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Inflammation Research

Screening of mimetic peptides for CD14 binding site with LBP and antiendotoxin activity of mimetic peptide *in vivo* and *in vitro*

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Abstract. *Objective and Design*: The study was aimed at screening out the mimetic peptides from the binding site of lipopolysaccharide binding protein and CD 14, and then observing if the mimetic peptide will inhibit *in vitro* LPS-induced inflammatory reaction and function as an anti-endotoxin in the model of LPS-induced acute lung injury.
Material and Methods: Human monocytic cell line (U937) was used *in vitro*. Thirty three-month-old SD rats were used. Phage display peptide library was adapted to screen mimetic peptide sequences.
Treatment: U937 cells were exposed to treatment with LPS and rhLBP and then were incubated with MP12 at three different concentrations after they were induced and differentiated by PMA. LPS intravenous injection was used to establish a model of rat acute lung injury which was later treated with intravenous injection of MP12.
Results: We successfully obtained the mimetic peptide of lipopolysaccharide-binding protein and CD 14 binding site, the gene sequence of which is FHRWPTWPLPSP (MP12). MP12 can markedly inhibit LPS induced TNF-α expression. MP12 can evidently increase PaO_2 of rats with acute lung injury and also increase the survival rate of these rats.
Conclusions: MP12 (FHRWPTWPLPSP) has the same function as mimetic of lipopolysaccharide-binding protein and CD 14 binding site. The application of MP12, both *in vitro* and *in vivo*, confers the biological activity required to antagonise LBP/CD14 and block LPS inflammatory signals, and it can markedly enhance PaO_2 of rats suffering from acute lung injury and also enhance their survival rate.

Key words: Lipopolysaccharide – Lipopolysaccharide-binding protein – CD14 – Antiendotoxin

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Introduction

Acute lung injury (ALI)/ Acute respiratory distress syndrome (ARDS), a leading cause of mortality in clinics [1], is characterized by extensive neutrophil influx into the lungs, production of proinflammatory mediators, and damage of lung epithelial and endothelial surfaces [2, 3, 4]. Lipopolysaccharide (LPS), or endotoxin, is the major structural and functional component of the outer membrane of gram-negative bacteria [5, 6], which has been recognized as the principal component responsible for causing ALI/ARDS. These complex macromolecules exhibit a variety of toxic and proinflammatory activities that are associated with the lipid A moiety and are causally related to the pathogenesis of gram-negative sepsis and ALI [7, 8].

Normally, the endotoxin level in human body appears to be very low. LPS concentration in the blood plasma is only 0.01~1 ng/ml, even when the individual is suffering from G-bacilli sepsis [9, 10]. The proinflammatory role of such low-level LPS relies on the endotoxin-sensitivity enhancing system, LBP/CD14, which is located upstream of the proinflammatory signal path and can pass on and proliferate the LPS proinflammatory signal [11, 12]. So antagonism of the endotoxin-sensitivity enhancing system, LBP/CD14, can efficiently inhibit the proinflammatory role of LPS [13, 14, 15].

During the course of the LPS proinflammatory reaction, LBP has double functions: on the one hand, LBP passes LPS to CD14 and activates the inflammatory signal path and, on the other hand, LBP carries LPS to HDL and then removes the LPS from the blood plasma through the liver [16, 17, 18]. So, it is necessary to find a proper antagonism target, which not only exerts an antagonism against LBP/CD14`s effect on the sensitization of LPS, but also does not influence LBP`s functions to remove and neutralize LPS, offering a more ideal strategy to prevent and cure endotoxin-induced acute lung injury.

After the N-terminus of LBP combines with LPS, the proinflammatory signal can be transmitted only by the combination of the C-terminal of LBP with mCD14 to form a ternary complex (LPS/LBP/CD14) [19, 20]. So, we assumed that the antagonism of the binding sites (blocking functional sites on mCD14 that combine LBP in the form of mimetic peptide) between LBP and mCD14 would not only block the LPS proinflammatory signal path, but also may not affect the fate of LBP/LPS evacuated through the HDL. Therefore, phage display peptide library, phages ELISA and LBP competitive inhibition experiments and DNA screening for testing sequence were jointly adopted, along with the attainment of mimetic peptide sequences (MP12). Using both in vivo and in vitro experiments, we have observed that the biological activity of LPS to cause inflammation was blocked by MP12 and that rats suffering from lipopolysaccharide-type acute lung injury were protected by MP12.

Materials and methods

Reagents

LPS (from *E. coli* 0111:B4), FITC-conjugated LPS (from *E. coli* 0111:B4), was purchased from Sigma (USA. 12 peptides phage display peptide library was purchased from New England Biolabs (USA). ELISA kit for tumor necrosis factor-alpha (TNF-α) was purchased from Jingmei Chemical (China). RT-PCR kit was purchased from TaKaRa (Japan). All materials used in cell culture were certified as LPS free and were tested by the Limulus amoebocyte lysate assay method to confirm the absence of detectable LPS.

Cell preparation and stimulation

Human monocyte-like cell line U937 cells were grown in RPMI 1640 modified to contain 2 mmol/L L-glutamine, 10 mmol/L HEPES, 1 mmol/L sodium pyruvate, 4500 mg/L glucose, 1500 mg/L sodium bicarbonate, and 10 % fetal calf serum (FCS) in cell culture plates. Cells were stimulated for 24 h with 20 ng/ml phorbol myristate acetate (PMA) in order to induce their differentiation to macrophage-like phenotype stage. The PMA-treated U937 cells became adherent and ceased to proliferate.

Cells were treated with PBS in the presence of 100 ng/ml rhLBP (control group), 100 ng/ml LPS in the presence of 100 ng/ml LBP (LPS group), and 0.1, 1 and 10 μg/ml MP12 followed by the addition of 100 ng/ml LPS in the presence of 100 ng/ml LBP (MP12-treated groups).

Murine AMs were harvested from SD rats by broncho-alveolar lavage (BAL) with 0.01 M phosphate buffer (PBS). The cells were spun at 200 × *g* to pellet cells and then washed twice with complete RPMI 1640. Total cells were counted from each sample using a hemocytometer. Cells were pooled, washed, and then resuspended in RPMI 1640 in a final concentration of 1×10^5 cells/ml. Cells were then cultured in cell culture plates for 2 h and were washed with PBS for removing the nonadherent cells. Adherent monolayer cells were stained with trypan blue to ensure the viability of AMs. AMs were used for fluorescence microscope observation and for flow cytometric analysis (FACS).

Immunohistochemistry

Immunohistochemistry was performed on cell smears. Endogenous peroxidase was quenched with 3 % hydrogen peroxide for 20 min at room temperature. After blocking with normal mouse serum for 20 min, the cell smears were incubated with a murine monoclonal anti-human CD14 (1:500; Novocastra, United Kingdom) for 1 h at room temperature. Cell smears were incubated with the secondary antibody, biotinylated anti-mouse immunoglobulin, for 20 min at 37 ºC. Following incubation with streptavidin-horseradish peroxidase for 25 min at 37 ºC, 3' 3'-diaminobenzidine was used as a chromogen. Negative controls consisted of cell smears incubated in the absence of the primary antibody.

Establishment of LPS-induced ALI model and anti-endotoxin therapy

The Animal Care and Use Committee of the Third Military Medical University approved all experiments. Kunming mice (three month of age, 180 ± 12 g) were purchased from the Animal Center of Third Military Medical University and were acclimatized for 1 wk in the animal housing facility before the experiments were performed. Rats in the 3 groups ($n = 10$ in each group) were treated with LPS (LPS group), LPS plus MP12 (LPS+MP12 group), and saline (control group), respectively. Rats in the LPS group were injected with LPS (6 mg/kg.BW) dissolved in 500 μl saline via the jugular vein. Those in the LPS + MP12 group were injected with LPS (6 mg/kg.BW) and were then immediately injected with MP12 (5 mg/kg.BW) via the jugular vein. Rats in the control group were injected with the same volume of saline. At 2 h after the LPS challenge, the arterial blood samples were collected by heparinized vacuum hemostix from rat arteria carotis communis and were used for detection of PaO2 (IL-1620, Instrumentation Lab, USA). After 3 days or after the rat died, a part of lung tissues was collected and put into 10 % buffered formalin for immediate fixation. Paraffin-embedded 5-μm sections of lungs were cut and stained with hematoxylin and eosin. The number of deaths was used to calculate the mortality of each group.

Measurement of endotoxin

One mL blood was taken from the jugular vein and immediately collected into an apyrogenic tube (containing heparin). Plasma was taken after the blood was centrifuged at 3000 r/min for 10 min (environmental temperature: 0 ºC). The levels of endotoxin were measured by limulus amebocyte lysate test.

Toxicity tests

Trypan blue exclusion indicated the toxicity of PMA and MP12 to U937 cells.

Panning of a phage display library

For the first round of phage selection, 1.5 ml of CD14 (100 μg/ml in 0.1 M $NaHCO_3$) were added to a 60-mm culture plate and incubated at 4 ºC overnight. The solution was then removed by suction and the plate was treated at room temperature with blocking solution (0.1 M $NaHCO_3$, 5 mg/ml BSA, 0.1 g/ml streptavidin, and 0.02 % w/v NaN_3) for 2 h. The plate was then washed 5 times with a TBS/Tween solution (0.5 % Tween 20 in 50 mM Tris–HCl, pH 7.5, 150 mM NaCl). After the last wash, 1.5 ml of phage library (4×10^{10} virions in 1.5 ml of TBS/Tween solution, a phage library kit, New England Biolabs, USA) were added and incubated at 4 ºC for 1 h. The plate was then washed 10 times with TBS/Tween, and bound phages were eluted by adding 1 ml of elution buffer (0.1 N HCl adjusted to pH 2.2 with glycine, 1 mg/ml BSA, and 0.1 mg/ml phenol red) and incubated at room temperature under stirring for 8 min. The solution of eluted phages was neutralized by adding Tris–HCl (pH 9.1) and kept in the dark at 4 ºC until its amplification. From the second to fourth rounds of phage selection, 1.5 ml of amplified phages from previous round, were submitted to the same protocol as in first round, with the exception that the final concentrations of CD14 were 10, 5, and 1 μg/ml, respectively. The bound phages in the third and fourth rounds were eluted by adding 1.5 ml of LBP (10 μg/ml , R&D Biolabs).

ELISA for selected phage clones binding to CD14

ELISA was performed as a standard protocol to check the binding specificity of 100 selected phage clones with CD14. The 96-well microtiter plates were coated with the CD14, and the plates were incubated at 4℃ overnight. Blocking was carried out with 1% BSA. The plates were washed 3 times with PBS. Isolated phage clones were added to each well and incubated for 1.5 h, followed by detection with horseradish peroxidase (HRP)-conjugated mouse anti-M13 phage monoclonal antibody (Amersham Pharmacia Biotech, Uppsala, Sweden) at a dilution of 1:5000. The substrate consisted of 0.22 mg/ml ABTS and 50 mM citric acid (pH 4.0). The optical density (OD) of the content in each well was determined compared with the blank at 495 nm using an ELISA reader. Binding of phage to CD14 was considered specific if the rate of OD in phage and OD in control was >5.

DNA sequencing and synthesis peptide

The DNA sequence of phage clones with higher affinity activity to CD14 was analyzed using a primer (5'-CCCTCATAGTTAGCGTAACG-3') with an ABI DNA sequencer 310. A peptide was synthesized by the solid phase method of Merrifield using the Fmoc. To increase the stability of the mimic peptide, an NH_2 residue was added to its carboxyl terminal and acetyl modifications were applied to its N-terminus. The modified peptide named MP12 was purified to homogeneity by reversed phase-HPLC. The molecular weight of MP12 was 1562.00 and its purity was higher than 95%.

Competitive Inhibition Assay for the synthetic peptide (MP12)

To test the competitive inhibition of the MP12 with LBP, a microtiter plate was coated with CD14 (1.0 μg/ml) and was incubated at 4℃ overnight. After the plates were blocked and washed, LBP (10 μg/ml) was added to the control well. LBP and MP12 were added to the test well and were incubated for 2 h (MP12 was tested at 0.1, 1.0, and 10 μg/ml), followed by detection with HRP-conjugated mouse anti-rhLBP monoclonal antibody. OD_{495} was measured by Lambda Bio-20 (PE). The inhibition was calculated using the following formula: [(OD control - OD test)/ OD control] × 100%.

Isolation of RNA and generation of cDNA by reverse transcription

Total RNA was isolated using TRIzol (Roche) from 5×10^5 U937 cells treated or not treated as previously described. Isolated RNA was purified using an RNeasy mini kit (TaKaRa) and was reverse-transcribed to cDNA as follows. For each reaction, 2 μg of

RNA was diluted to 11 μl with UltraPure distilled water (Gibco, USA) and mixed with 1 μl of 0.5 μg/μl oligo (dT)12–18 primer (Promega) and 1 μl of 10 mM dNTP mix (Promega), heated to 65 °C for 5 min, and quickly chilled on ice. Next, 4 μl of 5X first standard buffer and 2 μl of 0.1 M dithiothreitol were added, and the mixture was incubated for 2 min at 42 °C. For each reaction, 0.5 μl of M-MLV reverse transcriptase (Promega) was added, and the mixture was incubated for 60 min at 42 °C. Finally, each reaction was mixed with 0.5 μl of RNase H (MBI) and incubated for 20 min at 37 °C.

Reverse transcription polymerase chain reaction (RT-PCR)

PCR was carried out using cDNAs and AccuPrime SuperMix I (Invitrogen) with initial denaturation for 2 min at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s (for both TNF and -actin), annealing at 56 °C for 30 s (for both TNF-α and β-actine), and extension at 72 °C for 50 sec. A final annealing step was performed for 10 min at 72 °C. The PCR products were visualized using agarose gel electrophoresis, followed by ethidium bromide staining. The primers for TNF-α and β-actin were as follows: TNF-α (the length of production: 655 bp): F-5'CGA GTC TGG GCA GGT CTA CTT T 3', R-5' GCT ACA TGG GAA CAG CCT ATT GT 3'; β-actin (the length of production: 210 bp): F-5'GGA TCA GCA AGC AGG AGT ATG 3', R-5'CAC CTT CAC CGT TCC AGT TTT 3'. All primers were synthesized by Sangon (Shanghai, China).

TNF-αα assay

The number of U937 cells was adjusted to 1×10^6 cells/ml with culture medium and treated or not treated for 4 h as previously described. The cell suspension was transferred to the vials and centrifuged at 1,500 rpm for 5 min. The supernatants were removed and the concentrations of TNF-α in the supernatant were evaluated with a human TNF-α assay kit (Quantikine HS: R & D systems, Lambda Bio-20, PE).

Fluorescence microscopic observation and flow cytometric analysis (FACS)

In the LPS plus MP12 group, FITC-conjugated LPS (FITC-LPS, 100 ng/ml) and MP12 (1.0 μg/ml) were added into the culture of AMs (5×10^5 cells/ml) in presence of LBP (100 ng/ml). In the LPS group and control group, FITC-LPS (100 ng/ml) or the same volume of saline was added into the culture of AMs in presence of LBP (100 ng/ml), respectively. After 30 min at 37 °C, AMs were washed with phosphate buffer (PBS) and the binding of FITC-LPS was analyzed by fluorescence microscope observation and FACS, and the median fluorescence intensity (MFI) was determined, representing the LPS transfer to the surface of AMs.

Pathological examination and pathological score semi-quantitative analysis

Histological sections were processed in the usual manner: cut at 5 μm and stained with H&E. The change in pathological modality for lung tissue was observed under light microscopy and the integrity of the structure of lung was observed to see if there was pulmonary interstitial edema, inflammatory cell infiltrate, alveolar hemorrhage, hyaline-membrane formation, and atelectasis. Pathomorphic semi-quantitative analysis: done according to the grades of pulmonary interstitial edema, inflammatory cell infiltrate, alveolar hemorrhage, hyaline-membrane formation, atelectasis and by the degrees of "zero, light, moderate and severe", marking each as "0, 1, 2, 3", and then adding up to the total score.

Statistical analysis

Differences among groups were determined using one-way ANOVA test and Fisher exact test. A p value of 0.05 was considered to be of significance. Values are presented as mean ± SEM.

Results

Enrichment of the phage library

To monitor the enrichment of the bio-panning process, the input and output phage of each bio-panning round was titered. The titering process was performed according to the manufacturer's protocol without any modifications. With the above information, the recovery percentage of each round

Table 1. Enrichment of the phage library from rounds 1 to 4 expressed as recovery percentage (%)

Rounds	Input phage (pfu)	output phage (pfu)	Recovery (%)
1	4.0×10^{10}	1.4×10^{5}	3.5×10^{-6}
2	2.2×10^{12}	3.8×10^{7}	1.7×10^{-5}
3	6.8×10^{12}	5.3×10^{8}	7.8×10^{-3}
4	8.5×10^{12}	4.4×10^{9}	5.2×10^{-2}

Recovery (%) output phage/Input phage

can be derived by dividing the quantity of eluted phage by the quantity of input phage. The recovery percentage (%) was found to increase from 3.5×10^{-6} to 5.2×10^{-2} from rounds 1 to 4 (Table 1).

Selected phage clones binding to rhCD14

One hundred clones were randomly singled out among phages that had gone through four rounds of panning, and then ELISA was used to identify the binding activity between phage display polypeptide and CD14. The result showed that 73 phage clones had a higher binding power (OD_{405} value was over 5 fold higher than that of the control group), the OD_{405} values of 25 phage clones of which were found to be over 20 times higher than that of the control group (Fig. 1).

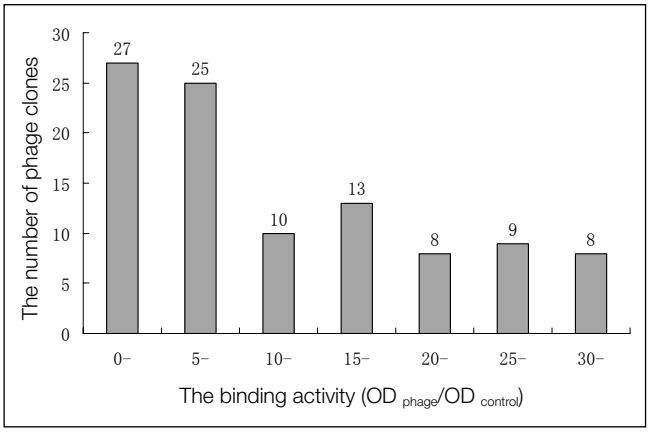

Fig. 1. Test results of binding activity between 100 randomly chosen phage clones and rhCD14. ELISA was used to identify the binding activity between phage display polypeptide and rhCD14. The OD phage/OD control of phage clones were used to express the binding activity between phage clones and rhCD14. Phage clones were found to have 5-fold higher rhCD14 binding activity than OD phage/OD control.

DNA sequencing of twenty-five phage clones with higher affinity activity with rhCD14

Twenty-five phage clones showing higher rhCD14 binding activity were subjected to DNA sequencing, through which 8 different peptide sequences were acquired (Table 2). The FHR amino acid residues from no.1 to no.3 were highly conserved, and the HPP from no.4 to no.6 and the amino

Table 2. DNA sequencing results of 25 phage clones with higher rhCD14 binding activity. Red letters represent the amino acid residues which are highly conservative and blue letters represent the amino acid residues which are less highly conservative.

No. of Sequence	Sequence														No.of clone
Sequence 1			**F**	**H**	**R**	W	**P**	T	W	**P**	L	P	S	P	15, 21, 33, 80 55, 71, 86, 90
Sequence 2	A	A	**F**	**H**	**R**	A	H	H	L	T	S	P			56, 17
Sequence 3			M	**H**	**R**	**H**	**P**	**P**	P	I	**T**	**L**	P	L	92, 72, 40
Sequence 4			**F**	**H**	S	N	W	**P**	K	G	S	T	S	L	30, 24
Sequence 5			**F**	P	W	**H**	F	F	S	**P**	Q	**L**	R	G	2, 18, 47
Sequence 6			M	**H**	**R**	**H**	**P**	**P**	P	I	**T**	T	A	A	66, 78, 53
Sequence 7			**F**	**H**	**R**	W	**P**	T	W	**P**	**T**	S	F	S	9, 12
Sequence 8			M	**H**	**R**	P	H	F	N	**P**	**T**	**L**	A	T	62, 67
Common			**F**	**H**	**R**	**H**	**P**	**P**	X	**P**	**T**	**L**	X	X	

100 µl of single-clone stored liquid was added to LB culture fluid, rocketing bed at 37 ºC, 225 rpm, breeding for 4.5 hours. After centrifuging at 4 ºC, 10000 rpm, for 1 min, 500 µl of supernatant fluid-containing phage, was transfered it to a new microcentrifuge tube, 200 µl of sedimentary fluid added and shaked up, then placed at room temperature for 10 minutes and later at 4 ºC, 10000 rpm, centrifuged for 10 min, and the supernatant fluid removed. The sediment weighed around 100 µl. 250 µl of alcohol was added to sodium iodide buffer, placed at room temperature for 10 min, and later at 4 ºC, 10000 rpm, centrifuged for 10 min, and the supernatant fluid removed. The sediment was washed away with alcohol of 70 % concentration, and subjected it to vacuum drying for a while. The sediment weighing around 30 µl TE was sent to Sangon Biology Company (Shanghai, China) obtain the test sequence. Sequencing primer was -96 g III 5'-GCCCTCATAGTTAGCGTAACG-3' (ph D: -12 TM kit self-prepared). The test results showed 8 peptides in total, of which FHRHPPXPTLXX was the common sequence. The no.1 peptide (FHRWPTWPLPSP) was shared by 8 phages. So, we have chosen the no.1 peptide to be artificially synthesed in order to make further study.

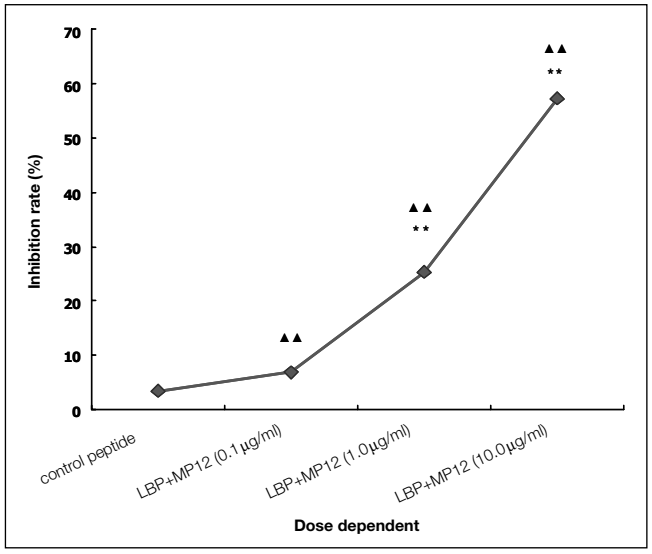

Fig. 2. MP12 competitive inhibition of binding of LBP to CD14 (concentration dependent). ELISA was used to test the competitive inhibitory activity of MP12. See specified testing methods in "Material and Methods". Competitive inhibitory activity of MP12 at three different concentrations was tested. LBP in the same concentration and random 12 peptides (final concentration 10.0 μg/ml, 50 μl) were added into the control polypeptide well and LBP added in the same concentration and diluted with 50 μl. Inhibition ratio was calculated according to the following formula: $[(OD_{495}$ control - OD_{495} test)/ OD_{495} control] × 100 %. The competitive inhibitory activity of MP12 (0.1–10.0 μg/ml) against LBP was markedly higher than that of control polypeptide. The inhibition ratio for MP12 increased with concentration. The inhibition ratio of 1.0 μg/ml and 10.0 μg/ml MP12 was evidently higher than that of 0.1 μg/ml MP12, and the inhibition ratio of 10.0 μg/ml MP12 reached 69.6 % at most, showing concentration dependence. **: $p<0.01$, versus LBP + MP12 (0.1 μg/ml) group; ▲▲: $p<0.01$, versus control peptide group. Data are presented as mean ± SEM.

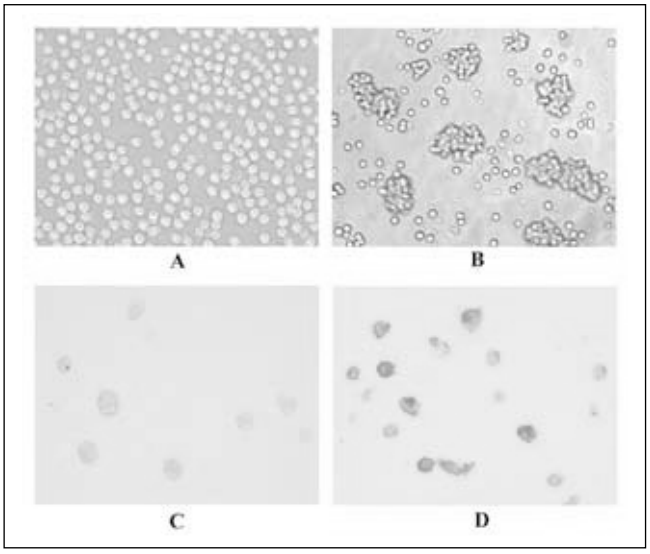

Fig. 3. PMA induced differentiation of U937 cells to macrophage-like phenotype stage
U937 cells were stimulated for 24 h with 20 ng/ml phorbol myristate acetate (PMA) in order to induce their differentiation to macrophage-like phenotype stage. Cell morphology was observed under inverted microscope. Immunohistochemistry was used to test the expression of CD14. (A) U937 cells were round and had suspended growth. (B) The PMA-treated U937 cells became adherent and ceased to proliferate. (C) U937 cells which had not been treated with PMA did not express CD14. (D) PMA-induced 24-hour U937 cells expressed a large amount of CD14.

residues from no.8 to no.10 were comparatively conserved. The 8 polypeptides shared a common sequence: FHRHPPX-PTLXX, which means that amino acid residues at 9 positions in the 12 peptides might be the framework for the binding surface between LBP and CD14. Interestingly, the no.1 polypeptide (FHRWPTWPLPSP) was shared by 8 phage clones (the clone no. included: 15, 21, 33, 80, 55, 71, 86, 90), the binding activity of the 8 clones was highly consistent with that of the rhCD14 ($OD_{phage}/OD_{control} > 25$). Hence, we have chosen the no.1 polypeptide (named MP2) to be artificially synthesised in order to make further study.

Competitive inhibition assay for MP12

A competitive inhibition assay was performed using ELISA to discover whether the synthetic peptide (MP12) and LBP competed for the same binding site on rhCD14. The results showed that the binding activity of LBP to rhCD14 was inhibited by MP12 in a dose-dependent manner. The inhibition by 1.0 and 10.0 μg/ml MP12 was 25.3 % and 57.2 %, whereas the control peptide had no effect. The peptide showed high competitive inhibition with LBP at the above-mentioned concentrations (Fig. 2).

PMA induced U937 cell differentiation to macrophage-like phenotype stage

U937 cells were round and had suspended growth and did not express CD14. They took the shape of a grape string after stimulation by PMA for 24 hours, and then had adherent growth and expressed a large amount of CD14 (Fig. 3).

Cytotoxicity tests

Cytotoxicity tests revealed that MP12 at the maximum concentration (10.0 μg/ml) and PMA at 20 ng/ml failed to display any toxicity towards U937 cells.

Influence of MP12 on TNF-α mRNA expression and protein Release

Differentiated and mature U937 ($CD14^+$) cells were used as experiment targets. On the basis of the levels of both gene and protein, we have tested how the changes in the concentrations (0.1, 1.0, 10.0 μg/ml) of MP12 affected the TNF-α expression induced by LPS. In the presence of LBP, the TNF-α mRNA expression by U937 cells and the concentration of TNF-α in the U937 cell culture supernatant were markedly increased. However, 1.0 and 10.0 μg/ml MP12 significantly inhibited the expression of TNF-α gene and protein induced by LPS. Interestingly,

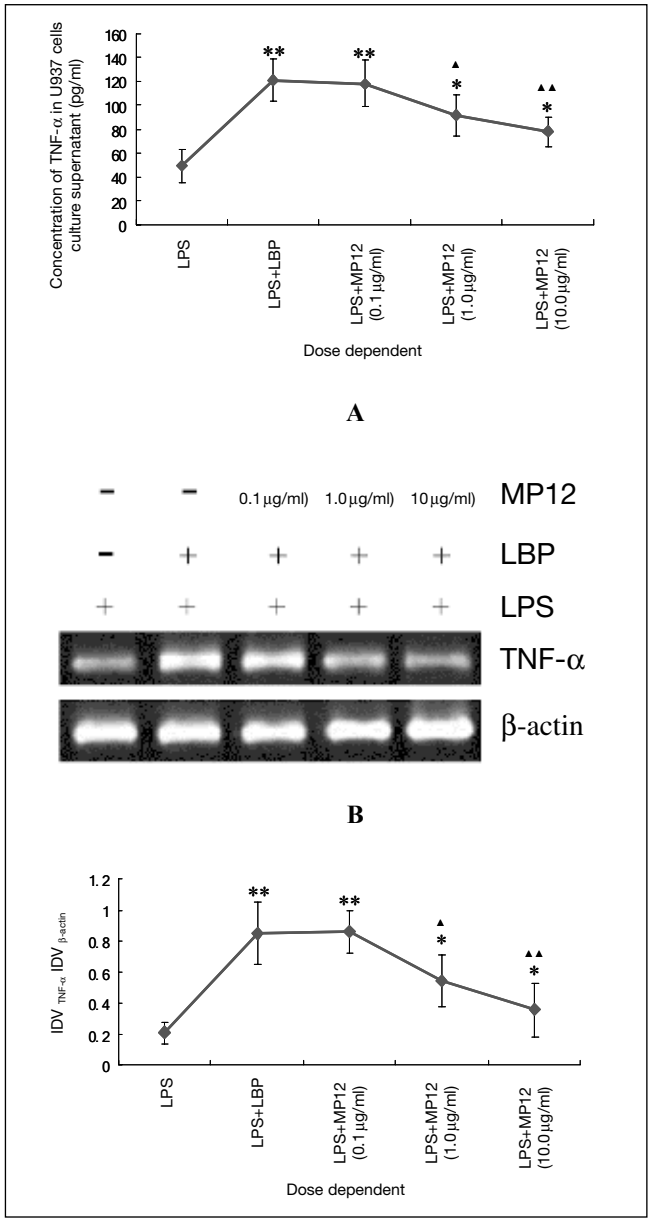

Fig. 4. Influence of MP12 on TNF-α mRNA expression and protein release

LPS (final concentration 100 ng/m) + rhLBP (final concentration 100 ng/ml) and different concentrations of MP12 (final concentration 0.1, 1.0, 10.0 μg/ml) were added to the PMA-induced mature U937 cells (1×10^6 cells/ml). The same concentration of LPS and rhLBP were added into the LBP group. The same final concentrations of LPS was added to the LPS group. Each group was incubated at 37℃ for 2 hours, with 5% CO_2 and the cells were separated by centrifugation and the supernatant was cultured.

(A) Cell culture supernatant was used to detect the concentration of TNF-α (with ELISA). LPS markedly amplified the inflammatory role of LPS, greatly increasing the secretion of TNF-α. The LPS-induced secretion of TNF-α was significantly inhibited by MP12 in a dose-dependent manner. (B) Cells were used to detect the expression of TNF-α mRNA by PCR. (C) Shooting with Gel Imager, the integrated density value (IDV) of bands was measured by EASY image analysis software. The semi-quantitative result of the TNF-α mRNA levels was expressed as $IDV_{TNF-\alpha}$/ $IDV_{\beta\text{-actin}}$. LBP can markedly enhanced LPS-induced expression of TNF-α mRNA. The LPS-induced expression of TNF-α mRNA was significantly inhibited by MP12 (1.0, 10.0 μg/ml) in a dose-dependent manner. Data are presented as mean ± SEM. **: $p<0.01$, *: $p<0.05$, versus control group; ▲▲: $p<0.01$, ▲: $p<0.05$, versus LPS+LBP group.

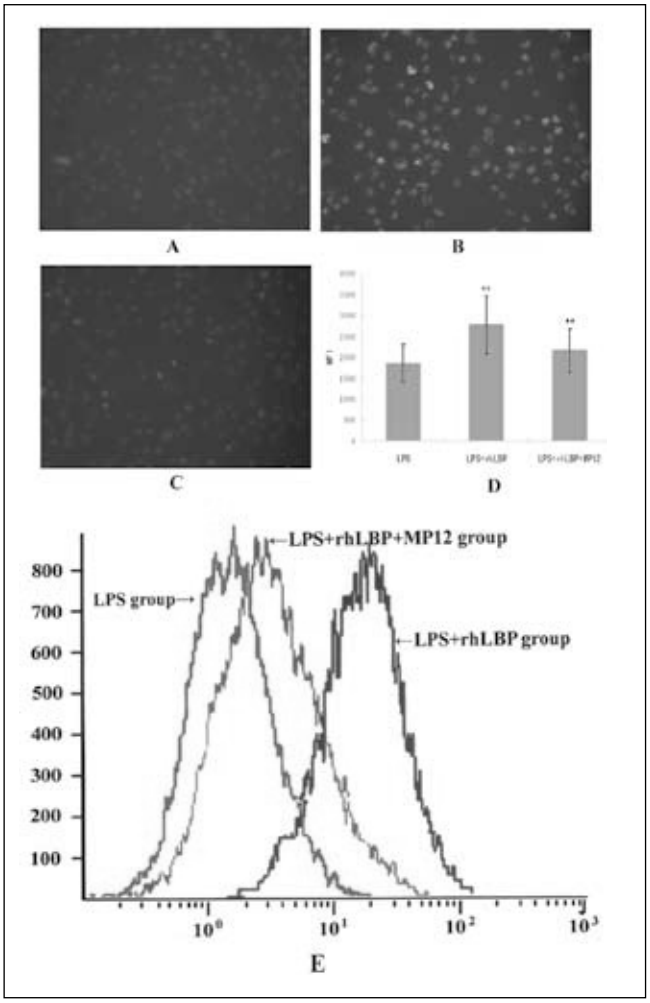

Fig. 5. Effects of MP12 on the binding of FITC-LPS to AMs

(A) Observation with fluorescent microscope. AM cells were incubated with FITC-LPS (A, LPS group), FITC-LPS in the presence of LBP (B, LPS+LBP group), and FITC-LPS in the presence of LBP together with 10.0 μg/ml MP12 (C, MP12-treated group). (D) Shooting with fluorescent microscope, and the average fluorescence intensity of image was measured by IPP image analysis software. LBP markedly enhanced the combination of LPS and AM cell. MP12 (10.0 μg/ml) significantly blocked the combination of LPS and AM cells mediated by LBP. (E) Flow cytometry testing was used. Red: LPS group, Green: LPS + rhLBP group, Blue: LPS + rhLBP + MP12 group. Data are presented as mean ± SEM. **: $p<0.01$, versus control group; ▲▲: $p<0.01$, versus LBP group.

TNF-α gene and protein expression induced by LPS was relatively low without LBP. Unfortunately, MP12 at low concentration (0.1 μg/ml) could not markedly influence the expression of TNF-α gene and protein induced by LPS (Fig. 4).

Effects of MP12 on the binding of FITC-LPS to AMs

We examined the effects of MP12 on the binding of FITC-LPS to AMs by fluorescent microscopy and flow cytometry testing. MP12 (10.0 μg /ml) apparently inhibited the binding of FITC-LPS to AMs when FITC-LPS was incubated with MP12 in the presence of LBP. These observations in-

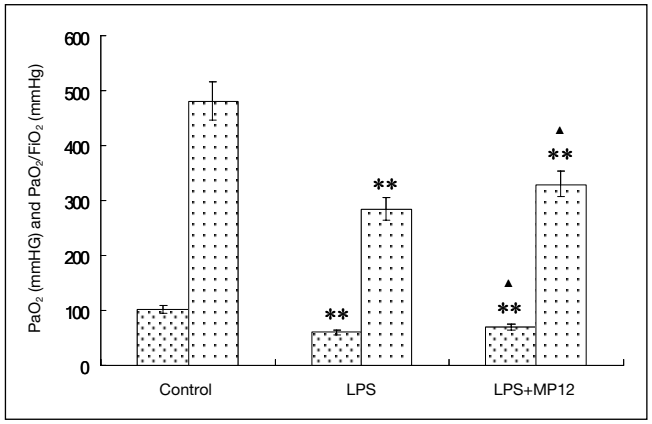

Fig. 6. Influence of MP12 on PaO_2 and PaO_2/FiO_2
LPS was injected into the jugular vein of a healthy 3-month-old rat to establish an ALI model. It shows that the model has been successfully established if the average PaO_2 was 60 mmHg. MP 12 (5 mg/kg.BW) was injected into the LPS + MP12 group rats immediately after the injection of LPS. PaO_2 was tested 2 hours later. The PaO_2 and PaO_2/FiO_2 of ALI rat can be markedly increased by MP12. Data are presented as mean ± SEM. **: $p<0.01$, versus control group; ▲: $p<0.05$, versus LBP group.

dicated that MP12 could bind to CD14 and thereby inhibit the interaction of FITC-LPS/LBP compound with CD14 (Fig. 5).

Influence of MP12 on endotoxin levels in the circulation

Plasma endotoxin level was evaluated at 2 h after treatment. There was a statistically significant difference in plasma endotoxin level between the control group and the LPS+MP12 group (0.78 EU/mL vs 1.82 EU/mL, $P<0.001$). There was no significant difference in plasma endotoxin levels between the LPS group and LPS + MP12 group (1.79 EU/mL vs 1.82 EU/mL, $P=0.61$). These observations indicated that the MP12 have no effect on the LPS.

Influence of MP12 on PaO_2 and PaO_2/FiO_2

The PaO_2 and PaO_2/FiO_2 of ALI rat can be markedly increased by MP12 (5 mg/kg.BW) . The average PaO_2 and PaO_2/FiO_2 of the LPS + MP12 group of rats were all higher than 60 mmHg and 300 mmHg, respectively (ALI diagnostic criteria is PaO_2 < 60 mmHg or PaO_2/FiO_2 < 300 mmHg) (Fig. 6).

Pathological Examination and Pathological score semi-quantitative analysis of Lung

Severe pulmonary interstitial edema, alveolar edema, pulmonary interstitial bleeding, hyaline-membrane formation, and a large amount of neutrophil infiltration were seen in ALI rat`s lung tissue which was induced by LPS. In the MP12 treatment group, lung edema showed an obvious decline, pulmonary interstitial bleeding was slight, and the amount of neutrophil infiltration was reduced apparently. Pathological score semi-quantitative analysis has shown that the pathological score of lung tissue of rat in the MP12 treatment group was apparently better than that in the LPS group ($p<0.01$) (Fig. 7).

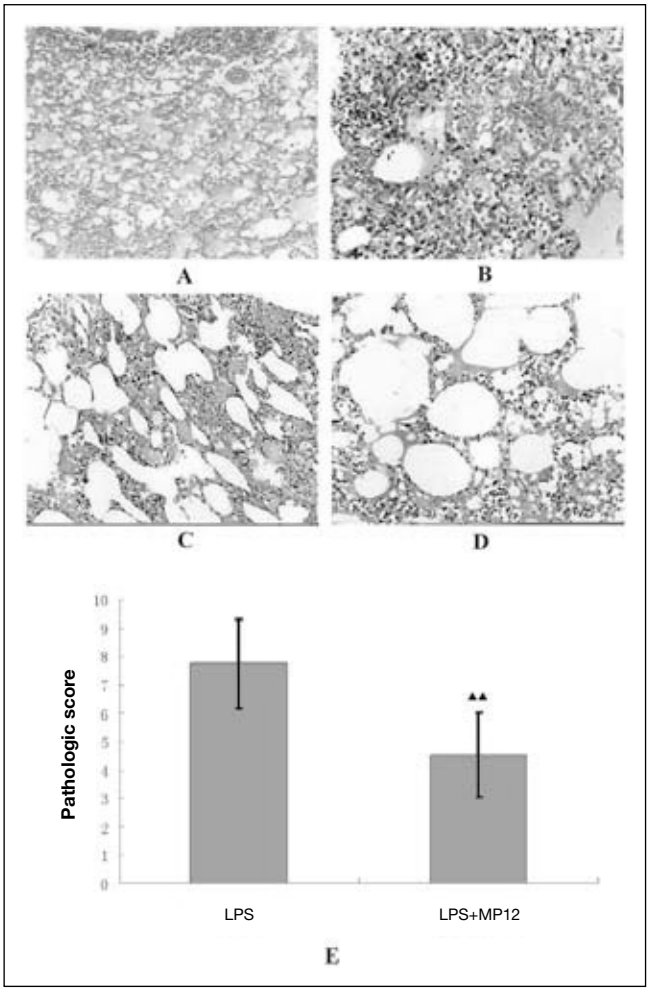

Fig. 7. Hematoxylin and eosin-stained histological lung sections of rat. A) Biopsy of the lung tissues taken from rats treated with LPS. Diffuse pulmonary and alveolar edema, pulmonary vascular peripheral edema, and bronchus peripheral neutrophil infiltration were seen. (B) A large amount of neutrophil infiltration, pulmonary interstitial bleeding, and hyaline-membrane formation were seen. (C) Biopsy of the lung tissues taken from rats treated with LPS plus MP12 (5 mg/kg.BW). Compared with LPS group, only slight pulmonary interstitial edema and neutrophil infiltration were seen. (D) Compared with LPS group, alveolar interval was not widened evidently. There was no edema fluid in the alveolar space. Neutrophil infiltration was reduced. (E) The pathological score in the MP12 treatment group was apparently better than that in the LPS group.

Mortality of LPS-treated rats with or without MP12

At 72 h after LPS treatment, 7 out of 10 rats died, whereas only 2 out of 10 rats treated with LPS and MP12 died. Therefore, MP12 reduced LPS-induced mortality ($p<0.05$ by Fisher exact test) in rats receiving MP12 compared with endotoxemic rats).

Discussion

Taking LBP/CD14 as a target, anti-endotoxin treatment has the advantage of blocking the transfer of the endotoxin inflammatory signal at an earlier period, which has in recent years been an ideal strategy to treat endotoxin diseases (such as ALI/ARDS and sepsis) [21, 22, 23]. In recent years, progress has been made in antagonising LBP/CD14 to inhibit the inflammatory reaction induced by LPS using methods such as LBP antibody, CD14 antibody, and sCD14 [24, 25]. However, there are several shortcomings. The key issue is that these methods inhibit LBP removal of LPS while exerting antagonism against LBP/CD14. With CD14 the target of the study was aimed at providing an ideal strategy to prevent and cure endotoxin ALI/ARDS by screening out a mimetic peptide from the binding site between LBP and CD14 and then using it to block CD14 in order to block the path of LPS inflammatory signalling and to remove the LPS/LBP compound through the HDL pathway.

With CD14 as the target molecule, we passed a phage display 12 peptide library through 4 rounds of affinity screening. The concentration of CD14 was reduced gradually, with the purpose of gaining the phage clones with high affinity. The first 2 rounds adopted acid cleaning and the latter 2 rounds adopted LBP competitive elution. The phage clones obtained in this way were able to compete with LBP binding to CD14. The result showed that the quantity of phages was high, with recovery rate rising from 3.5×10^{-6} in the first round to 5.2×10^{-2} in the fourth round. Twenty-five positive clones with extremely high binding activity were obtained through the ongoing binding activity testing of phage clones and CD14. Eight different polypeptide sequences were obtained after DNA sequencing of the 25 clones. The common sequence was: FHRHPPXPTLXX, which means that amino acid residues at 9 positions in the 12 peptides might represent be the framework of the binding surface between LBP and CD14. The no.1 polypeptide (FHRWPTWPLPSP) was shared by 8 phage clones (the clone no. included: 15, 21, 33, 80, 55, 71, 86, 90), and the binding activities of the 8 clones were highly consistent with that of the rhCD14 ($OD_{phage}/OD_{control} >25$). Hence, we have chosen the no.1 polypeptide (named MP12) to be artificially synthesised in order to make further study.

With the purpose to demonstrate the ability of MP12 to compete with LBP bind on to CD14, we embarkeding an experiment based on the following aspects: First, ELISA was adopted to test the competitive inhibiting activity of MP12 at different concentrations against LBP, with CD14 being the substrate molecule. The result showed that the competitive inhibition ratio of MP12 against LBP appeared to be concentration dependent. One and ten μg/ml MP12 had better inhibitory activity against LBP. The competitive inhibition ratio of 10.0 μg/ml MP12 reached 69.6 % at the most. Second, we have adopted fluorescence staining and flow cytometry to detect the ability of MP12 to block the combination of LPS/LBP compound and AMs. The result showed that 10.0 μg/ml MP12 can apparently block LBP-mediated combination of LPS and AMs. Due to the key role of AMs in endotoxin-induced ALI inflammatory reaction, they can block the combination of LPS/LBP compound and can greatly reduce the ALI inflammatory reaction. Our data also indicate that MP12 has potential for the treatment of endotoxin-induced ALI [26].

TNF-α is an inflammatory factor produced by most inflammatory cells stimulated by LPS [27, 28]. It can induce more inflammatory factors, which play a key role in the occurrence and development of SIRS (Systemic Inflammatory Response Syndrome) [29, 30]. PMA-induced mature $CD14^+$ U937 cells and LPS were cultured together, with different concentrations of MP12. The result showed that the LPS-induced expression of TNF-α mRNA and protein secretion was significantly inhibited by MP12 (1.0, 10.0 μg/ml) in a concentration-dependent manner.

An experiment conducted *in vivo* has shown that MP12 (5 mg/kg.BW) can markedly increase the PaO_2 and PaO_2/FiO_2 of rats suffering from endotoxin-induced ALI. The average PaO_2 and PaO_2/FiO_2 of rats in the treatment group were each higher than 60 mmHg and 300 mmHg, respectively, and were found to be higher than the values of ALI diagnositc criteria. The result of pathological observation and pathological score semi-quantitative analysis showed that lung edema of rats in the treatment group showed an obvious decrease and lung interstitial bleeding was slight and neutrophil infiltration apparently decreased. The pathological score of lung tissue was better in the treatment group than that in the LPS group. From the perspective of both pathology and physiology, MP12 proved to be protective for endotoxin-induced ALI.

In summary, the study has successfully screened out the mimetic peptides (MP12: FHRWPTWPLPSP) from the binding site of LBP and CD1. The polypeptide has strong biological activity to compete with LBP binding CD14. The mimetic peptide *in vitro* can evidently inhibit LPS-induced TNF-α secretion and block the LBP-mediated combination of LPS and AMs. Antiendotoxic activity *in vivo* can evidently improve the oxygenation index of rats suffering from endotoxin-induced ALI, preventing lung injury and reducing death rate. The study is helpful for understanding the role of LBP/CD14 in endotoxic inflammatory reactions and related diseases from a new point of view and aims to provide endotoxin-induced ALI/ARDS with novel prevention and cure strategies.

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