

# Role of vasoactive intestinal peptide in *Aspergillus fumigatus*-infected cornea

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Received: 2017-11-01 Accepted: 2017-12-13

## Abstract

• **AIM:** To investigate the anti-inflammatory role of vasoactive intestinal peptide (VIP) in *Aspergillus fumigatus* (*A. fumigatus*) keratitis.

• **METHODS:** Expression of VIP was tested by polymerase chain reaction (PCR) in C57BL/6 and BALB/c normal and *A. fumigatus* infected corneas. C57BL/6 mice were pretreated with recombinant (r) VIP, while BALB/c mice were pretreated with VIP antagonist, and then infected with *A. fumigatus*. Clinical score was recorded. Expression of pro- and anti-inflammatory cytokines, toll-like receptor 4 (TLR4), lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1), and neutrophil infiltration were tested by PCR, enzyme-linked immunosorbent assay (ELISA), and myeloperoxidase (MPO) assay.

• **RESULTS:** VIP mRNA expression in BALB/c cornea was higher than C57BL/6 cornea at 1 and 3d post infection (p.i.). rVIP treatment of C57BL/6 mice showed alleviated disease and down-regulated expression of interleukin-1 $\beta$  (IL-1 $\beta$ ) and tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), while IL-10 expression was up-regulated. Neutrophil infiltration and TLR4, IL-17 expression were decreased after rVIP treatment, while LOX-1 expression was up-regulated in C57BL/6. VIP antagonist pretreatment showed increased disease and higher IL-1 $\beta$ , TNF- $\alpha$ , TLR4, IL-17 and MPO levels, while IL-10 and LOX-1 levels were down-regulated in BALB/c mice.

• **CONCLUSION:** rVIP alleviate disease response of C57BL/6 mice. VIP antagonist resulted in worsened disease of BALB/c mice. VIP proposed anti-inflammatory role in *A. fumigatus* keratitis.

• **KEYWORDS:** fungal keratitis; vasoactive intestinal peptide; C57BL/6 mice; BALB/c mice.

**DOI:**10.18240/ijo.2018.02.01

**Citation:** Li C, Liu YY, Zhao GQ, Lin J, Che CY, Jiang N, Li N, Zhang J, He K, Peng XD. Role of vasoactive intestinal peptide in *Aspergillus fumigatus*-infected cornea. *Int J Ophthalmol* 2018;11(2):183-188

## INTRODUCTION

Fungal keratitis (FK) can lead to high rate of blindness<sup>[1-2]</sup>. FK still remains a therapeutic challenge for the ophthalmologists. The fungal infected corneas of BALB/c and C57BL/6 mice presented different manifestations. C57BL/6 mice had more severe and longer disease response than BALB/c mice<sup>[3]</sup>. Control of infection depends on the immune cells station and production of pro- and anti-inflammatory factors in cornea<sup>[4]</sup>.

Vasoactive intestinal peptide (VIP), as a neuropeptide, plays anti-inflammatory role in immune responses<sup>[5]</sup>. Studies have shown that VIP can regulate the production of cytokines, and have different role on regulating different cytokines<sup>[6-7]</sup>. Recombinant (r) VIP can down-regulate immune responses<sup>[4]</sup>. Studies showed that VIP significantly decreased tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interleukin-1 $\beta$  (IL-1 $\beta$ ) production while increased anti-inflammatory interleukin-10 (IL-10) by lipopolysaccharide (LPS) -infected monocytes<sup>[8]</sup>. VIP also protects epithelial barrier during bacterial infection<sup>[9]</sup>.

Recent studies showed that rVIP treatment lead to better disease responses by down-regulation of pro-inflammatory cytokines and up-regulation of anti-inflammatory cytokines<sup>[10]</sup>. VIP also can regulate defensins expression in the *Pseudomonas aeruginosa* (*P. aeruginosa*) keratitis<sup>[11]</sup>. rVIP treatment also downregulates the production of adhesion molecules and promotes healing and results in better disease outcome<sup>[12-13]</sup>.

Whereas VIP antagonist treatment can increase disease scores, elevate pro-inflammatory mediator expression, and reduce anti-inflammatory mediator expression in *P. aeruginosa* keratitis<sup>[14]</sup>.

Protein kinase (PK) C pathways are possibly involved in VIP pathway<sup>[15-16]</sup>. As to the effect of VIP on pattern recognition receptors, report showed that VIP can down-regulate TLR4 expression<sup>[10]</sup>. VIP elevated pulmonary surfactant protein A (SP-A) expression<sup>[17]</sup>. Lectin-like oxidized low-density lipoprotein receptor 1 (LOX-1) is an important receptor for fungal infection. Inhibition of LOX-1 results in reduced neutrophils infiltration and pro-inflammatory cytokines<sup>[18]</sup>. The effect of VIP on fungal infection related receptors, such as toll-like receptor 4 (TLR4), LOX-1 is still unclear.

The studies herein showed that VIP regulates expression of pro- and anti-inflammatory cytokines. Exogenous rVIP alleviated the disease response in C57BL/6 mice. VIP antagonist resulted in worsened disease in BALB/c mice. VIP can down-regulate TLR4 and IL-17, while up-regulate LOX-1 expression.

## MATERIALS AND METHODS

**Aspergillus Fumigatus Culture** *Aspergillus Fumigatus* (*A. fumigatus*) strain 3.0772, purchased from China General Microbiological Culture Collection Center, was inoculated and prepared according to the routine methods<sup>[18]</sup>. For *in vivo* experiment, yielded at  $1 \times 10^8$  CFU/mL without inactivation; for *in vitro* experiments, yielded at  $5 \times 10^6$  CFU/mL and inactivated overnight in 70% alcohol.

### In Vivo Experiment

**Corneal infection** Female BALB/c and C57BL/6 mice (8-week-old) were used for experiments. The left eyes were chosen for experiments. For experimental group, corneas were infected by routine method<sup>[18]</sup>. Control group only were removed the central corneal epithelium of left eye, covered soft contact lens and sutured the eyelids, without infection. Mice were treated in compliance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

**Corneal response to infection** Clinical score was recorded after 1, 3 and 5d infection. Fungal keratitis was graded by usage of clinical scores ranging from 0 to 12<sup>[19]</sup>.

**Recombinant vasoactive intestinal peptide treatment** C57BL/6 mice ( $n=5$ /group/time) were given rVIP (5 nmol/100 mL) (Bachem) by intraperitoneal injections from 1d before infection to 1, 3 or 5d post infection (p.i.) once one day. Control mice were similarly treated with control phosphate buffered solution (PBS)<sup>[14]</sup>.

**Vasoactive intestinal peptide antagonist treatment** BALB/c mice ( $n=5$ /group/time) were given VIP antagonist (10 mg/100 mL) (Bachem) by intraperitoneally injection from 1d before infection to 1, 3 or 5d p.i. once one day. Control mice were similarly treated with control PBS<sup>[14]</sup>.

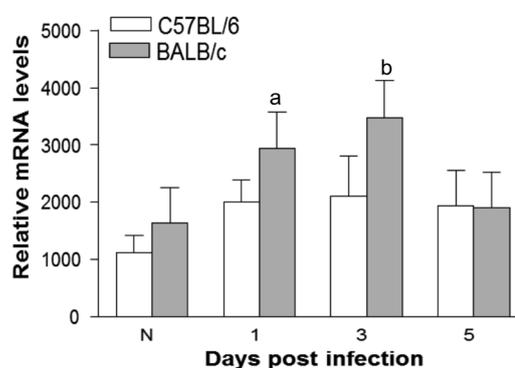
### Experimental Techniques

**Real-time polymerase chain reaction** After sacrifice, control and infected corneas were collected after 1, 3 and 5d infection for VIP mRNA detection. Corneas were collected after rVIP- or PBS-treatment and after VIP antagonist- or PBS- treatment at 3d p.i. Total RNA isolation, reverse transcription, and real-time polymerase chain reaction (PCR) reaction were done according to the routine methods<sup>[18]</sup>. The primers sequences are shown in Table 1.

**Enzyme-linked immunosorbent assay** After rVIP- or PBS-treatment for C57BL/6 mice, and VIP antagonist- or PBS-treatment for BALB/c mice, normal and infected corneas were collected after 3 and 5d infection ( $n=5$ /group/time).

**Table 1 Nucleotide sequences for real-time RT-PCR**

Gene	Gen bank No.	Primer sequence (5'-3')	Size (bp)
$\beta$ -Actin	NM_007393.3	F-GATTACTGCTCTGGCTCCTAGC R-GACTCATCGTACTCCTGCTTGC	147
VIP	NM_011702.2	F-AGAAGTCTGCTGTAATCGCTGGTG R-AGAAGTCTGCTGTAATCGCTGGTG	148
IL-1 $\beta$	NM_008361.3	F-CGCAGCAGCACATCAACAAGAGC R-TGTCCTCATCTGGAAGGTCCACG	111
TNF- $\alpha$	NM_013693.2	F-ACCCTCACACTCAGATCATCTT R-GGTTGTCTTTGAGATCCATGC	148
IL-10	NM_010548.2	F-TGTAACCGACTCCTTAATGCAGGAC R-CCTTGATTCTGGGCCATGCTTCTC	126
LOX-1	NM_138648.2	F-AGGTCCTTGCCACAAGACTGG R-ACGCCCTGGTCTTAAAGAATTG	273
IL-17	NM_010552.3	F-TTTAACTCCCTTGGCGCAAAA R-CTTTCCTCCGATTGACAC	165
TLR4	NM_021297.3	F-CCTGACACCAGGAAGCTTGAA R-TCTGATCCATGCATTGGTAGGT	71



**Figure 1 Expression of VIP** mRNA levels of VIP were higher in *A. fumigatus* infected BALB/c cornea than C57BL/6 corneas at 1 and 3d p.i. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

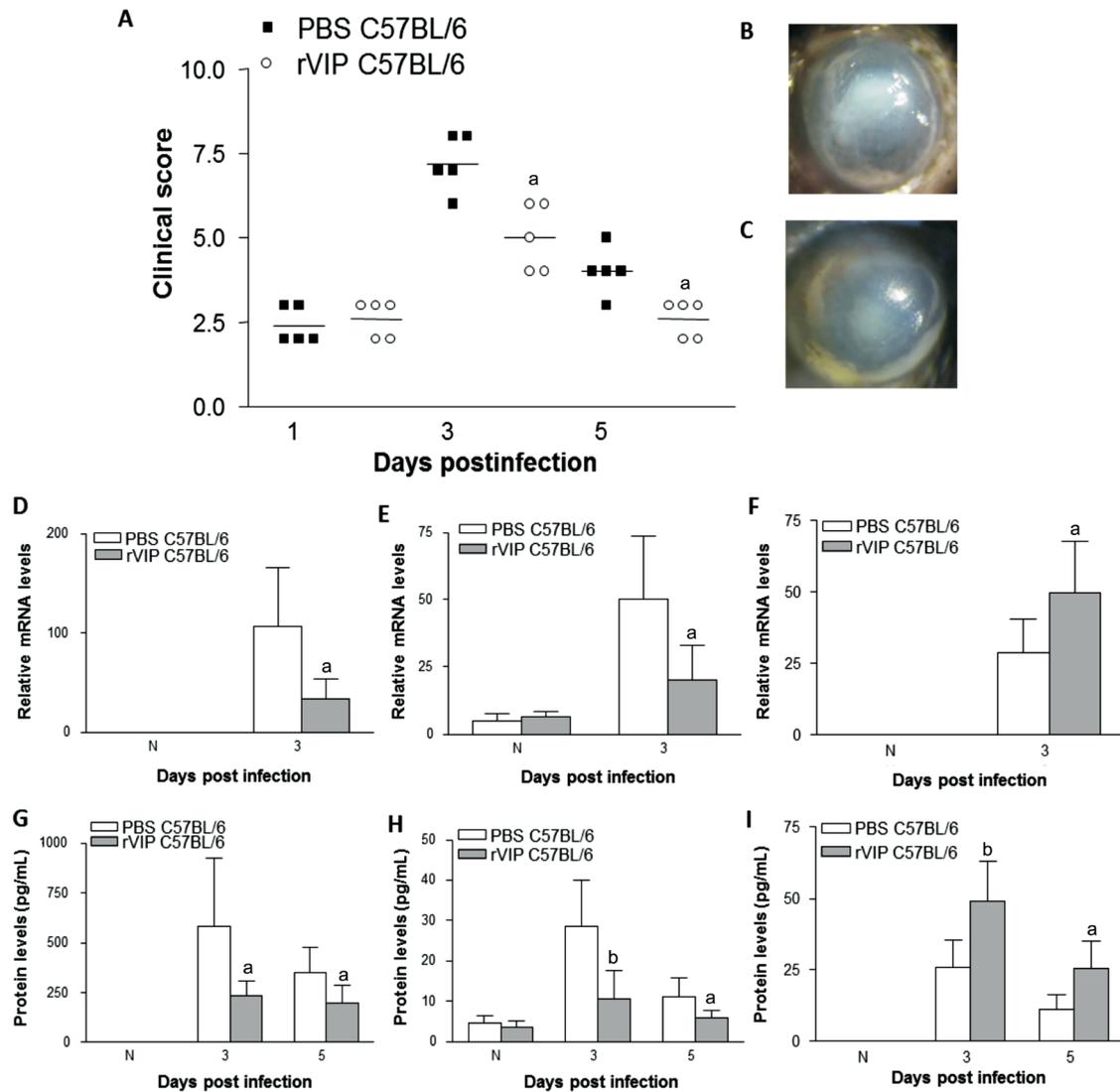
Individual corneas were homogenized according to the routine methods<sup>[18]</sup>. Totally 25 mL of each sample was used for IL-1 $\beta$  protein detection. Totally 50 mL of each sample was used for IL-10 and TNF- $\alpha$  protein detection (R&D Systems).

**Quantitation of corneal polymorphonuclear** Corneas ( $n=5$ /group/time) were collected after rVIP- or PBS-treatment, and also collected after VIP antagonist- or PBS- treatment at 3 and 5d p.i. Cornea was homogenized and the absorbency at 460 nm was monitored according to the routine methods<sup>[18]</sup>. The slope of the line was used to calculate units of myeloperoxidase (MPO) for each cornea.

**Statistical Analysis** Student's *t*-test was used to calculate the statistical difference of the PCR, enzyme-linked immunosorbent assay (ELISA) and MPO data. Values were considered significant at  $P \leq 0.05$ . All experiments were repeated once. Data are shown as mean  $\pm$  standard deviation (SD).

## RESULTS

**Vasoactive Intestinal Peptide Expression** VIP expression was tested by real-time RT-PCR in normal and infected corneas of BALB/c and C57BL/6 mice. Results showed that VIP mRNA was higher in BALB/c than C57BL/6 mouse corneas at 1 and 3d p.i. (Figure 1). VIP can be detected in normal and 5d p.i. corneas, but expression between the two mice strains were similar.

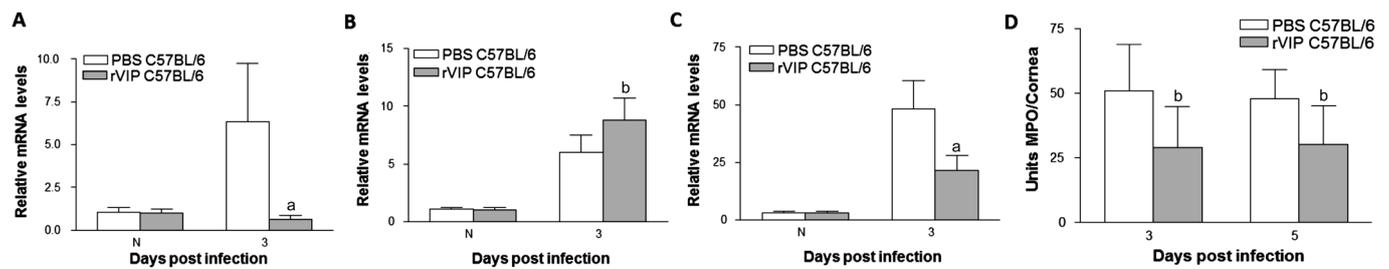


**Figure 2** rVIP treatment of C57BL/6 mice rVIP treatment significantly downregulated the clinical scores of C57BL/6 mice (A) after 3 and 5d infection. Photographs showed worsened disease after PBS treatment (B) than rVIP treatment (C) at 3d infection. mRNA levels of IL-1 $\beta$  (D) and TNF- $\alpha$  (E) were decreased in rVIP treated cornea after 3d infection. Protein expression of IL-1 $\beta$  (G) and TNF- $\alpha$  (H) were significantly decreased in rVIP treated cornea after 3 and 5d infection. IL-10 mRNA (F) was upregulated in rVIP treated cornea vs PBS treated cornea after 3d infection. IL-10 protein expression (I) was increased in rVIP treated cornea after 3 and 5d infection. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs PBS treatment.

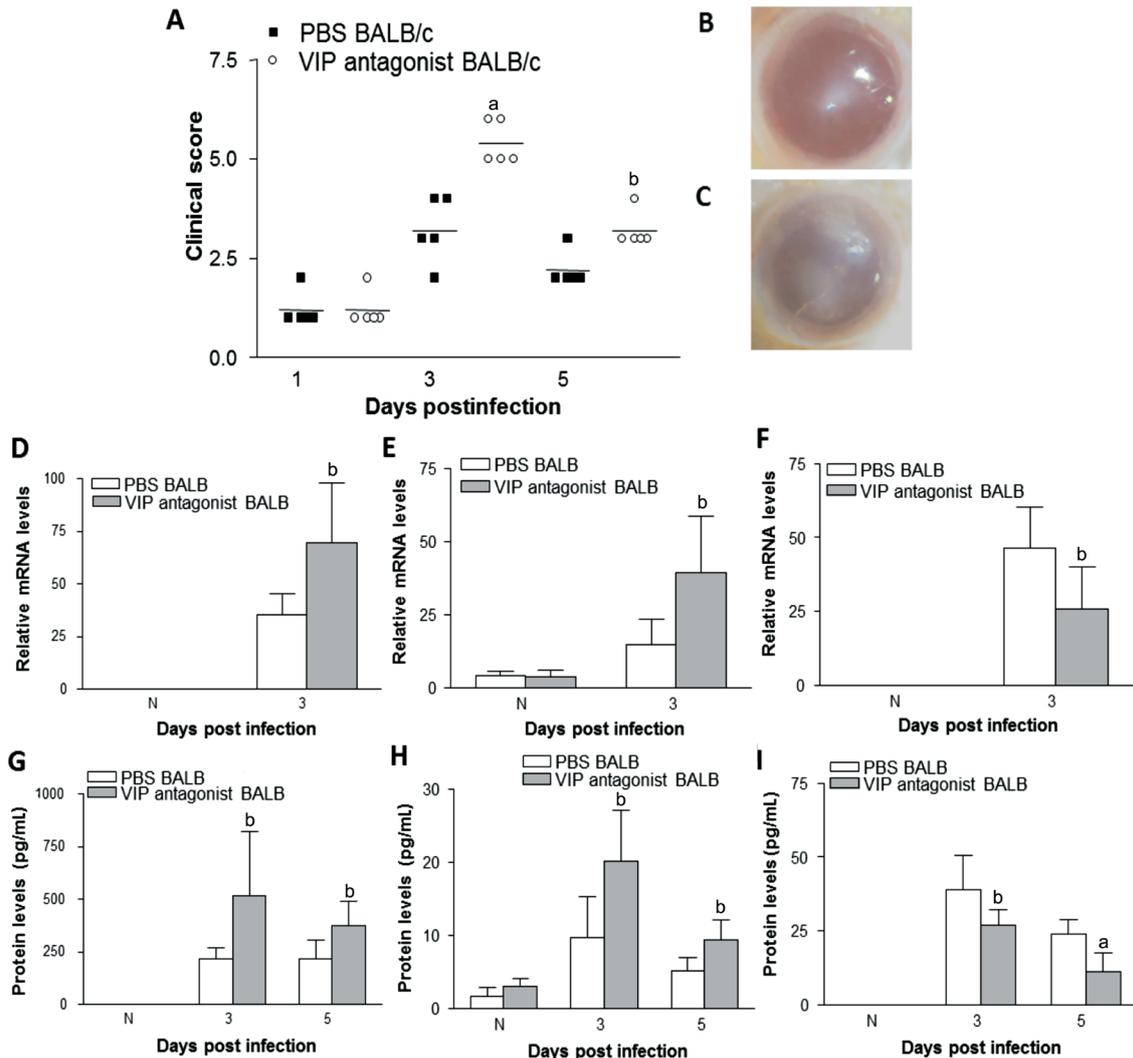
**Recombinant Vasoactive Intestinal Peptide Treatment of C57BL/6 Mice** C57BL/6 mice were given rVIP treatment to test whether exogenous VIP lead to better disease outcome. rVIP decreased the clinical score of C57BL/6 mice after 3 and 5d infection compared with PBS control (Figure 2A). Photographs showed worsened disease responses after control PBS control (Figure 2B) than rVIP treatment (Figure 2C) at 3d p.i. Next, we examined effect of rVIP treatment on cytokines expression after infection in C57BL/6 mice. After treatment with rVIP, corneal IL-1 $\beta$  (Figure 2D) and TNF- $\alpha$  (Figure 2E) mRNA levels were downregulated at 3d p.i. The protein levels of IL-1 $\beta$  (Figure 2G) and TNF- $\alpha$  (Figure 2H) also were downregulated in rVIP treated cornea after 3 and 5d infection when compared to control-treated mice. While anti-inflammatory mediator, mRNA levels of IL-10 (Figure 2F) were upregulated in rVIP treated cornea at 3d p.i. IL-10 protein

expression (Figure 2I) was increased in rVIP treated cornea at 3 and 5d p.i. rVIP treatment down-regulated the TLR4 (Figure 3A) and IL-17 (Figure 3C) expression at 3d p.i., while LOX-1 expression (Figure 3B) was up-regulated. In addition, MPO levels were down regulated in rVIP treated cornea compared with PBS control in C57BL/6 mice after 3 and 5d infection (Figure 3D).

**Vasoactive Intestinal Peptide Antagonist Treatment of BALB/c Mice** VIP antagonist treatment upregulated the clinical scores of BALB/c mice after 3 and 5d infection compared with PBS control (Figure 4A). Photographs showed worsened disease after VIP antagonist treatment (Figure 4C) compared with PBS control (Figure 4B) after 3d infection. Next, we examined effect of VIP antagonist treatment on pro- and anti-inflammatory cytokines expression. After VIP antagonist treatment, IL-1 $\beta$  (Figure 4D) and TNF- $\alpha$  (Figure 4E) mRNA



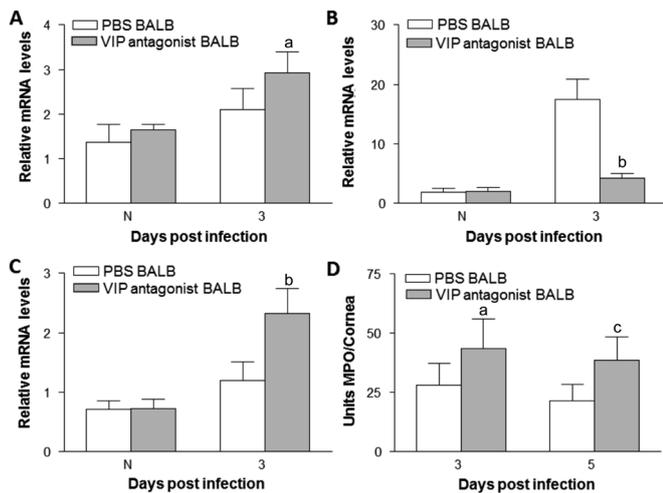
**Figure 3** rVIP treatment down-regulated the TLR4 (A) and IL-17 (C) expression at 3d p.i. LOX-1 expression (B) was up-regulated. MPO levels (D) were decreased in rVIP treated cornea compared with PBS treated cornea after 3 and 5d infection <sup>a</sup> $P < 0.001$ , <sup>b</sup> $P < 0.05$  vs PBS treatment.



**Figure 4** VIP antagonist treatment of BALB/c mice VIP antagonist treatment increased the clinical scores of BALB/c mice (A) after 3 and 5d infection. Photographs showed worsened disease after VIP antagonist treatment (C) compared with PBS (B) control after 3d infection. Relative mRNA levels of IL-1 $\beta$  (D) and TNF- $\alpha$  (E) were increased in VIP antagonist treated cornea at 3d p.i. The protein expression of IL-1 $\beta$  (G) and TNF- $\alpha$  (H) were upregulated in VIP antagonist treated cornea after 3 and 5d infection. IL-10 mRNA (F) was downregulated in VIP antagonist treated cornea compared with PBS control at 3d p.i. The protein levels of IL-10 (I) were decreased in VIP antagonist treated cornea after 3 and 5d infection. <sup>a</sup> $P < 0.01$ , <sup>b</sup> $P < 0.05$ .

levels were increased after 3d infection compared with PBS control. The protein levels of IL-1 $\beta$  (Figure 4G) and TNF- $\alpha$  (Figure 4H) were upregulated in VIP antagonist treated cornea after 3 and 5d infection when compared to control-treated mice. While mRNA levels of IL-10 (Figure 4F) were

downregulated in VIP antagonist treated cornea compared with PBS control after 3d infection. Protein levels of IL-10 (Figure 4I) were downregulated in VIP antagonist treated cornea after 3 and 5d infection. VIP antagonist treatment up-regulated TLR4 (Figure 5A) and IL-17 (Figure 5C) expression at 3d p.i.,



**Figure 5** VIP antagonist up-regulated TLR4 (A) and IL-17 (C) mRNA expression after 3d infection. LOX-1 expression (B) was down-regulated after 3d infection. VIP antagonist upregulated MPO levels (D) in BALB/c mice after 3 and 5d infection. <sup>a</sup>*P*<0.05, <sup>b</sup>*P*<0.001, <sup>c</sup>*P*<0.01 vs PBS treatment.

while LOX-1 expression (Figure 5B) was down-regulated. In addition, VIP antagonist upregulated MPO levels after 3 and 5d infection (Figure 5D).

## DISCUSSION

VIP is an important neuropeptide and has anti-inflammatory role<sup>[20]</sup>. VIP can rebalance the expression of cytokines<sup>[4]</sup>. It has been reported that VIP also can disrupt the surface membrane of pathogen, and kill various pathogenic bacteria, fungi, as well as the parasite<sup>[21]</sup>. VIP has important protective role for infection disease.

It has been reported that mouse showed constitutive expression of corneal VIP. However, VIP protein expression was higher in BALB/c than C57BL/6 mice in *P. aeruginosa* keratitis<sup>[4]</sup>. Data in this study revealed that both C57BL/6 and BALB/c mouse normal cornea can express VIP, and after *A. fumigatus* infection, BALB/c cornea can express more VIP than C57BL/6 corneas. This suggested protective role of VIP in fungal keratitis.

VIP plays protective role in many infectious diseases. VIP can reduce infected bacterial load and alleviated inflammation responses in mice with severe polymicrobial sepsis<sup>[21]</sup>. VIP could downregulate the expression of IL-6 and upregulate the production of IL-10 after LPS stimulation<sup>[22]</sup>. VIP regulates the balance of local immune responses by affecting various cytokines production<sup>[20]</sup>. Previous studies have reported that rVIP lead to better disease outcome and reduce corneal perforation in *P. aeruginosa* keratitis. rVIP also can down-regulate corneal expression of pro-inflammatory chemokines and up-regulate anti-inflammatory mediators<sup>[4]</sup>. rVIP protein was given to C57BL/6 mice to test the role of exogenous rVIP. Data showed that rVIP treatment down-regulate the clinical scores of C57BL/6 mice after 3 and 5d infection. rVIP treatment also decreased corneal IL-1 $\beta$  and TNF- $\alpha$

expression while increased anti-inflammatory mediator IL-10 in C57BL/6 mice. In addition, rVIP treatment also decreased polymorphonuclear (PMN) infiltrate of C57BL/6 mice. This is consistent with a previous study showing that VIP reduced TNF- $\alpha$  and increased IL-10 expression in zymosan-induced inflammation<sup>[23]</sup>. Our data suggest that rVIP alleviate disease response in C57BL/6 mice. rVIP treatment down-regulate corneal levels for pro-inflammatory cytokines, whereas anti-inflammatory mediators were up-regulated. rVIP also decreased PMN number in C57BL/6 mice.

Since treatment with rVIP can result in better disease outcome in C57BL/6 mice, VIP antagonist was used to test if blocking VIP in BALB/c mice had an opposite effect. Data showed that VIP antagonist treatment increased the clinical scores of BALB/c mice after 3 and 5d infection. IL-1 $\beta$  and TNF- $\alpha$  were increased in VIP antagonist treated cornea of BALB/c mice. While IL-10 was down-regulated in VIP antagonist treated cornea vs PBS control. MPO levels were up-regulated in VIP antagonist treated cornea. Our data showed that VIP is needed for the protective response of BALB/c mice. These findings are consistent with study showing that VIP significantly up-regulate expression of IL-4, IL-10, and TGF- $\beta$  compared with control group and down-regulate the expression of TLR2, TLR4<sup>[24]</sup>. This is consistent with our data showing that rVIP down-regulated the TLR4 expression while VIP antagonist can up-regulated TLR4 levels. Our *in vivo* data suggest that BALB/c mice treated with VIP antagonist showed increased disease, IL-1 $\beta$ , TNF- $\alpha$ , IL-17 and MPO levels, while IL-10 levels were down-regulated, which is consistent with Tan *et al*'s study<sup>[25]</sup>, showing that mice with VIP receptor 2 gene knock out exhibited exacerbated encephalomyelitis compared to wild type mice, and with increased TNF- $\alpha$ , IL-6, IL-17 production and reduced IL-10, IL-4 expression.

In summary, the data indicates that VIP is higher expressed in BALB/c vs C57BL/6 mouse cornea after infection. Exogenous rVIP alleviate disease response of C57BL/6 mice. VIP antagonist resulted in worsened disease of BALB/c mice. Continued studies of the role of VIP in fungal keratitis may enhance our understanding of protective role of VIP.

## ACKNOWLEDGEMENTS

**Foundations:** Supported by the National Natural Science Foundation of China (No.81470609; No.81500695); the Natural Science Foundation of Shandong Province (No. ZR2013HQ007; No.ZR2017BH025)

**Conflicts of Interest:** Li C, None; Liu YY, None; Zhao GQ, None; Lin J, None; Che CY, None; Jiang N, None; Li N, None; Zhang J, None; He K, None; Peng XD, None.

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