• **Basic Research** •

Expression and role of calcitonin gene-related peptide in mouse *Aspergillus fumigatus* keratitis

Min Yin, Cui Li, Xu-Dong Peng, Gui-Qiu Zhao, Yuan Wu, Heng-Rui Zheng, Qian Wang, Qiang Xu, Nan Jiang

Department of Ophthalmology, the Affiliated Hospital of Qingdao University, Qingdao 266003, Shandong Province, China
Co-first authors: Min Yin, Cui Li and Xu-Dong Peng
Correspondence to: Gui-Qiu Zhao. Department of Ophthalmology, the Affiliated Hospital of Qingdao University, 16 Jiangsu Road, Qingdao 266003, Shandong Province, China. zhaoguiqiu_good@126.com

Abstract

● **AIM: To investigate the expression and role of calcitonin gene-related peptide (CGRP) in the mouse models induced by *Aspergillus fumigatus* (*A. fumigatus*).**

● **METHODS: C57BL/6 mice were randomized into a control group and *A. fumigatus* keratitis group. The cornea photography was assessed under the slit lamp and the clinical score was recorded after infection. Western blot, real-time polymerase chain reaction (PCR) and immunohistofluorescence analysis were applied to detect CGRP expression in cornea of both groups. *In vitro*, tests were conducted with C57BL/6 mice macrophages to investigate CGRP expression after interaction with *A. fumigatus*. Cytokines expression induced by exogenous CGRP and the antagonist CGRP8-37 in *A. fumigatus*-exposed macrophages was evaluated by real-time PCR and ELISA.**

● **RESULTS: The cornea expression of CGRP was significantly elevated in C57BL/6 mice corneas and macrophages after *A. fumigatus* infection. After treatment with exogenous CGRP, the levels of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α) and IL-6 were reduced, and IL-10 level was increased in the *A. fumigatus* stimulated-macrophages. However, IL-1β, TNF-α and IL-6 levels were upregulated after pretreatment of CGRP8-37. But the mRNA levels of MIP-2, TGF-β and IL-10 were not changed.**

● **CONCLUSION: This study provides evidence that *A. fumigatus* increased CGRP expression. CGRP may play a protective role against inflammation in *A. fumigatus* keratitis.**

● **KEYWORDS:** calcitonin gene-related peptide; fungal keratitis; macrophages; inflammation; mice

INTRODUCTION

Fungal keratitis (FK) is recognized as a serious disease which can cause ocular pain, stromal destruction, corneal thinning, perforation and blindness especially in developing countries[1-2]. *Aspergillus* is one of the main pathogenic fungi. Especially, *Aspergillus fumigatus* (*A. fumigatus*) can induce a strong host immune inflammatory reaction of FK[3]. Currently, owing to the lack of efficacious antifungal drugs, increasing numbers of researchers are focusing on the mechanisms of uncontrolled overactive host innate immune inflammatory responses in FK[4]. Inflammation degree in the cornea is determined by the body immunocyte status and secretion of pro- and anti-inflammatory cytokines[5-6].

Calcitonin gene-related peptide (CGRP) is a 37 amino acid neuropeptide, which is widely distributed in the nervous systems[7-8]. Therefore, CGRP plays prominent immunoregulatory roles of macrophages[9], neutrophils[10], dendritic cells[11] and other immune cells in inflammation[12]. Results have shown that CGRP plays anti-inflammatory role in immune reactions. CGRP can inhibit the lipopolysaccharide (LPS)-induced release of interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α)[13]. CGRP also reduces neutrophil recruitment and opsonophagocytic killing of *Streptococcus pyogenes*[10].

Recent researches have shown that CGRP was obviously increased in different inflammatory diseases[9,14-16]. Studies have demonstrated CGRP treatment can up-regulate pro-inflammatory factors and down-regulate anti-inflammatory factors to reduce inflammation reactions[17-19]. Maruyama *et al*[20] found that CGRP inhibited β-glucan-induced inflammatory reactions by direct inhibition of nuclear factor-κB (NF-κB) in *Candida* osteomyelitis. In addition, Kashem *et al*[21] found that exogenous addition of CGRP can rescue the susceptibility of *C. albicans* cutaneous infection which was increased by ablation of sensory neurons. A previous study that described the nerve

architecture and distribution of sensory neuropeptides in mouse cornea indicated that CGRP is one of the most abundant in this tissue[22]. However, the role of CGRP has never been tested in experimental keratitis induced by *A. fumigatus*. Our study investigated the release of CGRP in C57BL/6 corneas and in cultured primary macrophages in response to *A. fumigatus* exposure. In addition, we investigated the effect of CGRP on inflammatory cytokine secretion to determine the role of CGRP in inhibition of inflammation.

MATERIALS AND METHODS

Ethical Approval This study was conducted in accordance with the Declaration of Helsinki and approved by the Research Ethics Committee of the Affiliated Hospital of Qingdao University. The treatments of mice were based on the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Mice and Reagents Adult (8-week) female C57BL/6 mice (specific pathogen-free; SPF) were purchased from Jinan Pengyue Experimental Animal Co., Ltd. (Jinan, Shandong Province, China). All the mice were weighed between 20-30 g and carefully housed in the laboratory conditions. Sabouroud culture was obtained from Babio biotech (Jinan, Shandong Province, China). Thioglycollate medium came from Hope Bio-Technology Co., Ltd. (Qingdao, Shandong Province, China). Sodium thioglycollate was from Xiya Chemical Industry Co., Ltd. (Shanghai, China). Fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) were derived from Gibco (San Diego, California, USA). RNAiso Plus, reverse-transcription polymerase chain reaction (RT-PCR) kit and TB Green™ Premix Ex Taq™ II were obtained from TaKaRa (Dalian, Liaoning Province, China). BCA Protein Assay Kit, ammonium persulfate, phosphate buffer saline (PBS), phenylmethylsulfonyl fluoride (PMSF) and radio immunoprecipitation assay (RIPA) lysis buffer were obtained from Solarbio (Beijing, China). Polyvinylidene difluoride (PVDF) membrane, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), sample loaded buffer, confining liquid and enhanced chemiluminescence (ECL) kit were derived from Beyotime Biotechnology (Shanghai, China). GADPH antibody, IL-1β and TNF-α ELISA kits were form Elabscience (Wuhan, China). CGRP was from GenScript Biotech Corp. (New Jersey, USA) and CGRP8-37 was from Abcam (Cambridge, UK). CGRP monoclonal antibody and TNF-α antibody were purchased from Cell Signaling Technology (CST, Danvers, MA, USA). Optimum cutting temperature (OCT) compound was from SAKURA Tissue-Tek (Torrance, CA, USA).

Preparation of *Aspergillus fumigatus* Standard strain *A. fumigatus* were purchased from China General Microbiological Culture Collection Center, species number 3.0772 (Beijing, China). *A. fumigatus* was inoculated in Sabouroud medium at 37℃ for 2-3d, collected with sterile PBS, sterilized by 75% alcohol, and then diluted with DMEM to yield an inoculum with $1×10^8$ colony-forming units (CFU)/mL with a hemacytometer. *A. fumigatus* conidia was also harvested in sterile PBS, reached the final concentrations of $2.5×10^7$ CFU/mL.

Animal Models of Fungal Keratitis The abrasion of all C57BL/6 mice corneal epithelium was caused by a 30-gauge needle during isoflurane inhalation anesthesia. Then 2 μL *A. fumigatus* conidia suspension ($2.5×10^7$ CFU/mL) was injected into corneal stroma of mice. And the control group was subjected to intra-stromal injection with 2 μL PBS. All corneas were evaluated under the slit lamp and the disease scores (1, 3 and 5d) were recorded according to an established scale. In a word, FK were categorized by turbidity level, ulcer area and morphology into mild (score 0-5), moderate (score 6-9) and severe (score 10-12) infection[23]. Mice corneas (*n*=6/ group/time), enucleation followed by cornea excision with a scalpel and microscissors, were prepared for RT-PCR and Western blot on 1, 3 and 5d post infection (p.i.), respectively. Then whole eyes (*n*=3/group/time) were also harvested to obtain frozen tissue sections for immunohistofluorescence (IHF) staining.

Primary Mice Peritoneal Macrophage Isolation Primary mice peritoneal macrophages were extracted in accordance with our previous reports[24]. In brief, female C57BL/6 mice (8-week) were given 1 mL of 3% thioglycollate medium by intraperitoneal injection. Mice were euthanized in 7d by cervical dislocation to harvest macrophages by peritoneal lavage with pre-cooled DMEM. Peritoneal macrophages were collected by centrifugation at 1200 rpm for 10min at 4℃.

Cell Culture and Stimulation Macrophages were cultured in DMEM and 10% FBS growth medium. Cellular suspension ($1×10^6$ cells/mL) was plated in 12-well-plates for mRNA detection and 6-well cell culture plates for protein expression, then cultured at 37℃ humidified 5% CO_2 incubator. After 2h, the culture medium was completely changed, the non-adherent cells were removed.

Macrophages were infected with *A. fumigatus* hyphae (final concentration of $5×10^6$ CFU/mL). An approximated proportion is equivalent to 5 CFU/macrophage. After hatching for 0, 4, 8 and 12h, macrophages were harvested to test the levels of CGRP mRNA and protein by real-time PCR and Western blot. Immunocytofluorescence (ICF) staining was also used to detect the CGRP protein level of control groups and *A. fumigatus*-infected groups (8h incubation).

Macrophages were treated with *A. fumigatus* suspension, after different concentrations of CGRP (1, 10 and 100 nmol/L) and CGRP8-37 (0.1 and 1 μmol/L) pretreatment for 30min respectively. All groups were cultured at 37℃ for 12 or 24h to detect the secretion of cytokines.

Table 1 Nucleotide sequence of mouse primers for real-time PCR amplification

Gene (mouse)	Gen Bank No.	Primer sequence (5′-3′)	Size (bp)
β-actin	NM_007393.3	F-GATTACTGCTCTGGCTCCTAG C	147
		R-GACTCATCGTACTCCTGCTTG C	
CGRP	NM_007587.2	F-GGACTTGGAGACAAACCACCA	118
		R-GAGAGCAACCAGAGAGGAACTACA	
IL-1β	NM_008361.4	F-CGCAGCAGCACATCAACA GAGC	111
		R-TGTCCTCATCCTGGAAGGTCCACG	
TNF-α	NM_013693.3	F-ACCCTCACACTCAGATCATCT T	148
		R-GGTTGTCTTTGAGATCCATGC	
IL-6	NM_001314054.1	F-CACAAGTCCGGAGAGGAGAC	141
		R-CAGAATTGCCATTGCACAAC	
IL-10	NM_010548.2	F-TGCTAACCGACTCCTTAATGCAGGAC	126
		R-CCTTGATTTCTGGGCCATGCTTCTC	
MIP-2	NM_009140.2	F-AAGTTTGCCTTGACCCTGAA	180
		R-AGGCACATCAGGTACGATCC	
TGF-β	NM_011577.2	F-CCCGAAGCGGACTACTATGC	69
		R-ATAGATGGCGTTGTTGCGGT	

PCR: Polymerase chain reaction; F: Forward; R: Reverse.

Real-time Polymerase Chain Reaction Total RNA in samples was separated by RNAiso plus reagent and quantified by spectrophotometry. The cDNA was synthesized by reverse transcription of total RNA (2 μg) which was used in the following real-time polymerase chain reaction (PCR; 20 μL reaction volumes) with TB Green™ Premix Ex Taq™ II using specific primers: 95℃ for 30s, followed by 40 cycles of 95℃ for 5s and 60℃ for 30s and a final stage of 95℃ for 15s, 60℃ for 30s, and 95℃ for 15s. DNA contamination was assessed by melting curve. Different sets of target gene products were quantified by the $2^{\Delta\Delta CT}$ method[25]. Table 1 lists are primer sequences in this study.

Western Blot Corneas of C57BL/6 and macrophages were lysed in RIPA lysis buffer containing 1 mmol/L PMSF for 2h. The lysates were centrifuged (12 000 rpm, 15min, 4℃) to collect supernatant. Then BCA Protein Assay Kit was used to measure protein concentration. After adding the SDS loading buffer, the supernatant was heated at 109.4℃ for 10 min in a metal bath. The total protein was separated from SDS-PAGE and transferred to PVDF membranes. The membranes were blocked by confining liquid and incubated with a monoclonal antibody to mouse GAPDH, and a monoclonal antibody to mouse CGRP at 4℃. After several PBST scrubbing, the PVDF membranes were incubated with the corresponding peroxidase-conjugated secondary anti-rabbit antibody. Signals were visualized by using ECL kit.

Immunohistofluorescence Staining IHF staining was tested as reported before[4]. Briefly, the eyeballs of C57BL/6 mice were removed. They were embedded in OCT, and then frozen in liquid nitrogen timely. Tissue specimens were cut into 3-μm thick tissue sections, placed on glass slides, stored at 37℃

for 6h, then fixed in acetone for 5min. Nonspecific binding was blocked with donkey serum diluted 1:100 with PBS at room temperature for 30min. Sections were incubated with monoclonal rabbit anti-mouse CGRP antibodies (CST, Danvers, MA, USA) diluted 1:400 overnight at 4℃, and followed by FITC-conjugated donkey anti-rabbit secondary antibody (Elabscience, Wuhan, China; 1:200, 1.5h, no light, room temperature). Isotype IgG was used as negative control. Cell nuclei were stained with DAPI. Slice digital images were taken with a fluorescence microscope (Zeiss Axiovert microscope, at 40× magnification).

Immunocytofluorescence Staining Macrophages were seeded on four-chamber slides (Thermo Fisher Scientifc, Massachusetts, USA) with or without *A. fumigatus* hyphae treatment for 8h to detect CGRP localization by using ICF assays. Briefly, after washing with PBS three times, macrophages were fixed with pre-cooled 4% formaldehyde for 15min. And then 0.5% Triton X-100 was used for permeabilization (20min, room temperature). After blocking with normal donkey serum for 30min, the cells were incubated at a 1:400 dilution of rabbit anti-mouse CGRP (CST, Danvers, MA, USA) overnight at 4℃. Donkey anti-rabbit antibody was used as secondary antibody, followed by washing with PBST for three times. Nuclei were stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) for 5min. Fluorescence was visualized using Zeiss Axiovert microscope at 40× magnification.

Enzyme-linked Immunosorbent Assay Macrophages culture medium was collected and centrifuged at 1000× g for 10min to separate the supernatant. According to manufacturer's instructions (Elabscience), ELISA kit for mouse IL-1β and

TNF-α was used to quantify the concentration of IL-1β and TNF-α protein from different treatment groups. The absorbance was tested at 450 nm (Molecular Devices, Sunnyvale, CA, USA).

Statistical Analyses All numerical data were presented as means±standard errors of the mean (SEM). All experiments were performed at least three times. One-way analysis of variance (ANOVA) test was used to make comparisons among three or more groups, and unpaired two-tailed Student's *t*-test also was used to identify between multiple groups. *P* value less than 0.05 was defined to be statistically significant.

RESULTS

CGRP Expression in Mouse Corneas in Response to *Aspergillus fumigatus* To observe the progression of *A. fumigatus* keratitis, C57BL/6 mouse corneas were inoculated with *A. fumigatus* conidia suspension. Corneal edema even ulceration was significantly at 1d after *A. fumigatus* infection. Thereafter, severity aggravated gradually and the clinical score reached the highest around 3d, after which the cornea began to alleviate or recover (Figure 1A-1B). Relative mRNA and protein expression of CGRP in uninfected control and *A. fumigatus*-infected mice corneas were tested by Western blot, IHF and RT-PCR. Results indicated that CGRP mRNA and protein levels were significantly increased in *A. fumigatus* groups and peaked at 3d p.i., when compared with control (Figure 1C-1D). To confirm these data, CGRP protein was also tested by IHF. CGRP was localized mainly to epithelium and stroma of *A. fumigatus*-infected mice corneas. While, lower amount of CGRP staining was detected in the control group (Figure 1E).

CGRP Expression in Primary Macrophages with the Challenge of *Aspergillus fumigatus* *In vitro*, we investigated whether CGRP were expressed in macrophages which were interacted with *A. fumigatus*. RT-PCR, Western blot and ICF staining were used to detect the mRNA and protein levels of CGRP. Data showed that CGRP were upregulated significantly at 4, 8 and 12h in *A. fumigatus*-infected group than in control group, and peaked at 8h (Figure 2A-2B). To determine these data, CGRP protein was tested by ICF. Comparison in ICF between the two groups at 8h (Figure 2C, green) revealed that *A. fumigatus*-exposed macrophages had increased CGRP staining.

Effect of Exogenous CGRP on Cytokines Production in Macrophages Interacted with *Aspergillus fumigatus* To assess the role of CGRP in *A. fumigatus* induced inflammatory responses, macrophages were pretreated with different concentrations of exogenous CGRP (a CGRP agonist; 1, 10 and 100 nmol/L) for 30min. The mRNA expression of IL-1β, TNF-α, IL-6, MIP-2, IL-10 and TGF-β in macrophages was tested by RT-PCR. We also used ELISA to examine IL-1β and TNF-α protein levels in macrophages supernatant after stimulation with *A. fumigatus* hyphae for 24h. We found that exogenous

CGRP pretreatment (10 and 100 nmol/L) significantly reduced IL-1β mRNA level induced by *A. fumigatus* hyphae (Figure 3A; *P*<0.01, *P*<0.001). And 100 nmol/L CGRP inhibited *A. fumigatus* hyphae-induced mRNA secretion of TNF-α (Figure 3B; *P*<0.05) and IL-6 (Figure 3C; *P*<0.05) at 12h. In contrast, results indicated that a series of concentrations of CGRP (1, 10 and 100 nmol/L) significantly enhanced the expression of IL-10 mRNA level after stimulation with *A. fumigatus* hyphae (Figure 3F; *P*<0.01, *P*<0.01, *P*<0.01). However, data showed that exogenous CGRP (1, 10 and 100 nmol/L) didn't alter MIP-2 and TGF-β mRNA levels. It indicates that CGRP has no effect on the expression of MIP-2 and TGF-β. IL-1β (Figure 3G; *P*<0.001) and TNF-α (Figure 3H; *P*<0.001) protein were reduced followed by CGRP (100 nmol/L) pretreatment when compared with *A. fumigatus* hyphae-exposed cells after 24h.

Effect of CGRP8-37 on Inflammatory Mediators in Macrophages after Stimulation with *A. fumigatus* To further investigate the effect of CGRP *in vitro*, macrophages were pretreated with various concentrations of CGRP8-37 (a CGRP antagonist, 0.1 and 1 μmol/L) for 30min, then followed by *A. fumigatus* hyphae stimulation. Data indicated that CGRP8-37 (0.1 μmol/L) significantly increased the expression of IL-1β mRNA (Figure 4A; *P*<0.01) after stimulation with *A. fumigatus* hyphae. We also found that the mRNA levels of TNF-α (Figure 4B; *P*<0.05, *P*<0.05) and IL-6 (Figure 4C; *P*<0.001, *P*<0.05) were increased with CGRP8-37 (0.1 and 1 μmol/L) pretreatment, as compared with *A. fumigatus* hyphae-exposed group at 12h, but MIP-2 (Figure 4D), TGF-β (Figure 4E) and IL-10 (Figure 4F) mRNA levels showed no difference after pretreatment, detected by RT-PCR. Results showed that CGRP8-37 (0.1 μmol/L) pretreatment increased the protein levels of IL-1β (Figure 4G, *P*<0.001) and TNF-α (Figure 4H, *P*<0.001) induced by *A. fumigatus* hyphae at 24h, tested by ELISA.

DISCUSSION

Neuropeptides play important roles in fungal infections[20,26]. The neuropeptide CGRP has an anti-inflammatory effect and regulates the expression of cytokines[18]. Its protective role has been demonstrated in infectious diseases caused by fungi and bacteria[13,19]. Using the C57BL/6 mouse *A. fumigatus* keratitis model and primary macrophages, we verified the hypothesis that CGRP plays a role in antifungal innate immune defense. In this study, we demonstrated that C57BL/6 cornea and macrophages release CGRP in response to *A. fumigatus*. Furthermore, in the mouse model of FK, CGRP expression was generally associated with the severity of keratitis. These findings were consistent with those previous studies showing that CGRP was upregulated in murine lungs and murine macrophages after LPS stimulation[9]. This suggests that CGRP may be an endogenous protective neuropeptide in the pathogenesis of FK.
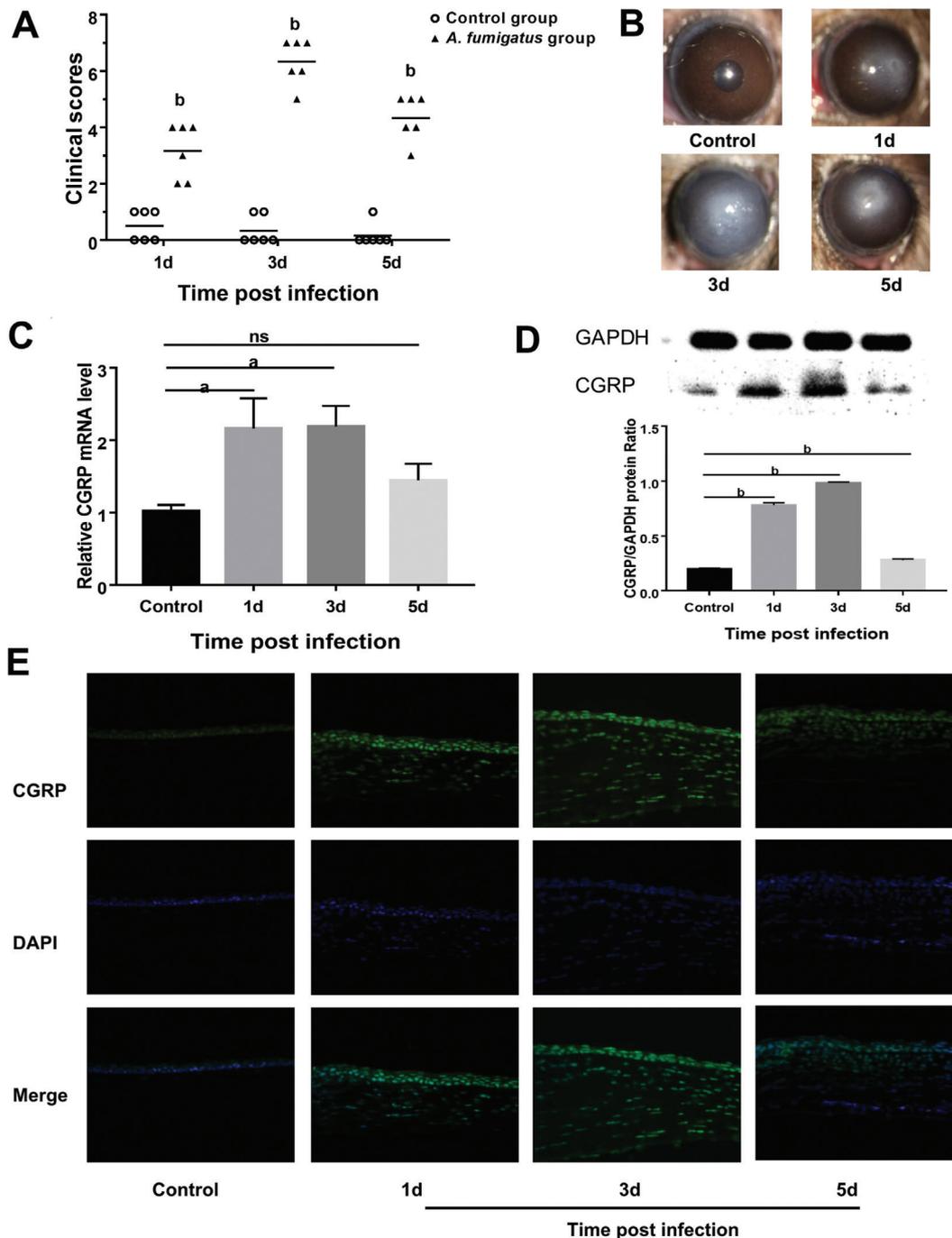
Figure 1 Expression of CGRP in *A. fumigatus*-infected C57BL/6 cornea A: Clinical scores indicated statistically significant differences at 1, 3 and 5d p.i. between control group and *A. fumigatus*-infected group; B: Representative eyes from *A. fumigatus*-treated mice were photographed with a slit lamp at 1 (score 4), 3 (score 7), 5d (score 5); C, D: mRNA and protein levels of CGRP were remarkably greater in the *A. fumigatus*-infected cornea than in control corneas; E: Greater staining intensity for CGRP was seen in *A. fumigatus*-infected group compared with control mice. Green: CGRP staining; Blue: Nuclear staining (DAPI). IHF images scale bar: 50 μmol/L. Values represent as means±SEM. $^aP<0.05$; $^bP<0.001$.

Under the circumstance of fungal infection, massive macrophages aggregate in the limbus or central cornea. These macrophages play a vital role in the innate immune system after infection of *A. fumigatus*[6]. Neuropeptides play anti-inflammatory roles in many acute and chronic diseases[14,26]. Several researches[18-19] have clearly shown that CGRP inhibited the release of pro-inflammatory factors. And increased endogenous CGRP can mitigate inflammatory reaction by increasing cAMP levels in the pulmonary inflammatory diseases[15]. Reinshagen *et al*[27] showed that subcutaneous injection of CGRP8-37 significantly increased the severity of colitis in the rat. Likewise, it has been demonstrated in two ways that CGRP acted as an anti-inflammatory mediator in antifungal innate immune defense. We found that CGRP inhibited the *A. fumigatus*-induced IL-1β, TNF-α, IL-6 secretion and promoted IL-10 secretion in macrophages. Second, CGRP8-37 significantly magnified
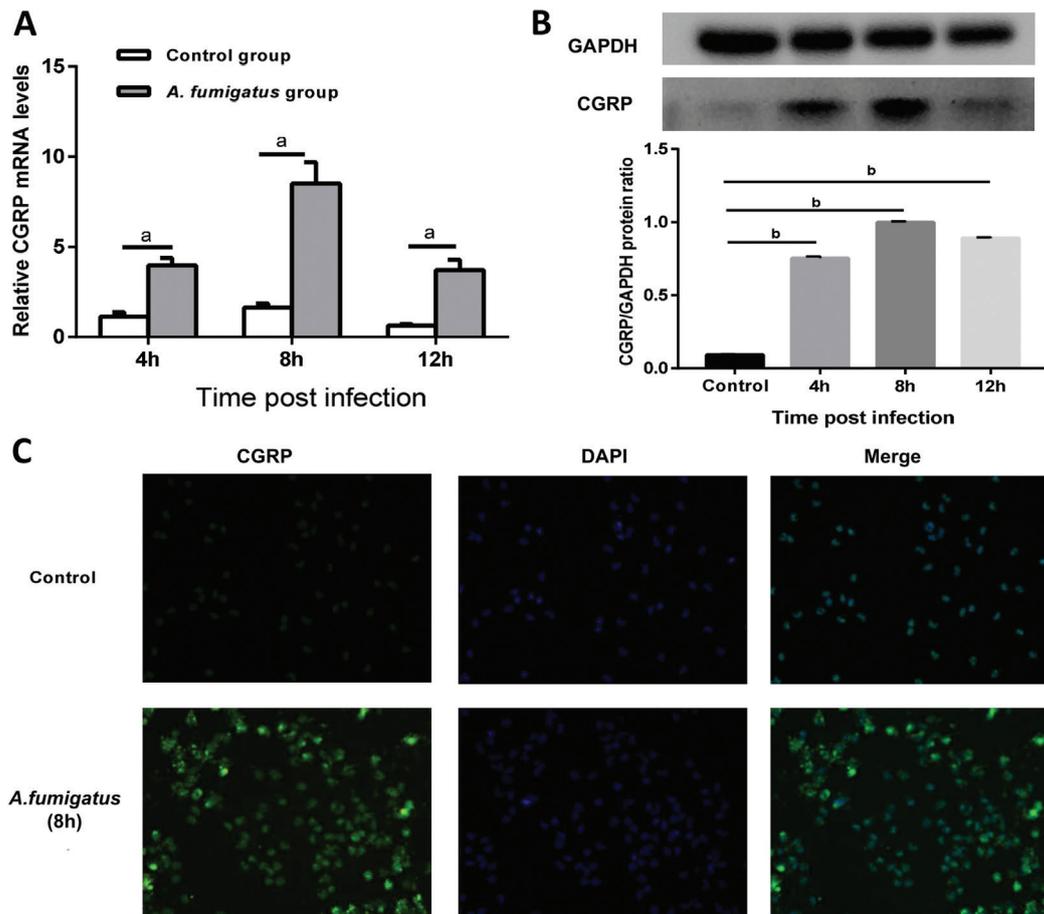
Figure 2 CGRP expression in primary macrophages with infection of *A. fumigatus* A, B: The levels of CGRP mRNA and protein in macrophages exposed to *A. fumigatus* were analyzed by RT-PCR and Western blot; C: ICF images showed CGRP protein in primary macrophages without or with exposure to *A. fumigatus*. Green: CGRP staining; Blue: Nuclear staining (DAPI). ICF images scale bar: 50 μmol/L. Values represent as means±SEM. [a]$P<0.05$; [b]$P<0.001$.
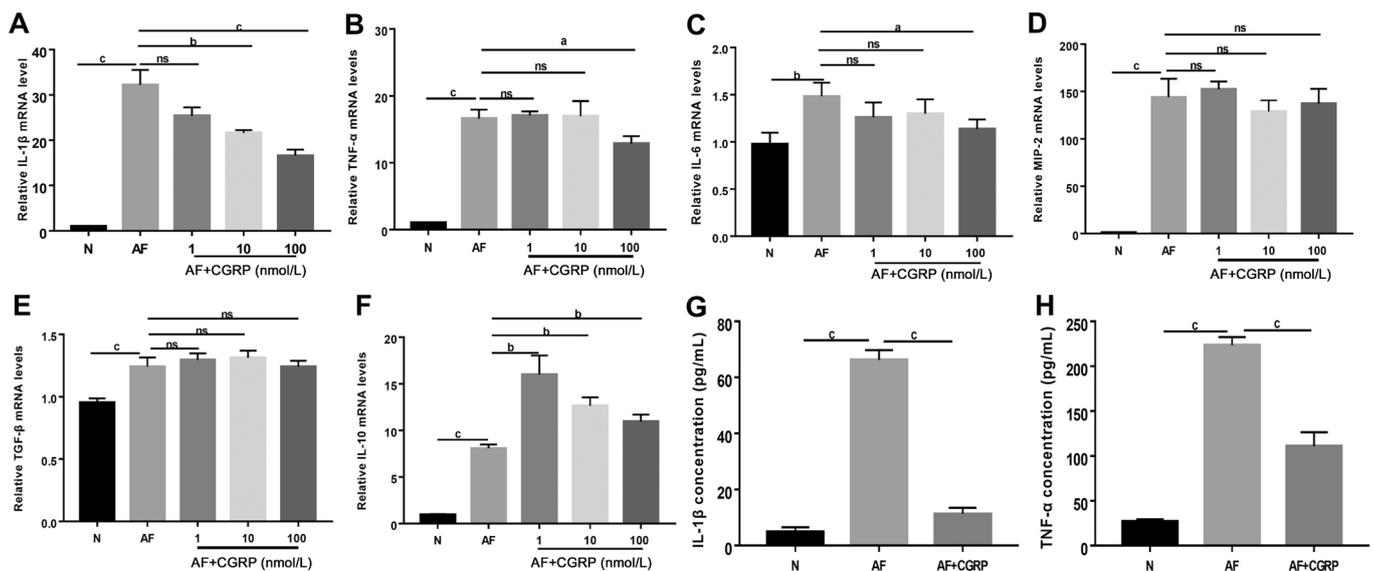
Figure 3 Exogenous CGRP attenuated inflammatory reaction in macrophages challenged with *A. fumigatus* A-C: The expression of IL-1β, TNF-α and IL-6 mRNA were reduced in different concentrations of CGRP after 12h stimulation with *A. fumigatus* hyphae; D, E: MIP-2 and TGF-β mRNA levels did not change significantly after various concentrations of CGRP pretreatment; F: Exogenous CGRP significantly increased *A. fumigatus* hyphae-induced secretion of IL-10 mRNA at 12h; G, H: IL-1β and TNF-α protein levels were significantly reduced at 24h after pretreatment of 100 nmol/L CGRP as compared with *A. fumigatus* hyphae-exposed control group. [a]$P<0.05$, [b]$P<0.01$, [c]$P<0.001$.
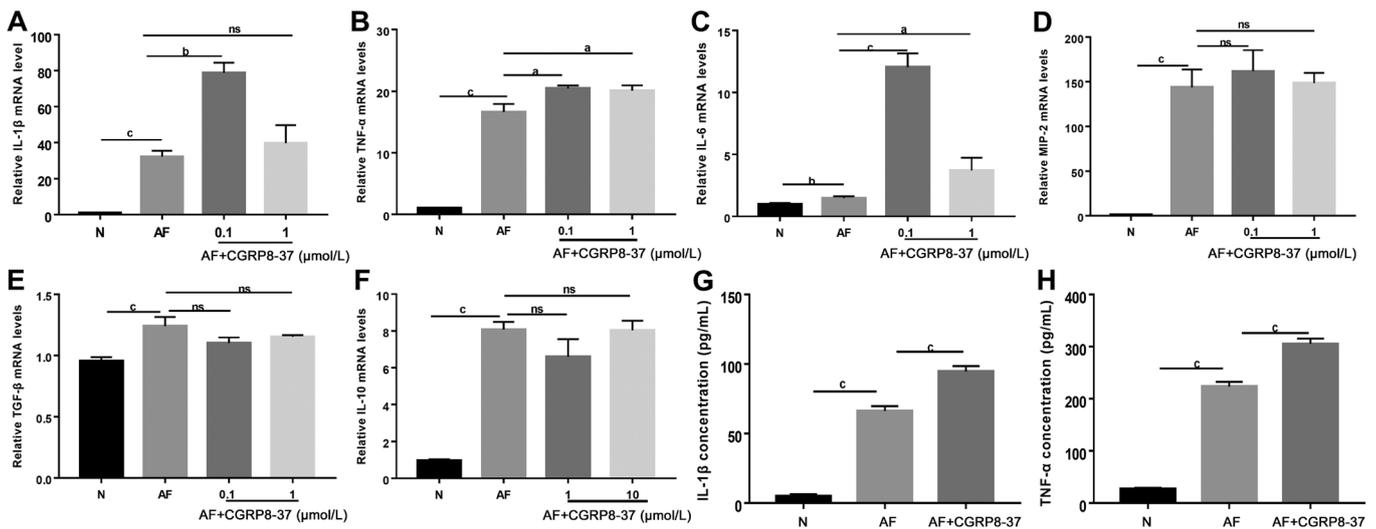
Figure 4 Effect of CGRP8-37 on cytokines in macrophages induced by *A. fumigatus* hyphae A-C: CGRP8-37 significantly increased relative mRNA levels of IL-1β, TNF-α and IL-6 compared with *A. fumigatus* hyphae-exposed control group at 12h; D-F: CGRP8-37 (0.1 and 1 μmol/L) did not change MIP-2, TGF-β and IL-10 mRNA levels, compared with *A. fumigatus* hyphae-exposed control group; G-H: CGRP8-37 (0.1 μmol/L) up-regulated the expression of IL-1β and TNF-α protein induced by *A. fumigates* hyphae at 24h. $^aP<0.05$, $^bP<0.01$, $^cP<0.001$.

the *A. fumigatus*-induced IL-1β, TNF-α, IL-6 secretion. This study is consistent with previous research result that intraperitoneal injection of exogenous CGRP obviously diminishes the histological injury and inflammatory factors levels in acute lung injury induced by LPS[28]. In addition, a similar observation has been reported that dendritic cells with CGRP pretreatment might attenuate allergic airway inflammation *in vivo*, and increase IL-10 levels[29]. Collectively, our data provided evidence that CGRP might be a protective factor against *A. fumigatus* by regulating the pro- and anti-inflammatory mediators.

However, we did not detect any differences in MIP-2/TGF-β levels in macrophages which were pretreated with exogenous CGRP or CGRP8-37 induced by *A. fumigatus* hyphae. While previous reports showed that levels of MIP-2/TGF-β were changed after pretreatment of CGRP or CGRP8-37. Wang *et al*[30] found that CGRP partially increased MIP-2 levels from the peritoneal macrophages, but did not change from alveolar macrophages in LPS-induced murine sepsis. Yoon and Kim[31] have reported that exogenous CGRP induced the secretion of TGF-β in tubulointerstitial fibrosis. However there is no research on the stimulated mouse macrophages with the infection of *A. fumigatus*. Discrepancies between our findings and previous might be caused by the difference of strains or disease types. Thus, the levels of MIP-2/TGF-β might be unaffected by CGRP after stimulation with *A. fumigatus*. Further researches are needed to clarify the importance of CGRP in fungal infections.

In summary, we first discovered that CGRP may function as a protector of FK. In the context of fungal infection, strategies to enhance CGRP secretion might be beneficial to prevent the *A. fumigatus*-associated inflammation. Thus, CGRP, as

an anti-inflammatory factor, might have the potential to be a new target in FK therapy. However, these conclusions were based on the responses of cell experiments. The specific mechanism of CGRP in cornea is still enigmatic, continued studies are needed to confirm further the role of CGRP. The study provided experimental basis for clinical application of neuro-immune regulation in FK. Furthermore, it emphasized the necessity of understanding and exploring the complex neuroimmunity mechanisms that contribute to treatment of FK.

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