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Effects of a novel peptide derived from human thrombomodulin on endotoxin-induced uveitis in vitro and in vivo

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ABSTRACT

Thrombomodulin (TM) is a single-transmembrane glycoprotein receptor for thrombin, which is best known as a cofactor for thrombin-mediated activation of anticoagulant protein C. C-type lectin-like domain (CTLD) of TM has distinct coagulation/fibrinolysis-independent anti-inflammatory properties. Here we found anti-inflammatory effects of a novel peptide (GC31) from CTLD of TM in endotoxin-induced uveitis, which was characterized by a reduction of leukocyte counts, protein concentration, tumor necrosis factor (TNF)-α and monocyte chemoattractant protein (MCP)-1 levels in aqueous humor. Through in vitro experiments, we further found that GC31 suppressed TNF-α and interleukin (IL)-6 expressions in lipopolysaccharide (LPS)-stimulated macrophage-like RAW264.7 cells and interrupted LPS-induced nuclear factor-κB (NF-κB) activation. These data indicate a beneficial role of peptide GC31 in preventing intraocular inflammatory response, especially uveitis.

1. Introduction

Macrophage activation by bacterial toxins such as lipopolysaccharide (LPS) induces secretion of a number of cytokines and chemokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, monocyte chemoattractant protein (MCP)-1 [1,2], which are key inflammatory mediators in many chronic inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, and ocular inflammatory diseases [2–4]. Uveitis, an ocular inflammatory condition, is one of the most common causes of blindness and accounts for 10–20% of cases of legal blindness per year [5]. Autoimmune diseases and infections are considered as the major causes of uveitis [6]. Corticosteroids are the most frequently prescribed medications for uveitis [5] and usually used as long-term therapy for severe uveitis to prevent recurrences. However, its supply is limited due to potentially serious adverse effects, such as induction of glaucoma, formation of cataracts, decreased resistance to infection, and decreased wound healing [7]. Therefore, additional steroid-sparing agents with less adverse effects are urgently needed.

Endotoxin-induced uveitis (EIU) is a widely accepted animal model of ocular inflammation, which is induced by a systemic injection of a sublethal dose of LPS [8,9]. It is characterized by the breakdown of blood–eye barrier and infiltration of leukocytes and protein in the eyes [10]. In this model, LPS may directly activate the vascular endothelium, macrophages, and other cells by interaction with toll-like receptor 4 (TLR4) [1] and induce cytokines and other inflammatory mediators production, such as TNF-α, IL-6, IL-1β, and MCP-1 [2,11]. TNF-α and IL-6 have been reported to be involved in the pathogenesis of LPS-induced uveitis [12]. MCP-1 has also been reported to be present in the plasma of patients with active Behçet's disease [13] and in the aqueous humor (AqH) of patients with active uveitis [14].

Thrombomodulin (TM) is well known for its natural anticoagulant function and anti-inflammatory properties [15]. Human TM consists of 557 amino acid residues arranged in five distinct domains including an N-terminal domain with weak sequence similarity to the family of C-type lectins, six tandemly repeated epidermal growth factor (EGF)-like domains, a Ser/Thr-rich region, a transmembrane domain, and a short cytoplasmic tail [16]. It is increasingly recognized that the C-type lectin-like domain (CTLD) of TM plays an important role in cell adhesion and inflammation

Abbreviations: TM, thrombomodulin; LPS, lipopolysaccharide; EIU, endotoxin induced uveitis; DXM, dexamethasone; TNF-α, tumor necrosis factor α; MCP-1, monocyte chemoattractant protein-1; NF-κB, nuclear factor kappaB

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control. The possible mechanisms involved in anti-inflammatory properties of CTLD may be that the lectin-like module protects the vasculature and tissues from pro-inflammatory stimuli by interfering with leukocytes adhesion through suppressing adhesion molecules expression via NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways, overall dampening the inflammatory response [17]. It is reported that mice lacking the N-terminal domain of TM have an augmented response to lung injury, myocardial ischemia–reperfusion, endotoxemia, and arthritis and that administration of recombinant lectin-like domain of TM significantly attenuates the development of arthritis [17,18].

In the present study, we identified and characterized a novel 31-amino acid peptide with the sequence of GFQWVTGDNNTSYSRWARLDLNGAPLCGPLC (named GC31) from the CTLD of TM. We assessed the peptide's anti-inflammatory properties in EIU. In addition, to further elucidate the mechanism of the effect of GC31 on EIU, we determine the anti-inflammatory effects of GC31 using LPS-induced mouse macrophage-like RAW264.7 cells as the model.

2. Materials and methods

2.1. Preparation of peptides

According to the characteristics of CTLD structure of the C-type lectins superfamily [19], we focused on one conserved amino acid sequence from CTLD of TM (GC31: GFQWVTGDNNTSYSRWARLDLNGAPLCGPLC, molecular weight: 3412.8 Da) in our study. A control peptide, named VP30 (VISLLLNGDGGVGRRRLWIGLQLPPGCGDP, molecular weight: 3129.6 Da), was simultaneously synthesized from the same domain of TM and used in all the following experiments in vitro and in vivo.

A high-efficiency solid-phase peptide synthesis was performed by ChinaPeptides Co., Ltd. in Shanghai, PR China with an automatic peptide synthesizer (Symphony; Protein Technologies, Tucson, AZ). The purified peptide was characterized by analytical high-performance liquid chromatography (HPLC; Shimadzu, Kyoto, Japan) and mass spectrometry (MS, Finnigan TSQ 7000; Thermo, Waltham, MA) with the purity over 95%. All the synthesized peptides were freeze-dried and stored at −20 °C until used.

2.2. Animals for EIU induction and treatment

Eight-week-old male Wistar rats (weighing 160–220 g), procured from Sino-Brithish SIPPR/BK LAB.ANIMAL LTD., CO were used in this study. They were maintained under a 12-h light/12-h dark cycle. Food and water were supplied ad libitum. All animal experiments were in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

EIU was induced by a single subcutaneous injection of LPS (200 μg/100 μL, *Escherichia coli,* 055:B5, Sigma–Aldrich, St. Louis, MO, USA) in saline into the right hind footpad. Rats were anesthetized by intraperitoneal injection of 0.15 mL pentobarbital (5.47 g/100 mL saline). Pupils were dilated by instillation of one drop of tropicamide 5% and one drop of tetracaine 1% was administered for local anesthesia prior to intravitreal injection. Intravitreal injections (10 μL) were performed in both eyes using sterile syringes fitted with 29-gauge needle (Ultra-Fine™; Becton Dickinson AG, USA) under a surgical microscope, as previously described [20]. Injections were performed 1 mm behind the limbus, and the needle was left in the eye for 30 s to prevent the liquid to reflux. Rats were injected intravitreally in both eyes with phosphate-buffered saline (PBS, pH 7.4), GC31 (2, 10 and 20 μg), VP30 (20 μg), DXM (20 μg, Sigma–Aldrich, St. Louis, MO) diluted in PBS immediately after LPS injection. Rats of control group or GC31-alone group were injected intravitreally with PBS or 20 μg GC31 and without LPS injection.

There were 10 rats in each group. Aqueous humor of six rats and eyeballs of the other four rats were collected when rats were under deep anesthesia and then they were euthanized by cervical dislocation.

2.3. Infiltrating cells and proteins in aqueous humor

Twenty-four hours after LPS injection, the aqueous humor (AqH) was collected from the eyes immediately by an anterior chamber puncture with a 30-gauge needle under a surgical microscope. AqH was collected from both eyes of each animal and pooled.

For cell counting, the AqH samples were suspended in the Trypan-blue solution (1:5), and applied to a hemocytometer. The number of cells per field (an equivalent of 0.1 μL) was manually counted under a light microscope (Olympus Optical Ltd., London, UK), and the number of cells per microliter was obtained by averaging the results of four fields from each sample. Total protein concentration in AqH was measured with a bicinchoninic acid (BCA) protein assay reagent kit (Pierce, Rockford IL, USA).

2.4. Levels of TNF-α, MCP-1 and IL-6

The levels of TNF-α and MCP-1 in AqH and concentrations of TNF-α and IL-6 in the culture medium were assessed with a commercially available enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's instructions. The ELISA assay was performed in duplicate.

2.5. Histopathological examination

Animals were examined under slit lamp at 24 h after LPS injection and eyes were then enucleated immediately and fixed in 4% paraformaldehyde for 48 h at room temperature, after which they were kept in 70% alcohol at 4 °C until they were embedded in paraffin. Sagittal sections (5 μm) were cut near the optic nerve (eight eyes of four rats in each group) and stained with hematoxylin and eosin (H&E). The iris–ciliary body complex, anterior chamber, vitreous, and retina were observed under light microscope. Infiltrating leukocytes in the anterior chamber and vitreous cavity were counted in a masked fashion by an ocular pathologist [11].

2.6. Cell culture

RAW264.7, a murine macrophage-like cell line purchased from the CBCAS (Cell Bank of the Chinese Academic of Sciences, Shanghai, China) was maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Biochrom, AG, Germany) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin). The cells were maintained at 37 °C in an atmosphere of 5% CO_2.

2.7. Cell viability assay

The cell viability of RAW264.7 cells was assessed using the Cell Titer 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, Madison, WI, USA). Briefly, cells were plated in the 96-well plate and allowed to adhere for 24 h. Then cells were treated with various concentrations of peptides for 24 h. Cells in the control group were left untreated. After then 20 μL MTS solution was added to each well and incubated for another 3 h at 37 °C. Absorbance was detected at 490 nm with a microplate reader (Bio-Rad; Model 680; USA).

2.8. Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted with Trizol reagent (Invitrogen), according to the manufacturer's instructions. The fluorescence quantitative RT-PCR was performed on the Rotor-Gene 3000 (Corbett Research, Australia) instrument with a One-Step SYBR PrimeScript RT-PCR kit (TaKaRa Bio Inc., Japan) according to the protocol. Specific sense and antisense primers used were as follows: TNF-α, sense: 5′-GACGTGGAACTGGCAGAAGAG-3′, anti-sense: 5′-TTGGTGGTTTGTGAGTGTGAG-3′; IL-6, sense: 5′-CTGCAAGAGACTTCCATCCAGTT-3′, anti-sense: 5′-GAAGTAGGGAAGGCCGTGG-3′; GAPDH, sense: 5′-CCAAGGAGTAAGAAACC-3′; anti-sense: 5′-GCAGCGAACTTTATTGA-3′. GAPDH was chosen as a housekeeping gene where indicated.

2.9. Immunofluorescence staining for NF-κB

RAW264.7 cells were pretreated with GC31 or VP30 (10 μM) for 30 min, and then stimulated with 100 ng/mL LPS for 1 h. After incubation, cells were washed with Tris-buffered saline, fixed in cold methanol/acetone (1:1) for 20 min and permeabilized with 0.1% Triton X-100 for 10 min at room temperature. Cells were incubated with NF-κB p65 antibody (1:50, Cell Signaling Technology, Beverly, MA) in a humid chamber overnight at 4 °C. After incubated with Alexa555-conjugated secondary IgG antibody (1:500; Invitrogen) for 45 min, the coverslips were mounted in medium containing 4,6-diamino-2-phenylindole (DAPI) (Dako Cytomation) onto the glass slides. Slides were visualized and photographed under a confocal fluorescence microscope (LSM 510, Carl Zeiss, Gottingen, Germany). The same exposure was used for photographing all samples.

2.10. Cytoplasmic and nuclear protein extracts and Western blotting

After cells were rapidly harvested using a cell scraper, nuclear and cytoplasmic extracts were prepared on ice with ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas Life Science, Opelstrasse, Germany). Protein concentration was measured by the Bradford method. Cell homogenates (30–50 μg of protein) were separated by 10% SDS–PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membranes. After blocking with 5% bovine serum albumin (BSA), the membrane was incubated overnight 4 °C with the target antibody. Each membrane was further incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1;1000, R&D Systems, Inc.), respectively. Lamin A/C (1:1000, Epitomics, Inc., California, USA) was chosen as a nuclear housekeeping protein in the nuclear extract. The bands were visualized using the ECL system detection system (Pierce, Rockford, IL, USA), and the band density was determined by Image J software (NIH, USA).

2.11. Statistical analysis

All the experiments were repeated at least three times. The results were expressed as mean ± standard deviation (S.D.) and analysed using one-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test using SPSS 16.0 software for multiple comparisons of mean values. $p < 0.05$ was considered statistically significant.

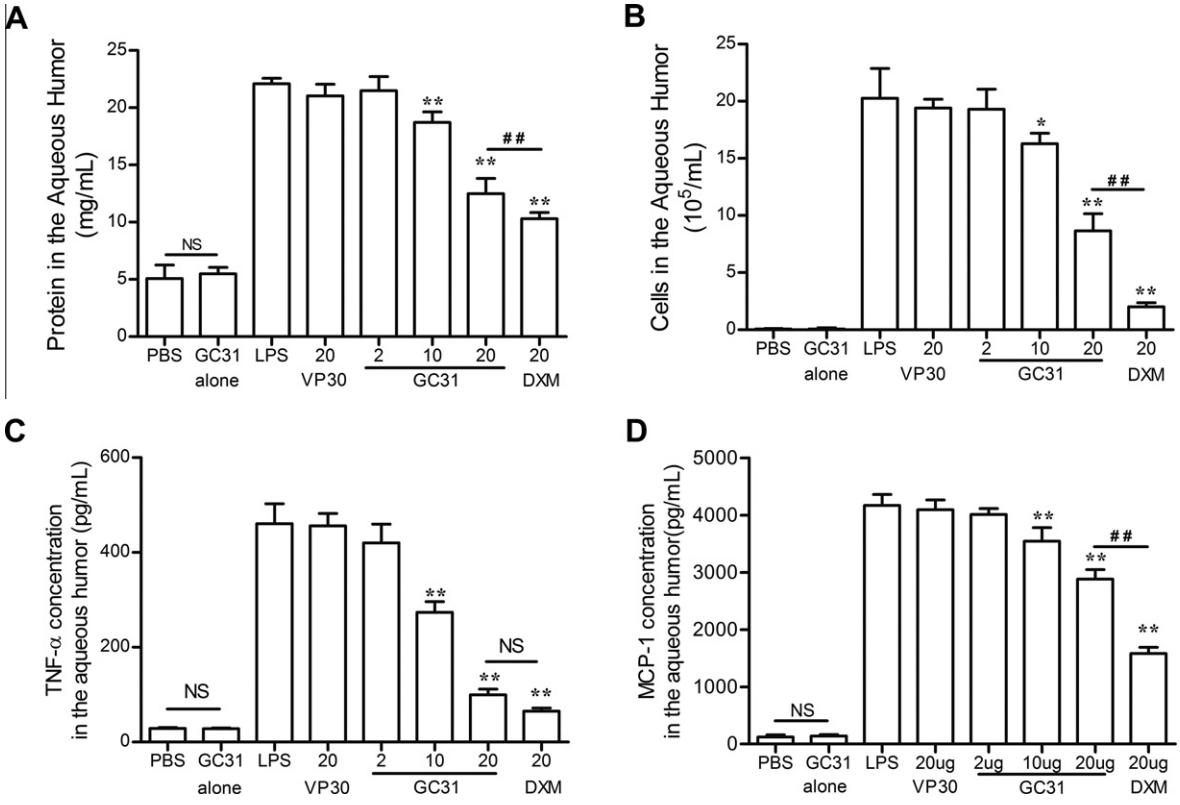

Fig. 1. Effect of GC31 peptide on LPS-induced protein concentration (A), cell counts (B), TNF-α (C), and MCP-1 concentration (D) in the aqueous humor. The aqueous humor was collected in rats treated with PBS, GC31, VP30 or DXM alone or combined simultaneously with LPS (200 μg) challenge for 24 h. The levels of TNF-α (C) and MCP-1 (D) were measured using ELISA method. Each value represents the mean ± S.D. of six rats. $^{*}p < 0.05$ and $^{**}p < 0.01$ versus the LPS group. $^{\#\#}p < 0.01$ versus the DXM group.

3. Results

3.1. Effect of GC31 on cellular infiltration and protein concentration in aqueous humor

Twenty-four hours after LPS injection, the protein concentration in AqH of rats increased to 22.09 ± 0.49 mg/mL (n = 6) and GC31 administration significantly decreased protein concentrations in a dose-dependent manner (Fig. 1A). The group treated with 20 μg/eye DXM exhibited a bit stronger inhibitory ability than 20 μg/eye GC31 group (GC31: 12.48 ± 1.35 mg/mL; DXM: 10.31 ± 0.53 mg/mL, Fig. 1A). The level of protein concentration detectable from the GC31-alone group was 5.49 ± 0.57 mg/mL and showed no difference with the control group. Likewise, the number of leukocytes in the groups treated with GC31 or DXM was significantly diminished than that in the LPS group (Fig. 1B). No infiltrating cells were detected in AqH of the control group and GC31-alone group. However, compared with LPS-alone group, treatment with VP30 (20 μg/eye) or GC31 (2 μg/eye) combined with LPS injection resulted in no obvious reduction in the cell counts and protein concentration (Fig. 1A and B).

3.2. Levels of TNF-α and MCP-1 in aqueous humor

Secretion of TNF-α and MCP-1 in the AqH was remarkable in the LPS group (TNF-α: 460.5 ± 94.96 pg/mL; MCP-1: 4168 ± 191.6 pg/mL). Treatment with GC31 significantly reduced TNF-α and MCP-1 production, compared with the LPS group (in 10 μg and 20 μg GC31 group, $p < 0.01$, Fig. 1 C and D). The effect of 20 μg GC31 on TNF-α level was almost the same as that for DXM group

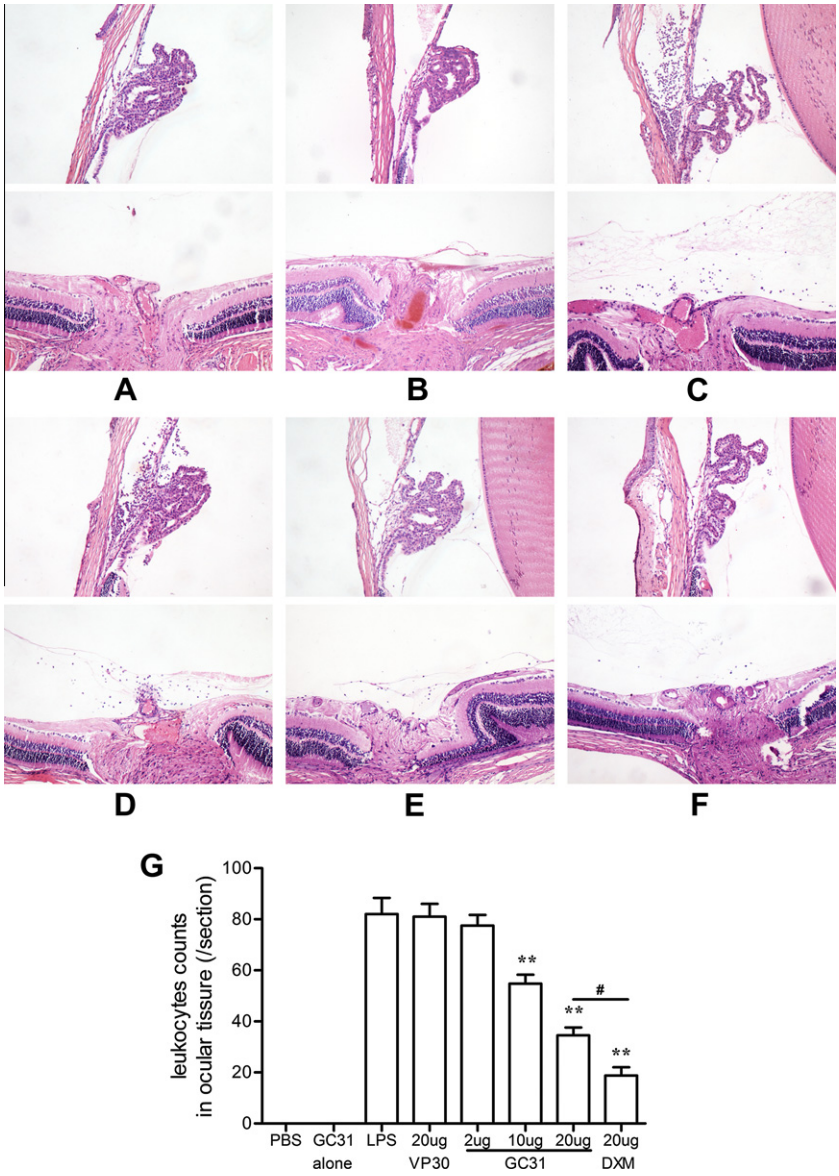

Fig. 2. Histolopathologic features of inflammatory cells infiltration in ocular tissues 24 h after LPS injection. Rats without LPS injection (A) or with GC31 (20 μg) injection alone (B) showed no inflammation in the anterior chamber and posterior vitreous cavity. Severe inflammatory cells infiltration was observed in LPS-alone (200 μg) injected rats (C) and 20 μg VP30 treated rats (D). A significant attenuation of inflammation was observed in rats treated with 20 μg GC31 (E) or 20 μg DXM (F) and LPS simultaneously. Hematoxylin and eosin staining; original magnification ×100. (G) Effect of GC31, VP30 or DXM at indicated concentrations on leukocytes infiltration in histological sections. Mean ± S.D. (n = 8 eyes). $^{**}p < 0.01$ versus the LPS group. $^{\#}p < 0.05$ versus the DXM group.

($p > 0.05$, Fig. 1C). Treatment with VP30 (20 μg/eye) or GC31 (2 μg/eye) showed no inhibitory effects on the TNF-α and MCP-1 concentrations (Fig. 1 C and D).

3.3. Histopathologic findings

Histological examination showed that severe cellular infiltration was observed in the anterior chamber, iris-ciliary bodies, and posterior vitreous (particularly around the optic nerve head). In the LPS group, 82 ± 13 inflammatory cells per histological section were detected ($n = 8$, Fig. 2C), while inflammatory cells in the GC31 or DXM treated groups were significantly decreased (Fig. 2 E and F). But treatment with VP30 (20 μg) or GC31 (2 μg) did not induce any decreased grading (Fig. 2 D and G). No inflammation was observed histopathologically in rats treated with PBS or GC31 (20 μg) alone (Fig. 2 A and B).

3.4. GC31 suppresses LPS-induced expression of IL-6 and TNF-α in RAW264.7 cells

The peptides did not decrease the cell viability of RAW264.7 cells when these cells were incubated with GC31 (0.1 μM, 1 μM, and 10 μM) or VP30 (10 μM) for 24 h (data not shown).

TNF-α and IL-6 are known as inflammatory cytokines which are correlated with LPS stimulation [2,12]. To assess the effect of GC31 on LPS induced pro-inflammatory cytokines up-regulation, RAW264.7 cells were pretreated with 0.1–10 μM GC31 for 30 min and incubated for 24 h with LPS (100 ng/mL). We observed that LPS remarkably increased levels of TNF-α and IL-6 in RAW264.7 cells, whereas GC31 significantly reduced TNF-α and IL-6 productions in a dose-dependent fashion (Fig. 3 A and B). GC31 (0.1 μM) and VP30 (10 μM) did not influence TNF-α and IL-6 protein level compare to the LPS group.

We then performed RT-PCR to analyze the effects of GC31 on LPS-induced TNF-α and IL-6 mRNA levels. As shown in Fig. 3 C and D, LPS (100 ng/mL) treatment significantly elevated TNF-α and IL-6 mRNA expression, which was suppressed by GC31. However, VP30 (10 μM) did not demonstrate any inhibitory effect on TNF-α and IL-6 productions. These data indicated that GC31 might inhibit the production of TNF-α and IL-6 at the gene transcription and translation levels in LPS-activated RAW264.7 cells.

3.5. Effects of GC31 on NF-κB activation in LPS-stimulated RAW 264.7 cells

To further characterize the mechanism underlying the effects of GC31, the activation and translocation of NF-κB were examined. Firstly, we chose GC31 10 μM to perform immune-fluorescence staining of NF-κB p65. Nuclear staining was evident after LPS stimulation, indicating that NF-κB had been activated and translocated to the nucleus (Fig. 4A). In PBS-treated and GC31-treated cells, cytoplasmic staining with nuclear sparing was evident, whereas VP30 group had the nuclei stained obviously. Next, we tested the expression of nuclear NF-κB p65 following 1 h after LPS treatment

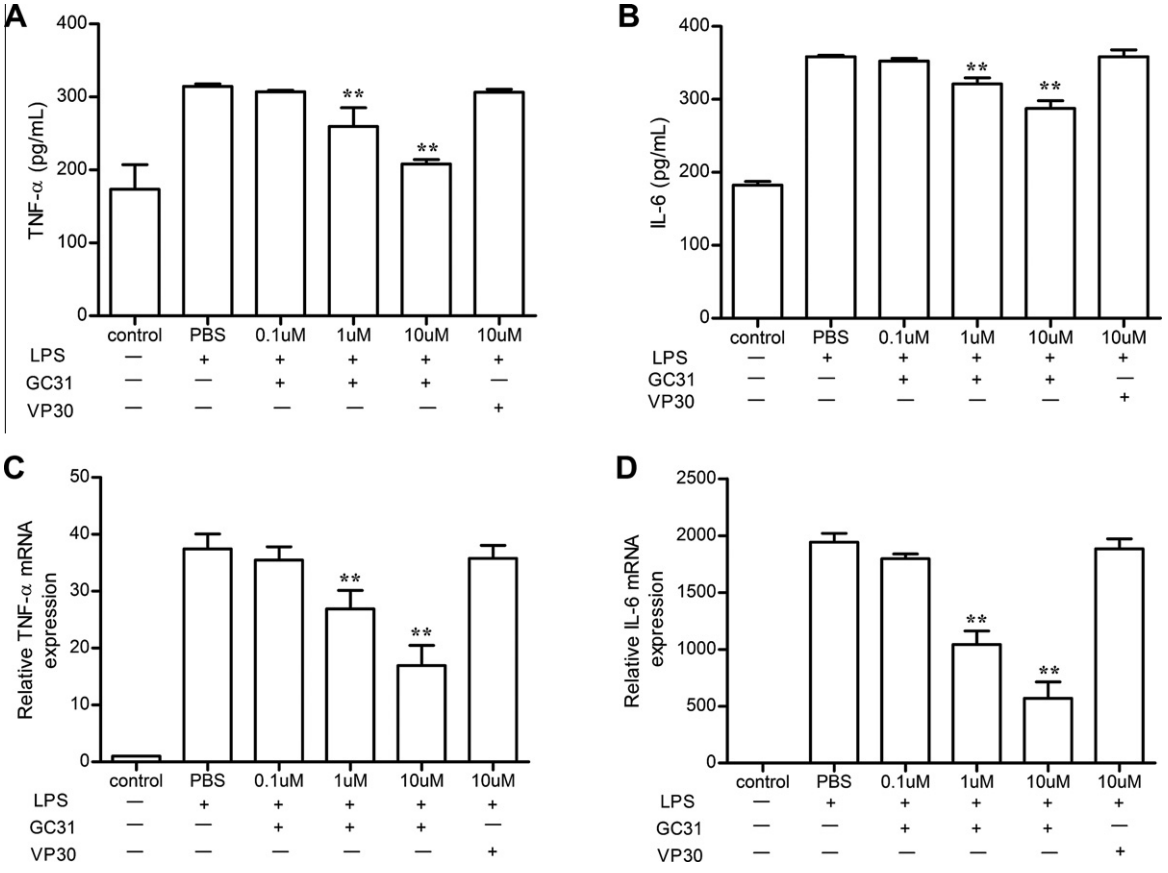

Fig. 3. Effects of GC31 on LPS-induced TNF-α and IL-6 production in RAW264.7 cells. The cells were treated with GC31 (0.1 μM, 1 μM, 10 μM) or VP30 (10 μM) simultaneously combined with LPS (100 ng/mL). (A, B) The amounts of TNF-α and IL-6 in the culture medium 24 h after LPS stimulation were determined using ELISA assay kits. (C, D) The mRNA levels of TNF-α and IL-6 were assessed by RT-PCR analysis 6 h after LPS stimulation. GAPDH mRNA was measured as the internal control. The data represent means ± S.D. of triplicate measurements. $^{**}p < 0.01$ versus the LPS group.

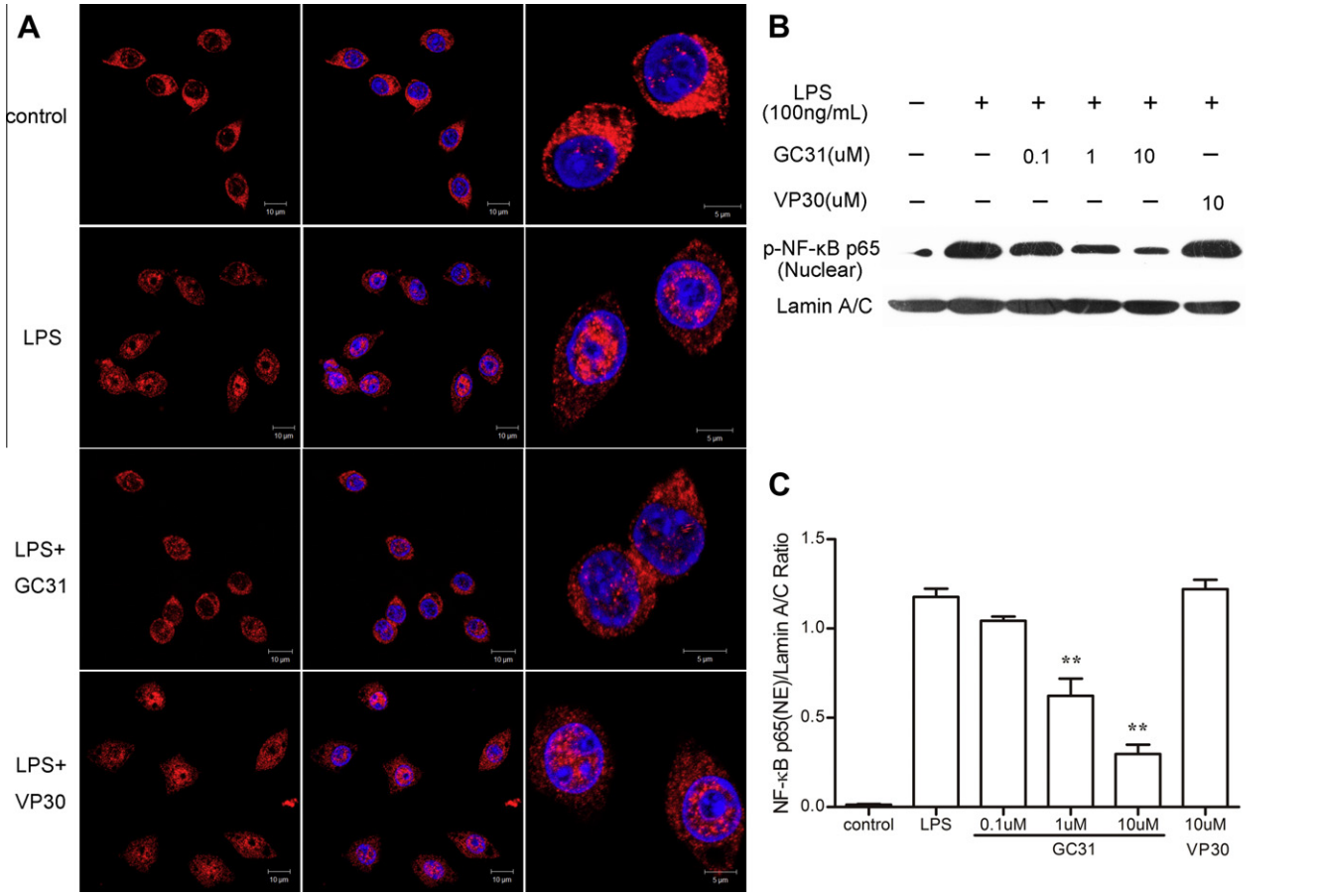

Fig. 4. Effects of GC31 on NF-κB activity in LPS-activated RAW264.7 cells. (A) Cells were pre-treated with 10 μM GC31 or VP30 for 30 min and then stimulated with 100 ng/mL LPS for 1 h. The cells were subjected to an immunofluorescent staining method with anti-NF-κBp65 antibody. (Red: NF-κB p65; Blue: nuclear.) (B) Cells were pre-incubated with GC31 or VP30 for 30 min and then with 100 ng/mL LPS for 1 h. Nuclear proteins were analyzed by western blot analysis using antibody against NF-κB p65. Lamin A/C was used as the internal control. (C) The band density was determined by Image J software and the relative level was calculated as the ratio of NF-kB p65 to Lamin A/C protein expression. The results represent means ± S.D. of three independent experiments. $^{**}p < 0.01$ versus the LPS group.

by western blotting analysis. Pretreatment with GC31 at 1.0 μM, and 10 μM significantly inhibited NF-κB p65 protein translocation compared with the LPS alone group (Fig. 4 B and C). The result suggested that GC31 suppressed LPS-induced activation of NF-κB in the nuclear fraction in a dose-dependent fashion.

4. Discussion

In view of many side effects of traditional treatments and repeated occurrences of intraocular inflammation, uveitis remains a challenging field to most ophthalmologists. Additional immunosuppressive drugs including T-cell inhibitors, anti-metabolites, alkylating agents and new immunomodulatory agents that target specific inflammatory cytokines such as TNF-α are also associated with many adverse effects, such as nephrotoxicity [21], malignancy, multiple sclerosis [22] and high medical cost are concerned. Moreover, in certain ocular tissues, tight junctions reduce the passage of drugs from the blood to the eye, thus limiting the bioavailability of immunosuppressors in ocular tissues. As a result, short peptides become ideal alternatives for treating uveitis, owing to their sufficient penetration capability, potentially low immunogenicity, and less toxicity [23].

As mentioned before, the CTLD of TM plays a distinct role in anti-inflammation by interfering with leukocyte adhesion, complement activation and cytokine generation [17,18,24]. In this study, we synthesized a novel peptide, GC31, from a conserved sequence of TM to investigate their potential anti-inflammatory activity in vitro and in vivo.

It has been well documented that many inflammatory mediators, such as TNF-α, IL-6, MCP-1, and macrophage inflammatory protein (MIP)-2, have been involved in rabbits, rats, and mice with EIU, which can induce increased protein concentration in the aqueous humor [2,11,12,25]. In the present study, we found that GC31 significantly inhibited LPS-induced TNF-α and MCP-1 secretion in AqH and level of IL-6 and TNF-α in RAW264.7 cell medium, which demonstrated the anti-inflammatory effects of GC31 on preventing the increase of pro-inflammatory factors stimulated by LPS. TNF-α is a pivotal inflammatory mediator by inducing the synthesis of other cytokines, such as MCP-1, IL-8, intercelluar adhesion molecule (ICAM)-1, enhancing inflammatory cells adherence to the endothelium, and promoting inflammatory responses [2,25–27]. MCP-1 is uniquely essential for monocyte recruitment during inflammation and the number of ocular inflammatory cells was significantly reduced in MCP-$1^{-/-}$ mice of EIU [11,14,27]. Therefore it indicates that GC31 reduction of leukocyte infiltration in the AqH and vitreous cavity is caused, at least in part, by its inhibition of MCP-1 and TNF-α expression. In addition, Conway [17] showed that the lectin-like domain of TM could interfere with leukocyte adhesion to ECs by ICAM-1 – dependent pathway. ICAM-1 is upregulated and expressed on vascular endothelial cells of the iris and the ciliary body shortly after LPS injection [28]. Therefore, another possible mechanism for the suppression of inflammatory cells exu-

dation by peptide GC31 could be due to inhibition of LPS induced ICAM-1 production.

NF-κB, a ubiquitously expressed family of transcription factors of various genes [29], is known to play a crucial role in macrophage activation and regulation of inflammatory cytokine genes in response to TLR4 signals [30]. TNF-α is activated through NF-κB, also activates NF-κB, thus promoting its own secretion and generating a positive loop that amplifies the cytokine cascade and the inflammatory response [29]. In the in vitro study, we found that GC31 could significantly suppress NF-κB nucleus translocation in LPS-stimulated RAW264.7 cells, which raised a possibility that the positive loop between TNF-α and NF-κB might be interfered by GC31. Based on some studies which showed that TNF-α-induced ICAM-1 and MCP-1 expression required NF-κB activity [25,26] and NF-κB activation in EIU tissue specimens was largely detected [31], we concluded that the anti-inflammatory properties of peptide GC31 were performed by blocking LPS-activated NF-κB translocation, inhibiting pro-inflammatory mediators expression and then dampening the EIU.

The CTLD of C-type lectin superfamily has a double-loop structure and the long loop region which lies within the domain is involved in Ca^{2+}-dependent carbohydrate binding [19]. The location of GC31 in CTLD of TM corresponds to the long loop region. GC31 structure analysis by the Expert Protein Analysis System (ExPASy) proteomics server (http://heliquest.ipmc.cnrs.fr/) shows that some part of the sequence (TSYSRWARLDLNGAPLCGPLC) is amphipathic and retains cationic and hydrophobic faces. This structure is thought to be important for interaction with a negatively charged amphipathic LPS [32]. Meanwhile, Shi CS [24] demonstrated that CTLD of TM inhibited LPS-induced inflammatory mediator production via binding to LPS and specifically interacting with Lewis Y, a tetrasaccharide expressed on the surface of pathogens. Accordingly, this novel peptide GC31 rather than VP30 might have a possible site for interaction between CLTD and LPS antigen and may play an anti-inflammatory role in LPS-induced inflammation.

We performed gross examination for all experimental animals, and no corneal edema, cataract, vitreous hemorrhages, or retinal detachment was found in all groups 24 h after EIU induction. And light microscopy demonstrated that the iris-ciliay body and retinal morphology in GC31-alone group had no distinguishable findings from the control group; no evident signs of inflammation or immune reaction were found in GC31-alone group. This information primarily indicated that its effective concentration (10 and 20 μg) in the study had no obvious tissue toxicity and immunogenicity. Although its biologic activity is not as strong as that of DXM, the latter has many impressive side effects described above.

Some limitations of the present study need to be considered. First, the study focused on parameters of inflammatory response, but not data concerning the exact mechanism of action of GC31. We can ascertain that the peptide exerts its effects through the inhibition of inflammatory mediators production mediated by the inhibition of NF-κB. However, the interaction between GC31 and LPS deserves further studies. Second, the used model (EIU) has certainly been useful in elucidating a variety of mediators that are activated in the eye, and testing a wide variety of potential pharmacologic inhibitors [8,20,31,32], but there are various immune abnormalities including genetic elements, infectious agents, and autoimmune components implicated in uveitis. The effect of peptide GC31 in the LPS-induced uveitis was demonstrated in the current study, however its ability to counteract ocular inflammation induced by other causes needs to be elucidated in the future researches by other animal models (experimental autoimmune uveitis for instance).

In summary, the present study demonstrated that in vivo administration of GC31 reduced intraocular inflammatory response in EIU by inhibition of protein and inflammatory cells infiltration and pro-inflammatory cytokines production. Moreover, GC31 inhibited the activation of NF-κB and suppressed the transcription and production of TNF-α and IL-6 cytokines in LPS-stimulated RAW264.7 cells. These findings suggest that the novel peptide GC31, a sequence of TM lectin-like domain, may be addressed as a promising therapeutic agent in LPS induced intraocular inflammation.

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