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Screening for a Peptide That Inhibits Expression of a Broad-spectrum of Chemokines Using Models of Endotoxin Tolerance and LPS-induced Pro-inflammation*

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Abstract The goal of this study was to screen bioactive peptides to identify an efficient antagonist of multiple pro-inflammatory chemokines that inhibits the pathological process of inflammatory diseases. A phage display library was sequentially screened by binding phages. The binding properties of individual phage clones to LPS-activated PBMCs were determined using cell-based ELISAs. The positive clones were selected and determined by chemotaxis assays. A high-activity peptide was determined to inhibit carrageenan-induced paw oedema and formaldehyde-induced arthritis in Wistar rats *in vivo*. A possible mechanism of inflammation inhibition involving chemokine mRNA by the peptide was determined by analyzing mRNA expression levels of chemokines and tristetraprolin (TTP) by SqRT-PCR.Nineteen phage clones were selected after four rounds of biopanning with a cut-off of 3-fold higher binding to LPS-activated PBMCs than to normal PBMCs. Nine of the phage clones inhibited IL-8, MCP-1, and MIP-1β production *in vitro*. Five clones displayed the same peptide(CI-S5)most robustly inhibited the chemotactic activity *in vitro* and reduced paw oedema and arthritis in Wistar rats *in vivo*. SqRT-PCR results indicated that mRNA expression of IL-8, MCP-1, and MIP-1β were reduced and TTP mRNA expression was increased in the CI-S5 treatment group. Our data demonstrate that CI-S5 is a broad-spectrum antagonist of pro-inflammatory chemokines as it enhances the expression of TTP to reduce chemokine mRNA expression. This study provides a basis for the development of new peptide-based therapies for the treatment of inflammatory diseases.

Key words endotoxin tolerance, mixed-target phage display, broad-spectrum chemokine antagonist, bioactive peptide, tristetraprolin (TTP)
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In recent years, increasing evidence indicates that chemokines play a substantial role in the pathology of inflammatory diseases[1]. In the early stage of inflammation, stimulating factors induce inflammatory cells to produce large amounts of chemokines, leading to the accumulation of a large number of inflammatory cells and continued high chemokine levels. Leukocyte recruitment increases significantly, resulting in destructive damage. Meanwhile, the injury induced by the recruited leukocytes promotes high expression levels of chemokines, thus further aggravating the tissue damage. This "vicious cycle" destroys the ability of leukocytes to protect the organism and results in inflammatory diseases [2-3]. Therefore, targeting the chemokines and their receptors to prevent excessive inflammatory responses is valuable for inflammation therapy. Several studies have shown that targeting only a single chemokine or receptor often results in unsatisfactory clinical trials due to the narrow range of indications and high eliminatory rates[4]. Redundancy is

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a remarkable feature of the chemokine system, and most inflammatory diseases involve many chemokines and their receptors. Consequently, identification of novel broad-spectrum inhibitors of inflammatory chemokines is of great significance. The broad-spectrum inhibitors have great potential to reduce or prevent inflammatory diseases, but few studies of this nature have been reported.

Lipopolysaccharide (LPS) is a potent inflammatory mediator and can induce the production of pro-inflammatory cytokines and chemokines by activating transcription factors[5-6]. Endotoxin tolerance is thought to be an adaptive mechanism of the innate immune system to protect against septic shock[7]. After re-stimulation with a lethal dose of endotoxin, the secretion of inflammatory factors and the inflammatory injury of the organism is reduced, thus resulting in increased survival [8]. Thus, the pro-inflammatory process and the endotoxin tolerance phenomenon are instructive and meaningful for developing anti-inflammation therapies.

In the present study, we developed two cell models using a new sequential screening strategy utilizing whole-cell phage display to isolate peptides that bind selectively to LPS-induced pro-inflammatory chemokines. Future studies will focus on developing the positive peptides as efficient antagonists of pro-inflammatory chemokines. This study lays the foundation for identifying new broad-spectrum chemokine inhibitors.

1 Materials and methods

1.1 Animals and reagents

Male (n = 30) and female (n = 30) Wistar rats weighing (140.0 ± 7.8) g were obtained from the Experimental Animal Centre of Southern Medical University (Guangzhou, China). All experimental protocols were approved by the Animal Care and Use Committee of Guangzhou Medical College in accordance with the National Guidelines for Animal Care and Use.

A Ph.D.-C7C library and the *E. coli* host strain ER2738 were purchased from New England Biolabs (Beverly, MA). Human lymphocyte separation medium was purchased from HaoYang Biological (Tianjin, China). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Gibco BRL (Grand Island, NY). Horseradish peroxidase (HRP)-conjugated anti-M13 antibody (anti-M13/HRP) and unconjugated anti-M13 antibody were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Human PBMCs isolated on Ficoll (Pharmacia Biotech) gradients from the buffy coats of healthy donors were cultured for 72 h in RPMI 1640 medium from Invitrogen (USA). LPS was purchased from Sigma. Stock solutions were diluted appropriately in RPMI 1640 before experiments.

1.2 Isolation and culture of PBMCs

PBMCs were isolated from buffy coats by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences). The composition of the adherent cell population was analyzed by FACS[9].

1.3 Cell stimulation and development of *in vitro* models

To examine pro-inflammatory conditions, PBMC populations were adjusted to cell densities of 1×10^6 cells/ml and cultured in RPMI 1640 medium at 37℃ for 2 h. Unattached cells were removed. Attached cells were stimulated with LPS (10 mg/L) in RPMI 1640 media for 24 h at 37℃. Cells cultured under the same conditions in the absence of LPS stimulation were used as unmanipulated controls.

To examine LPS tolerance conditions, one set of adherent cells was treated with 1 mg/L LPS, while the other set was left untreated. After 16 h of the initial LPS stimulation, the supernatants of all cells were removed and the cells were washed once with warm RPMI 1640 to remove residual LPS. After addition of fresh RPMI 1640, cells were incubated for 2 h prior to the addition of 10 mg/L LPS or medium alone as a mock treatment. Six hours after the second stimulation, supernatants and cells were harvested for protein and RNA examination, respectively. Cell supernatant were analyzed for TNF-α and NO content using ELISA Kits to make sure the model established was successful.

1.4 Screening of peptides by phage display

Inflammatory chemokines produced by LPS-activated PBMCs were concentrated by ultrafiltration. A strategy of sequential screening was applied by binding the phage library to unmanipulated PBMCs, binding the pre-screened phage library to the endotoxin-tolerant PBMCs, and finally binding the flow-through phages to LPS-activated PBMCs. The bound phages were eluted and combined with the concentrated chemokine supernatants for initiation of four rounds of biopanning as described previously[10]. Four rounds of screening were performed from which

40 phage clones that bound the LPS-activated PBMCs were randomly selected for further analysis.

1.5 Phage binding assay

The binding properties of the selected individual phage clones to LPS-activated PBMCs were determined using cell-based ELISAs [11]. The LPS-activated and untreated PBMCs were cultured in RPMI 1640 medium in 96-well plates for 2 h. After gentle washing with DPBS, the cells were treated with 0.3% hydrogen peroxide in DPBS for 10 min and blocked with 1% BSA in DPBS (PBSB). Cells were incubated with individual phage clones in PBSB for 2 h in triplicate. After washing, the bound phages were probed with anti-M13/HRP (1∶5 000) and detected with TMB. The reaction absorbence (A) was read at 450 nm on a microplate reader.

1.6 Chemokine secretion assay

Positive clones were selected considering the inhibitory activity of chemokine secretion assay with three different chemokines. The concentrated chemokine-containing supernatants from LPS-activated PBMCs were treated in triplicate with binding clones in RPMI 1640 medium at 37℃ for 24 h. The supernatants from unactivated and LPS-activated PBMCs were assigned as the blank and positive control groups. Interleukin-8 (IL-8), MCP-1 and MIP-1β protein levels were determined in the supernatants using the LiquiChip (Qiagen, Hilden, Germany) as described previously[12].

1.7 DNA sequencing

After four rounds of biopanning, binding assays, and inhibitory assays, positive phage clones were randomly chosen from the titration plates and amplified. Individual phage clones were amplified in *E. coli* ER2738 and the amplified phages were collected by treatment with PEG 8000/NaCl and centrifugation at 10 000 g for 10 min. Phage pellets were suspended in iodide buffer. Single-stranded phage DNA was separated on and extracted from agarose gels and further purified. The DNA was sequenced using a –96 p Ⅲ sequencing primer (5′ HOCCCTCATAGTTAGCGTAA CG 3′). DNA sequencing was performed by the Yingjun Biotechnology Company (Guangzhou, China), and the DNA sequences were analyzed using Bioedit, DNAman, and Protparam software.

1.8 Peptide synthesis

The four positive peptides displayed by the phage clones were chemically synthesized by Huatuo Biotechnology Company (Guangzhou, China) and were characterized by high performance liquid chromatography and mass spectrometry. Peptides with purities ⩾95% were used for our experiments.

1.9 Chemotaxis assays using positive peptides

Transwell experiments were performed to quantify the effect of the positive peptides on blocking LPS-induced PBMC migration. PBMC migration was assayed in 24-well cell culture chambers using inserts with 3-μm pore membranes. The lower chambers contained 250 μl culture medium. The blank wells included only RPMI 1640 chemotaxis buffer (containing 0.5% fetal bovine serum). The negative control group contained only media supplemented with supernatant from 10 mg/L LPS-activated PBMCs. The peptides test group contained media supplemented with supernatant from LPS-activated PBMCs and the four positive peptides with 10 mg/L in media. The viral macrophage inflammatory proteins-Ⅱ (vMIP-Ⅱ), which presumably has a structure similar to that of CC chemokines has been shown to inhibit many chemokine receptors to block the chemotaxis migration. The positive control group contained 10 mg/L VMIP-Ⅱ (maintained in laboratory). The upper chambers were inserted and incubated for 90 min at 37℃. The cell density was counted using a hemocytometer. All assays were performed three times with triplicate wells.

1.10 Competitive binding activity with positive clones

A competitive binding assay was performed to test the CI-S5 peptide (PFLNTWS) and its mutants could compete for the binding of the phage clone 15 that displayed the peptide. LPS-activated PBMCs were incubated with phage clone 15, the original source of CI-S5, in 96-well plates in the presence of various concentrations (0.1, 1, 10 and 100 mg/L) of the PFLNTWS peptide or individual mutant peptides (VFLNTWS, PTLNTWS, PFRNTWS, PFLMTWS, PFLNGWS, PFLNTFS, and PFLNTWA). The mixtures were incubated simultaneously with LPS-activated PBMCs for 2 h on ice. The phages bound to LPS-PBMC were quantified by the cell-based ELISA.

1.11 Carrageenan-induced paw oedema

Wistar rats were divided into five groups (n=6). Rats were pre-treated with 0.1 ml of a 1% suspension of carrageenan (Sigma) in normal saline by injection into the subplantar region of the left hind paw to

induce oedema. Subsequently, each group received different formulations of the treatment. Group A was the control group without any further treatment, groups B, C, and D received CI-S5 (10, 50, and 100 μg/kg body weight, respectively), and group E received indomethacin (Sigma) at 5 mg/kg body weight as the positive control group. Paw size was measured immediately before carrageenan injection and 3 and 5 h after injection.

1.12 Formaldehyde-induced arthritis

Wistar rats were divided into five groups (n=6) and baseline ankle joint diameters were measured using a micrometer screw gauge. The five groups were treated as in the carrageenan-induced paw oedema model and monitored for 10 days. Arthritis was induced by injecting with 0.1 ml formaldehyde (4% v/v) into the left hind paw of each animal on the first and third days[13]. Joint diameters of the treatment groups were compared with those of the control group.

1.13 Analysis of chemokine mRNA levels by SqRT-PCR

The oligonucleotide primer sequences used were: IL-8, 5′ TTGGCAGCCTTCCTGATTTC 3′ (forward) and 5′ AACTTCTCCACAACCCTCTG 3′ (reverse), MIP-1, 5′ CCAAACCAAAAGAAGCAAGC 3′ (forward) and 5′ AGAAACAGTGACAGTGGACC 3′ (reverse); MCP-1β, 5′ TCCAGCATGAAAGTCTCT-GC 3′ (forward) and 5′TGGAATCCTGAACCCAC-TTC 3′ (reverse); GAPDH, 5′ ACCACAGTCCATG-CCATCAC 3′(forward) and 5′ TCCACCACCCTG-TTGCTGTA 3′(reverse).

Human PBMCs were divided into four groups: the control group of RPMI 1640 medium alone, the LPS-activated group, the LPS+CI-S5 peptide group, and the CI-S5 peptide group . The concentration of both LPS and CI-S5 peptide contained 10 mg/L. All the groups were cultured 12 h after added the reagent, and then centrifuged to the cultured medium. After that, washed with sterile PBS, and abandoned the waste. Finally, all the groups were respectively RNA extraction, reverse transcription and PCR reactions to detect the expression of chemokine in each group[14]. The PCR conditions were: 95℃ for 5 min and 40 cycles of 95℃ for 15 s, 60℃ for 15 s, and 72℃ for 32 s. Amplification specificity was evaluated by melt-curve analysis from 60℃ to 95℃ by 0.2℃ intervals. All reactions were performed in triplicate.

1.14 Analysis of tristetraprolin (TTP) mRNA levels by SqRT-PCR

The primers used for TTP analysis were 5′ TCT-CTGCCATCTACGAGAGCCTC 3′ (forward) and 5′ GCTGATGCTTTGTCGCAGCACATG 3′ (reverse). Human PBMCs were divided into four groups: the blank control group, the LPS group, the LPS+CI-S5 peptide group, and the LPS+IL-10 (positive control) group. The concentration of LPS IL-10 and CI-S5 peptide contained 10 mg/L. All groups were cultured for 12 h after treatments, centrifuged, and washed with sterile PBS. SqRT-PCR was performed as described above for chemokine mRNA analysis.

1.15 Statistical analysis

Data are expressed as $\bar{x} \pm s$. Differences between experimental and control groups were statistically analyzed using ANOVA. $P < 0.05$ were considered to be statistically significant.

2 Results

2.1 *In vitro* pro-inflammatory and endotoxin-tolerance models

Human PBMCs were stimulated with LPS to develop an *in vitro* inflammation model. Analysis of culture supernatants indicated that secretion of IL-8, MCP-1 and MIP-1β was elevated in the LPS-activated group as compared to the media-alone control group. Thus, a successful model was established. In the endotoxin-tolerance model, secretion of TNF-α and NO into the media of the LPS-activated group was significantly higher than in the media-alone control group, the LPS+LPS group, and the LPS+media group. However, there were no significant differences between the three groups, indicating that establishment of the model was successful.

2.2 Screening for phages that bound LPS-activated PBMCs

The biopanning efforts resulted in an enrichment of phages that specifically bound LPS-activated PBMCs. After four rounds of screening, the number of phages recovered from LPS-activated PBMCs was 167 times higher than that from the first round (2.84×10^{-7} to 4.75×10^{-5}).

2.3 Selecting phage clones that specifically bound LPS-activated PBMCs

Forty phage clones were randomly selected after the four rounds of screening. We measured the abilities of the phages to bind to LPS-activated PBMCs and

normal PBMCs (Figure 1). With a cut-off of 3-fold higher binding to LPS-activated PBMCs than to normal PBMCs, 19 out of 40 phage clones were selected.

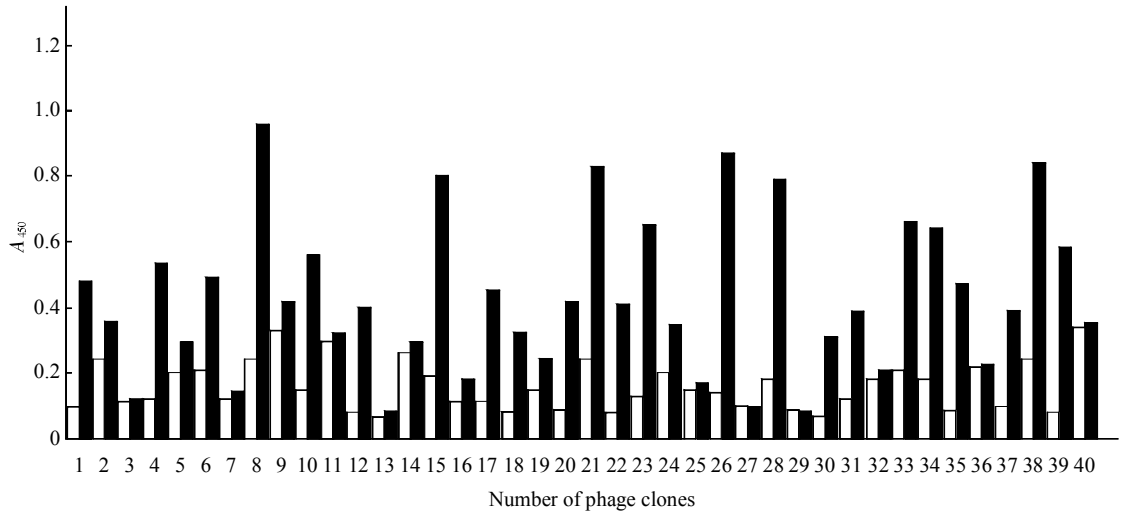

Fig. 1 Selective binding of the phage clones to LPS-activated PBMCs

Forty phage clones were randomly selected for further examination of their ability to bind to LPS-activated PBMCs (filled bars) and normal PBMCs (open bars) on an automated ELISA plate reader. Data are expressed as means of three independent experiments. □: PBMC; ■: LPS-PBMC.

2.4 Effect of the positive clones on the production of chemokines by PBMCs

Phage clones displaying the peptides that bound LPS-activated PBMCs were examined to determine if they could modulate chemokine production in response to LPS stimulation. LPS-activated PBMCs were treated with the 19 positive clones individually for 24 h and the protein levels of IL-8, MCP-1, and MIP-1β in the culture supernatants were measured. Nine out of 19 positive clones inhibited the production of chemokines by LPS-activated PBMCs as compared to PBMCs treated with LPS alone (Figure 2). The peptides that were displayed on the nine positive clones could be candidates for efficient antagonism of pro-inflammatory chemokines.

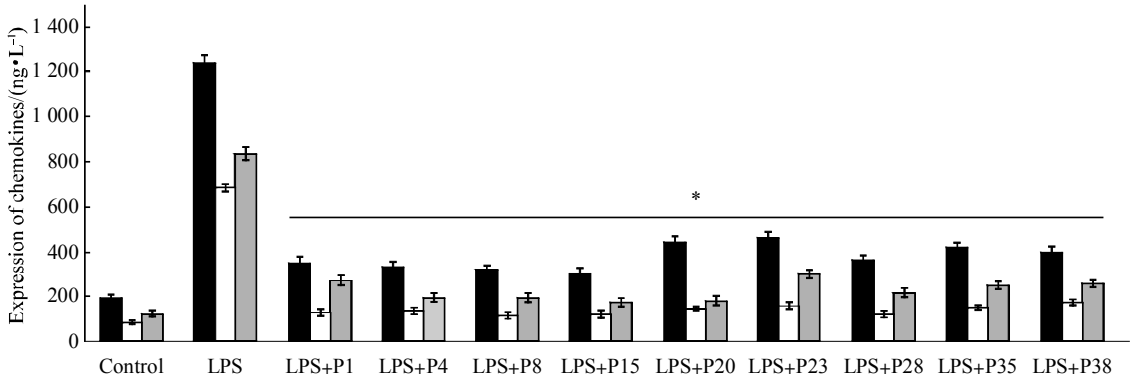

Fig. 2 Effect of the positive clones on the production of chemokines by PBMCs

Nine out of 19 positive clones inhibited the production of chemokines by LPS-activated PBMCs as compared to PBMCs treated with LPS alone (*P < 0.05). Data are expressed as means of three independent experiments. ■: IL-8; □: MCP-1; ▨: MIP-1β.

2.5 Analysis of displayed peptides

Analysis of the peptides displayed by the nine phage clones identified four unique peptides. Five of the nine phage clones displayed the same peptide, PFLNTWS (CI-S5). Two of the nine clones displayed the peptide KSNTHAA. The remaining two clones displayed the peptides GSPTELT and NHTLSAQ. Therefore, specific phage clones were selected during the biopanning process.

2.6 The ability of the four peptides to inhibit chemokine activity

Chemotactic assays had a predictive value for assessing the anti-inflammatory nature of the peptides. The inhibitory rate of the four peptides showed that the most common peptide, CI-S5, had the strongest inhibitory effect on cell migration following LPS activation compared with the other three peptides. The inhibitory rate of CI-S5 reached 48.72%, and the rates of the other three peptides were 32.48%, 27.76% and 24.60%.

2.7 Competitive binding activity with positive clones

Next, we tested whether the CI-S5 peptide (PFLNTWS) and its mutants could compete for the binding of the phage clone 15 that displayed the peptide through a competitive binding assay. The binding of phage clone 15 to LPS-activated PBMCs was inhibited by CI-S5, but not by any of the point mutants(Figure 3a). The inhibitory effects of CI-S5 on the binding of phage clone 15 to LPS-activated PBMCs were concentration dependent.

2.8 Effect of CI-S5 on chemokine production

We tested whether CI-S5 could inhibit the secretion of chemokines by LPS-activated PBMCs. Compared with the LPS-activated PBMCs without peptide treatment, the LPS-activated and CI-S5-treated group had significantly reduced levels of IL-8, MCP-1 and MIP-1β ($P < 0.05$) in their culture supernatants (Figure 3b). However, there were no significant differences between the control group and the CI-S5+ LPS group. The mutant peptides had no inhibitory effect on the secretion of these chemokines by LPS-activated PBMCs(data not shown in the Figure 2). The results indicate that CI-S5, but not its point mutants, inhibited chemokine production by LPS-activated PBMCs.

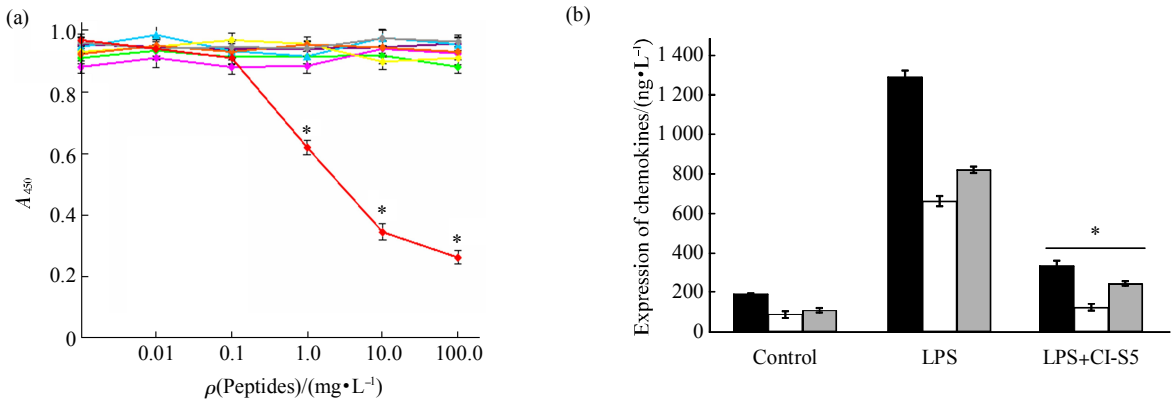

Fig. 3 Competitive binding activity with positive clones and effect of CI-S5 on chemokine production

(a) After washing, the binding of phage was evaluated using HRP-conjugated anti-M13 monoclonal antibodies, TMB substrate, and absorbance measurements at 450 nm. : PFLNTWA; : PFLNTFS; : PFLNGWS; : PFLMTWS; : PFRNTWS; : PTLNTWS; : VFLNTWS; : PFLNTWS. (b) Data are expressed as $\bar{x} \pm s$ from three independent experiments. *$P < 0.05$ compared with the untreated control group. : IL-8; : MCP-1; : MIP-1β.

2.9 Effect of CI-S5 on carrageenan-induced oedema in Wistar rats

Wistar rats were separated into five groups. Group A was a negative control group. Groups B, C, and D were treated with CI-S5 at 10, 50, and 100 μg/kg body weight, respectively. Group E was treated with indomethacin at 5 mg/kg body weight as a positive control.The carrageenan-induced oedema test showed that CI-S5 significantly reduced the paw oedema from (10.8±1.1) mm in the control to (2.5±0.5) mm in the

50 μg/kg peptide group ($P < 0.05$). The inhibition rate of CI-S5 after 5 hours reached 76.8%, a level similar to that of the indometacin positive control group (Table 1).

Table 1 Effect of CI-S5 peptide on carrageenan-induced paw oedema in Wistar rats

Group	Initial paw size before carrageenan dministratio/mm	Increase in paw size 3 h after carrageena administration/mm	Increase in paw size 5 h after carrageena administration/mm	Inhibition 3 h after carrageenan administration/%	Inhibition 5 h after carrageena administration /%
A(Control)	25.0±0.02	12.3±1.2	10.8±1.0	–	–
B(CI-S5, 10 μg/kg)	24.8±0.06	6.8±0.6*	3.8±1.4*	41.4	65.8
C(CI-S5, 50 μg/kg)	24.4±0.05	4.5±0.4*	3.2±1.1*	56.8	70.4
D(CI-S5, 100 μg/kg)	24.3±0.06	4.9±1.2*	2.5±0.5*	56.1	76.8
E(Indomethacin, 5 mg/kg)	24.7±0.04	3.6±1.1*	1.6±0.8*	68.2	85.2

n=6, compared with control group, *$P < 0.05$.

2.10 Effect of CI-S5 on formaldehyde-induced arthritis in Wistar rats

The results of the formaldehyde-induced arthritis test are shown in Table 2. All three doses of CI-S5 (10, 50, and 100 μg/kg body weight) and indomethacin treatments significantly ($P < 0.05$) inhibited arthritis during the 10-day period of the study.

Table 2 Effects of CI-S5 peptide on formaldehyde-induced arthritis in rats

Group	Increase in paw size/mm									
	Day 1	Day 2	Day 3	Day 4	Day 5	Day6	Day7	Day 8	Day 9	Day 10
A(Control)	7.0±0.2	8.3±0.3	10.2±0.5	11.7±0.5	10.8±0.4	8.6±0.6	7.4±0.7	6.7±0.2	6.0±0.3	5.8±0.2
B(CI-S5, 10 μg/kg)	6.4±0.4	4.7±0.1*	6.2±0.5*	4.6±0.6*	3.6±0.4*	3.0±0.3*	2.6±0.2*	2.2±0.2*	1.8±0.4*	1.6±0.2*
C(CI-S5, 50 μg/kg)	5.6±0.2*	6.1±0.4*	7.4±0.4*	4.8±0.3*	2.4±0.2*	1.6±0.2*	1.5±0.3*	1.4±0.2*	1.2±0.2*	1.2±0.2*
D(CI-S5, 100 μg/kg)	3.8±0.4*	3.9±0.4*	5.7±0.3*	3.1±0.3*	2.0±0.2*	1.6±0.2*	1.4±0.2*	1.4±0.4*	1.2±0.2*	1.2±0.2*
E(Indomethacin, 5 mg/kg)	3.6±0.4*	4.1±0.4*	5.3±0.2*	3.2±0.3*	2.1±0.3*	1.8±0.2*	1.6±0.2*	1.8±0.2*	1.6±0.2*	1.4±0.2*

n=6, compared with control group, *$P < 0.05$.

2.11 Analysis of chemokine and TTP mRNA levels

Agarose gel electrophoresis results are shown in Figure 4. Compared with the LPS-activated PBMC group, the mRNA expression levels of IL-8, MIP-1β, and MCP-1 were reduced in LPS-activated PBMCs treated with CI-S5. Therefore, CI-S5 treatment altered the expression of chemokine mRNA subsequently reducing the generation of the chemokines, which then could have an inhibitory effect on inflammation. Thus, we hypothesized that low levels of endogenous TTP may be responsible for the relative stability of the chemokine mRNA. As predicted, increased expression of TTP resulted in reduced stability of the chemokine mRNA. Furthermore, treatment with CI-S5 enhanced

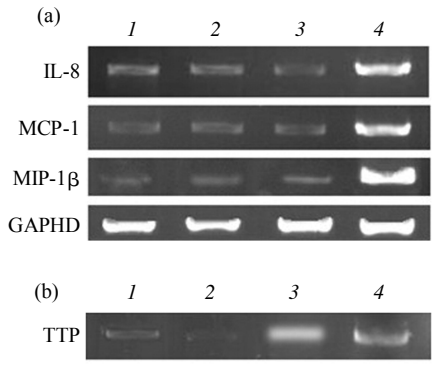

Fig. 4 The effect of CI-S5 on PBMC expression of chemokine and TTP mRNA

(a) Analysis of IL-8, MCP-1and MIP-1β mRNA. *1*: Negative control; *2*: CI-S5 treatment; *3*: LPS +CI-S5 treatments; *4*: LPS only treatment. (b) Analysis of TTP mRNA. *1*: Negative control; *2*: LPS only treatment; *3*: LPS+CI-S5 treatments; *4*: LPS+IL-10 treatments.

the expression of TTP and TTP-induced enhancement of deadenylation that causes reduction of chemokine mRNA[15].

3 Discussion

In recent years, with the applications of phage display technology expanding, phage display has played an increasing role in the selection of bioactive peptides for the treatment of inflammatory diseases[16-17]. In our study, we created two different *in vitro* cell models relating to the inflammatory process. In the pro-inflammatory and endotoxin-tolerance models, we chose to use PBMCs rather than monocytes/ macrophages. PBMCs are a mixed group that includes monocytes, lymphocytes, and other mononuclear cell populations. These conditions reflect a more realistic environment for immune system responses to external stimuli. This study was designed to examine pro-inflammatory chemokines produced by the mixed cell population while screening bioactive peptides for those with broad-spectrum inhibitory effects to block the inflammatory process. Negative and positive selection using PBMCs delivers a broader and more comprehensive strategy for identifying bioactive peptides. Because of the broad target cell base, the selected peptides may be able to overcome the disadvantages of conventional macromolecular drugs and single-target anti-inflammatory drugs. Compared with steroidal drugs, the bioactive peptide has similar inhibitory activities with fewer toxic side effects [18]. The bioactive peptide has the potential to become a new prodrug for the treatment of inflammatory diseases in clinical tests.

In this study, we developed two *in vitro* cell models (anti-inflammatory and pro-inflammatory) and employed a screening strategy to identify new peptides that selectively bind to LPS-activated PBMCs. The CI-S5 peptide significantly inhibited the production of IL-8, MCP-1, and MIP-1β. Based on classifications, the four chemokines were separated into CC and CXC categories. The receptors of the two chemokine categories are different, so we hypothesize that the inhibitory activity of CI-S5 was not specific to the locus where the receptor and its ligand bind each other. We also examined the mRNA expression of IL-8, MCP-1, and MIP-1β. CI-S5 reduced the chemokine mRNA expression levels, which resulted in decreased chemokine production, thus playing an inhibitory role in inflammation.

Numerous mechanisms for regulating mRNA decay have been identified and are subject to regulation by extracellular factors [19]. One *cis*-acting factor that confers mRNA instability is the AU-rich element (ARE). AREs in many chemokines mediate posttranscriptional regulation of proinflammatory genes when bound by ARE-binding proteins such as TTP. Low endogenous levels of TTP may be responsible for the relative stability of chemokine mRNA. However, TTP has a suppressive effect on the promoter elements of TNF-α and IL-8, and LPS stimulation can release this suppression[20]. Thus, TTP may be a more general regulator of ARE-containing mRNAs. Because IL-8, MCP-1, and MIP-1β all contain AREs, we hypothesized that CI-S5 may enhance the expression of TTP to reduce chemokine mRNA stability. Fortunately, analysis of TTP mRNA expression indicated that CI-S5 can increase TTP expression. The increased TTP levels may be sufficient to mediate the expression of the chemokine mRNA. Investigation into the mechanism has preliminarily revealed that CI-S5 may moderate chemokine expression by increasing TTP expression.

This study indicates that the CI-S5 peptide may function as a broad-spectrum inhibitory prodrug for the treatment of inflammatory diseases. CI-S5 treatments enhanced TTP expression and reduced production of inflammatory chemokines, which could then inhibit inflammatory progress and prevent or reduce the pathological processes of inflammation. Our findings provide a basis for the development of new peptide-based therapies for the treatment of inflammatory diseases.

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炎症及炎症耐受模型筛选广谱趋化因子抑制肽 *

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摘要 通过减少炎性组织或细胞趋化因子及炎性因子的表达量能将炎症性病理过程抑制在起始阶段. 我们通过体外构建人外周血单个核细胞 LPS 激活的急性炎症模型及内毒素耐受模型，进行噬菌体肽库亲和筛选，ELISA 检测与炎性 PBMC 的结合能力，分泌抑制实验筛选抑制性噬菌体克隆，经趋化抑制、竞争结合及生成抑制实验检测体外活性，大鼠足肿胀及关节炎模型检验多肽体内作用，SqRT-PCR 检测趋化因子及 TTP 的 mRNA 水平，探讨其作用机制. 筛选到的目标多肽 CI-S5 趋化抑制率达到 48.72%，明显抑制噬菌体阳性克隆 P15 与 LPS 激活 PBMC 的结合，动物试验能明显降低大鼠足肿胀及关节炎症. 机制研究显示，CI-S5 多肽能降低 3 种趋化因子的表达量，并调节 TTP 使其表达增加，提示 CI-S5 能够靶向炎症前期 PBMC，为炎症治疗提供了针对早期急性炎症反应的广谱小分子抑制肽.

关键词 内毒素耐受，混合靶标噬菌体展示，广谱趋化因子抑制，生物活性肽，TTP

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