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Kallikrein-Binding Protein Inhibits LPS-Induced TNF-α by Upregulating SOCS3 Expression

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ABSTRACT

Kallikrein-binding protein (KBP) was previously identified as a serpin family member with specific inhibitory effect on tissue kallikrein and angiogenesis, while there is little knowledge about the effects on inflammation. The aim of this study is to investigate whether KBP can suppress LPS-induced inflammatory process. Our results showed that both recombinant KBP and KBP overexpression inhibited LPS-stimulated TNF-α transcription and translation in macrophage cell line RAW264.7 and primary macrophages. Furthermore, KBP treatment protected mice from endotoxin shock and repressed serum TNF-α production, increasing survival rate of mice from 10% to 50% when compared to LPS alone. Moreover, qPCR and Western blot analysis demonstrated that both suppressor of cytokine signaling 3 (SOCS3) transcription and translation were induced by KBP treatment in the present of LPS. RNA interference assay and luciferase assay showed that SOCS3 was responsible for the down-regulation of TNF-α by KBP, rather than NF-κB subunit p65 and β-catenin. Therefore, we demonstrated that KBP suppressed LPS-induced TNF-α production via upregulating SOCS3 expression. These results present the protective effects of KBP on LPS-induced inflammation and provide novel information for the anti-inflammation mechanism. J. Cell. Biochem. 114: 1020–1028, 2013.

KEY WORDS: SERPINA3K; ANTI-INFLAMMATION; ENDOTOXIN; TNF-α; MACROPHAGE

Zhiyu Dai and Lei Lu contributed equally to this study.

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Lipopolysaccharide (LPS), known as endotoxin, is the principal component of gram-negative bacterial outer membrane. It is a highly immunogenic antigen with the ability to induce host inflammatory response and tissue damage. Over-reacting to LPS may lead to septic shock. One of the target cells of LPS is macrophage, LPS firstly binds to LPS-binding protein (LBP) in plasma, and then it is delivered to CD14 in cell membrane of macrophage and forms complex with its receptor Toll-like receptor 4 (TLR4). Activation of LPS-TLR4 axis triggers NF-κB pathways and various MAPK pathways, which lead to releasing a variety of pro-inflammatory factors, such as tumor necrosis factor-α (TNF-α) [Guha and Mackman, 2001]. Investigation on LPS responsible inflammatory cytokines may gain new insight into the drug discovery [Swantek et al., 1999].

Suppressor of cytokine signaling (SOCS) is a negative regulator for various cytokines. There are eight members of SOCS family protein, all of them contain SH2 domain and SOCS box. The best-characterized members are SOCS1 and SOCS3 [Alexander and Hilton, 2004]. Both SOCS1 and SOCS3 have the kinase inhibitory region (KIR) in N-terminal, which is essential for JAK tyrosine kinase inhibition. In addition, SOCS box in the C-terminal is important for their function as E3 ubiquitin ligases [Yoshimura et al., 2007]. Studies showed that both SOCS1 and SOCS3 participated in negative regulation of LPS-TLR4 response. SOCS1-deficient mice were hypersensitive to LPS, and macrophages isolated from SOCS1-deficient mice produced elevated level of TNF-α [Nakagawa et al., 2002]. Moreover, studies also showed that SOCS1 facilitated p65 undergoing ubiquitin-mediated degradation via directly binding to p65 [Yoshimura et al., 2007]. Overexpression of SOCS3 in macrophage was showed to suppress LPS-induced TNF-α release [Qasimi et al., 2006]. Others reported that SOCS3 played an important role in the regulation of IL-10 signal triggered by TLR activation, and SOCS3 was strongly induced by IL-10 in the present of LPS [Qasimi et al., 2006]. Recent studies also suggested that SOCS3 inhibited TLR-stimulated response by targeting TNF-receptor-associated factor 6 (TRAF6) [Frobose et al., 2006].

Kallikrein-binding protein (KBP), also named SERPINA3K, is firstly identified as a member of the serine proteinase inhibitor (serpin) family [Chai et al., 1991]. KBP is a plasma protein and mainly synthesized and secreted by liver, while it is also expressed at lower level in kidney, pancreas, and retina. Previous studies showed that KBP specifically formed a covalent complex with tissue kallikrein, and inhibited proteolytic activities of kallikrein, therefore modulated the kinin formation in vivo [Chao et al., 1990]. Importantly, KBP has other functions independent of binding to kallikrein. It has been showed that KBP-transgenic mice were less sensitive to LPS-induced endotoxin shock, however the molecular mechanism is unclear [Chen et al., 1997]. Our previous studies have shown that KBP inhibited angiogenesis and vascular permeability via down-regulating VEGF [Gao et al., 2003]. Our studies also revealed that KBP exhibited anti-angiogenic activity in retina and solid tumor, such as gastric carcinoma and hepatocellular carcinoma [Lu et al., 2007; Zhu et al., 2007]. Recently, KBP has been showed to have potent anti-inflammatory and anti-oxidant activities in retina and cornea [Zhang and Ma, 2008; Zhang et al., 2009; Liu et al., 2011]. Furthermore, the anti-angiogenic and anti-inflammatory effect of KBP in retina has been demonstrated via blocking the Wnt signaling [Zhang et al., 2010].

In the present study, we have investigated the protective effects and the underlying mechanism of KBP on mice endotoxin shock. Our study provides novel information for the anti-inflammation mechanism of KBP.

MATERIALS AND METHODS

ETHICS STATEMENT

Care, use, and treatment of all animals in the present study were in strict agreement with the institutionally approved protocol according to the United States Public Health Service (USPHS) Guide for the care and use of laboratory animals, as well as the guidelines set forth in the Care and Use of Laboratory Animals by the Sun Yat-sen University. The animal use protocol has been reviewed and approved by the institutional animal care and use committee of Sun Yat-sen University (IACUC SYSU, no. 20061211005).

EXPERIMENTAL ANIMAL

Male BALB/c mice (18–22 g) were obtained from Center of Experimental Animal, Sun Yat-sen University (Guangzhou, China). Mice were allowed to acclimate to local conditions for at least 1 week and maintained under a 12 h dark, 12 h light cycle with food and water ad libitum.

CELL CULTURE

For peritoneal exudate cell isolation, mice were injected with 3% thioglycollate 5 days prior to euthanasia with chloralic hydras, and the peritoneal exudate cells were lavaged and collected by 5 ml of cold Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) from the peritoneal cavity of mice, then cells were washed twice and suspended in culture medium with 10% (v/v) fetal bovine serum (FBS; Gibco BRL), 100 U/ml penicillin, and 100 U/ml streptomycin (Gibco BRL). The peritoneal exudate cells suspension was dispensed onto 24-well culture plates at 1×10^6 cells cells per well and incubated at 37°C in a humidified incubator at 5% CO_2. The adherent macrophages were determined by morphology and non-specific esterase staining.

RAW264.7 murine macrophages were purchased from the Cell Bank of China Science Academy (Shanghai, China) and maintained in DMEM supplemented with 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin and incubated at 37°C in a humidified incubator at 5% CO_2. The human macrophage-like cell line THP-1 was cultured in RPMI 1640 with 25 mM HEPES buffer, 10% (v/v) FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2. To induce the conversion of THP-1 cells into mature macrophages cells, THP-1 cells were induced by 50 nM PMA for 48 h.

EXPRESSION AND PURIFICATION OF RECOMBINANT KBP AND PLASMID CONSTRUCTION

The expression and purification of KBP were carried out as described previously [Gao et al., 2003]. In brief, the pET28a(+)/KBP construct was expressed by BL21 (DE3) *Escherichia coli* strain (Novagen, Madison, WI) induced by 1 mmol/L isopropyl-β-D-triogalactoside

(IPTG) for 12 h. The cells were harvested by centrifugation at 10,000*g* for 30 min and completely resuspended with cold 10 mmol/L imidazole diluted by binding buffer (6.9 g/L NaH_2PO4, 18 g/L NaCl, pH 8.0), then lysed by sonication. The supernatant fractions were collected by centrifugation at 15,000*g* for 30 min, and filtered using 0.45 μm filter (PALL). The supernatant was loaded onto a column filled with Ni-NTA binding resin (Novagen), the flow was collected and reloaded for two more times. Bound his-tagged KBP was eluted by elution buffer (40 mmol/L imidazole diluted by binding buffer) after washing by 300 ml washing buffer (20 mmol/L imidazole diluted by binding buffer). Then the eluted KBP were dialyzed for 12 h twice using10% glycerol diluted by PBS. Finally, the endotoxin in recombinant proteins is removed to be less than 0.1 EU/ml by commercial ToxinEraser Endotoxin Removal kit (Genescript, Piscataway, NJ) according to the manual. Recombinant KBP was confirmed by SDS–PAGE and Western blot analysis.

Plasmid expressing KBP was constructed by cloning the full-length KBP cDNA into the pTriEx 1.1 vector (a gift from professor Jianxing Ma, University of Oklahoma). In brief, the cDNA of KBP was amplified from the total RNA of rat liver by reverse transcription-PCR. The PCR product of KBP was cloned into pTriEx 1.1 vector using *BamH* I and *Xho* I restriction sites and subjected to sequencing analysis (Invitrogen, Carlsbad, CA).

ENDOTOXIN SHOCK MODEL

LPS from *E. coli* O111:B4 strain was purchased from Sigma (L2630, St. Louis, MO), and dissolved by PBS at 1 mg/ml. For in vivo survival experiment during endotoxemia, mice were injected intraperitoneally with KBP (20 mg/kg body weight per time) 2 h prior to LPS administration (10 mg/kg body weight, defined as 0 h), and the mortality rate was accessed at 72 h. For testing the counteract effect of KBP on TNF-α in vivo, mice were administrated with KBP (20 mg/kg body weight per time) 2 h before and the same time point as LPS administration (10 mg/kg body weight, defined as 0 h), then the mice serum was collected at 2, 4, and 8 h from retro orbital plexus using chloralic hydras anesthesia, then centrifuged 5 min at 2,000 × rpm, the supernatant was collected and subjected to determine TNF-α using commercial TNF-α ELISA kit (eBioscience, San Diego, CA).

MEASUREMENT OF TNF-α SECRETION

The primary murine macrophages or RAW264.7 murine macrophages seeded in 24-well culture plates were cultured in the growth medium until 90% confluence. The cells were washed thrice with PBS and the growth medium was replaced by DMEM. KBP (28.8 μg/ml) was added to the medium 2 h prior to LPS (1 μg/ml) treatment and incubated with the cells for the time indicated, PBS was added as control. The conditioned medium was harvested and TNF-α concentration in the supernatant was determined using a mouse TNF-α ELISA kit (eBioscience).

WESTERN BLOT ANALYSIS AND CELLULAR FRACTIONATION

RAW264.7 cells were seeded in 60-mm plates and cultured in the growth medium until 80% confluence. The culture medium was replaced with DMEM supplemented with KBP at concentrations 28.8 μg/ml or PBS for 2 h, then treated with LPS (1 μg/ml). After incubation for indicated time, the cells were harvested and lysed for total protein extraction. Protein concentration was determined using Bio-Rad *DC* protein assay kit (Bio-Rad Laboratories, Hercules, CA) according to manufacturer's protocol. Aliquots of equal amounts of protein (80 μg) from the cell lysate were subjected to Western blot analysis for the SOCS3 (rabbit polyclonal antibody, ProteinTech Group, Chicago, IL) or TNF-α (mouse monoclonal antibody, Santa Cruz, CA), or KBP rabbit polyclonal antibody (Genscript, Nanjing, China) as described previously. The same membrane was stripped and reblotted with an antibody specific to β-actin or GADPH (mouse monoclonal antibody, Sigma).

For cellular fractionation, the cytoplasm and nuclear fraction were isolated using NE-PER Nuclear and Cytoplasmic Extraction Kit (Thermo Fisher, IL), and the cytoplasm and nuclear protein concentration was measured as described above. Equal amounts of proteins were blotted with a polyclonal antibody for p65 (Cell Signaling, Beverly, MA) and then reblotted with a mouse antibody for Histone H3 (Abcam, Cambridge, MA) and GAPDH (mouse monoclonal antibody, Sigma) as an internal control.

QUANTITATIVE REAL-TIME PCR

Total RNA was extracted from RAW264.7 macrophage cells according to the manufacturer's instructions for Trizol reagent (Invitrogen). Five hundred nanograms total RNA was used for reverse transcription using PrimeScript® RT reagent Kit Perfect Real Time kit (Takara Bio, Inc., Shiga, Japan) and then subjected to quantitative real-time PCR analysis (qPCR) using SYBR® Premix Ex Taq™ (Perfect Real Time) (Takara Bio, Inc.) and a Roche's capillary-based LightCycler® 2.0 Systems (Roche Diagnostics Corporation, Indianapolis, IN). Mouse cDNA were amplified with specific primers for TNF-α (sense primer: 5′-agcccccagtctgtatcctt-3′, antisense primer: 5-′ctccctttgcagaactcagg-3′) and SOCS1 (sense primer: 5′-gttgtggagggtgagatg-3′, antisense primer: 5′-atggagaggtaggagtgg-3′), SOCS3 (sense primer: 5′-cctttgacaagcggactctc-3′, antisense primer: 5′-gccagcataaaaaccccttca-3′), IL-10 (sense primer: 5′-tttgaattccctgggtgagaa-3′, antisense primer: 5′-acaggggagaaatcgatgaca-3′) and β-actin (sense primer: 5′-actcttccagccttccttc-3′, antisense primer: 5′-atctccttctgcatcctgtc-3′) (Invitrogen). Target mRNA was determined using the comparative cycle threshold method of relative quantitation. The calibrator sample was isolated from untreated RAW264.7 cells with β-actin used as an internal control. All samples were assayed in triplicate.

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RAW264.7 cells were plated on culture slides, and incubated with PBS or KBP (28.8 μg/ml) for 2 h, then stimulated with LPS (1 μg/ml) for 8 h. The cells were washed, and fixed in 4% paraformaldehyde. Then, cells were blocked with normal non-immunone goat serum at 37°C for 1 h. After washing three times, they were incubated with rabbit SOCS3 polyclonal antibody or p65 antibody at 37°C for 2 h, then were incubated with FITC conjugated goat anti-rabbit IgG (Dako, Glostrup, Denmark) at 37°C for 1 h after three times washing. Finally, The cell nucleus was stained with 4,6-di-amino-2-phenylindole (DAPI) (Sigma). Cells were visualized under a confocal microscope (Carl Zeiss, Oberkochen, Germany). In negative-control staining, the primary antibodies were omitted.

PLASMID TRANSFECTION AND SIRNA TRANSFECTION

KBP overexpression plasmids were transfected into RAW264.7 cells using Lipofectamine 2000 transfection regent (Invitrogen) according to the manual. Thirty-six hours post-transfection, cells were incubated with LPS (1 μg/ml) for another 8 h. For knockdown experiment, siRNA oligonucleotides matching selected region of mouse SOCS3 were obtained from Ribobio Company (Shanghai, China). RAW264.7 cells were transfected with siRNA oligonucleotides at a final concentration of 50 nmol/L using Hiperfect reagent (Qiagen, Hilden, Germany). Thirty-six hours later, cells were harvested for analyzing knock-down efficiency, or treated with LPS (1 μg/ml) or KBP (28.8 μg/ml) for the time indicated.

LUCIFERASE ASSAY

RAW264.7 Cells were seeded in 24-well plates 24 h prior to transfection, then transiently co-transfected with pNFκB-Luc (This vector contains multiple copies of κB element fused to the HSV-TK promoter, a gift from Professor Jun Li from Sun Yat-sen University) or TOPFLASH (β-catenin/TCF reporter plasmid, a gift from Professor Jian-xing Ma from University of Oklahoma Health Science Center) and pRL-TK *renilla* luciferase plasmids by using Attractene Transfection Reagent (Qiagen). A negative-control vector pGL3-basic was added at identical DNA to subtract the background fluorescence. Forty-eight hours after transfection, cells were pre-stimulated with KBP (28.8 μg/ml) or PBS for 2 h, then stimulated with LPS (1 μg/ml) for another 4 h. Cells were then lysed using 100 μl of passive lysis buffer (Promega, Madison, WI), and the firefly luciferase (NFκB-Luc or TOPFLASH) activity and *renilla* luciferase activity were measured by using the Dual-Luciferase Reporter system (Promega) according to user's manual in Luminometer (Berthold Centro LB 960, Germany), and normalized by *renilla* luciferase activity.

STATISTICAL ANALYSIS

All data were expressed as mean ± standard deviation. SPSS 13.0 software was used for statistical evaluation using one-way ANOVA firstly for comparison of more than two groups, and multiple comparisons amongst these groups was performed using LSD-t test. A *P*-value less than 0.05 were significantly considered.

RESULTS

KBP SUPPRESSES LPS-INDUCED TNF-α PRODUCTION

KBP was expressed and purified by affinity chromatography, and further confirmed by Coomassie-stained SDS–PAGE and Western blot using specific anti-His tag antibody (Fig. S1). We next demonstrated that the inhibition effect of KBP on pro-inflammatory factor TNF-α in LPS-stimulated murine macrophages cell lines RAW264.7. As shown in Figure 1A, LPS (1 μg/ml) significantly induced TNF-α secretion by ELISA analysis ($^*P < 0.05$) at 4- and 8-h point, and the effect was attenuated by KBP (28.8 μg/ml) pre-treatment ($^*P < 0.05$). In order to evaluate the effect of KBP on LPS-induced TNF-α expression, murine primary peritoneal macrophages were isolated from BALB/c mice, and pre-exposed to media containing PBS or KBP, followed by stimulating with LPS. TNF-α concentration in the supernatant was significantly elevated by LPS treatment at 2-, 4-, and 8-h point, compared with PBS treated control ($^*P < 0.05$), while KBP remarkably repressed the LPS-stimulated TNF-α secretion, compared with LPS-only treatment ($^*P < 0.05$, Fig. 1B). In addition, similar effect was observed in another human macrophage-like cell line THP-1 when pretreated with KBP (Fig. S2). To further validate the observation by recombinant KBP, we directly overexpressed KBP in RAW264.7 cells prior to LPS treatment. Western blot analysis showed that KBP was successfully expressed in cells as shown in Figure 1C, and KBP overexpression significantly attenuated the TNF-α level stimulated by LPS.

Furthermore, qPCR analysis was performed to verify whether KBP suppressed TNF-α secretion is at transcriptional level. As shown in Figure 1D, LPS induced TNF-α mRNA expression by more than 220% at 4-h point, and KBP treatment normalized TNF-α mRNA to nearly PBS treated level in the present of LPS, while KBP alone had no effect on TNF-α transcript compared to PBS group. Overall, these results suggested that KBP inhibits LPS-induced TNF-α transcription and expression in macrophages, indicating that KBP may contribute to inflammatory process.

KBP IMPROVES SURVIVAL OF MICE BY ENDOTOXIN INJECTION

To test the anti-inflammatory effect of KBP in vivo during endotoxin injection, mice were pre-administrated with KBP, then serum TNF-α level at 2-, 4-, and 8-h point after LPS injection were measured. LPS injected mice exhibited dramatic high level of serum TNF-α, compared with PBS treated group ($^*P < 0.05$, Fig. 2A), while KBP pretreatment reduced the level of serum TNF-α significantly ($^*P < 0.05$, Fig. 2A).

Moreover, we examined whether KBP modulated the survival of endotoxin mice. Notably, KBP pretreatment protected mice from endotoxin shock by injecting a lethal dose of LPS. As shown in Figure 2B, LPS injection significantly induced mortality in mice, only with 10% survival by 72 h ($^*P < 0.05$). In contrast, administration of KBP increased survival from 10% to 50% ($^*P < 0.05$).

KBP INDUCES SOCS3 EXPRESSION IN MACROPHAGES DURING LPS TREATMENT

It has been reported that either SOCS1 or SOCS3 activation was responsible for suppression of LPS-induced inflammatory cytokine transcripts [Kinjyo et al., 2002; Nakagawa et al., 2002; Alexander and Hilton, 2004; Jo et al., 2005; Fujimoto and Naka, 2010]. SOCS1 and SOCS3 mRNA expression in macrophages were evaluated by qPCR analysis. Interestingly, SOCS1 mRNA was unchanged both in LPS or LPS + KBP treatment, SOCS3 mRNA was mildly induced in response to LPS treatment, while KBP significantly increased SOCS3 mRNA by about fourfold in 6 h ($^*P < 0.05$, Fig. 3A), and KBP alone also enhanced SOCS3 transcripts by about threefold ($^*P < 0.05$, Fig. S3). Meanwhile, KBP treatment also upregulated SOCS3 protein expression in the presence of LPS at 8 h by Western blot analysis in both RAW264.7 cells (Fig. 3B) and THP-1 cells (Fig. S2). To obtain further insight into the function of SOCS3, we determined its subcellular localization by confocal microscopy analysis. As shown in Figure 3C, SOCS3 displayed a pattern in the cytoplasm in unstimulated state (upper panel), while SOCS3 showed a little in the nucleus by LPS treatment, and the cells turned to be much more outstretched than control (middle panel). Apparently, cells morphol-

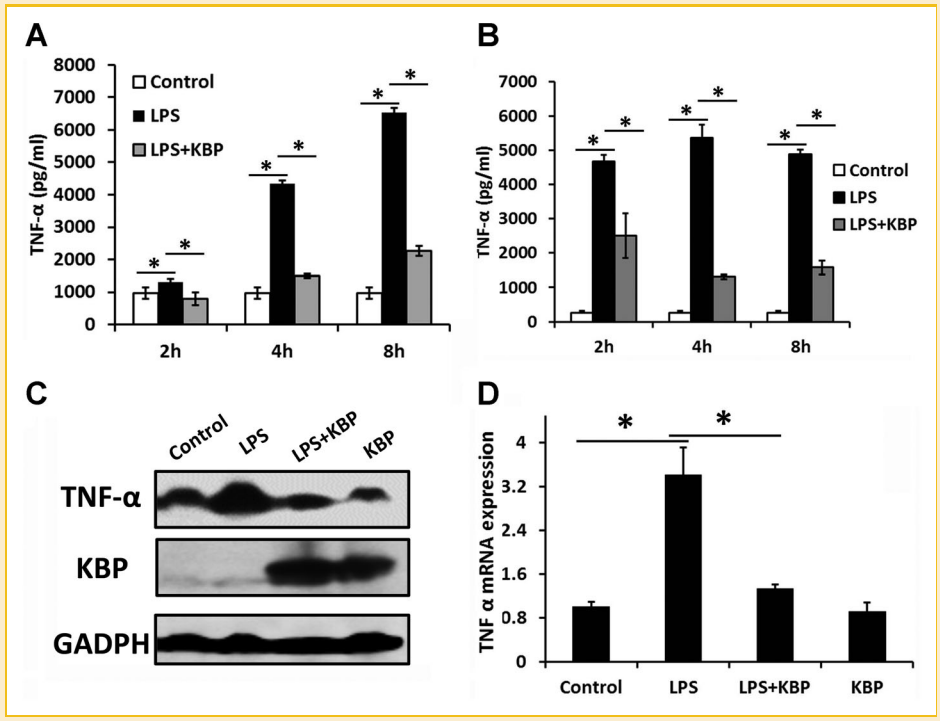

Fig. 1. KBP suppresses LPS-induced TNF-α production in murine macrophages. A: Murine macrophages cell lines RAW264.7 were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, then exposed to 1 μg/ml LPS for indicated times, the supernatant were harvested and TNF-α concentration was determined by commercial ELISA kit, PBS treated cells were used as control (mean ± SD, n = 4, $^*P < 0.05$). B: Murine primary peritoneal macrophages were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, then treated with LPS (1 μg/ml) for indicated times, then TNF-α concentration in the supernatant was determined (mean ± SD, n = 4, $^*P < 0.05$). C: RAW 264.7 cells were transfected with KBP plasmids for 36 h, then stimulated with LPS (1 μg/ml) for another 8 h. Total protein was subjected to Western blot analysis, and blotted with TNF-α and KBP antibody. D: Murine macrophages cell lines RAW264.7 were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, respectively, then exposed to LPS (1 μg/ml) for another 4 h. The cells were collected for RNA extraction, then TNF-α mRNA was analyzed relative to β-actin mRNA by qPCR (mean ± SD, n = 3, $^*P < 0.05$).

ogy treated by KBP was much the same as that of control cells, but the total staining of SOCS3 were stronger than that of other groups (lower panel), indicating that more SOCS3 proteins was induced. These results suggested that KBP might perform anti-inflammatory effect via inducing transcription and expression of SOCS3.

SOCS3 IS RESPONSIBLE FOR THE ANTI-INFLAMMATORY EFFECT OF KBP

Since SOCS3 contributes to LPS-TLR4 signaling and increased SOCS3 by KBP treatment, we next evaluated that whether SOCS3 participated in the anti-inflammatory effect of KBP by knocking

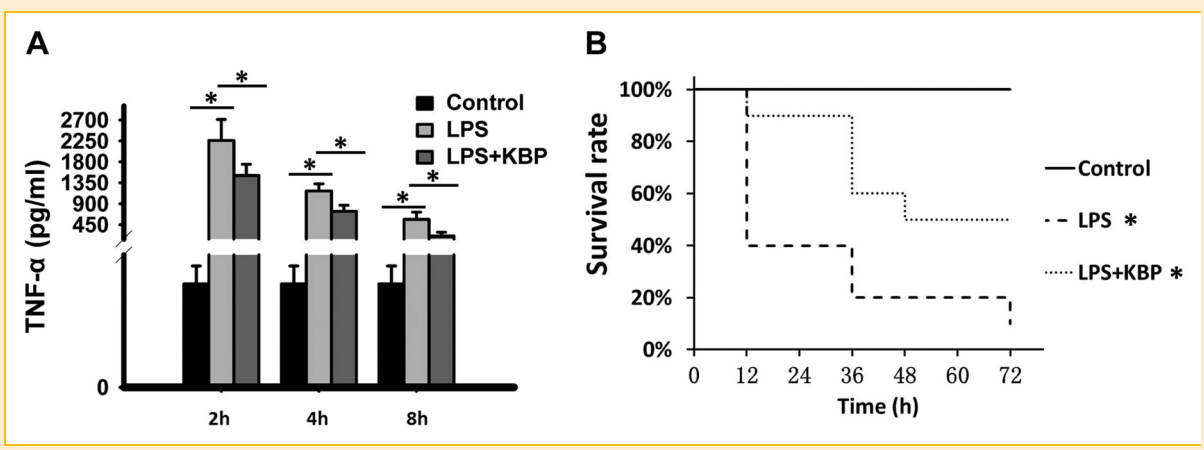

Fig. 2. KBP represses the inflammatory effect of endotoxin in mice. A: BALB/c mice were treated with an intraperitoneal injection of LPS or KBP as described in method. Serum TNF-α level was determined as describe previously (mean ± SD, n = 5, $^*P < 0.05$). B: Mice were intraperitoneally injected with LPS or KBP as described in method, the mortality was assessed at 72 h, and the data were plotted as a Kaplan–Meier plot (n = 10, $^*P < 0.05$).

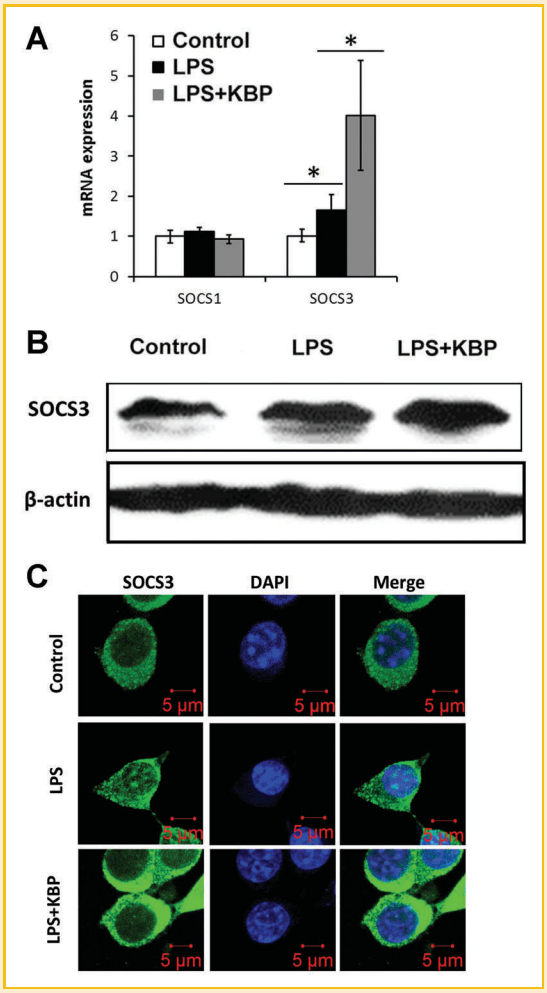

Fig. 3. KBP induces SOCS3 transcription and protein expression in macrophages. A: RAW264.7 cells were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, then incubated with LPS (1 μg/ml) for 4 h, then SOCS1 and SOCS3 mRNA was analyzed relative to β-actin mRNA by qPCR analysis (mean ± SD, n = 3, $^{*}P < 0.05$). B: RAW264.7 cells were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, followed by incubation with LPS (1 μg/ml) for additional 8 h, and then total protein were extracted and subjected to Western blot analysis. Samples were blotted with SOCS3 antibody. C: RAW264.7 cells were seeded on coverslip for appropriate time, then pretreated with KBP (28.8 μg/ml) or PBS for 2 h, followed by incubating with LPS (1 μg/ml) for another 8 h, then cells were fixed and immunostained with SOCS3 antibody (green). Nucleus was co-stained with DAPI fluorescence dye (blue).

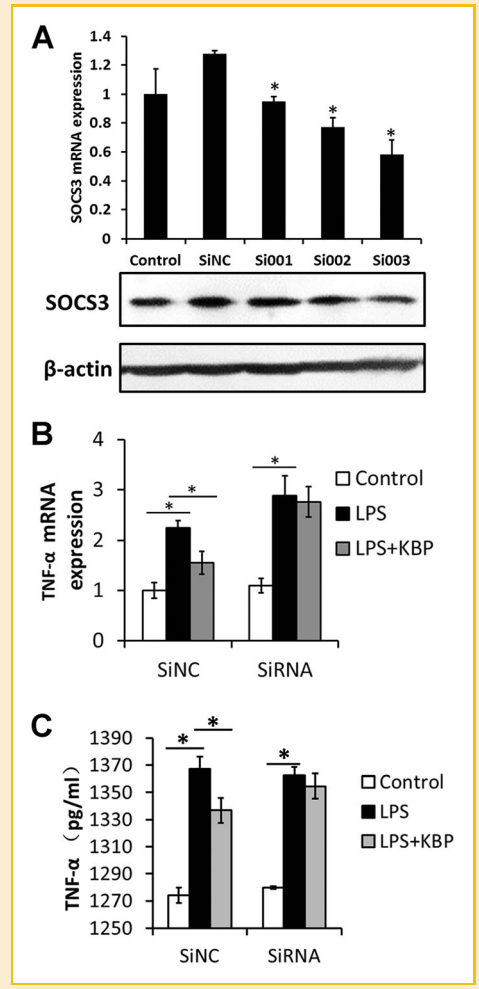

Fig. 4. SOCS3 is responsible for the KBP's suppression of LPS-stimulated TNF-α expression. A: RAW264.7 cells were transfected with either scramble or three paired SOCS3 specific siRNA oligonucleotides. Thirty-six hours later, cells were extracted, TNF-α mRNA was analyzed relative to β-actin mRNA by qPCR analysis. When performed with Western blot analysis, cells were extracted and subjected to SDS-PAGE, and blotted with SOCS3 antibody (mean ± SD, n = 3, $^{*}P < 0.05$). B: RAW264.7 cells were transfected with either scramble or SOCS3 specific siRNA oligonucleotides. Thirty-six hours later, cells were pretreated by KBP (28.8 μg/ml) or PBS for 2 h, followed by incubating with LPS (1 μg/ml) for 4 h, TNF-α mRNA was analyzed as describe previously (mean ± SD, n = 4, $^{*}P < 0.05$). C: RAW264.7 cells were transfected with either scramble or SOCS3 specific siRNA oligonucleotides. Thirty-six hours later, cells were pretreated by KBP (28.8 μg/ml) or PBS for 2 h, then incubated with LPS (1 μg/ml) for 8 h, TNF-α concentration was determined as describe above (mean ± SD, n = 4, $^{*}P < 0.05$).

down SOCS3. Three paired oligonucleotides were synthesized and transfected into RAW264.7 cell lines, qPCR and Western blot analysis were performed to verify the efficiency of SOCS3 knocking down. As shown in Figure 4A, SOCS3 mRNA was decreased by all small interference RNA compared with scramble oligonucleotide (SiNC)($^{*}P < 0.05$); Apparently, Si003 specific oligonucleotides knocked down more than 40% SOCS3 mRNA when compared with the SiNC group, and these results were further confirmed by Western blot analysis.

Furthermore, Si003 specific oligonucleotides were subjected to knock down SOCS3 when exposed to LPS and KBP. Our results showed that there was no significant difference in TNF-α transcription between control group transfected with scrambled and Si003 oligonucleotides, while LPS alone after SOCS3 knocking down displayed elevated TNF-α transcription ($^{*}P < 0.05$, Fig. 4B), suggested that SOCS3 involved in LPS-TLR4 axis. However, LPS + KBP group pre-incubated with Si003 oligonucleotides exhibited elevated TNF-α transcription ($^{*}P < 0.05$, Fig. 4B), compared with scrambled oligonucleotides transfection, but did not exhibited significant difference when compared with LPS treated alone (Fig. 4B). Similarly, TNF-α in the medium was measured by ELISA assay, the results showed that Si003 oligonucleotides

transfection completely prevented the KBP-suppression of TNF-α secretion (Fig. 4C).

KBP HAS NO EFFECT ON NF-κB AND β-CATENIN/TCF IN MACROPHAGES

It is well documented that NF-κB is one of the most important transcription factors in regulating TNF-α transcription, hence we performed luciferase assay to evaluate whether NF-κB contribute to the anti-inflammation activity of KBP in macrophages. Luciferase plasmids containing NF-κB response element were transfected into RAW264.7 cells, as shown in Figure 5A, LPS significantly induced NF-κB activity ($^{*}P < 0.05$), while KBP treatment had no effect on NF-κB activity when compared to LPS treatment. To further gain insight into whether canonical NF-κB subunit p65 contributes to the effect of KBP, cytoplasm and nucleus fraction of cells were extracted in the presence of KBP, as shown in Figure 5B, LPS significantly stimulated p65 translocation into nucleus, while KBP seem not to counteract the effect of LPS. Moreover, further study using confocal microscope analysis confirmed that KBP did not restore the p65 translocation into nucleus induced by LPS (Fig. 5C). These results suggested that NF-κB subunit p65 might not participate in the anti-inflammation activity of KBP in macrophages.

Recent study showed that KBP attenuated retinal inflammation via suppression of Wnt pathway, but whether β-catenin participates in KBP-suppression of TNF-α is still unknown. As shown in Figure 5D, LPS dramatically induced β-catenin/TCF activity when performed luciferase assay ($^{*}P < 0.05$), while KBP treatment showed no effect on β-catenin/TCF transcription activity when compared to LPS treated. Overall, β-catenin probably did not contribute to KBP's function in macrophages.

DISCUSSION

Although a number of studies have shown that KBP has anti-angiogenesis and anti-inflammation activities, the direct effect of KBP on LPS-induced macrophage is largely unexplored. The present study demonstrated the anti-inflammatory activity of KBP in macrophage and endotoxin mice, and revealed a novel pathway of anti-inflammation.

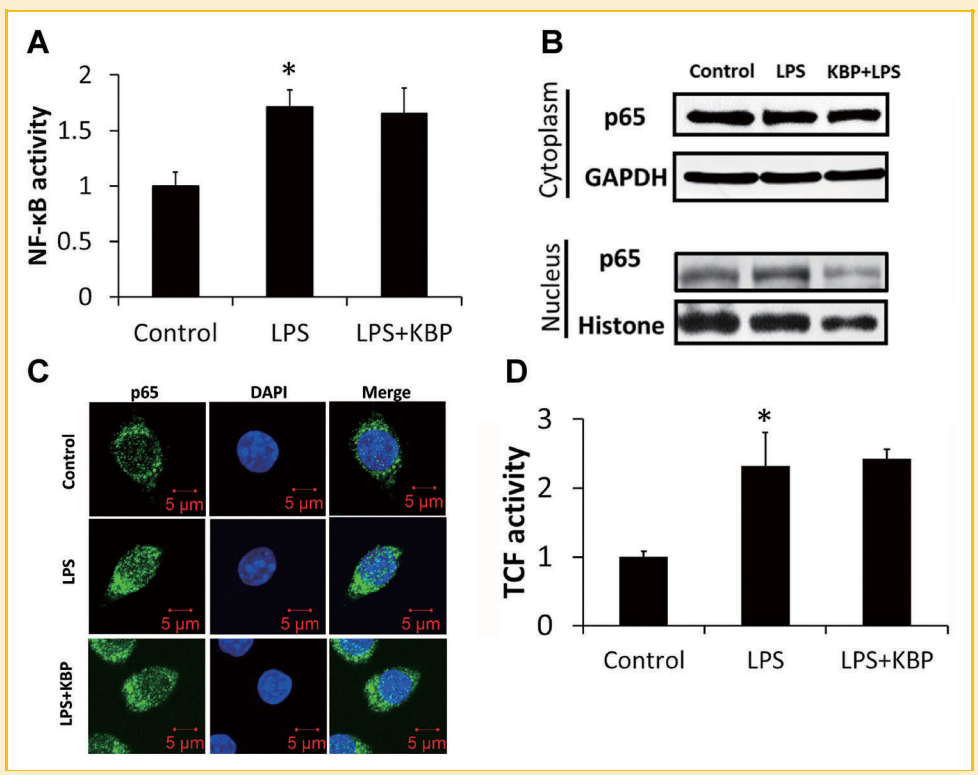

Fig. 5. KBP has no effect on NF-κB activity and β-catenin/TCF in macrophages. A: RAW264.7 cells were co-transfected with pNFκB-Luc plasmid and pRL-TK plasmid. Forty-eight hours later, cells were pre-stimulated with KBP (28.8 μg/ml) or PBS for 2 h, then stimulated with LPS (1 μg/ml) for 4 h. Their luciferase activities were measured, data represented luciferase activity and was expressed as percentages of the respective control (mean ± SD, n = 5, $^{*}P < 0.05$). B: RAW264.7 cells cultured in 10 cm plates were pre-stimulated with KBP (28.8 μg/ml) or PBS for 2 h, then stimulated with LPS (1 μg/ml) for 4 h. Cytoplasm and nucleus fraction of cells were extracted using commercial kit. Equal mount of protein was subjected to Western blot analysis, and blotted with p65 antibody and Histone H3 antibody. C: RAW264.7 cells seeded on coverslip were pretreated with KBP (28.8 μg/ml) or PBS for 2 h, followed by incubating with LPS (1 μg/ml) for another 8 h, then cells were fixed and immunostained with p65 antibody (green). Nucleus was co-stained with DAPI fluorescence dye (blue). D: RAW264.7 cells were co-transfected with TOPFLASH plasmid and pRL-TK plasmid. Forty-eight hours later, cells were pre-stimulated with KBP (28.8 μg/ml) or PBS for 2 h, then stimulated with LPS (1 μg/ml) for 4 h. Then their luciferase activities were measured and calculated as described above (mean ± SD, n = 5, $^{*}P < 0.05$).

Previous studies reported that KBP expression was decreased during acute phase inflammation [Chao et al., 1990], whereas the significance of the down-regulation levels during inflammation responses is poorly explored. Our results showed that KBP down-regulated TNF-α transcription and expression in RAW264.7 cells, primary macrophages and human macrophage-like cell line THP-1 when exposed to LPS (Fig. 1 and Fig. S2). We also found that KBP attenuated TNF-α transcripts stimulated by poly(I:C), a typical TLR3 ligand (data not shown). TNF-α is mainly produced by macrophages, large amounts of TNF-α are released in response to LPS and other bacterial products, which is considered to be a key player in the development of septic shock [Wajant et al., 2003]. Our data demonstrated that KBP treatment decreased serum TNF-α production in mice during endotoxemia (Fig. 2A), which may be responsible for its effect on protecting mice from endotoxin injection (Fig. 2B). Moreover, we previously found that KBP inhibits VEGF, a major pro-angiogenic and pro-inflammatory factor [Gao et al., 2003; Lu et al., 2007; Zhu et al., 2007]. Overall, these results suggest that KBP have anti-inflammatory activities.

Inflammation is delicately regulated by the balance between endogenous pro-inflammatory factors and anti-inflammatory factors, and one of the simplest ways to attenuate cascade pro-inflammatory cytokine signal is via a negative feedback, one of the most important class negative feedback inhibitors is SOCS protein family [Alexander and Hilton, 2004]. SOCS proteins function broadly in immune cells as negative inhibitors of both TLR and cytokine-receptor signaling [Jo et al., 2005; Yoshimura et al., 2005; Rothlin et al., 2007]. Both SOCS1 and SOCS3 are participated in LPS-TLR4 signaling, either induced SOCS1 or SOCS3 is sufficient to suppress LPS-induced TNF-α release, which is consistent with our result that SOCS3 knockdown elevated minor TNF-α transcripts (Fig. 4B). Importantly, we found that SOCS3 mRNA and protein were induced in response to LPS combined with KBP treatment, instead of SOCS1 (Fig. 3). Our data showed that KBP reduced inflammatory factor TNF-α production induced by LPS via upregulating SOCS3, since SOCS3 silencing completely aborted KBP's effect on TNF-α production (Fig. 4). Taken together, these results indicate that SOCS3 is the key mediator participating in the anti-inflammatory activity of KBP.

SOCS3 can be induced by different classes of cytokines, hormone and infectious agents in different tissue, and one of the most frequently investigated proteins is IL-10. IL-10 prevents the host from overreacting to inflammation response, and protects against endotoxin-induced lethality [Howard et al., 1993]. It is reported that SOCS3 is required in IL-10 inhibition of LPS-induced TNF-α transcription and translation via IL-10R/JAK1/STAT3 pathway [Moore et al., 2001]. Activation of STAT3 leads to a transcriptional response of increasing cytosol SOCS3. Therefore, SOCS3 dampens signal cascade triggered by LPS-TLR4 and other cytokine receptors, resulting in decreased pro-inflammatory factors such as TNF-α. However, upregulation of IL-10 by KBP alone was not observed in our study, while IL-10 was increased by KBP in the present of LPS when compared to LPS alone (Fig. S4). It is possible that KBP upregulates SOCS3 expression via IL-10/STAT3 axis, which needed further investigation.

A number of studies have implicated several transcription factors in TNF-α gene regulation, such as NF-κB and NFA, and others including interferon regulatory factor (IRF) families, Ets, C/EBPα [Falvo et al., 2010; Shebzukhov and Kuprash, 2011]. In macrophages, NF-κB is undoubtedly considered the most important transcription factor, on the other hand, TNF-α is described as one of the classical NF-κB target pro-inflammatory cytokine [Blackwell and Christman, 1997]. Several researches provided evidence that the binding of NF-κB, typically p65, to TNF-α promoter region in response to LPS stimuli [Tsytsykova et al., 2007; Falvo et al., 2010]. Thus, we evaluated whether KBP might suppress NF-κB activities, which represent the down-regulation effect of TNF-α. Our results showed that KBP had no effect on NF-κB transcription activity and p65 nucleus translocation (Fig. 5A–C). Moreover, it is also documented that the suppression of TNF-α production of IL-10 is not involved inhibition of NF-κB [Denys et al., 2002]. It is possible that KBP affects other NF-κB members such as p50, c-Rel, or other transcription factors, which is worth further investigation.

KBP is a secreted glycoprotein that might exhibit anti-inflammatory effect via a receptor. The anti-angiogenic effect of KBP is proved to be independent of its interactions with kallikrein-kinin system from previous study [Gao et al., 2003]. Bin Zhang found that KBP protected cells from oxidative stress via binding to Müller cells in a specific and saturable manner [Zhang and Ma, 2008]. Recently, KBP has been reported to inhibit Wnt pathway by blocking low-density lipoprotein receptor-like protein 6 (LRP6) [Zhang et al., 2010]. In this study, they found that KBP binds to LRP6 with a high specificity and affinity, and function as an antagonist of LRP6. The Wnt pathway is known to participate in multiple physiological and pathological processes, and is also involved in inflammatory response via bacterial component including LPS [Duan et al., 2007; Kim et al., 2010]. However, whether Wnt pathway participates in macrophage inflammatory factor regulation is largely unexplored. Recent studies reported that LPS causes glycogen synthase kinase 3 β (GSK3β) phosphorylation, induces β-catenin accumulation and nuclear translocation, and β-catenin silencing elevates IL-6 mRNA expression during LPS treatment [Duan et al., 2007; Kim et al., 2010; Schaale et al., 2011; Lee et al., 2012]. Although KBP is found to bind to LPR6 in ARPE19 cells, it is unclear whether KBP binds to LRP6 of macrophage and attenuates Wnt pathway during LPS treatment. Our luciferase data showed that LPS significantly induced β-catenin/TCF reporter activity, whereas there was no significant difference between LPS alone and

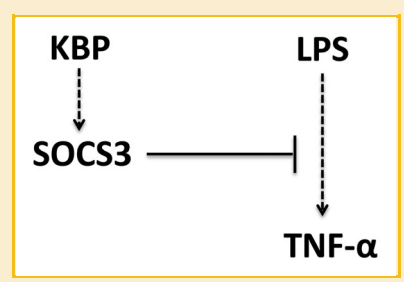

Fig. 6. The schematic pathway of anti-inflammatory effect of KBP in LPS-induced macrophages. Through binding to unknown receptor, KBP increases SOCS3 transcription and translation, which in turn suppresses LPS-mediated TNF-α product and the pro-inflammatory signaling.

LPS + KBP treatment (Fig. 5D). These results suggest that β-catenin might not involve in KBP's anti-inflammatory activity, since that different cell types may represent various mechanisms.

In conclusion, we demonstrate that KBP-mediated TNF-α suppression is involved in upregulating SOCS3 transcription and expression, which in turn exhibits negative regulation of the pro-inflammatory signaling (Fig. 6). Via attenuating acute inflammation, KBP could be implicated in therapy for septic shock.

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