on the production of inflammatory cytokines *in vivo.* The effect of B-HA on the production of inflammatory cytokines *in vivo* was assessed in a mouse model of LPS-induced inflammation. Animals were treated with LPS, B-HA, or LPS + B-HA. The serum levels of TNF-α (A), IL-1 (B), IL-6 (C), and IL-10 (D) were measured using ELISA. L, LPS; B-HA, bioactive hyaluronan. * $P < 0.05$, ** $P < 0.01$ compared with the LPS group.

inflammatory efficacy of B-HA. However, the IL-10 production is increased in the macrophages treated with LPS alone. In inflammatory response, anti-inflammatory factors are increased to avoid excessive inflammation.

In conclusion, B-HA demonstrates the inhibitory effect on LPS-induced inflammatory response by reducing proinflammatory cytokines and increasing anti-inflammatory cytokine. The TLR4 signaling pathway is involved in the anti-inflammatory effects of B-HA. A new therapeutic strategy for sepsis and validation studies are required using other types of immune cells and animal models to provide further evidence.

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Compliance with ethics guidelines

Na You, Sasa Chu, Binggang Cai, Youfang Gao, Mizhou Hui, Jin Zhu, and Maorong Wang declare that they have no conflict of interest. All institutional and national guidelines for the care and use of laboratory animals were followed.

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