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RESEARCH PAPER

Kukoamine B, a novel dual inhibitor of LPS and CpG DNA, is a potential candidate for sepsis treatment

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BACKGROUND AND PURPOSE

Lipopolysaccharides (LPS) and oligodeoxynucleotides containing CpG motifs (CpG DNA) are important pathogenic molecules for the induction of sepsis, and thus are drug targets for sepsis treatment. The present drugs for treating sepsis act only against either LPS or CpG DNA. Hence, they are not particularly efficient at combating sepsis as the latter two molecules usually cooperate during sepsis. In this study, a natural alkaloid compound kukoamine B (KB) is presented as a potent dual inhibitor for both LPS and CpG DNA.

EXPERIMENTAL APPROACH

The affinities of KB for LPS and CpG DNA were assessed using biosensor technology. Direct interaction of KB with LPS and CpG DNA were evaluated using neutralization assays. Selective inhibitory activities of KB on pro-inflammatory signal transduction and cytokine expression induced by LPS and CpG DNA were analysed by cellular assays. Protective effects of KB in a sepsis model in mice were elucidated by determining survival and circulatory LPS and tumour necrosis factor-alpha (TNF-α) concentrations.

KEY RESULTS

KB had high affinities for LPS and CpG DNA. It neutralized LPS and CpG DNA and prevented them from interacting with mouse macrophages. KB selectively inhibited LPS- and CpG DNA-induced signal transduction and expression of pro-inflammatory mediators without interfering with signal pathways or cell viability in macrophages. KB protected mice challenged with heat-killed *Escherichia coli*, and reduced the circulatory levels of LPS and TNF-α.

CONCLUSIONS AND IMPLICATIONS

This is the first report of a novel dual inhibitor of LPS and CpG DNA. KB is worthy of further investigation as a potential candidate to treat sepsis.

Abbreviations

CFU, colony formation units; CpG DNA, oligodeoxynucleotides containing CpG motifs; DAPI, 4′, 6-diamidino-2-phenylindole; EC, heated killed *Escherichia coli*; KB, kukoamine B; LAL, limulus amebocyte lysate; LPS, lipopolysaccharide; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di phenytetrazoliumromide; MyD88, myeloid differentiation primary response gene (88); Pams3CSK4, Pam3Cys-Ser-(Lys) 4. 3HCl; PAMPs, pathogen-associated molecular patterns; poly I : C, polyinosinic: polycytidylic acid; PMB, polymyxin B; TLR, Toll-like receptor

Introduction

Sepsis is a generalized inflammatory response to infections triggered mainly by the presence of invasive bacteria and their pathogen-associated molecular patterns (PAMPs) (Bianchi, 2007). Despite improvements in supportive care and effective antibacterial agents, morbidity and mortality for sepsis has not decreased over recent decades (Martin *et al.*, 2003; Dombrovskiy *et al.*, 2007). Hence, it is still important to investigate and develop effective agents for the treatment of sepsis.

Bacterial lipopolysaccharide (LPS) and oligodeoxynucleotides containing CpG motifs (CpG DNA), the structural basis of bacterial DNA, are two well-recognized PAMPs. Upon recognition by their pattern recognition receptors, Toll-like receptor (TLR)4 or TLR9, which are commonly expressed on macrophages (Sparwasser *et al.*, 1997; Kawai and Akira, 2010), LPS or CpG DNA can separately activate nuclear factor κB (NF-κB) and mitogen-activated protein kinases (MAPK) (including c-Jun N-terminal kinase and p38) signalling pathways. Then, the expression of various oncogenes and pro-inflammatory cytokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), as well as enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) are upregulated (Kagan and Medzhitov, 2006; Rowe *et al.*, 2006; Kawagoe *et al.*, 2008). These mediators act sequentially or synergistically to generate uncontrolled cascades of sustained inflammatory reactions and eventually cause sepsis (Hotchkiss and Karl, 2003; Ulloa and Tracey, 2005; Medzhitov and Horng, 2009). Recently, LPS and CpG DNA were shown to synergistically activate signalling pathways, induce the release of large amounts of pro-inflammatory cytokines and eventually cause more serious sepsis (Hume *et al.*, 2001; De Nardo *et al.*, 2009). Additionally, pretreatment with either CpG DNA or bacterial DNA was also demonstrated to significantly potentiate the toxicity of lipopolysaccharide in mouse models (Cowdery *et al.*, 1996).

Considering that LPS and CpG DNA play pivotal and synergistic roles in triggering sepsis, sepsis will be prevented or attenuated more effectively if LPS and CpG DNA are simultaneously neutralized. However, there is no report of an investigation or development of a double-target drug for sepsis that simultaneously neutralizes the effects of LPS and CpG DNA (Parrish *et al.*, 2008; Leone *et al.*, 2010).

Traditional Chinese herbs have been widely used in the treatment of infection and inflammatory diseases for thousands of years. Interestingly, we have found that extractions and fractions of several herbs have a high affinity for both LPS and CpG DNA, as detected by biosensors (data not published). To obtain the active compound of these traditional Chinese herbs, column chromatography and high-performance liquid chromatography (HPLC) was used to isolate the active monomers. As expected, kukoamine B (KB), a natural alkaloid compound with high affinity for both LPS and CpG DNA, was isolated from a traditional Chinese herb *cortex Lycii*; this was screened out from over one hundred traditional Chinese herbs.

KB is a pure spermine alkaloid (purity over 99%) with polyamine backbone and dihydrocaffeic acid appendage, as determined by structural analysis. It is a strong polar alkaloid with a positive charge. Although KB had been purified from *cortex Lycii* previously, it was isolated by use of an affinity screening test (Funayama *et al.*, 1995). Additionally, its biological characteristics are unclear and need to be elucidated. Therefore, in the present study, we verified whether KB has a direct antagonistic effect against LPS and CpG DNA and evaluated its protective effects in a mouse model of sepsis.

Methods

Reagents and materials

Lipopolysaccharide from *Escherichia coli* O111:B4 (LPS), fluorescein isothiocyanate-labelled LPS (FITC-LPS), polyinosinic: polycytidylic acid (poly I : C), polymyxin B (PMB), 4′, 6-diamidino-2-phenylindole (DAPI), 3-(4,5)- dimethylt hiahiazo(-z-y1)-3,5-di phenytetrazoliumromide (MTT) and n-octyl β-D-glucopyranoside (OG) were purchased from Sigma Chemicals (St. Louis, MO, USA). Pam3Cys-Ser-(Lys) 4 × 3HCl (Pams3CSK4) was obtained from Invivogen (San Diego, CA, USA). CpG DNA 1826 (CpG, 5′-TCCATGACGTTCCTGATGCT′ -3′, the optimal murine sequence and abbreviated as CpG DNA), 5′ -biotinylated CpG DNA 1826, 5-FAM-labelled CpG DNA 1826 (5-FAM-CpG DNA) and primers for real-time polymerase chain reaction (PCR) were all synthesized by SBS Genetech (Beijing, China). Recombinant Murine TNF-α and interleukin-1β (IL-1β) were obtained from PeproTech Inc. (Rocky Hill, NJ, USA).

Animals

Kunming (KM) mice (4–6 weeks old, weighing 18–20 g, male and female in equal number) were obtained from the Experimental Animal Center of the Third Military Medical University (Chongqing, China) and housed under specific pathogen – free conditions with free access to standard pellet food and distilled water. All animal experiments were performed in accordance with the National Guidelines for Animal Care and Use.

Preparation and identification of KB

KB was isolated and identified from a traditional Chinese herb *Cortex Lycii* by coupling affinity biosensor with chromatography in our laboratory, its purity was over 99%. The structure of KB was determined at the National Center of Biomedical Analysis (Beijing, China).

Preparation of murine peritoneal macrophages

Peritoneal cells were lavaged from the peritoneal cavity of normal KM mice as previously reported (Nathan and Terry, 1975). In brief, 5 mL precooled Dulbecco's modified eagle's medium (DMEM) was injected i.p and withdrawn with a 25-gauge needle. Cells were washed twice before being cultured with DMEM medium supplemented with 10% endotoxin – free foetal bovine serum (Gibco, Grand Island, NY, USA), 2 mM glutamine, 100 $U \cdot mL^{-1}$ penicillin, and 100 $\mu g \cdot mL^{-1}$ streptomycin. After 2 h of incubation at 37°C in a moist atmosphere of 5% CO_2, non-adherent cells were removed by washing with culture medium. The adherent cells were stained with Wright's stain for morphological identification.

Cells culture

The purified murine peritoneal macrophages and murine macrophage-like cell line, RAW 264.7 cells (purchased from

ATCC Manassas, VA, USA) were cultured at 37°C in a 5% CO_2 humidified incubator and maintained in the same culture medium as mentioned above. The cells were diluted with 0.4% trypan blue in phosphate-buffered saline (PBS, 0.1 mM, pH 7.4) and live cells were counted by a haemacytometer. The concentration of the cells was adjusted to 1×10^6 mL^{-1} before stimulation by LPS and CpG DNA.

Preparation of bacterial strain

Bacterial strain of *E. coli* ATCC 35218 were kept in our laboratory and prepared as follows: single colonies from viable, growing LB agar plates were transferred to 50 mL sterile liquid of LB broth (Oxoid, Cambridge, UK) and cultivated aerobically at 37°C in a shaker for 12 h. These cultures were then transferred to 500 mL of fresh LB medium and shaken for another 12 h, after which the bacteria would reach the log phase of growth. The suspension was then centrifuged at 9391× *g* for 5 min at 4°C, the supernatant was discarded, and the bacteria were resuspended and diluted into sterile saline to achieve a concentration of approximately 1×10^{10} colony formation units (CFU)·mL^{-1}. Finally, bacterial suspensions were incubated in a water bath at 100°C for 30 min to inactivate the bacteria.

Affinity assessment and calculation of K_d value using affinity biosensor

LPS and 5′ biotinylated CpG DNA were, respectively, immobilized on the reacting surfaces of cuvettes in an IAsys plus affinity biosensor (Farfield, Cheshire, UK), according to the manufacturer's instructions. Briefly, LPS was immobilized on a non-derivatized cuvette via the linkage of PBS/OG [(0.02 M PBS, pH 7.4)/1.25% OG (w/v)], which formed a lipid-coated surface. 5′-biotinylated CpG DNA was immobilized on the surface of a biotin cuvette by linking to avidin, which had been coated on the surface of biotin cuvette. For affinity detection, 1 μL of KB dissolved in PBS was added, respectively, into the LPS cuvette or CpG DNA cuvette containing 49 μL PBS and incubated for 3 min. The cuvette was then rinsed with 50 μL of PBS and 50 μL of 0.01 M HCl for regeneration and ready for the next cycle of affinity test. Data analysis was performed using the FASTplot software package (Farfield). The K_d value was calculated using the FASTfit software (Farfield) based on results of affinity tests of KB in serial concentrations (0.25, 0.5, 1, 2, 4 μM).

LPS-neutralization assessment by use of the limulus amebocyte lysate (LAL) test

KB and PMB were diluted in LPS-free water and incubated with equal volume of LPS (2 ng·mL^{-1}) at 37°C for 30 min. Subsequently, 100 μL of each incubated sample was added to 100 μL of the quantitative LAL reagents (A & C Biological Ltd, Zhanjiang, China) dissolved in LPS-free water and reacted at 37°C for 60 min in an ATI 320-06 kinetic tube reader (Lab Kinetics Ltd, Bruton, UK). The gel clotting formation of LAL products induced by the existence of non-neutralized LPS was measured. Half-maximal (50%) inhibitory concentration (IC_{50}) was obtained and calculated as an index for LPS neutralization assessment.

LPS and CpG DNA binding assay using flow cytometry

RAW264.7 cells stained with FITC-LPS (200 ng·mL^{-1}) or 5-FAM-CpG DNA (10 μg·mL^{-1}) in the presence or absence of KB (dissolved in culture medium) were washed twice with warm sterilized PBS (0.01 M, pH 7.4), resuspended gently with cold PBS and treated with 4% paraform for 10 min for fixation. Then they were centrifuged and washed with clean PBS to eliminate the paraform. The cells were resuspended in PBS and each sample was examined on a FACScalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence intensity was detected and calculated using the Cellquest Software.

LPS and CpG DNA binding assay using immunofluorescence imaging/confocal microscopy

RAW264.7 cells cultured in 20 mm non-pyrogenic cell culture dishes were incubated with or without KB (dissolved in culture medium), immediately prior to treatments of FITC-LPS (200 ng·mL^{-1}) or 5-FAM-CpG DNA (10 μg·mL^{-1}) at 37°C for 30 min. The cells were then fixed with 4% paraform for 10 min, followed by the nucleus stain with DAPI (100 ng·mL^{-1}) for 2 min. Each sample was examined under a 510 Meta confocal microscope (Zeiss, Göttingen, Germany) at appropriate wavelengths. Images were captured and processed using the LSM Image Examiner software.

Gene expression assessment of pro-inflammatory cytokines using real-time fluorogenic PCR assays

Total RNA extracted from RAW 264.7 cells using a Trizol reagent (Roche, Basel, Switzerland) was reverse transcribed into cDNA with a ReverTra Ace-α-RNA easy kit (TOYOBO, Osaka, Japan). Transcribed cDNA template was mixed with SYBR Green PCR mastermix (TOYOBO, Osaka, Japan) and the following primers: β-actin, sense, 5′-G GGAAATCGTGCGTGACATCAAAG-3′, antisense, 5′-CATACC CAAGAAGGAAGGC TGGAA-3′; TNF-α, sense, 5′-CAGGTTC TGTCCCTTTCACTCACT-3′, antisense, 5′-GTTCAGTAGAC AGAAGAGCGTGGT-3′; IL-6, sense, 5′-TGGAGTACCATAGCT ACCTGGAGT-3′, antisense, 5′-TCCT-TAGCCACTCCTTC TGTGACT-3′; TLR4, sense, 5′-AAGGCATGGCATGGCTT ACAC-3′, antisense, 5′-GGCCAATTTTGTCTCCACAGC-3′; TLR9, sense, 5′-TCGCTCAACAAGTACACGC-3′, antisense, 5′-GCTCTGCATCATCTGCCTC- 3′; iNOS, sense, 5′-TCCTAC ACCACACCAAAC-3′, antisense, 5′-CTCCAATCTCTGC CTATCC-3′; COX-2, sense, 5′-TAGCAGATGACTGCCCAACT-3′, antisense, 5′- CACCTCTCCACCAATGACCT-3′. Quantitative real-time PCR was performed using an iCycler Thermal Cycler (Bio-Rad, Hercules, CA, USA), with the reaction conditions as follows: (i) denature at 94°C for 300 s (1 cycle); and (ii) denature at 94°C for 30 s → anneal at 59°C for 30 s → extend at 72°C for 45 s (40 cycles). After the PCR was finished, dissociation curve analysis was performed to see if there is any bimodal dissociation curve or abnormal amplification plot. For each sample, mRNA expression levels for specific transcripts were normalized to the amount of β-actin and the

$2^{-\Delta\Delta}$CT method was used to analyse the gene expression data as described previously (Livak Schmittgen, 2001).

Important proteins expression assessment using Western blot analysis

Equal amounts of protein lysates extracted from RAW 264.7 cells by RIPA lysis buffer kit (Thermo Pierce, Rockford IL, USA) were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Blots on the membranes were blocked in 5% dry skim milk and probed with the anti- p-p38, p38, tubulin (1:100 dilutions, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), NF-κB inhibitor α (IκB-α) and p-IκB-α (1:1000 dilutions, Cell Signaling, Danvers, MA, USA). The blots were then incubated with secondary goat anti-mouse or-rabbit IgG antibodies (1:2000 dilutions, Cell Signaling) and developed with SuperSignal West Femto Maximum Sensitivity Substrate kit (Thermo Pierce) for chemiluminescence assay under a ChemiDoc XRS gel imaging system (Bio-Rad).

Evaluation of NF-κB activity using enzyme-linked immunosorbent assay (ELISA)

The nucleic protein was extracted from RAW264.7 cells with the RIPA Lysis and Extraction Buffer kit (Thermo Pierce) and adjusted to the same amount. The DNA binding activity of NF-κB in the nucleic protein of each sample was quantified by ELISA using the Trans-AM NF-κB p50 and p65 Transcription Factor Assay Kit (Active Motif, Tokyo, Japan) according to the instructions of the manufacturer. Briefly, each protein sample was incubated in 96-well plates coated with immobilized oligonucleotide containing a consensus (5′-GGGACTTTCC-3′) binding site. The active NF-κB binding to the target oligonucleotide was detected by incubation with a primary antibody specific for the activated form of p50 or p65, visualized by anti-IgG horseradish peroxidase conjugate and Developing Solution, and detected and quantified at 450 nm.

Transient transfection and luciferase assay

RAW 264.7 cells were co-transfected with plasmid pGL-luc2P/NF-κBRE (Promega, Madison, WI, USA) and pGL-hRluc (control plasmid) using lipofectamine reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Then LPS and CpG DNA were added in the presence or absence of KB. After incubation for 6 h, luciferase activity was analysed with the Dual-Glo assay kit (Promega) and detected in a luminometer. Relative luciferase light units were normalized to Renilla luminescence.

Measurement of cytokine concentrations using ELISA

RAW264.7 cells were treated with various stimuli in the presence or absence of KB or PMB (stimuli and KB were added simultaneously unless stated otherwise). The supernatants were harvested at indicated time-points, and TNF-α and IL-6 levels were measured using ELISA kits (R&D, Minneapolis, MN, USA).

Cytotoxicity assay using the MTT method

Cytotoxicity was detected using the MTT assay. RAW264.7 cells or murine peritoneal macrophages pretreated with KB alone or together with LPS and CpG DNA were added with 180 μL of fresh DMEM, plus 20 μL of MTT solution (5 mg·mL^{-1} in 0.01 M PBS) and incubated for 4 h. The supernatant was removed and 150 μL of dimethyl sulphoxide was added to each well for the dissolution of the formazan crystals, which was assayed at 550 nm in a Model 550 microplate reader (Bio-Rad).

Survival analysis in mouse model of sepsis

For survival analysis, three batches of experiments were carried out. Mice were injected i.v. with heat-killed *E. coli* (EC, 1.0×10^{11} CFU·kg^{-1}) in order to establish the sepsis model. The volume of a single injection was 0.2 mL per 20 g bodyweight. The survival of mice was observed up to 7 days.

Firstly, 80 KM mice were randomly divided into five groups (16 mice per group) in order to observe the effect of KB (60 mg·kg^{-1}) with only one injection. Each group was treated, i.v., with 60 mg·kg^{-1} KB, EC or EC in combination with 15, 30 and 60 mg·kg^{-1} KB, respectively. KB was injected simultaneously with EC.

Secondly, 100 mice were randomly divided into five groups (20 mice per group) in order to observe the effect of a lower dose of KB administered for 3 days. Each group was treated i.v. with 5 mg·kg^{-1} KB, EC or EC in combination with 1.25, 2.5 and 5 mg·kg^{-1} of KB, respectively. KB was administered every 8 h for 3 days.

Thirdly, 96 mice were randomly divided into six groups (16 mice per group) in order to observe the time-dependent effect of KB (60 mg·kg^{-1}). Each group was treated i.v. with EC or EC in combination with KB (60 mg·kg^{-1}). KB was given once at 0, 2, 4, 6, 8 h after injection of EC.

Measurement of circulatory levels of LPS and TNF-α

One hundred and twelve KM mice were randomly divided into two groups (56 in each). Group 1 were given sublethal dose of EC (1.0×10^{10} CFU·mL^{-1}) and Group 2 were simultaneously given EC and KB (60 mg·kg^{-1}). Eight mice in each group were killed at the indicated time-points, and blood was drawn i.v. For LPS detection, the whole blood sample was diluted instantly into LPS-free NS, and then measured by use of an LAL assay. For serum TNF-α, the serum was harvested after centrifugation at 835× *g* for 10 min, and then TNF-α was assayed using an ELISA kit.

Statistics and presentation of data

The Kaplan–Meyer log-rank test was used for the survival analysis of the mouse model of sepsis. Cytokine concentrations and other data are expressed as means ± SD. Student's *t*-test was used for paired comparisons, and one-way ANOVA and post hoc Bonferroni correction were used for multiple comparisons. Differences with a P value less than 0.05 were considered to be statistically significant.

Results

KB binds to both LPS and CpG DNA with high affinity

After the structure and purity of KB had been determined (Figure 1A), the binding affinity of KB for LPS and CpG DNA

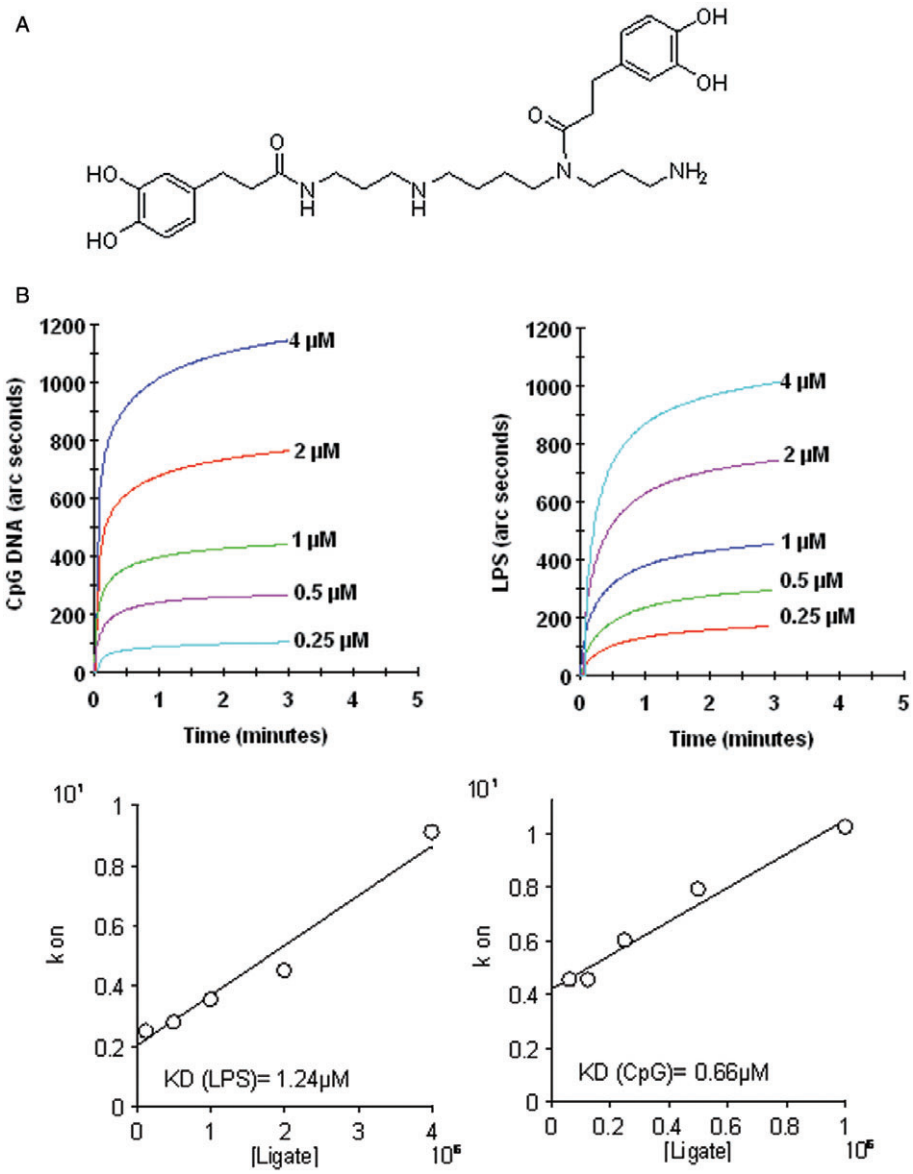

Figure 1

Structure of KB and its affinities for LPS and CpG DNA. (A) The structure of KB. (B) Effect of different concentrations of KB on the biosensor affinity tests for LPS and CpG DNA (CpG). K_d values shown in the linear plot below the binding curves were from one representative test of two. In (B) arc second is the unit of angular measurement applied by biosensor to represent affinity response. One arc second is equal to 1/3600 radian or 0.01592°. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; LPS, lipopolysaccharide; PMB, polymyxin B.

was firstly measured using affinity biosensor as it was isolated from *Cortex Lycii*. The K_d values of KB for LPS and CpG DNA were 1.23 μM and 0.66 μM, respectively (Figure 1B).

KB is a dual neutralizer of both LPS and CpG DNA

Herein, PMB, a specific classic LPS-neutralizer with high affinity, was introduced as a positive tool drug for LPS-binding and -neutralization analysis (Cavaillon and Haeffner-Cavaillon, 1986; Jiang *et al.*, 2004). In an affinity assay, KB exhibited high affinities not only for LPS but also for CpG DNA. PMB had an even higher affinity for LPS, but it was unable to bind to CpG DNA (Figure 2A). In the LAL test, which analysed the direct antagonistic effects on LPS, KB was shown to act in a similar way to PMB as it dose-dependently neutralized LPS (2 ng·mL^{-1}), with a neutralizing ratio of up to 70.26%. The IC_{50} values for inhibitory effects on LPS were 4.80 μM for PMB and 14.93 μM for KB, respectively (Figure 2B). Above results show that KB acts as a dual-neutralizer for both LPS and CpG DNA, but PMB only neutralizes LPS.

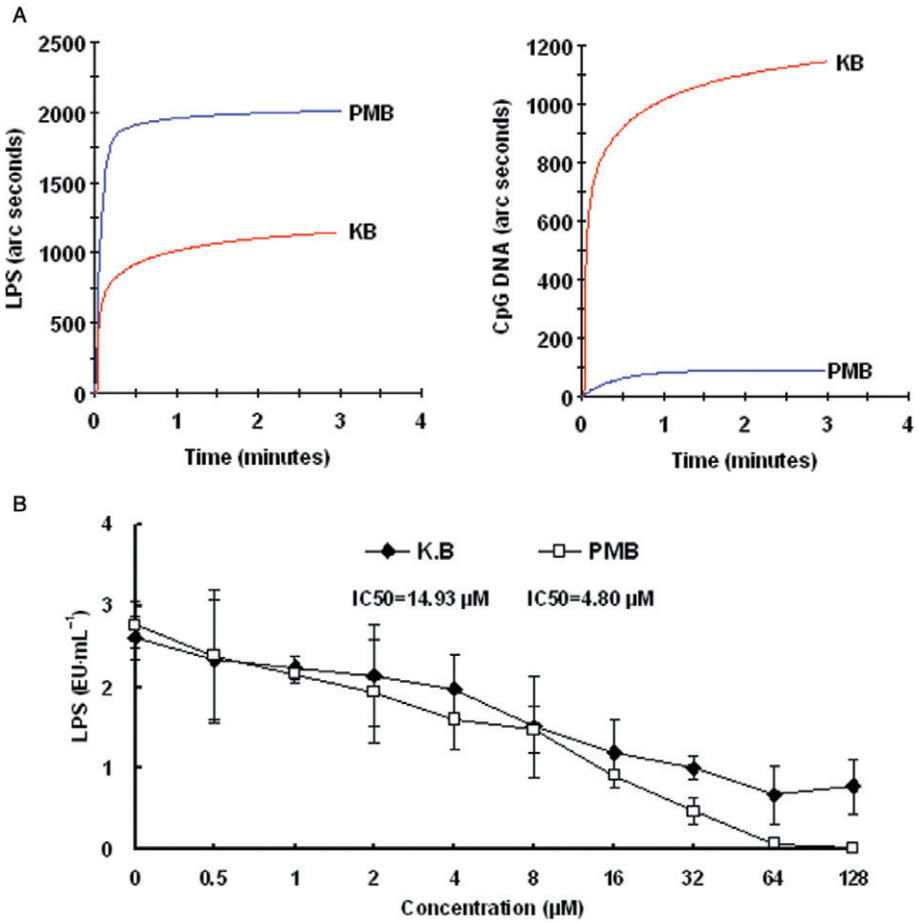

Figure 2

KB neutralized both LPS and CpG DNA *in vitro*. (A) 4 μM of KB or PMB were added into the cuvette of biosensor and incubated for 3 min, and then the affinities of KB or PMB for LPS and CpG DNA were tested. Results shown were from one of three repeated tests. (B) LPS (2 ng·mL^{-1}) was incubated with PMB or KB (0–128 μM), respectively, in equivalent volumes for 30 min. The remaining free LPS were detected by LAL test. This plot was also revealed a half-maximal inhibitory concentration. (IC_{50}) calculated via the inhibitory rate. Results expressed as mean ± SD are representative of three with identical outcomes. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; LPS, lipopolysaccharide; PMB, polymyxin B.

KB is a dual inhibitor of LPS- and CpG DNA-induced TNF-α and IL-6 release

The cytokine assays showed that both KB and PMB significantly inhibited LPS-induced TNF-α and IL-6 release from RAW 264.7 cells ($P < 0.05$, Figure 3A$_1$) and murine peritoneal macrophages ($P < 0.01$, Figure 3A$_2$). However, only KB reduced CpG DNA-induced TNF-α and IL-6 release ($P < 0.01$, Figure 3A$_1$,A$_2$). For cytokines mRNA expressions, KB also reduced the mRNA expressions of TNF-α and IL-6 upregulated by LPS and CpG DNA from RAW 264.7 cells ($P < 0.05$, Figure 3B$_1$) and murine peritoneal macrophages ($P < 0.05$, Figure 3B$_2$). These results show that KB acts as a dual-inhibitor of both LPS and CpG DNA, but PMB only inhibits the effects of LPS.

KB was also found to inhibit LPS- and CpG DNA-induced TNF-α release in a dose- and time-dependent manner. Firstly, KB (0–200 μM) was shown to dose-dependently inhibit LPS- and CpG DNA-induced TNF-α release. The inhibitory effect of KB increased significantly at concentrations over 50 μM ($P < 0.01$, Figure 3C). Secondly, KB (200 μM)-induced inhibition of LPS- and CpG DNA-induced TNF-α release was shown to be time-dependent. Preincubation of KB with LPS or CpG DNA for 20 and 40 min markedly decrease LPS- and CpG DNA-induced TNF-α release ($P < 0.01$, Figure 3D$_1$). Preincubation of KB alone with RAW 264.7 cells for 20 and 40 min did not further inhibit subsequent LPS- and CpG DNA-induced TNF-α release compared with the simultaneous addition of KB with LPS and CpG DNA to RAW 264.7 cells ($P > 0.05$, Figure 3D$_2$). Importantly, inhibitory effects of KB were also observed if the treatment with KB was delayed at several time-points. However, no obvious effect was observed if KB was added at 120 min after the addition of LPS or CpG DNA (Figure 3D$_2$).

In order to exclude the possibility that KB-induced inhibition of cytokine release was due to its cytotoxicity, the cytotoxic effects of KB on RAW264.7 cells and murine peritoneal macrophages were investigated by MTT assay. The results show that KB alone (ranging from 25 to 800 μM) or in

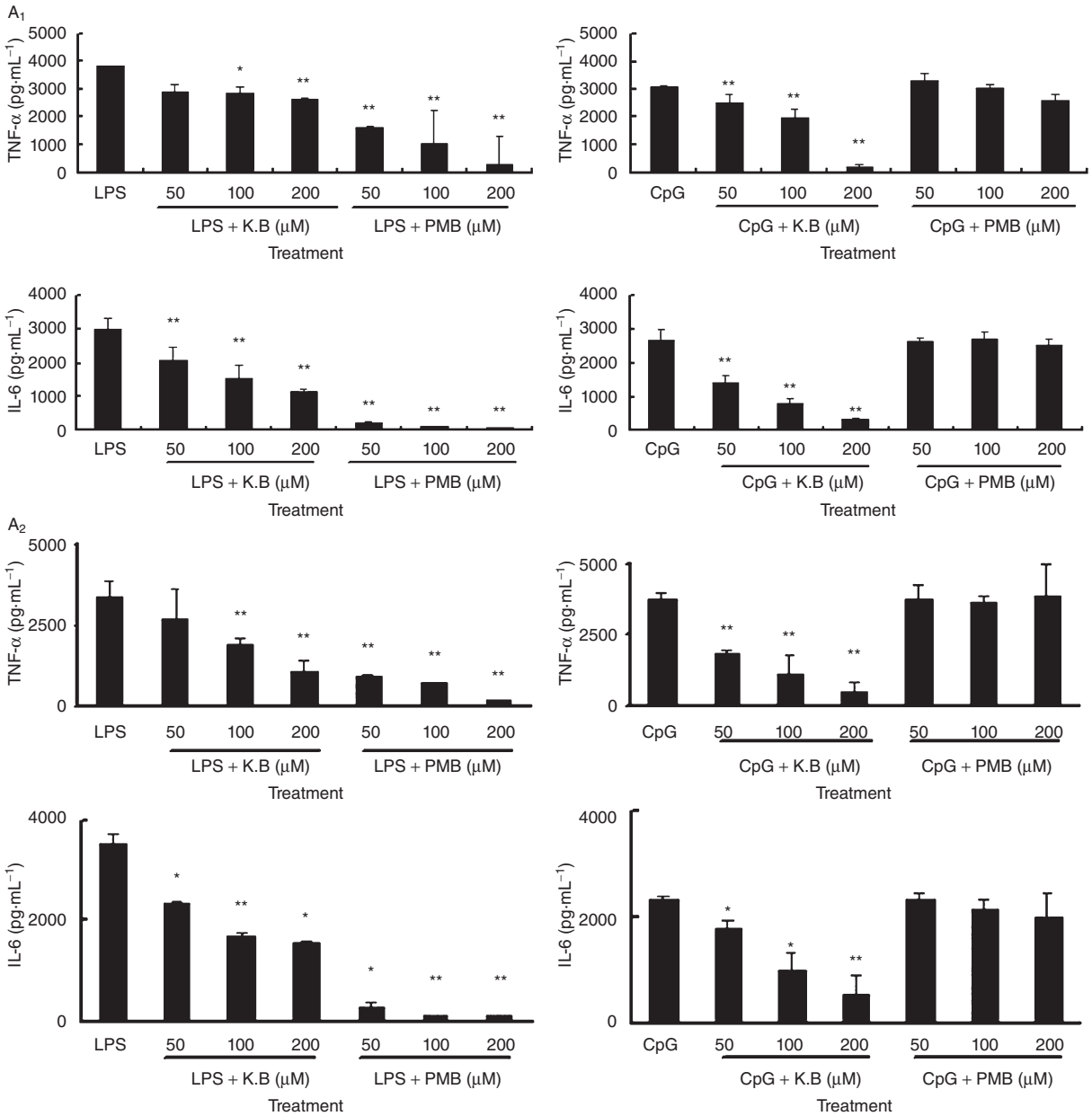

Figure 3

KB inhibited cytokine expression and release from RAW 264.7 cells and murine peritoneal macrophages. (A) 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG DNA, and KB or PMB were simultaneously added to RAW264.7 cells (A_1) or murine peritoneal macrophages (A_2). After incubation for 12 h, TNF-α and IL-6 in the supernatants were detected by enzyme-linked immunosorbent assay (ELISA). The tests were repeated four times and one representative result was shown. *$P < 0.05$, **$P < 0.01$ versus LPS or CpG DNA alone. (B) The mRNA expressions of TNF-α and IL-6 in RAW 264.7 cells (B_1) or murine peritoneal macrophages (B_2) simultaneously treated with LPS (100 ng·mL^{-1}) or CpG DNA (CpG, 10 μg·mL^{-1}) in the presence of absence of KB (100, 200 μM) for 4 h were assayed by real-time polymerase chain reaction and normalized by the expressions of β-actin. The fold change of mRNA expression was expressed as mean ± SD and from three tests with similar outcomes. *$P < 0.05$, ** $P < 0.01$ was statistical differences for comparisons indicated. (C) LPS (100 ng·mL^{-1}) or CpG DNA (10 μg·mL^{-1}) was incubated with KB (0–200 μM) simultaneously. TNF-α release in the supernatants at 12 h were measured by ELISA and expressed as mean ± SD. Results are from one representative experiment of four with similar outcomes. * $P < 0.05$, ** $P < 0.01$ versus LPS; a $P < 0.05$, b $P < 0.01$ versus CpG. (D_1) LPS (200 ng·mL^{-1}) or CpG DNA (20 μg·mL^{-1}) was preincubated with KB (400 μM) or sterile PBS in equal volumes for 20 min or 40 min (as noted in the figure) before their addition to cultured RAW 264.7 cells for stimulation. TNF-α release in the supernatants at 12 h were measured by ELISA and are expressed as mean ± SD. Data are representative of three with identical results. (D_2) RAW 264.7 cells were treated with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG, and KB (200 μM) was added at each indicated time-point (noted as –40, –20, 0, 20, 40, 60 and 120 min). Cytokines released in the culture supernatants at 12 h were measured by ELISA. Data expressed as mean ± SD were from one of three independent experiments. * $P < 0.05$ and **$P < 0.01$ represented statistical differences compared with groups of LPS or CpG alone (–). CpG DNA, oligodeoxynucleotides containing CpG motifs; IL-6, interleukin-6; KB, kukoamine B; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-alpha.

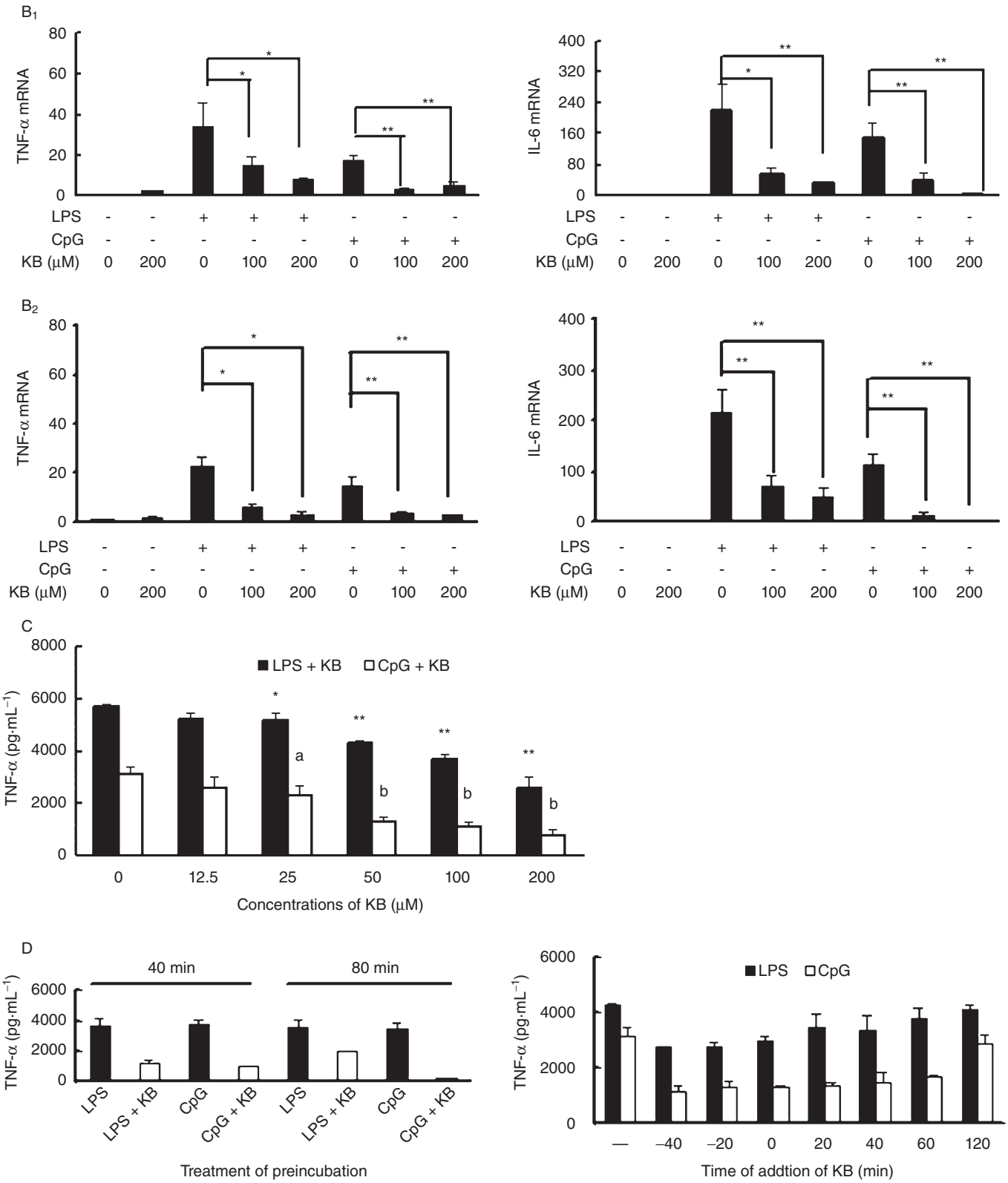

Figure 3
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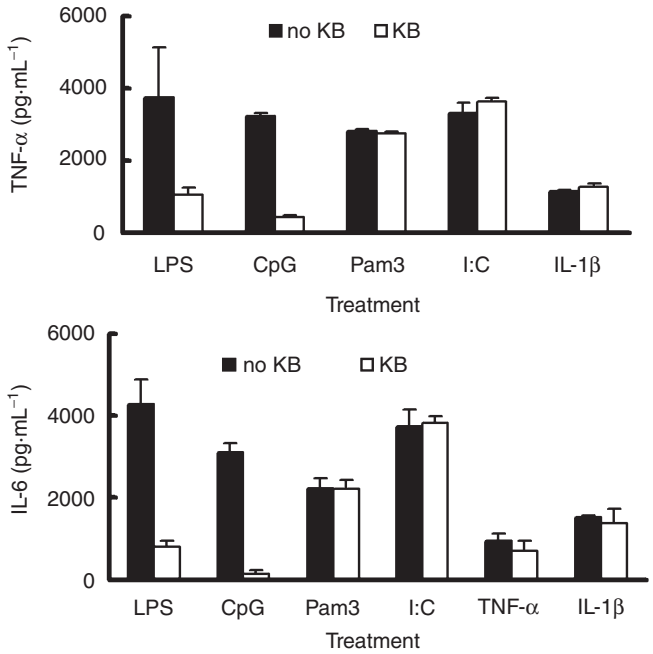

Figure 4

KB selectively inhibited cytokine release from RAW 264.7 cells induced by LPS and CpG DNA but not other stimuli. RAW264.7 cells were treated with 100 ng·mL^{-1} LPS, 10 μg·mL^{-1} CpG DNA (CpG), 10 μg·mL^{-1} Pam3CSK4 (Pam3), 20 μg·mL^{-1} Poly I : C (I : C), 50 ng·mL^{-1} TNF-α and 50 ng·mL^{-1} IL-1β, respectively, in the presence or absence of 200 μM KB for 12 h. TNF-α and IL-6 in the supernatants were collected at 12 h after the stimulation and detected by enzyme-linked immunosorbent assay. Levels of the cytokines are expressed as mean ± SD. Results from one test of three repeats are presented. CpG DNA, oligodeoxynucleotides containing CpG motifs; IL-1β, interleukin-1β; KB, kukoamine B; LPS, lipopolysaccharide.

combination with LPS (100 ng·mL^{-1}) and CpG DNA (10 μg·mL^{-1}) for 24 h did not have a toxic effect on either RAW264.7 cells or murine peritoneal macrophages (Supporting Information Figure S1), indicating that KB-induced inhibition of cytokine release is not due to the cytotoxicity of KB.

KB is a selective inhibitor of LPS and CpG DNA

Cytokines release could also be induced by stimuli from other origins (Kawai and Akira, 2010). In order to investigate whether the inhibitory effects of KB on cytokine release are only limited to LPS and CpG DNA, the influence of KB on cytokine release induced by several other pathogens or cytokines were observed in RAW 264.7 cells. The results showed that KB only significantly reduces TNF-α and IL-6 release induced by LPS and CpG DNA, and does not affect cytokine release induced by either pathogen-derived stimuli such as Pam3CSK4 (TLR1/2), Poly I : C (TLR3) or cytokines like TNF-α and IL-1β (Figure 4). These results show that KB is a selective inhibitor of LPS and CpG DNA.

KB inhibits LPS and CpG DNA binding onto the cell surface and internalization within RAW 264.7 cells

Binding of LPS and CpG DNA onto the cell surface of macrophages via their receptors is a prerequisite for cell activation and cytokine release. Because KB can bind to LPS and CpG DNA, the influence of KB on the distribution of LPS and CpG DNA onto macrophages was observed. Using flow cytometry, KB was found to dose-dependently reduce the mean fluorescence intensity (MFI) on the cell surface of RAW 264.7 cells incubated with FITC-LPS or 5-FAM-CpG DNA for 30 min, the reduced MFI induced by KB (200 μM) was as high as 43.30% for LPS and 46.27% for CpG DNA, respectively ($P < 0.05$, Figure 5A).

Internalization of CpG DNA within the cells is necessary for CpG DNA-induced cell activation and cytokine release, and this internalization has recently been found to be also significant for LPS-induced cell activation and cytokine release (Kagan *et al.*, 2008). Therefore, the effect of KB on the internalization of FITC-LPS or 5-FAM-CpG was observed using confocal microscopy. The results show that KB not only attenuated fluorescence on the cell surface dose-dependently but also reduced fluorescence within the cells incubated with FITC-LPS (Figure 5B1) or 5-FAM-CpG DNA for 30 min (Figure 5B2).

KB down-regulates two receptors (TLR4 and TLR9) and two important enzymes (iNOS and COX-2) mRNA expressions upregulated by LPS and CpG DNA

TLR4 and TLR9 are receptors that recognize LPS and CpG DNA, respectively. The results from real-time PCR experiments showed that KB down-regulates TLR4 and TLR9 mRNA expression upregulated by LPS and CpG DNA in RAW 264.7 cells, respectively ($P < 0.05$) (Figure 6A).

iNOS and COX-2 are respective enzymes necessary for the synthesis of two major secondary inflammatory mediators, NO and prostaglandin. KB significantly reduced iNOS and COX-2 mRNA expressions induced by LPS and CpG DNA in RAW 264.7 cells ($P < 0.01$) (Figure 6B). These results further demonstrate that the anti-LPS and anti-CpG DNA activity of KB is associated with its down-regulation of the mRNA expression of two receptors (TLR4 and TLR9) and two important enzymes (iNOS and COX-2).

KB inhibits IκB-α and p38 phosphorylation and NF-κB activation induced by LPS and CpG DNA

Myeloid differentiation primary response gene (88) (MyD88)-dependent pathway is common and important for signal transduction mediated by LPS/TLR4 and CpG DNA/TLR9 to induce robust inflammatory reactions (Kawai and Akira, 2010). IκB-α and p38 are two important signal molecules in the MyD88-dependent pathway, the phosphorylation of which accounts for activation of downstream transcription factors like NF-κB and AP-1 (O'Neill and Bowie, 2007). Therefore, the effects of KB on LPS- and CpG DNA-induced IκB-α and p38 phosphorylation and NF-κB activation were observed.

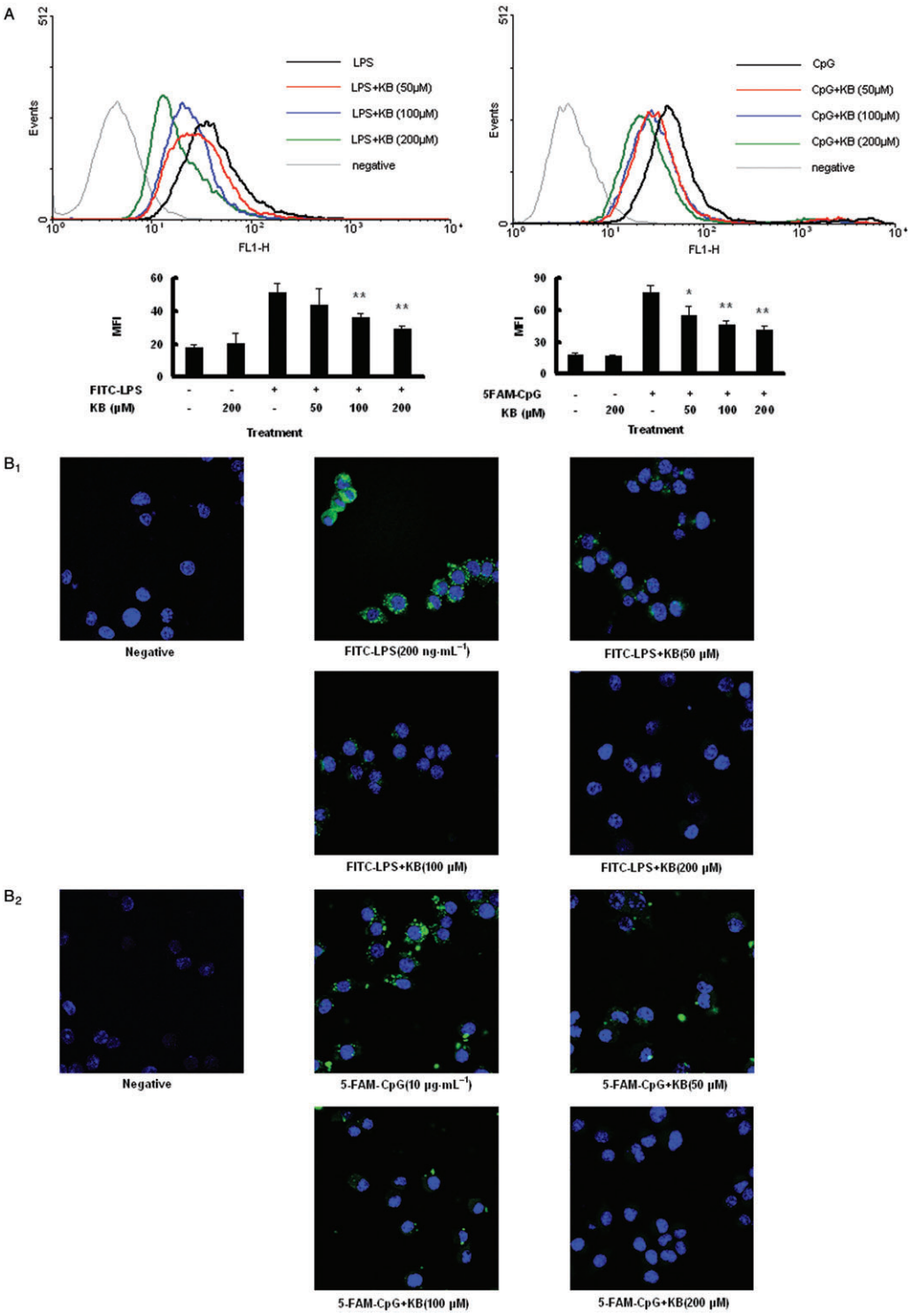

Figure 5

KB interfered with the binding and internalization of FITC-LPS and 5-FAM-CpG DNA in RAW 264.7 cells. (A) Effects of KB (50, 100, 200 μM) on mean fluorescence intensity (MFI) of 200 ng·mL^{-1} LPS or 10 μg·mL^{-1} 5-FAM-CpG DNA (5-FAM-CpG) on RAW 264.7 cells detected by flow cytometry after 30 min incubation for binding process. MFI data are expressed as mean ± SD. Comparison of MFI was made between the presence and absence of KB (*$P < 0.05$; ** $P < 0.01$). (B) Effects of KB (50, 100, 200 μM) on immunofluorescence imaging of 200 ng·mL^{-1} FITC-LPS (B_1) or 10 μg·mL^{-1} 5-FAM -CpG (B_2) on RAW 264.7 cells captured under confocal microscopy for 30 min incubation. Respective green fluorescence denoted the amounts and locations of FITC-LPS or 5-FAM-CpG and blue fluorescence represented nucleus stained by DAPI. Plots and images of flow cytometry and confocal microscopy were from one representative experiment of three with similar results. CpG DNA, oligodeoxynucleotides containing CpG motifs; FITC-LPS, flourescein isothiocyanate-labelled lipopolysaccharide; KB, kukoamine B; LPS, lipopolysaccharide.

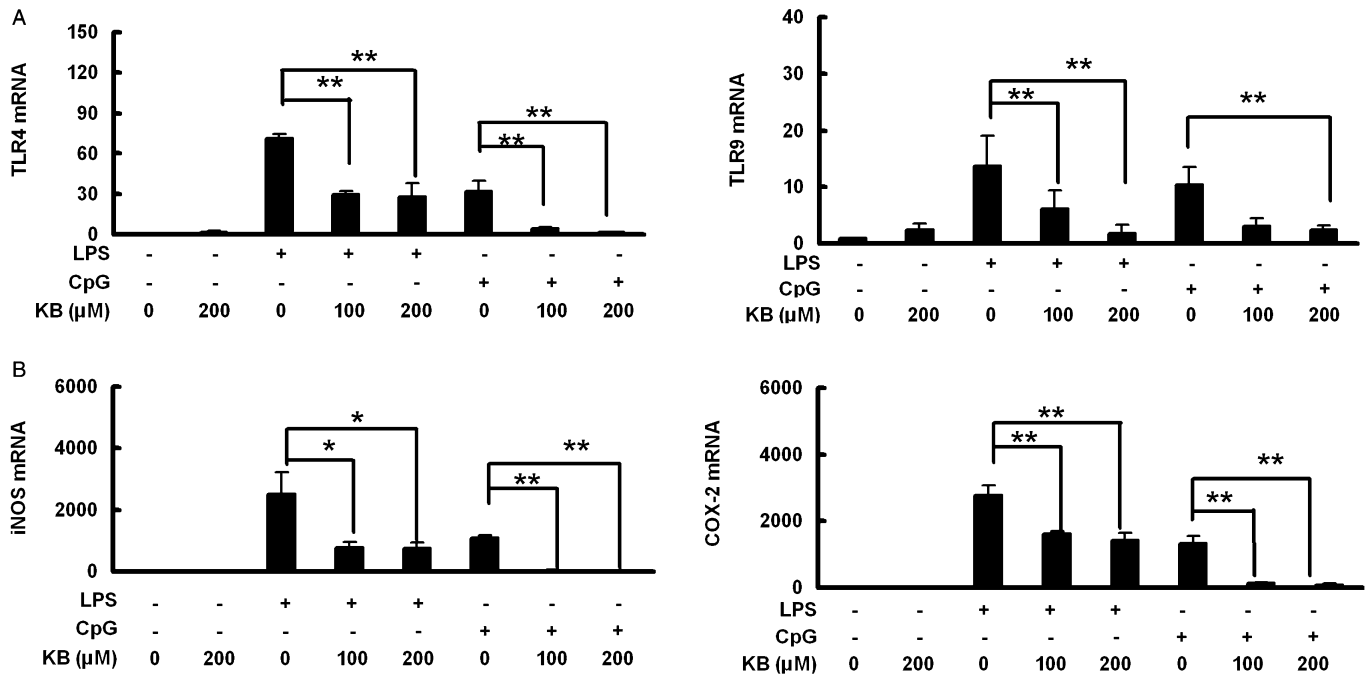

Figure 6

KB down-regulated mRNA expressions of TLR4, TLR9, iNOS and COX-2 upregulated by LPS and CpG DNA in RAW 264.7 cells. RAW 264.7 cells were treated with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG DNA with KB (100, 200 μM) or without KB for 12 h. mRNA expressions of TLR4 and TLR9 (A) and iNOS and COX-2 (B) were assayed by real-time polymerase chain reaction and normalized by β-actin. Fold changes in mRNA expression are shown and expressed as mean ± SD. Statistical differences were made of comparisons as indicated (*P < 0.05; **P < 0.01). Each experiment was repeated three times and representative results are presented. COX-2, cyclooxygenase-2; CpG DNA, oligodeoxynucleotides containing CpG motifs; iNOS, inducible nitric oxide synthase; KB, kukoamine B; LPS, lipopolysaccharide; TLR4, toll-like receptor 4; TLR9, toll-like receptor 9.

Firstly, the phosphorylation levels of IκB-α and p38 in RAW 264.7 cells were determined by Western blot analysis. As the degradation of IκB-α was detectable within a short time, the IκB-α level at 15, 30, 60 and 90 min after stimulation by LPS or CpG DNA were detected. The results showed IκB-α degradation was not detected after 45 min of LPS or CpG DNA stimulation (Supporting Information Figure S2). Therefore, the time-point selected was at 30 min after stimulation. The results demonstrated that KB alone did not induce the phosphorylation of IκB-α and p38. In cells treated for 30 min with LPS or CpG DNA, the levels of phosphorylation of IκB-α and p38 (p-IκB-α and p-p38) were significantly increased as well as the degradation of IκB-α. However, the presence of KB (100 μM, 200 μM) suppressed the IκB-α and p38 phosphorylation as well as the degradation of IκB-α (Figure 7A). In contrast, KB had no effect on the phosphorylation of IκB-α and p38 or on the degradation of IκB-α induced by TNF-α or IL-1β (Figure 7B).

Secondly, NF-κB activation was tested using ELISA and a reporter activity assay. The ELISA results showed that KB (200 μM) suppresses the activation of p50 and p65 (two subunits of NF-κB) induced by LPS or CpG DNA (P < 0.01) (Figure 7C). The results from the luciferase assays further showed that KB also inhibits the NF-κB-dependent luciferase activity induced by LPS and CpG DNA in RAW 264.7 cells transfected with pNF-κB-Luc plasmid (P < 0.01) (Figure 7D).

KB protects mice challenged with EC and reduces the circulatory levels of LPS and TNF-α

To further confirm KB's ability *in vivo*, mice were injected with lethal EC (1.0 × 10^{11} CFU·kg^{-1}) with KB or with sterilized NS. The results showed that a single injection of KB (15, 30, 60 mg·kg^{-1}) with *E. coli* significantly decreases the mortality rate from 87.5% to 62.5%, 62.5%, or 37.5%, respectively (P < 0.05, Figure 8A$_1$), suggesting that KB protects sepsis mice from death in a dose-dependent manner. Significantly, KB still decreased the mortality rate even if it was given 2 h after the bacterial challenge. However, no significant protection was observed if KB was given 2 h later after the bacterial challenge (Supporting Information Figure S3).

In order to make sure that a lower dose of KB also produces an anti-sepsis effect, 1.25, 2.5 and 5 mg·kg^{-1} of KB was administered every 8 h for 3 days. KB given in this way was also able to decrease the mortality rate from 95% to 65%, 60% and 45%, respectively (P < 0.05, Figure 8A$_2$).

In mice challenged with sublethal doses of EC (1.0 × 10^{10} CFU·kg^{-1}), simultaneous injection of KB (60 mg·kg^{-1}) significantly decreased the circulatory LPS and TNF-α levels in a

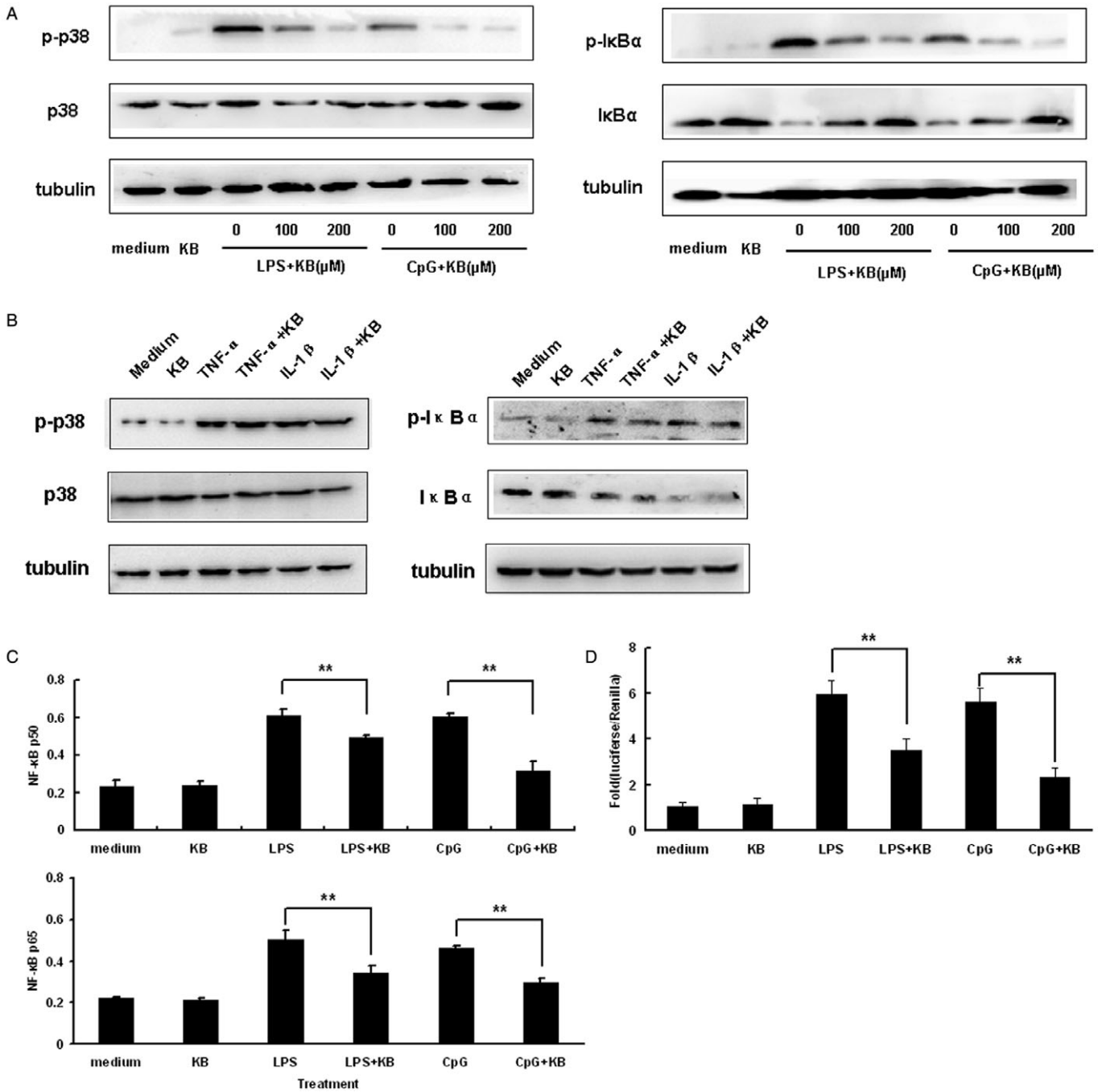

Figure 7

KB inhibited IκB-α and p38 phosphorylation and NF-κB activation induced by LPS and CpG DNA in RAW 264.7 cells. (A) RAW 264.7 cells were treated with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG DNA with or without KB (100, 200 μM) for 30 min. Total protein was extracted and the expression of p-IκB-α and p-p38 were detected by Western blot analysis and normalized to response to tubulin. (B) RAW 264.7 cells were treated with 50 ng·mL^{-1} TNF-α or 50 ng·mL^{-1} IL-1β in the presence or absence of 200 μM KB for 30 min. Total protein was extracted. Expression of p-IκB-α and p-p38 were detected by Western blot analysis and normalized to response to tubulin. (C) RAW 264.7 cells were treated with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG in the presence or absence of 200 μM KB for 2 h. The nucleic protein of each sample was extracted and adjusted to equal amounts. Enzyme-linked immunosorbent assay was used to detect the amounts of active p50 and p65. ** $P < 0.01$ versus LPS or CpG DNA alone. (D) RAW 264.7 cells seeded in a 96-well culture plate were cotransfected with pGL-luc2P/NF-κBRE and pGL-hRluc using Lipofectamine 2000 reagent. After transfection for 48 h, LPS and CpG DNA were added in the presence or absence of KB. After incubation for another 6 h with LPS and CpG DNA, the luciferase activity was detected and relative luciferase light units were normalized to Renilla luminescence. **$P < 0.01$ versus LPS or CpG DNA alone. Each experiment was repeated three times and representative results are presented. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; LPS, lipopolysaccharide.

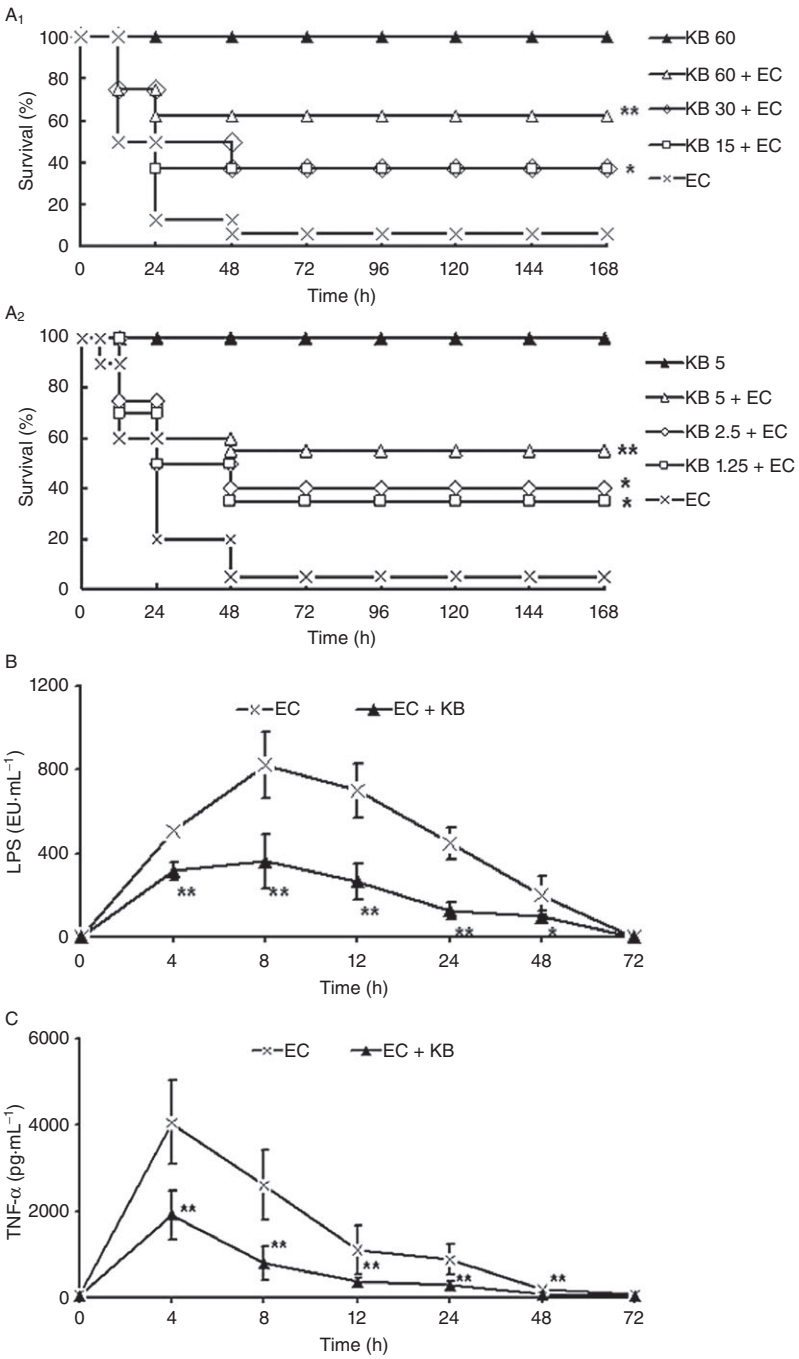

Figure 8

KB increased the survival rate and reduced circulatory levels of LPS and TNF-α of KM mice challenged with heat-killed *Escherichia coli* (EC). (A_1) Eighty KM mice were randomly divided into five groups (16 mice per group).Each group was treated with 60 mg·kg^{-1} KB, heat-killed *E. coli* (EC, 1.0×10^{11} CFU·kg^{-1}) or EC in combination with 15, 30 and 60 mg·kg^{-1} KB, respectively. The injections were simultaneous. * $P < 0.05$ and ** $P < 0.01$, statistical differences compared with heat-killed *E. coli* group. (A_2) KM mice were randomly divided into five groups (20 mice per group). Each group was treated with 10 mg·kg^{-1} KB, EC (1.0×10^{11} CFU·kg^{-1}) or EC in combination with 1.25, 2.5 and 5 mg·kg^{-1} KB respectively. Administration of KB was performed every eighth-hour after injection of EC for 3 days. The survival experiments were repeated three times each. (B) One hundred and twelve mice were divided randomly into two groups (56 mice per group) and treated with EC (1.0×10^{10} CFU·kg^{-1}) alone or in combination with KB (60 mg·kg^{-1}) simultaneously. Eight mice in each group were killed randomly at each time-point (0, 4, 8, 12, 24, 48, 72 h). The whole blood samples (10 μL) were collected intravenously and diluted in 190 μL of endotoxin-free saline. Levels of LPS were detected by the LAL test. (C) For circulatory TNF-α measurement, mice were treated in the same way described in (B). Serum TNF-α was assayed with enzyme-linked immunosorbent assay kits. Results are expressed as mean ± SD. *$P < 0.05$ and ** $P < 0.01$, statistical differences compared with EC group at each time-point. Data are one of two with identical results. CpG DNA, oligodeoxynucleotides containing CpG motifs; EC, heat-killed *Escherichia coli*; KB, kukoamine B; KM, Kunming; LPS, lipopolysaccharide.

time-dependent manner ($P < 0.05$) (Figure 8B,C) These results demonstrate that KB not only neutralizes LPS and decreases the release of pro-inflammatory cytokines *in vitro* but also has the same effect *in vivo*.

Discussion

In this study, KB, an active alkaloid compound isolated from traditional Chinese herb *Cortex Lycii*, was firstly characterized as a selective dual inhibitor of LPS and CpG DNA. It was verified to be a novel dual inhibitor of LPS and CpG DNA and to inhibit the inflammatory response in mouse macrophages and protect mice from the lethal effects of sepsis via its direct binding and neutralizing effect on LPS and CpG DNA.

It is well known that there are several PAMPs which have been found to activate the host defense network and induce sepsis. LPS and CpG DNA are two pivotal PAMPs that trigger sepsis (Crouser *et al.*, 2008). Importantly, a potential synergistic interaction was found recently between LPS and CpG DNA for the pathogenesis of sepsis (De Nardo *et al.*, 2009). Nevertheless, the current drugs used to treat sepsis only target one of either of the two PAMPs. For example, lipid A analogues (including E5531, E5564) and synthetic cationic peptides derived from PMB or bactericidal/permeability increasing protein are antagonists of LPS (Christ *et al.*, 1995; Wiese *et al.*, 2003). The benefits of these drugs are still debatable as they might not be effective for all types of sepsis (Wagner, 1993; Nahra and Dellinger, 2008). Therefore, to improve the strategy for treating sepsis, it would be better to use a drug that targets LPS and CpG DNA simultaneously, and this should prevent or reduce the inflammatory response from the beginning.

Recently, renewed interest in finding new drugs to treat sepsis has focused on investigating and evaluating compounds from traditional Chinese herbs, which have been widely used clinically for thousands of years. Guided by this strategy, in our previous work, traditional Chinese herbs were screened, and several active anti-sepsis monomers were isolated by targeting a single pathogen molecular such as LPS or CpG DNA (Genfa *et al.*, 2005; Liu *et al.*, 2009). In the present experiments, by targeting both LPS and CpG DNA, KB was isolated and identified from the traditional Chinese herb *Cortex Lycii* by coupling affinity biosensor with chromatography.

The biosensor utilized in our study is an optical sensor based on surface plasmon resonance technique. It is widely used as a label-free method for the detection of interactions between immobilized molecules and sampling substances (Quinn *et al.*, 2000). To create the dual targets-guided affinity detection method, LPS and CpG DNA was, respectively, immobilized on the reacting surfaces of the sensor on which samples were added, and then their affinities for LPS and CpG DNA were measured. Our results obtained using this technique showed that several extractions and fractions from traditional Chinese herbs have high affinities for both LPS and CpG DNA. Therefore, it is possible to discover contributing compounds from traditional Chinese herbs using affinity biosensor technology, and this was the method used to reveal KB.

Although KB was first isolated from *Cortex Lycii* in 1995 (Funayama *et al.*, 1995; Potterat, 2010), there has been no report about its bioactivities until now. Herein, KB was identified as a novel dual inhibitor for LPS and CpG DNA. There has been no compound reported with such activities previously.

To verify the dual inhibitory roles of KB, it we first compared with PMB, a typical classic LPS-neutralizer. Previously, PMB was reported to have a high affinity for LPS of 25.8 nM using the same method as that used in our laboratory (Jiang *et al.*, 2004). Herein, KB was shown to have high affinity (1.24 μM) for LPS, but the affinity was lower than that of PMB (25 nM) (Jiang *et al.*, 2004). However, KB had a similar IC_{50} as PMB in the LPS neutralization test, indicating that KB's LPS-neutralization activity is similar to that of PMB. Importantly, KB was also found to have high affinity (0.66 μM) for CpG DNA and this was not found for PMB.

The K_d value is a descriptive concept for affinity between purely interactive molecules (like KB with LPS or CpG DNA). The smaller the K_d value, the more tightly bound the ligand is, or the higher the affinity between two molecules. However, the K_d value could not be thought to be absolute amount or concentration of ligand binding to a receptor/molecule, especially in bioactivity studies, so concentrations used in cellular tests are commonly higher than the K_d value. In previous experiments, the usual concentration of PMB used was 10 $\mu g \cdot mL^{-1}$ (7.7 μM) in cytokine assays, approximately 300-fold higher than its K_d value (25 nM) (Saemann *et al.*, 2005; Paromov *et al.*, 2008). Herein, the maximum concentration of KB was set at 200 μM, which is only 140- fold or 300-fold higher than its K_d value for LPS or CpG DNA, respectively. Importantly, KB showed no cytotoxicity towards RAW 264.7 cells at these concentrations, as determined by MTT assay, suggesting that KB-induced inhibition of cytokine release was not due to a cytotoxic effect of KB. Additionally, although lower concentrations (12.5, 25 μM) of KB were tested (data not shown), at these lower concentrations KB was less effective (Figure 3C).

In the cellular tests, KB, unlike PMB, inhibited the release of cytokines induced by both LPS and CpG DNA, suggesting that KB is more bioactivite than PMB. Additionally, our results also showed that KB is a dual and selective inhibitor of LPS- and CpG DNA-induced TNF-α and IL-6 release because it was unable to inhibit the release of cytokines induced by other pathogens such as Pam3CSK4, Poly I : C or by other cytokines such as TNF-α and IL-1β.

It is necessary for LPS and CpG DNA to bind to macrophages via their pattern recognition receptors (TLR4 and TLR9) before the signalling pathways are activated and cytokines are released (Hoshino *et al.*, 1999; Hemmi *et al.*, 2000). KB might neutralize the effects of LPS and CpG DNA by interfering with its binding duration to macrophages. By use of flow cytometry and confocal microscopy, KB was found to reduce the fluorescence of LPS and CpG DNA on RAW 264.7 cells; this provides indirect evidence that KB neutralizes the effects of LPS and CpG DNA by inhibiting their binding to macrophages. KB was also shown to inhibit mRNA upregulation of TLR4 and TLR9, and thus prevent the activation of macrophages by LPS and CpG DNA. The above results suggest that the pharmacological activities of KB depend on its binding directly to the two PAMPs.

It is well known that the relevant targets for the effects of LPS include molecules like LPS binding protein (LBP), CD14 and TLR4, and it is possible that the inhibitory effects of KB are induced by targeting these molecules. However, our results showed that these molecules are not involved in the effects of KB observed in our experiments. Firstly, LBP can be excluded as target because KB is still active in the serum-free culture medium (Supporting Information Figure S4). Secondly, CD14 and TLR4 can be excluded as direct targets as in our cytokine experiments, preincubation of KB with RAW 264.7 cells did not strengthen the inhibitory effect of KB itself on the cytokine release induced by LPS and CpG DNA, indirectly suggesting that KB probably does not produce its effect by directly interfering with the interaction of LPS with CD14, TLR4 and other relevant targets on the cell surface (Figure 3D2). Instead, it functions by direct neutralization of LPS and CpG DNA.

After binding to receptors, all PAMPs elicit conserved inflammatory reactions via the activation of two major kinase-mediated signalling pathways, which transduce upstream signals to activate transcription factors like NF-κB (Hacker and Karin, 2006; Kumar *et al.*, 2009; Medzhitov and Horng, 2009). It is obvious that if KB neutralizes LPS and CpG DNA, it would affect the signalling pathway activated by LPS or CpG DNA. In this study, KB was shown to attenuate the phosphorylation of p38 and IκB-α, two representative signal molecules in the signal pathways as well as to inhibit the degradation of IκB-α (Yamamoto *et al.*, 2004; Neuder *et al.*, 2009). However, KB did not interfere with the p38 and IκB-α phosphorylations, or the degradation of IκB-α upregulated by TNF-α and IL-1β, indicating that KB does not directly target the signal pathway. In addition, KB was shown to inhibit the activation of NF-κB. These data provide further evidence that KB affects the signal transduction pathway upregulated by LPS and CpG DNA via direct neutralization of the two PAMPs.

To further verify the inhibitory effects of KB on LPS- and CpG DNA-induced activation of other inflammatory mediators, we determined the expression of iNOS and COX-2 upregulated by LPS and CpG DNA, and obtained similar results as those described above. The above data collectively indicate that KB is an inhibitor of both LPS and CpG DNA *in vitro*.

Heat-killed *E. coli* are known to lack viability, but still contain large quantities of CpG DNA and LPS molecules within their cells. Therefore, EC can be used to replace LPS and CpG DNA challenge to induce of model of sepsis that can be used to evaluate the anti-LPS and anti-CpG DNA abilities of KB *in vivo* (Buras *et al.*, 2005; Gasse *et al.*, 2007; Liu *et al.*, 2009). Herein, both single administration of KB (60 mg kg^{-1}) and multiple injections of KB (1.25, 2.5 and 5 $mg \cdot kg^{-1}$) every eighth hour for 3 days were observed to decrease the mortality rate of the mice challenged with heat-killed *E. coli*. In mice administered with a single dose of KB (60 $mg \cdot kg^{-1}$), plasma levels of LPS and TNF-α were reduced, demonstrating the neutralizing ability of KB against LPS and CpG DNA *in vivo*. Importantly, KB was effective only when given within 2 h of the injection of *E. coli*, further suggesting that KB needs to be administered soon after LPS and CpG DNA if it is to have an effect in this acute sepsis model.

Compared with the dose of recombinant human-activated protein C (rhAPC) and hydrocortisone (glucocorticoid), two anti-sepsis drugs recommended by surviving sepsis campaign guidelines, used to treat sepsis the dose of KB needed to have an effect was not high. rhAPC is used at 0.576 $mg \cdot kg^{-1}$ daily in humans, and the maximum dose of hydrocortisone is 5 $mg \cdot kg^{-1}$ daily in humans (Abraham *et al.*, 2005; Dellinger *et al.*, 2008). In this study, daily injections of KB at doses of 3.75, 7.5 and 15 $mg \cdot kg^{-1}$ were used in sepsis mice, equivalent to daily injections of 0.42, 0.83 and 1.70 $mg \cdot kg^{-1}$ in adult humans, according to the drug conversion principle applied in pharmacology studies (the dose·kg^{-1} of mice is about ninefold that for adult humans). The doses of KB were in the similar order of magnitude to that recommended for clinical use of rhAPC and lower than that of hydrocortisone. Importantly, KB proved to be non-toxic to mice because it did not affect either the survival rate of control mice (Figure 8A) or cause pathological changes in major organs like the heart, lung, liver and kidney (Supporting Information Figure S5).

How KB interacts with LPS or CpG DNA is an interesting question. Herein, we hypothesize that KB probably interacts with LPS and CpG DNA via a physical interaction. LPS and CpG DNA are chemicals with negative charges (Ferguson *et al.*, 2000; Agrawal and Kandimalla, 2002). Most of the neutralizers of LPS, reported until now, are chemicals with positive charges. The chemicals with positive charges can decrease the negative charges of LPS, leading to a change in the bioactivities of LPS. For example, PMB was thought to play its neutralizing-LPS role via its positive charges (Brandenburg *et al.*, 2005). KB is a positively charged spermine alkaloid compound with a polyamine backbone and two dihydrocaffeic acid appendages (Figure 1A). Therefore, we suppose that KB decreases the bioactivity of LPS by a physical interaction just like PMB. CpG DNA is also a chemical with negative charges on the three phosphates on the 5′ side as well as on that linking C and G (Agrawal and Kandimalla, 2002). Therefore, KB probably interacts with CpG DNA in a similar physical interaction to that with LPS. As to why PMB is unable to interact with CpG DNA and how KB interacts with LPS or CpG DNA remain to be investigated in the future, these tasks need the cooperation of chemists and pharmacologists.

In conclusion, KB neutralizes LPS and CpG DNA and selectively inhibits LPS- or CpG DNA-mediated inflammation *in vitro* and *in vivo*. KB should be considered as a suitable candidate for the treatment of sepsis.

Acknowledgements

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Conflicts of interest

The authors state no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 The effect of KB on the cell growth of RAW 264.7 cells and murine peritoneal macrophages. RAW 264.7 cells (A) or murine peritoneal macrophages (B) were incubated with KB (0, 25, 50, 100, 200, 400 and 800 μM) alone or with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG DNA for 24 h, MTT assays was used to measure the cell viability, which is expressed as mean ± SD (OD value). Differences were compared among groups with of without KB. Data were one of three with identical results. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; LPS, lipopolysaccharide; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-di phenytetrazoliumromide.

Figure S2 Time-dependent detection of IκB-α degradation in RAW 264.7 cells. RAW 264.7 cells were treated with 100 ng·mL^{-1} LPS or 10 μg·mL^{-1} CpG DNA (A) or 50 ng·mL^{-1} TNF-α or 50 ng·mL^{-1} IL-1β (B) for 15, 30, 45 and 60 min. Total protein was extracted and degradation of IκB-α was detected by Western blot analysis and response normalized to that of tubulin. Data are one of two with identical results. CpG DNA, oligodeoxynucleotides containing CpG motifs; IL-1β, interleukin-1β; KB, kukoamine B; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-alpha.

Figure S3 KB increased survival of KM mice challenged with heat-killed *Escherichia coli* (EC) in a time-dependent manner. Ninety-six KM mice were randomly divided into six groups (16 mice per group). Each group was treated with heat-killed EC (1.0×10^{11} CFU·kg^{-1}) and heat-killed EC in combination with 60 mg·kg^{-1} KB, respectively, administered at 0, 2, 4, 6, 8 h after the *E. coli* injection. *$P < 0.05$, **$P < 0.01$ versus heat-killed EC group. Data are one of two with identical results. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; KM, Kunming; LPS, lipopolysaccharide.

Figure S4 Inhibitory effects of KB on LPS- and CpG DNA-induced TNF-α release in RAW 264.7 cells cultured in serum-free Dulbecco's modified eagle's medium (DMEM). LPS 100 ng·mL^{-1} or 10 μg·mL^{-1} CpG DNA was added to RAW264.7 cells cultured in foetal bovine serum (FBS) – free DMEM. KB (50, 100, 200 μM) was added immediately following LPS or CpG DNA. After incubation for 4 h, TNF-α in the supernatants was detected by enzyme-linked immunosorbent assay. *$P < 0.05$, **$P < 0.01$ versus LPS or CpG DNA alone. The tests were repeated three times and one representative result is shown. CpG DNA, oligodeoxynucleotides containing CpG motifs; KB, kukoamine B; LPS, lipopolysaccharide; TNF-α, tumour necrosis factor-alpha.

Figure S5 The histological morphology of lung, liver, kidney and heart in mice treated with NS or with KB. KB 60 mg·kg^{-1} or the same volume of NS was i.v. injected into mice. The mice were killed 24, 48, and 72 h after injection of KB or NS. Tissue specimens were sampled to receive tissue section examination by H&E staining. The images from the two treatments were captured and compared.