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A synthesized cationic tetradecapeptide from hornet venom kills bacteria and neutralizes lipopolysaccharide in vivo and in vitro

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Abstract

Sepsis is a complex clinical syndrome that results from a harmful host response to infection, in which foreign bacteria and lipopolysaccharide (LPS) are potent activators of different immune cells, including monocytes and macrophages. To date, there are currently few effective adjuvant therapies in clinical use except activated protein C focusing on the coagulation system. Mastoparans (MPs) are wasp venom cationic amphiphilic tetradecapeptides; these are capable of modulating various cellular activities, including stimulation of GTP-binding protein, phospholipase C and can bind to a phospholipid bilayer. Masroparan-1 (MP-1, INLKAIAA-LAKKLL-NH_2), a tetradecapeptide toxin isolated from hornet venom, was synthesized chemically. In this study, *Escherichia coli* 25922 (*E. coli* 25922) and LPS were used to induce sepsis in an animal model. We found that MP-1 treatment at 3 mg/kg protected mice from otherwise lethal bacteria and LPS challenges. MP-1 has antibacterial capabilities against Gram-negative and Gram-positive bacteria. Its antibacterial action against *E. coli* may result from the destruction of bacterial membrane structures. In addition, treatment of murine peritoneal macrophages with MP-1 potently inhibited the respiratory burst. This effect maybe related to an inhibition of NADPH oxidase in the membrane. Furthermore, MP-1, bound with high-affinity to LPS and lipid A with dissociation equilibrium constants of 484 and 456 nM, respectively, and neutralized LPS in a dose-dependent manner. MP-1 also significantly reduced the expression of TLR4, TNF-α and IL-6 mRNA and the release of cytokines in LPS-stimulated murine peritoneal macrophages. Our results shows that the MP-1-mediated protection of mice from lethal challenge by live bacteria and LPS was associated with its bactericidal action and inhibition of inflammatory responses by macrophages to both bacteria and LPS (the release of cytokines and reactive oxygen species).

Keywords: Mastoparan-1; Lipopolysaccharide; Sepsis; Macrophage; Respiratory burst; Pro-inflammatory cytokines

1. Introduction

Sepsis is a complex clinical syndrome that results from a harmful and damaging host response to infection. This condition may result in septic shock, multiple organ dysfunction syndrome (MODS) and ultimately death [1]. An epidemiological study found that the incidence of sepsis was approximately three cases per 1000 people. The overall mortality rate is about 30%, rising to 40% in the elderly and more than 50% in patients with septic shock [2].

Lipopolysaccharide (LPS; known also as endotoxin) is an outer membrane component of Gram-negative bacteria, and is thought to be a major mediator of sepsis and septic shock. Pro-inflammatory cytokines, nitrogen monoxide and superoxide anions are induced by LPS, which may damage cells and lead to organ injury. Despite improved care, the hospital mortality rate from severe sepsis and septic shock has not improved significantly over recent decades. Unfortunately, many experimental inflammatory antagonist-based therapies have failed in sepsis trials, and there are currently no effective anti-LPS drugs in clinical use. Thus, it is important to investigate new anti-LPS drugs to potentially identify a clinically relevant anti-sepsis drug.

A large number of peptides isolated from a variety of sources such as insect venom [3,4] and insect haemolymph [5], comprising approximately 14–40 residues, have been

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shown to have potent antibacterial activities. There have been extensive studies on the biological activities and modes of action of these toxins. Mastoparans (MPs) are a group of toxic peptides found in venom sacs of wasps. They have comprehensive biological activities, including stimulation of GTP-binding proteins (G proteins) [6,7], phospholipase A2 and phospholipase C [8,9], as well as a membrane-perturbing activity [10]. Mastoparan appears to stimulate G proteins via peptide binding in the phospholipid bilayer and the formation of a α-helix that resembles the intracellular loops of G-protein-coupled receptors [11]. Recently, Solomon et al. reported that mastoparan protected rats from LPS-induced mortality [12]. This finding suggested the presence of a functional linkage between CD14, heterotrimeric G proteins and mastoparan. This linkage most likely affects intracellular G protein signaling.

As the LPS molecule was characterized by a negative charge, the mechanism of biological activity of LPS probably involves specific binding of lipid A moiety to the cationic residues of a receptor molecule. Several agents that antagonize the effects of LPS exhibit extensive physicochemical properties (i.e., hydrophobicity and cationic charge) within their binding domains. These include polymyxin B (PMB), bactericidal/permeability increasing protein (BPI) and Limulus anti-LPS factor (LALF). Thus, we presume that such a functional association exists between mastoparan and LPS.

Based on this supposition, we synthesized seven kinds of mastoparans and observed their role in the treatment of septic mice model. Among them, mastoparan-1 (MP-1), a cationic tetradecapeptide isolated from the venom of the hornet, was most effective at protecting mouse models (data not shown). In this study, we report the protection of mice from septic shock by MP-1 induced by bacterial infection and LPS and provide a possible mechanism for this protection.

2. Materials and methods

2.1. Materials

2.1.1. Reagents and strains

LPS from *Escherichia coli* (*E. coli*) O55:B5 and *E. coli* O111:B4, lipid A from Salmonella Re 595 and polymyxin B sulfate salt were purchased from Sigma Chemicals (St. Louis, MO, USA). Benzylpenicillin, cefazolin, cefaradin and cefetaxime were purchased from North China Pharmaceutical Group Corp. (Shijiazhuang, China). Imipenem was purchased from Merck & Co. Inc. (Whitehouse Station, NJ, USA). Mouse TNF-α and IL-6 ELISA kits were purchased from Biosource International (Camarillo, CA, USA). The kinetic turbidimetric Limulus amebocyte lysate (LAL) kit was from Zhanjiang A & C Biological Ltd. (Zhanjiang, PR China). Bacterial strains of *E. coli* ATCC 25922 were kindly provided by Dr. Zhuo Chao. *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 25923 were kept in our laboratory, and other strains used in these experiments were isolated from clinical wards and identified by the clinical laboratory.

2.1.2. Peptide synthesis and purification

Mastoparan-1 (INLKAIAALAKKLL-NH_2) was synthesized and purified by Hybio Engineering Co. Ltd. (Shenzhen, PR China). It was synthesized with the Pioneer^{TM} peptide synthesis system (American Biosystem Inc.), which utilized Fmoc chemistry via stepwise solid phase peptide assembly starting from an Fmoc-Lys (Boc)-Wang resin. After drying, the resin was cleaved by a TFA cocktail solution, and the peptide was purified to 98.5% by high performance liquid chromatography (prepared by Shenzhen Hybio Engineering Co. Ltd., PR China). No endotoxin was detected in the peptide solutions, as determined by the LAL test.

2.1.3. Isolation of peritoneal macrophage from mice

The peritoneal macrophages were isolated and purified as described previously [13]. They were isolated from the abdominal cavity of Kunming (KM) mice, washed two times and cultured in RPMI 1640 supplemented with 10% newborn calf serum (NCS), 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were adjusted to the concentration of 1.0×10^6/ml and grown in 24-well polystyrene plates about 1 ml/well at 37 °C in 5% CO_2. After 2 h, the wells were washed with RPMI1640 and the macrophages were purified.

2.2. Methods

2.2.1. In vitro studies

2.2.1.1. Determinations of MIC and MBC. Drug solutions were made fresh on the day of assay, or were stored at −70 °C for no longer than 2 weeks. The ranges of concentration assayed for MP-1 and other antimicrobial agents were 0.5–512 and 0.25–512 μg/ml, respectively.

Minimal inhibitory concentration (MIC) was assayed at 5.0×10^5 cfu/ml on Mueller–Hinton (MH) broth by the microbroth dilution method. This was done according to procedures outlined by the National Committee for Clinical Laboratory Standards (National Committee for Clinical Laboratory Standards, 2003) [14]. Polypropylene 96-well plates (Sigma–Aldrich) were incubated for 18 h at 37 °C in an incubator. The MIC was taken at the lowest drug concentration at which observable growth was inhibited. The minimum bactericidal concentration (MBC) values were determined by plating 0.01 ml samples from clear tubes onto plant extract-free agar plates. The MBC was taken at the lowest concentration of each drug of which 99.9% reduction of the initial inoculums was observed.

2.2.1.2. Preparation of E. coli ATCC 25922 sample for thin section electron microscopy. *E. coli* ATCC 25922 was cultured in nutrient LB. Normal morphology was observed

when the cell form exhibited exponential growth phase from 4 to 6 h. Bacteria (6.0×10^6 cfu/ml) from the exponential phage were cultured with MP-1 (100 μg/ml) for 15 min, then harvested by centrifugation at 3000 rpm for 10 min. After washing three times with physiological saline and 2.5% buffered glutaraldehyde (pH 7.4) for 2 h at room temperature, the bacteria were post-fixed in 1.5% osmium tetroxide. Next, the bacteria were dehydrated through graded alcohols, embedded in Epon 812, thin-sectioned, stained with uranyl acetate and lead citrate, and examined under transmission electron microscope (TEM).

2.2.1.3. Assay of affinities of MP-1 for LPS/lipid A. LPS or lipid A was immobilized on the surface of a hydrophobic cuvette (Thermo Labsystem, USA) as described previously [15]. The hydrophobic cuvette was pretreated with 2-propanol and PBS/AE (containing 0.025% (w/v) sodium azide and 1 mM EDTA, pH 7.4). 50 μl of 2-propanol and 10 μl of LPS or lipid A (20 mg/ml in chloroform) were mixed in the cuvette and allowed to bind for 1 min. The cuvette was washed seven times with 60 μl PBS/AE and data were collected for 5 min. The cuvette was washed with 0.1 M HCl, PBS/AE and 10 mM NaOH. Data were collected after the final PBS/AE wash. After the LPS or lipid A was immobilized on the surface of the hydrophobic cuvette, various concentrations of MP-1 or PMB were added. Next, a binding curve was generated, and the dissociation equilibrium constants (K_d values) were measured with Affinity Sensors Iasys.

2.2.1.4. Determination of Limulus lysate assay inactivation. The ability of the peptide neutralizing LPS was assayed with the LAL test, which is an extremely sensitive indicator of the presence of free, non-neutralized LPS. Free LPS activates the Limulus coagulation cascade, leading to gel clotting. A range of concentrations of peptide (0, 10, 20 and 40 μM) were incubated with 2 ng/ml LPS in 500 μl of LPS-free distilled water at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent. The kinetic turbidity was measured using a Tube Reader ATi-321 (Lab Kinetics Ltd., UK).

2.2.1.5. Assay for superoxide anion (O_2^-). Superoxide anion generation was measured as the superoxide dismutase (SOD) inhibited reduction of ferricytochrome [16]. The peritoneal macrophages suspended in RPMI1640 (1.0×10^6 cells/ml) containing 10% NCS were added into wells of a 24-well plate and incubated with different concentrations of MP-1 in 5% CO_2 for 2 h, and then washed twice with PBS. After warming to 37 °C, ferricytochrome *c* was added (final concentration = 0.8 mg/ml), followed rapidly by LPS O111:B4 (400 ng/ml). Superoxide dismutase was added into each well (30 μg/ml), and some cells received no treatment. After cells were incubated at 37 °C for 1 h, they were read on an ultraviolet spectrophotometer (550 nm). The data were expressed as means ± standard deviation (S.D.) of triplicate determination, in nanomoles of $O_2^-/10^6$cells after correction for any spontaneous or nonspecific superoxide anion generation. Calculations were based on differences in the reactions with and without SOD (30 μg/ml) divided by the extinction coefficient for the reduction of ferricytochrome *c* (ε = 21.1/mM/10 mm).

2.2.1.6. Assay for hydrogen peroxide (H_2O_2) release. The 460 nm emission from reduced scopoletin, excited by light at 350 nm, is extinguished when scopoletin is oxidized by H_2O_2 in the presence of horseradish peroxidase (HRP). Under the assay conditions, the loss of fluorescence was proportional to the concentration of H_2O_2, and was recorded. Peritoneal macrophages (1.0×10^6 cells/ml) were suspended in RPMI1640 containing 10% NCS with one of several concentrations of MP-1 in 5% CO_2 for 2 h, and then washed twice with PBS. The generation of extracellular H_2O_2 was determined, as described, by monitoring the decrease in fluorescence of scopoletin during its oxidation, when catalyzed by HRP [17]. Cells were harvested and resuspended at 1.0×10^6/ml in PBS without Ca^{2+} and Mg^{2+} in the presence of 3 μM scopoletin and 20 μg/ml HRP in the dark. Cells were added to the 24-well plates, which were pre-warmed at 37 °C and contained 1 ml of reaction mixture with buffer or LPS O111:B4 (400 ng/ml). Fluorescent measurement was taken with the Hitachi F-2500 fluorometer at time zero and at intervals over the next 30 min (excitation/emission wavelengths: 350/460 nm). H_2O_2 release was determined from a standard curve prepared under the above condition.

2.2.1.7. Assay for NADPH oxidase. The peritoneal macrophages were incubated with different concentrations of MP-1 for 2 h, washed with cold PBS and centrifuged at 1000 rpm for 10 min. After the suspensions were discarded, cells in 10 mM PBS (pH 7.0) containing 1 mM EGTA, 7 mM phenylmethane-sulfonvifluoride (PMSF) and 15 μg/ml leupeptin, were disrupted by sonication (3×20 s). Fractionation was conducted [18,19]. The supernatants were centrifuged at 100,000 g for 1 h to separate the low-density membranes. The membranes were resuspended in PBS/0.34 M sucrose (sucrose–PBS). Activation of the oxidase with SDS (50 μM) was carried out in 0.8 ml (final volume) of 10 mM pH 6.7 Hepes-buffered saline (HBS)/10 μM flavin adenine dinucleotide/80 μM cytochrome *c*/1 mM EGTA. The activation medium contained $1.0–2.0 \times 10^6$ cell equivalents of membranes and $2.0–4.0 \times 10^6$ cell equivalents of cytosol. After 5 min of preincubation at 34 °C, 0.2 mM NADPH and LPS O111:B4 (400 ng/ml) were added and incubated for 1 h at 37 °C in 5% CO_2. The reduction rates of cytochrome were measured at 550 nm before and after the addition of 30 μg of superoxide dismutase (SOD). The difference between the rates represents the superoxide-dependent reduction of cytochrome *c*.

2.2.1.8. Assay for cytokines. One millilitres of purified peritoneal macrophages (1.0×10^6 cells/ml) was pre-incubated with MP-1 for 0.5 h, then stimulated with LPS (100 ng/ml). After cells were incubated for another 4 h, the levels of TNF-α and IL-6 in the supernatants were analyzed using the appropriate ELISA kits.

2.2.1.9. Analysis for TNF-α, IL-6 and TLR4 mRNA expression. Three millilitres of purified peritoneal macrophages (1.0×10^6 cells/ml) were grown in 6-well polystyrene plates and incubated with different concentrations of MP-1 for 0.5 h before the addition of LPS O111:B4 (100 ng/ml) for 4 h to extract the RNA. After removing the medium and washing the cells once with sterile PBS, the RNA was purified according to the manufacturer's instructions and 2 μg of total RNA from duplicate wells of each treatment group were reverse transcribed. A master mix containing reaction buffer, dNTPs, Taq polymerase, and 2 μl cDNA/25 μl reaction mixture was distributed into different PCR tubes. Forward and reverse primers corresponding to different individual genes were added to the PCR tubes, and subjected to PCR amplification using primer sets designed against each of β-actin, TLR4, TNF-α and IL-6. These reactions were run for 40 cycles. The annealing temperature that was maintained at 51 °C for TLR4 and 55 °C for TNF-α and IL-6; the rest of the conditions included 94 °C for denaturing followed by extension at 72 °C for 40 s. The PCR products were determined using 1.5% agarose gel electrophoresis and ethidium bromide staining, and photographs were analyzed by Quantity One software, which compares the relative density between objective strap and β-actin. The PCR primers were designed by Primer Premier5 software and synthesized by Bioasia biotechnology Ltd. (Shanghai, China). The PCR primers were: TLR4 (285 bp product): forward, 5′-TTT ATT CAG AGC CGT TGG-3′; reverse, 5′-AGT TGC CGT TTC TTG TTC-3′; TNF-α (349 bp product): forward, 5′-TTC TGT CCC TTT CAC TCA CTG G-3′; reverse, 5′-TTG GTG GTT TGC TAC GAC GTG G-3′; IL-6 (329 bp product): forward, 5′-GAG GAG ACT TCA CAG AGG ATA C-3′; reverse, 5′-GAC TCT GGC TTT GTC TTT CTT G-3′; mouse β-actin (455 bp product): forward, 5′-CCC TGT ATG CCT CTG GTC-3′, reverse, 5′-GTC TTT ACG GAT GTC AAC G-3′.

2.2.1.10. Cytotoxicity assay. Cytotoxicity was established using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [20]. Peritoneal macrophages, screened on a regular basis for mycoplasma infections, were plated in a 96-wells culture dish in a concentration of 5.0×10^5 cells/well in RPMI-1640. After an overnight culture period, cells were washed with RPMI-1640 medium and incubated with various concentrations of MP-1 for 2 h. Subsequently, 20 μl of MTT solution (5 mg/ml in PBS) were added in a total volume of 200 μl medium. Cells were incubated for 4 h at 37 °C and 5% CO_2 whereafter, the supernatant was removed and 150 μl DMSO was added to each well to dissolve produced formazan crystals. The extinction was measured at 490 nm using a microplate reader.

2.2.1.11. Hemolytic activity. The hemolytic activity of MP-1 was evaluated by determining the released hemoglobin of 8% suspensions of fresh human erythrocytes at 414 nm [21]. Human red blood cells (RBC) were centrifuged and washed three times with PBS. The 100 μl of RBC suspended 8% (v/v) in PBS was plated into 96-well plates, and then 100 μl of MP-1 solution (from 5 to 80 μmol/L) was added to each well. The plates were incubated for 1 h at 37 °C and centrifuged at 150 g for 5 min. 100 μl of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader. The observed hemolysis of RBC in PBS solutions and in 0.1% Triton X-100 solution were used as negative and positive controls, respectively. Zero percentage and 100% hemolysis was determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation:

$$\%\text{Hemolysis} = \frac{(A_{\text{MP-1}} - A_{\text{negative}})}{(A_{\text{positive}} - A_{\text{negative}})} \times 100$$

2.2.2. In vivo studies

2.2.2.1. Experimental sepsis animal models and MP-1 treatment. One hundred KM mice (4–6 weeks old), with equal numbers of male and female, were obtained from the Experimental Animal Center of the Chongqing Medical University (Chongqing, China). The weight of mice on the day of the experiments was (20.9 ± 1.4) g. They were maintained in specific pathogen free (SPF) condition until used.

The mice were randomly divided into five groups (20 mice/group) and were intravenously injected as follows: MP-1 alone (3 mg/kg) in group 1, LPS O55:B5 (20 mg/kg) in group 2, living *E. coli* ATCC 25922 (1.0×10^8 cfu/g bodyweight) in group3, LPS O55:B5 (20 mg/kg) plus MP-1 (3 mg/kg) in group 4, and living *E. coli* ATCC 25922 (1.0×10^8 cfu/g bodyweight) plus MP-1 (3 mg/kg) in group 5. The total injection volume was 0.2 ml/20 g bodyweight. The general conditions and mouse mortalities were observed for 3 days.

2.2.2.2. Serum cytokine assay. Forty mice were randomly divided into five groups (8 mice/group). Group 1 was given 10 mg/kg LPS O111:B4, groups 2, 3 and 4 were given 0.75, 1.5 and 3 mg/kg MP-1 followed by infusion with LPS O111:B4, respectively and group 5 was infused with Ringer's solution as a negative control. Blood samples (0.5 ml) were drawn at 0.5 h after inception of the experiment and assayed using the appropriate ELISA kits.

2.3. Statistics and presentation of data

The Chi-squared exact test was used to analyze the differences in mouse mortality among the groups. Other

Table 1
MIC and MBC of MP-1, PMB and other antibiotics against select bacteria

Bacteria	Antibiotics (μg/ml)						
	MP-1	PMB	Benzylpenicillin	Cefazolin	Cefaradin	Cefataxime	Imipenem
E. Coli 1	64/128	1	>512	512	512	>512	1
E. Coli 2	32/128	4/32	>512	512	512	>512	1
E. Coli 3	>512	>512	>512	>512	>512	>512	1/2
E. Coli 4	32	16/64	>512	16/64	16	1	1/2
E. Coli 5	64	4/16	>512	>512	>512	>512	1/4
E. Coli 35218	16/32	4/16	>512	64	32/128	1	1
E. Coli ATCC25922	8/16	2/32	128/256	8/128	16/32	1/2	1/2
S. aureus ATCC25923	8/16	64/256	512	2	4/8	1/2	1/2
S. aureus 1	128	32	>512	>512	>512	512	8/16
S. aureus 2	16	128	32/256	8	4/32	2	1
S. aureus 3	4/8	128	256	512	2	2	1
P. aeruginosa ATCC27853	64/128	>512	>512	>512	>512	32/64	2/8
P. aeruginosa 1	64/128	32/64	>512	>512	>512	256/512	16/64
K. pneumoniae 1	128	8/16	>512	>512	>512	>512	2/4
K. pneumoniae 2	64/128	8/32	>512	>512	>512	512/>512	4/32
K. baumanii	16/32	256/512	>512	>512	>512	128/512	4/8
E. cloacae	64	1	>512	>512	>512	512/>512	2/8
Bacillus proteus	>512	>512	256/512	16/64	16/32	2	4/8

Bacteria were inoculated in an MH broth at a concentration of 10^5 cfu/ml. MP-1 were added into each culture at a concentration range of 0.5–512 μg/ml. Polypropylene 96-well plates (Sigma–Aldrich) were incubated for 18 h at 37 °C in incubator. The MIC was taken as the lowest drug concentration able to inhibit any visible bacterial growth. In addition, MIC values for benzylpenicillin, cefazolin, cefaradin, cefataxime, PMB and imipenem were also determined. The MBC was taken as the lowest concentration of each drug that resulted in more than 99.9% reduction of the initial inoculums.

results were expressed as mean ± S.D. Each experiment was repeated at least twice and each data point represents the mean of at least four parallel samples. A Student's *t*-test was used to examine the differences between groups. A (double-sided) *p*-value less than 0.05 was considered significant, and a *p*-value less than 0.01 was considered very significant.

3. Results

3.1. MP-1 has antibacterial activities

MP-1 had an antibacterial effect against Gram-positive and Gram-negative bacteria. On the whole, the antimicrobial activity of MP-1 was more powerful than penicillin and first generation cephalosporin, and was even better than cephalosporin for some bacteria. The results are summarized in Table 1. Among these bacteria, MP-1's effect on *E. coli* 25922 was significant. Thus, we selected this bacteria to further observe MP-1's influence on morphology.

E. coli 25922 untreated with MP-1 was rod-shaped with some flattening or a slight dimpled surface (Fig. 1A). Exposure to MP-1 (100 μg/ml) for 15 min resulted in considerable morphologic abnormalities of the bacteria. For example, bacteria became swollen and damaged (Fig. 1B).

3.2. MP-1 has high-affinities for LPS/lipid A

To examine whether synthetic MP-1 bound specifically to LPS and lipid A, FASTplot was used to generate binding curves, and K_d values were measured by FASTfit. The affinities of the peptide for LPS and lipid A were compared with that of PMB, a high-affinity binder of LPS. Data

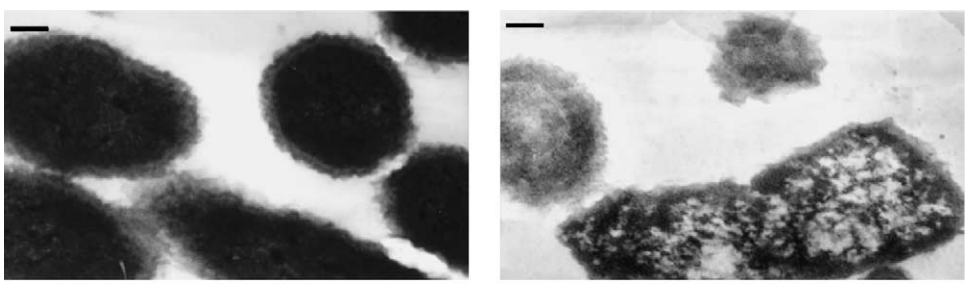

Fig. 1. Thin section electron micrographs of *E. coli* ATCC 25922 exposed to MP-1. Cells of *E. coli* from exponential phase growth were incubated with MP-1 (100 μg/ml) for 15 min and then harvested by centrifugation at 3000 rpm for 10 min. After washing with buffered glutaraldehyde (pH 7.4) for 2 h, the bacteria were post-fixed in 1.5% osmium tetroxide, dehydrated through a graded alcohol, embedded in Epon 812, thin-sectioned, stained with uranyl acetate and lead citrate and examined under TEM. (A) No MP-1 treatment; (B) MP-1 (100 μg/ml) treatment. Each figure magnified ×50,000. Scale bars = 100 nm.

Table 2
Binding affinities of MP-1 and PMB for LPS and lipid A

Peptides	K_d values (nM)	
	LPS	Lipid A
MP-1	484	456
PMB	19	11

LPS or lipid A was immobilized on the surface of a hydrophobic cuvette as described in Section 2. Different concentrations of MP-1 and PMB were added to the immobilized cuvette. A binding curve was generated and K_d values were determined.

demonstrated that MP-1 bound LPS and lipid A had K_d values of 484 and 456 nM, respectively (Table 2). The affinities of PMB were 19 and 11 nM for LPS and lipid A, respectively (Table 2). The affinities of PMB for LPS and lipid A were significantly higher than those of MP-1.

3.3. MP-1 neutralizes LPS activity in in vitro experiments

As described previously, the LAL test is an extremely sensitive indicator of the presence of free, non-neutralized LPS, allowing detection of free LPS at the pg/ml level. LAL test results generally represent the ability of a molecule to neutralize LPS. Thus, we used LAL testing to assess the ability of MP-1 to neutralize LPS. As shown in Fig. 2, both MP-1 and PMB inhibited LPS activity in a dose-dependent manner. PMB more strongly inhibited LPS activity as compared to MP-1 at the same concentrations.

3.4. MP-1 inhibits respiratory bursts of peritoneal macrophages

A small amount of superoxide anions were found in normal macrophages without the LPS stimulus. When cells were stimulated with LPS, superoxide anion production

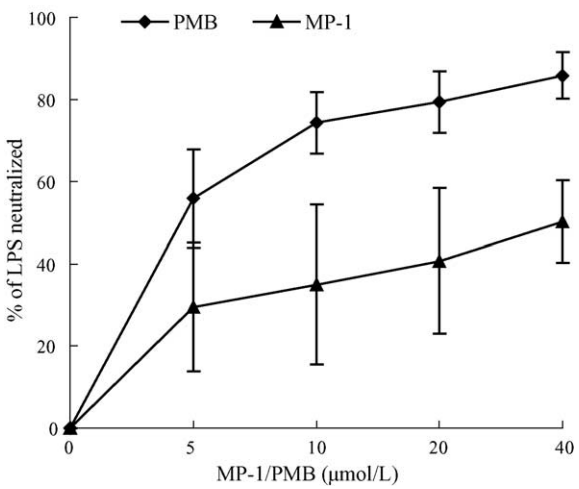

Fig. 2. Neutralization of LPS in vitro by MP-1 and PMB. Indicated concentrations of MP-1 and PMB were incubated with 2 ng/ml LPS O55:B5 in 500 μl of LPS-free distilled water at 37 °C for 30 min. Subsequently, 100 μl of this mixture was added to an equal volume of the LAL reagent and the kinetic turbidity was measured. The test was repeated four times.

Table 3
Effect of MP-1 on active oxygen and NADPH oxidase in murine peritoneal macrophages stimulated with LPS

MP-1 (μmol/L)	LPS (100 ng/ml)		
	O_2^- (μmol/L)	H_2O_2 (nmol/L)	NADPH oxidase activity (%)
0	3.22 ± 0.18	72.0 ± 4.8	100.0 ± 12.1
5	2.32 ± 0.16**	60.9 ± 6.5*	77.8 ± 12.8*
10	1.38 ± 0.36**	49.9 ± 3.5*	55.4 ± 6.6*
20	1.23 ± 0.31**	45.0 ± 3.8**	32.9 ± 8.9**
40	0.69 ± 0.23**	36.2 ± 7.0**	25.0 ± 7.7**
Medium	0.89 ± 0.26**	15.1 ± 4.5**	17.3 ± 4.8**

The peritoneal macrophages (1.0×10^6 cells/ml) were pretreated with indicated concentrations of MP-1 for 2 h, and then incubated with 0.8 mg/ml ferricytochrome *c* and 400 ng/ml LPS O111:B4. SOD was added into each well (30 μg/ml), and some cells received no treatment. After cells were incubated for 1 h, cell-free supernatants were collected and read on an ultraviolet spectrophotometer (550 nm). An extinction coefficient of 21.1 mM/cm was used for ferricytochrome *c*. The data were expressed in nanomoles $O_2^-/10^6$ cells after correction for any spontaneous or nonspecific superoxide anion generation. The peritoneal macrophages (1.0×10^6 cells/ml) were re-suspended in RPMI1640 with MP-1 for 2 h. Next, 3 μM scopoletin and 20 μg/ml HRP were added; H_2O_2 production, triggered by 400 ng/ml LPS O111:B4, was measured according to the loss of fluorescence at 460 nm after excitation at 350 nm. NADPH oxidase activities were measured in a cell-free system. Cells were pretreated with the above solution. After sonication and centrifugation, the membranes and cytosol were re-suspended in the assay mixture (0.8 ml) containing Hepes-buffered saline (pH 6.7), combined with 80 μM cytochrome, 50 μM SDS, 10 μM FAD and 1 mM EGTA. The action was initiated by the addition of 0.2 mM NADPH and 400 ng/ml LPS O111:B4. O_2^- generation was observed for 1 h during the assembly of the active NADPH oxidase complex. Results were expressed as the rate of superoxide production. The data were expressed as means ± standard deviation (S.D.) of more than triplicate determination.
* $p < 0.05$ compared to the group without MP-1.
** $p < 0.01$ compared to the group without MP-1.

increased. MP-1 inhibited superoxide anion production in peritoneal macrophages stimulated with LPS in a dose-dependent manner (Table 3).

As shown in Table 3, H_2O_2 was released when LPS was added in peritoneal microphages, but MP-1 inhibited release of H_2O_2 in a dose-dependent manner. The difference was significant ($p < 0.05$).

Among the respiratory macrophages, the NADPH oxide of the membranes was a key element involved in the release of superoxide anion and H_2O_2. Table 3 summarizes the rates of superoxide production exhibited by the NADPH oxidase. It was evident that superoxide production increased when LPS was added. Data demonstrated that the introduction of MP-1 interfered with the superoxide-mediated reduction of cytochrome *c*. MP-1 inhibited the NADPH oxidase activity of LPS-stimulated peritoneal microphages in a dose-dependent manner. The group differences between LPS-stimulated macrophages without MP-1 treatment and those with MP-1 treatment were significant ($p < 0.05$).

3.5. MP-1 inhibited expression of TLR4 mRNA

Signaling by TLR family members is required for LPS to induce cytokine release [22,23]. In macrophages and

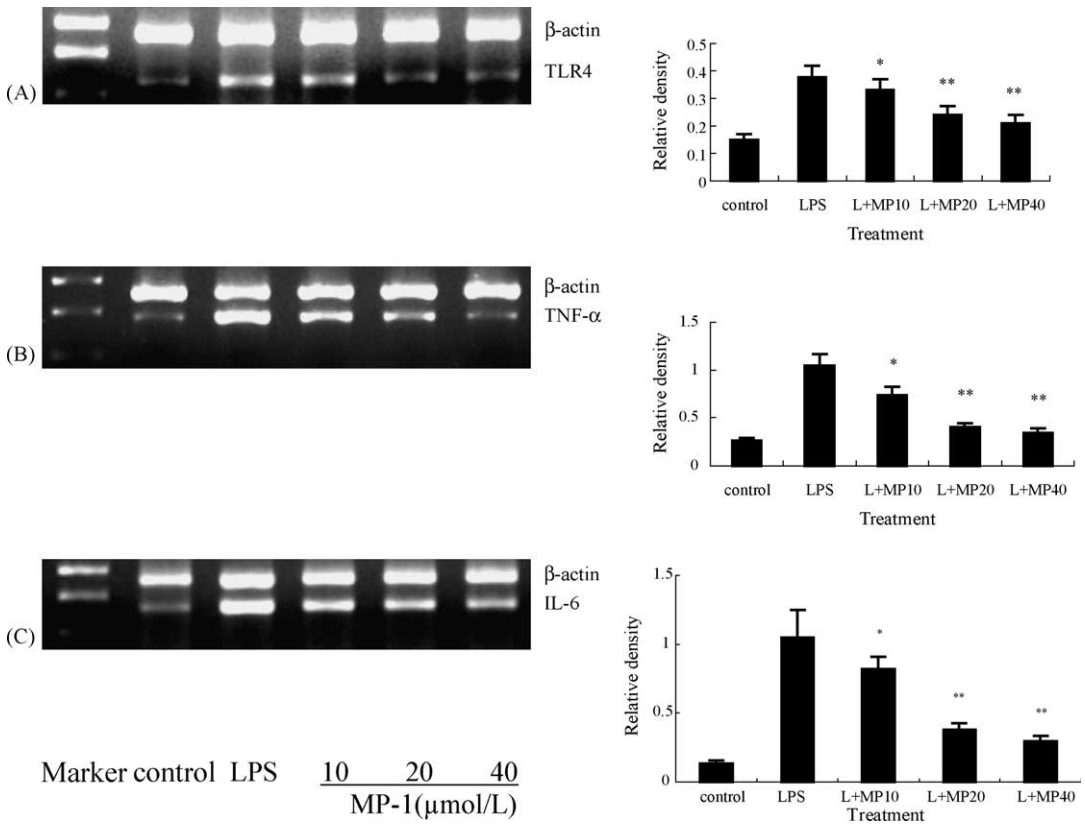

Fig. 3. Effect of MP-1 on the stimulation of mRNA expression of: (A) TLR4, (B) TNF-α and (C) IL-6 in murine peritoneal macrophages by LPS. The peritoneal macrophages (1.0×10^6/ml) were incubated with indicated concentrations of MP-1 for 0.5 h in 6-well polystyrene plates and then cultured for 4 h with LPS O111:B4 (100 ng/ml). Total RNA was extracted, reverse transcribed and cDNA was subjected to PCR using cytokine-specific primers. Densitometric analysis was used to quantify the amount of PCR product. β-Actin was used as a standard to allow semi-quantitative comparison between samples. Values represent the means from three independent experiments. Molecular weight markers were 377 bp and 237 bp from upper to lower. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to LPS group.

monocytes, TLR4 is a pattern-recognition receptor (PRR) for LPS. We investigated the mRNA expression of TLR4 in cells treated with MP-1. In non-stimulated macrophages, TLR4 mRNA was expressed at a low level (Fig. 3A). LPS elevated mRNA expression of TLR4, which was potently reduced by pretreatment of the cells with MP-1.

3.6. MP-1 reduced TNF-α and IL-6 mRNA expression and release in vitro

To investigate whether MP-1 was capable of inhibiting cytokine expression at the mRNA level, mRNA expression of TNF-α and IL-6 were measured (Fig. 3B and C). MP-1 decreased mRNA expression of TNF-α and IL-6 in a dependent manner. Treatment with MP-1 (40 μM) almost completely reduced the mRNA expression of TNF-α and IL-6 to the level of the control group. During these experiments, the viability and morphology of macrophages was monitored. There were no obvious toxic effects of MP-1 on the cells.

The release of various cytokines acts as a good indicator of sepsis [24–26]. TNF-α was thought to be an early cytokine, whereas both IL-6 and IL-12 were considered later cytokines. Therefore, we tested TNF-α and IL-6 levels in vitro. As shown in Fig. 4, LPS-induced macro-

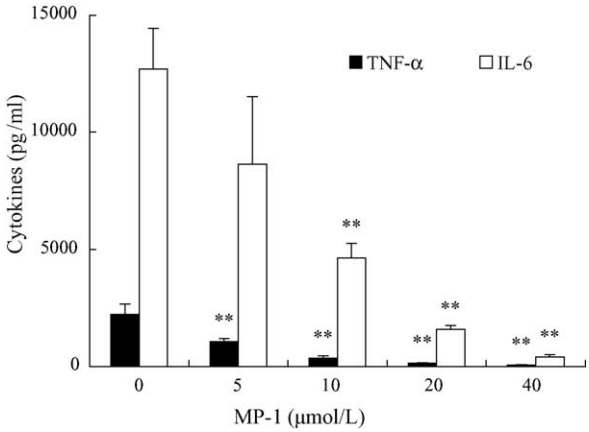

Fig. 4. Effect of MP-1 on release of pro-inflammatory cytokines from murine peritoneal macrophages induced by LPS. One millilitres of purified peritoneal macrophages (1.0×10^6 cells/ml) was pre-incubated with indicated concentrations of MP-1 for 0.5 h, then stimulated with LPS O111:B4 (100 ng/ml). After cells were incubated for another 4 h, the levels of TNF-α and IL-6 in the supernatants were analyzed using the appropriate ELISA kits. Cytokine levels were expressed as mean ± S.D. in pg/ml. Student's *t*-test was used to examine the differences. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared to the group without MP-1. The levels of TNF-α and IL-6 in medium were (40.6 ± 7.7) pg/ml and (243.8 ± 85.7) pg/ml, respectively.

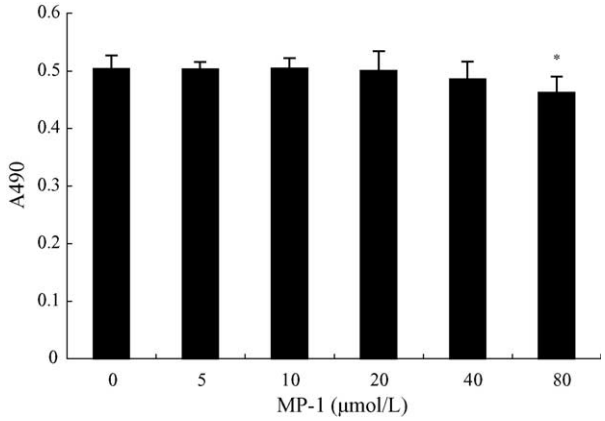

Fig. 5. Cytotoxicity of MP-1 on peritoneal macrophages. Peritoneal macrophages were plated in a 96-wells culture dish in a concentration of 5×10^5 cells/well in RPMI-1640. After an overnight culture period, cells were washed and incubated with various concentrations of MP-1 for 2 h. Subsequently, 20 μl of MTT solution (5 mg/ml in PBS) were added in a total volume of 200 μl medium. Cells were incubated for 4 h at 37 °C and 5% CO_2 whereafter the supernatant was removed and 150 μl DMSO was added to each well to dissolve produced formazan crystals. The extinction was measured at 490 nm using a microplate reader. $^{*}p < 0.05$ as compared to control.

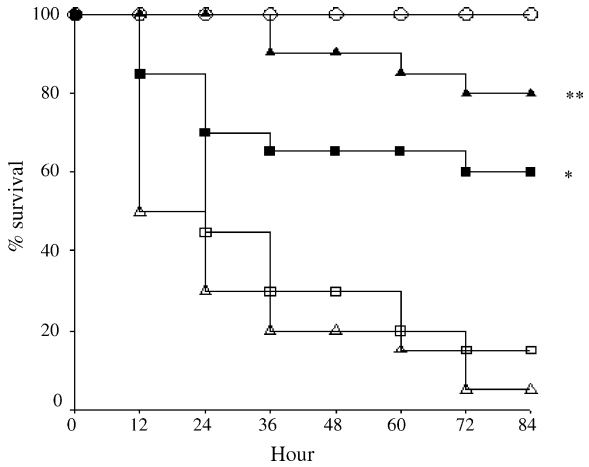

Fig. 6. Effect of MP-1 on the survival of mice challenged with LPS and live bacteria. Survival of LPS O55:B5 and bacteria challenged mice. Mice were randomly divided into five groups ($n = 20$). Groups of mice were treated with ○, MP-1 (3 mg/kg); ▲, LPS (20 mg/kg) + MP-1 (3 mg/kg); ■, living bacteria (1.0×10^8 cfu/g bodyweight) + MP-1 (3 mg/kg); □, living bacteria (1.0×10^8 cfu/g bodyweight); △, LPS (20 mg/kg). The mice were observed for 3.5 days. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to LPS group.

phages to release TNF-α and IL-6. However, pretreatment of cells with MP-1 inhibited cytokine release in response to the presence of LPS, in a dose-dependent manner.

3.7. Cytotoxic effect of MP-1 on peritoneal macrophages and RBC

Cytotoxic effects of MP-1 on peritoneal macrophages and RBC were studied in vitro with MTT assay and hemolytic activity, respectively. Macrophages treated with MP-1 (0–40 μmol/L) for 2 h did not show significant deference with normal cells (Fig. 5). In addition, when mixed with human RBC, no hemolysis was detected with a high concentration (40 μmol/L) of MP-1 (Table 4). The results indicated that no toxic effect was found at our experimental concentrations.

Table 4
Hemolytic activity of MP-1 on human red blood cells ($n = 3$)

Treatment		Hemolytic activity (%)
MP-1 (μmol/l)	80	6.5 ± 0.7
	40	0
	20	0
	10	0
	5	0
PBS solutions		0
Triton X-100 solution		100

The hemolytic activity of MP-1 was evaluated by determining the released hemoglobin of 8% suspensions of fresh human erythrocytes at 414 nm. Hundred microlitres human red blood cells (RBC) were suspended 8% (v/v) in PBS was plated into 96-well plates, and then 100 μl of MP-1 solution (from 5 to 80 μmol/L) was added to each well. The plates were incubated for 1 h at 37 °C and centrifuged at 150 g for 5 min. Hundred microlitres of the supernatant were transferred to 96-well plates. Hemolysis was measured by absorbance at 414 nm with an ELISA plate reader. The observed hemolysis of RBC in PBS solutions and in 0.1% Triton X-100 solution were used as negative and positive controls, respectively. Zero percentage and 100% hemolysis was determined in PBS and 0.1% Triton X-100, respectively. The hemolysis percentage was calculated using the following equation: $\%\text{hemolysis} = \frac{(A_{\text{MP-1}} - A_{\text{negative}})}{(A_{\text{positive}} - A_{\text{negative}})} \times 100$.

3.8. MP-1 protected sepsis mouse models and reduced cytokine release in vivo

In vitro data suggests that MP-1 has the ability to protect mice from lethal challenge. In order to investigate whether MP-1 could protect mice from lethal challenge by LPS and *E. coli* in vivo, we made sepsis mouse models using a lethal

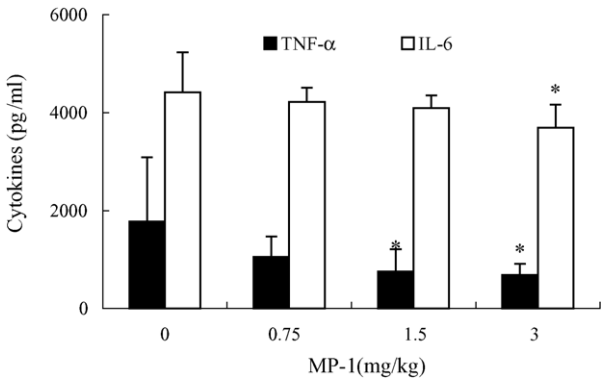

Fig. 7. Effect of MP-1 on pro-inflammatory cytokines (pg/ml) release in mice challenged with lethal LPS O55:B5. Forty mice were randomly divided into five groups (8 mice/group). Group 1 was given 10 mg/kg LPS O55:B5, groups 2, 3 and 4 were given 0.75, 1.5 and 3 mg/kg, respectively. MP-1 followed by infusion with LPS O55:B5. Group 5 was infused with Ringer's solution as a negative control. Blood samples (0.5 ml) were drawn at 0.5 h after inception of the experiment and assayed using the appropriate ELISA kits. Cytokine levels were expressed as mean ± S.D. Student's *t*-test was used to examine the differences. $^{*}p < 0.05$ and $^{**}p < 0.01$ as compared to the LPS group. The levels of TNF-α and IL-6 in medium were (27.5 ± 7.6) pg/ml and (99.1 ± 100.0) pg/ml, respectively.

dose of LPS and *E. coli*. Mice challenged with LPS O55:B5 and *E. coli* showed progressive lethargy or restlessness, and eventually died. Most mice challenged with LPS and *E. coli* died on the first day, and most died within 3 days. In contrast, only 20 or 40% of mice in the groups pretreated with MP-1 prior to the LPS or *E. coli* challenge died within 2 days (Fig. 6). These results indicate that MP-1 had the ability to protect mice from LPS and bacteria.

In vitro, MP-1 has been shown to inhibit the release of cytokines in macrophages. In our in vivo experiments, MP-1 also inhibited release of TNF-α and IL-6, in a dose-dependent manner, in LPS-stimulated mice (Fig. 7).

In summary, our results demonstrate that MP-1 could potently suppress LPS-induced release of TNF-α (an early cytokine) and IL-6 (a later cytokine) both in vitro and in vivo.

4. Discussion

The primary aim of this study was to investigate the activity of MP-1 in bacterial death and the neutralization of LPS in vitro and in vivo. Here, we found that mice treated with heat-inactivated bacteria or LPS alone usually died within 24 h. Mice that lived beyond day 3 survived; this is possibly due to the rapid metabolism of bacterial components and LPS [27]. In contrast, mice treated with MP-1 were significantly more likely to survive past 3 days. This effect is correlated with the ability of MP-1 to kill bacteria and to inhibit the LPS-induced release of inflammatory mediators such as TNF-α, IL-6 and superoxide anion by immune cells. Thus, MP-1 plays an important regulatory role in the production of early and later pro-inflammatory cytokines that are known to be involved in sepsis.

Previous studies have shown that MPs exhibit antimicrobial activity against bacteria [28,29]. As a homolog of MPs, MP-1 also revealed its antimicrobial activity. MIC and MBC demonstrated that MP-1 exhibited bactericidal activity for some Gram-negative and Gram-positive bacteria (Table 1). The observation by thin section EM suggest the mechanism of this bactericidal effect. The bactericidal effect on *E. coli* 25922 may be related to an increased membrane permeability (Fig. 1). Li et al. found that swelling appeared on the surface of some *E. coli* cells after addition of this peptide to the cultures for 2 h, and indicated that it was possible for MP-1 to modulate antibacterial activity on the cellular surface that led to cell lysis [29]. In our study, we get similar results to those in Li's experiments. But MP-1 seemed exert earlier effect on the surface of *E. coli*.

The helical structure of MP-1 could bind to lipid bi-layer membranes, and lead to a change in bacterial membrane permeability [29]. Solomon et al. reported that mastoparan could protect Wistar rats from LPS-induced lethality and that mastoparan inhibited cytokine production from LPS-stimulated human peripheral blood mononuclear cells (hPBMC), but its mechanism remained unclear [12]. Therefore, we hypothesized that amphiphilic antibacterial peptides kill bacteria by binding to LPS. Our data demonstrated that MP-1 pretreatment of murine macrophages inhibited the release of TNF-α and IL-6, induced by LPS (Fig. 4). Because pro-inflammatory cytokines such as TNF-α and IL-6 are well-known markers of sepsis, our data suggests that cytokine inhibition may be an important mechanism of MP-1 that protects mice challenged with LPS.

LPS consists of three chemical parts, one of which is lipid A, an evolutionary conserved region. This has been identified as the toxic component of LPS. The two phosphate groups of lipid A exhibiting negative charges interact with the four positive charges in MP-1 produced by one asparagines and three lysine residues. In addition, over 70% of the amino acids that are hydrophobic which leads to binding of the fatty acyl groups of lipid A to MP-1 in hydrophobic interaction.

In our in vitro experiments, the affinities of MP-1 for LPS and lipid A were measured by affinity sensor technology, and were 484 and 456 nM, respectively (Table 2). To characterize the activity of this peptide, it was compared with that of PMB, which is one of the strongest binders of LPS and is a potent anti-endotoxin peptide [30]. PMB is a positively charged amphipathic cyclic oligopeptide linked to a single fatty acid, and has been shown to bind lipid A stoichiometrically (1:1) with a K_d of approximately 10–100 nM (as assessed by different methods) [31]. Our data demonstrated that the affinity of MP-1 for LPS and lipid A was lower than that of PMB, which bound to LPS and lipid A with high-affinity (K_d of 19 and 11 nM, respectively) (Table 2). This suggests a binding action between MP-1 and the LPS molecule, which could result in the reduction of LPS activity.

To confirm that the binding ability of MP-1 to LPS and lipid A represents the LPS-neutralizing activity, we used LAL tests to reveal the neutralizing activities of MP-1 on LPS. Our data demonstrated that the ability of MP-1 to neutralize LPS was equal to about one-third of PMB's ability to neutralize LPS (Fig. 2). Because the amount, position of negative charge and space conformation are closely related to the activity of LPS, we hypothesized that MP-1 binds the negative phosphate groups, thus changing the negative characteristic of LPS and leading to the loss of its activity.

LPS-induced signal transduction in macrophages is mediated by TLR4, as shown by the failure of TLR4-deficient mice to respond to LPS [32]. Most often, anti-LPS agents inhibit TLR4 mRNA transcription and its protein expression, as well as TNF-α and IL-6. Here, we investigated whether MP-1 inhibits TLR4, TNF-α and IL-6 mRNA transcriptions. RT-PCR results demonstrated that mRNA transcription of TLR4, TNF-α and IL-6 increased in LPS-stimulated murine peritoneal macrophages (100 ng/ml), but MP-1 inhibited these mRNA transcriptions in a dose-dependent manner (Fig. 3).

The mechanism of MP-1 effecting on LPS is not similar to some other current investigational anti-LPS drugs, such as E5564, a structural analog of the lipid A portion of LPS. Mullarkey et al. reported that in in vitro experiment, E5564 dose-dependently inhibited LPS-mediated TNF-α release from primary cultures of human myeloid cells and murine macrophage cell lines. In in vivo experiment, E5564 blocked induction of LPS-induced cytokines and LPS or bacterial-induced lethality in primed mice [33]. E5564 and LPS might share a common mechanism of cell activation by interacting with TLR4, resulting in inhibition of the biological effects of LPS [34]. However, the mechanism of MP-1 effecting on LPS is similar to some other current investigational anti-LPS drugs, such as the BPI, a natural protein stored within the neutrophil granules. BPI also had potent antibacterial and anti-LPS activities in vitro and in vivo [35]. A recombinant BPI (rBPI) protects animals against the effects of Gram-negative bacteria and LPS. The affinity of rBPI for LPS/lipid A was approximately 2–5 nM [36], higher than MP-1.

Solomon et al. reported a functional association between GPI-anchored protein and G proteins [12]. Since MPs activate G proteins [11,12], we wondered whether MP-1 inhibits the signal transduction of G protein. Macrophage respiratory burst is the representative result of G proteins activated by LPS. NADPH oxidase is key enzyme in macrophage respiratory burst; therefore, it could produce superoxide anions in the presence of NADPH. In the current study, we used a detergent-induced cell-free system. With this system, the assembly of an active NADPH oxidase required the participation of at least five protein, including p22 (phox), gp91 (phox) and cytosolic components as follows: p47 (phox), p67 (phox), p40 (phox) and the small GTPase Rac (1 or 2) [37,38]. Tisch et al. hypothesized that p67-phox may be the main mastoparan-binding component. The binding site on p67-phox was localized on the 1–238 amino terminal fragment of the molecule and NADPH oxidase activation, supported by this fragment, was inhibited by mastoparan [39,40]. Our results showed that MP-1 significantly inhibited superoxide anion formation, hydrogen peroxide formation and the activity of NADPH oxidase in a dose-dependent manner. Additional studies are required to assess whether or not MP-1 binds to the same site as NADPH.

The inhibitory effects of MP-1 on LPS-induced peroxide, oxidase, cytokines and mRNA activities/amounts were not due to a direct toxic effect of MP-1 on the macrophages. Firstly, 40 μM MP-1 did not affect the expression of β-actin housekeeping gene (Fig. 3). Secondly, macrophages viability was measured by morphology and MTT assays was unaffected by MP-1. More importantly, in mice treated with MP-1, we did not observe any side effects such as liver or kidney dysfunction (data not shown).

In conclusion, MP-1 is a potent anti-LPS agent with a high-affinity for LPS/lipid A and a strong inhibitory effect on the production of radical oxygen species in vitro. It is also an effective bactericidal agent. MP-1 treatment protected mice from LPS challenge and decreased LPS-triggered TNF-α and IL-6 release by decreasing blood endotoxin in vivo, suggesting that MP may warrant future study as a clinical anti-sepsis agent.

Acknowledgments

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References

[1] Titheradge MA. Nitric oxide in septic shock. Biochim Biophys Acta 1999;1411:437–55.
[2] Angus DC, Linde-Zwirble WT, Lidicker J, Clermont G, Carcillo J, Pinsky MR. Epidemiology of severe sepsis in the United States: analysis of incidence, outcome, and associated costs of care. Crit Care Med 2001;29:1303–10.
[3] Bernheimer AW, Rudy B. Interactions between membranes and cytolytic peptides. Biochim Biophys Acta 1986;864:123–41.
[4] Degrado WF. Design of peptides and proteins. Adv Protein Chem 1988;39:51–124.
[5] Steiner H, Hultmark D, Engstrom A, Bennich H, Boman HG. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature 1981;292:246–8.
[6] Gilman AG. G proteins: transducers of receptor-generated signals. Annu Rev Biochem 1987;56:615–49.
[7] Higashijima T, Uzu S, Nakajima T, Ross EM. Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). J Biol Chem 1988;263:6491–4.
[8] Argiolas A, Pisano JJ. Facilitation of phospholipase A2 activity by mastoparans, a new class of mast cell degranulating peptides from wasp venom. J Biol Chem 1983;258:13697–702.
[9] Okano Y, Takagi H, Tohmatsu T, Nakashima S, Kuroda Y, Saito K, et al. A wasp venom mastoparan-induced polyphosphoinositide breakdown in rat peritoneal mast cells. FEBS Lett 1985;188:363–6.
[10] Suh BC, Song SK, Kim YK, Kim KT. Induction of cytosolic Ca^{2+} elevation mediated by Mas-7 occurs through membrane pore formation. J Biol Chem 1996;271:32753–9.
[11] Higashijima T, Wakamatsu K, Takemitsu M, Fujino M, Nakajima T, Miyazawa T. Conformational change of mastoparan from wasp venom on binding with phospholipid membrane. FEBS Lett 1983;152:227–30.
[12] Solomon KR, Kurt-Jones EA, Saladino RA, Stack AM, Dunn IF, Ferretti M, et al. Heterotrimeric G proteins physically associated with the lipopolysaccharide receptor CD14 modulate both in vivo and in vitro responses to lipopolysaccharide. J Clin Invest 1998;102:2019–27.
[13] Lebrun I, Cavallaro V, Juliano L, Juliano MA, de Sousa e Silva MC. Effects of 'casoparan', a peptide isolated from casein hydrolysates with mastoparan-like properties. Mediat Inflamm 2004;13:263–8.
[14] Oh H, Hedberg M, Wade D, Edlund C. Activities of synthetic hybrid peptides against anaerobic bacteria: aspects of methodology and stability. Antimicrob Agents Chemother 2000;44:68–72.
[15] Zheng J, Zhou H, Gong XY, Lv GF, Wei G, Lu YL. A synthetic peptide derived from bactericidal/permeability increasing protein (BPI) neutralizes endotoxin in vitro and in vivo. Int Immunopharm 2004;4:533–43.

[16] Pick E, Mizel D. Rapid microassays for the measurement of superoxide and hydrogen peroxide production by macrophages in culture using an automatic enzyme immunoassay reader. J Immunol Methods 1981;46:211–26.
[17] Nathan CF, Root RK. Hydrogen peroxide release from mouse peritoneal macrophages: dependence on sequential activation and triggering. J Exp Med 1977;146:1648–62.
[18] Aviram I, Sharabani M. Inositol lipids and phosphatidic acid inhibit cell-free activation of neutrophil NADPH oxidase. Biochem Biophys Res Commun 1989;161:712–9.
[19] Fujita I, Takeshige K, Minakami S. Characterization of the NADPH-dependent superoxide production activated by sodium dodecyl sulfate in a cell-free system of pig neutrophils. Biochim Biophys Acta 1987;931:41–8.
[20] Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Immunol Methods 1983;65:55–63.
[21] Putsep K, Branden CI, Boman HG, Normark S. Antibacterial peptide from *H. pylori*. Nature 1999;398:671–2.
[22] Guha M, Mackman N. LPS induction of gene expression in human monocytes. Cell Signal 2001;13:85–94.
[23] An H, Xu H, Yu Y, Zhang M, Qi R, Yan X, et al. Up-regulation of TLR9 gene expression by LPS in mouse macrophages via activation of NF-kappaB, ERK and p38 MAPK signal pathways. Immunol Lett 2002;81:165–9.
[24] Harbarth S, Holeckova K, Froidevaux C, Pittet D, Ricou B, Grau GE, et al. Diagnostic value of procalcitonin, interleukin-6, and interleukin-8 in critically ill patients admitted with suspected sepsis. Am J Respir Crit Care Med 2001;164:396–402.
[25] Lipford GB, Sparwasser T, Bauer M, Zimmermann S, Koch ES, Heeg K, et al. Immunostimulatory DNA: sequence-dependent production of potentially harmful or useful cytokines. Eur J Immunol 1997;27:3420–6.
[26] Yao YM, Redl H, Bahrami S, Schlag G. The inflammatory basis of trauma/shock-associated multiple organ failure. Inflamm Res 1998;47:201–10.
[27] Zhou H, Zheng J, Wang LX, Ding GF, Luo P, Lu YL, et al. Chloroquine protects mice from challenge with CpG ODN and LPS by decreasing proinflammatory cytokine release. Int Immunopharma 2004;4:223–34.
[28] Ho CL, Hwang LL. Structure and biological activities of a new mastoparan isolated from the venom of the hornet *Vespa basalis*. Biochem J 1991;274:453–6.
[29] Li ML, Liao RW, Qiu JW, Wang ZJ, Wu TM. Antimicrobial activity of synthetic all-D mastoparan M. Int J Antimicrob Agents 2000;13:203–8.
[30] Ghiselli R, Giacometti A, Cirioni O, Circo R, Mocchegiani F, Circo R, et al. Neutralization of endotoxin in vitro and in vivo by Bac7 (1–35), a proline-rich antibacterial peptide. Shock 2003;19:577–81.
[31] Ried C, Wahl C, Miethke T, Wellnhofer G, Landgraf C, Schneider-Mergener J, et al. High affinity endotoxin-binding and neutralizing peptides based on the crystal structure of recombinant Limulus anti-lipopolysaccharide factor. J Biol Chem 1996;271:28120–7.
[32] Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, et al. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999;162:3749–52.
[33] Mullarkey M, Rose JR, Bristol J, Kawata T, Kimura A, Kobayashi S, et al. Inhibition of endotoxin response by e5564, a novel Toll-like receptor 4-directed endotoxin antagonist. J Pharmacol Exp Ther 2003;304:1093–102.
[34] Hawkins LD, Ishizaka ST, McGuinness P, Zhang H, Gavin W, DeCosta B, et al. A novel class of endotoxin receptor agonists with simplified structure, toll-like receptor 4-dependent immunostimulatory action, and adjuvant activity. J Pharmacol Exp Ther 2002;300:655–61.
[35] Elsbach P, Weiss J. Role of the bactericidal/permeability-increasing protein in host defence. Curr Opin Immunol 1998;10:45–9.
[36] de-Haas CJ, Haas PJ, van-Kessel KP, van-Strijp JA. Affinities of different proteins and peptides for lipopolysaccharide as determined by biosensor technology. Biochem Biophys Res Commun 1998;252:492–6.
[37] Abo A, Boyhan A, West I, Thrasher AJ, Segal AW. Reconstitution of neutrophil NADPH oxidase activity in the cell-free system by four components: p67-phox, p47-phox, p21rac1, and cytochrome b-245. J Biol Chem 1992;267:16767–70.
[38] Rotrosen D, Yeung CL, Katkin JP. Production of recombinant cytochrome b558 allows reconstitution of the phagocyte NADPH oxidase solely from recombinant proteins. J Biol Chem 1993;268:14256–60.
[39] Tisch D, Sharoni Y, Danilenko M. The assembly of neutrophil NADPH oxidase: effects of mastoparan and its synthetic analogues. Biochem J 1995;310:715–9.
[40] Tisch-Idelson D, Fridkin M, Wientjes F. Structure-function relationship in the interaction of mastoparan analogs with neutrophil NADPH oxidase. Biochem Pharmacol 2001;61:1063–71.