Efficacy of VIP as Treatment for Bacteria-Induced Keratitis Against Multiple *Pseudomonas aeruginosa* Strains

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Purpose. Previous studies have demonstrated the efficacy of vasoactive intestinal peptide (VIP) treatment in regulating inflammation following bacterial keratitis induced by the *P. aeruginosa* strain 19660. However, in the current study we assessed whether disease outcome is specific to 19660 or if VIP treatment is effective against multiple *P. aeruginosa* strains.

Methods. B6 mice received daily IP injections of VIP from −1 through 5 days post injection (p.i.). Control mice were similarly injected with PBS. Corneal infection was induced using PA 19660, PAO1 or KEI 1025. Disease response was documented and bacterial plate counts and myeloperoxidase assays were performed. Expression of select inflammatory mediators as well as enzymes associated with lipid mediator production was assessed after VIP treatment. KEI 1025 was characterized by cytotoxicity and invasion assays and then confirmed for ExoS/ExoU expression.

Results. VIP treatment converted the susceptible response to resistant for the three *P. aeruginosa* strains tested. Disease response was significantly reduced with no corneal perforation. Anti-inflammatory mediators were enhanced after VIP treatment, while pro-inflammatory molecules were reduced compared to controls. Furthermore, VIP reduced inflammatory cell persistence in the cornea after infection with each of the *P. aeruginosa* strains.

Conclusions. VIP treatment is effective at ameliorating disease pathogenesis for multiple *P. aeruginosa* strains, both cytotoxic and invasive. This study is also the first to indicate a possible role for VIP regarding lipid mediator expression in the eye. In addition, the clinical isolate, KEI 1025, was characterized as an invasive strain. Overall, this study strengthens the preclinical development of VIP as a therapeutic agent for ocular infectious disease.

Keywords: neuropeptide, cornea, infection, treatment

Pseudomonas aeruginosa continues to be among the most common pathogens resulting in bacterial keratitis. With over 125 million contact lens wearers worldwide, overnight or extended lens wear continues to be the major risk factor.[1] However, recent evidence has revealed that water sources, lens care products, and hygiene are particularly relevant to the pathogenesis of these infections, as well.[2–4] In addition, patients who are immunocompromised (secondary to malnutrition, alcoholism, diabetes, steroid use), and those who have undergone refractive corneal surgery are also at increased risk.[5,6] As a result, there are roughly one million office/outpatient clinic and emergency department visits annually with over 75% of keratitis visits resulting in antibiotic prescriptions, ultimately translating into an economic burden of approximately $175 million per year in treatment costs.[7]

Currently, treatment of bacterial keratitis focuses on the pathogen instead of the host response. Fourth-generation ophthalmic fluoroquinolones are effective against both gram-positive and -negative bacteria; they penetrate fairly well into ocular tissue, and even demonstrate better mutant prevention characteristics than older generation agents.[6] Although control of the bacteria is of utmost importance, antibacterial treatment does not guarantee good visual outcome. Restoration of host immune homeostasis is integral in regards to ocular function. Treatment involving corticosteroids does address the host response to an extent; however, there remains indecision regarding use of this class of hormones.[7] In fact, a review evaluating the effects of corticosteroids from animal experiments, case reports and series, case-comparison and cohort studies, as well as clinical trials from 1950 to 2000 found that the role of corticosteroids in the adjunctive treatment of bacterial keratitis remains unsubstantiated.[7] Although experimental models suggested likely advantages, clinical studies did not show any significant effect of topical corticosteroid therapy on the outcome of bacterial keratitis.[7] In this regard, endogenous peptides have emerged as attractive therapeutic options. From previous studies using a well-established model of bacterial keratitis, it has been shown that treatment with the neuropeptide, vasoactive intestinal peptide (VIP) results in markedly reduced disease severity and conversion from a susceptible to resistant phenotype.[8] It has also been reported in this same modeling system that VIP exerts a robust immunomodulatory effect (predominately pro-resolving in nature) following infection.[8–12] However, while VIP has been shown to be efficacious following bacterial keratitis induced by American Type Culture Collection (Manassas, VA, USA) *P.*

aeruginosa strain 19660,[8–12] it is imperative to assess whether these effects are specific to this particular strain or if it would be equally effective against other strains of *P. aeruginosa*. Therefore, in the current study, we compared VIP treatment after infection with cytotoxic *P. aeruginosa* strain 19960 (PA 19960) against both an invasive strain (PAO1) and a clinical isolate (KEI 1025) in a mouse model of bacterial keratitis.

Methods

Bacterial Preparation

Bacterial cultures were prepared as previously described.[13] In brief, each strain was grown in peptone-tryptic soy broth (PTSB) containing 5% peptone (BD Biosciences, Franklin Lakes, NJ, USA) and 0.25% trypticase soy broth (Sigma-Aldrich, St. Louis, MO, USA) at 37°C in a circulating water bath (150 rpm) for 18 hours to an optical density (OD) reading of 1.4 to 1.6 at 540 nm. Cultures were centrifuged at 6000*g* for 10 minutes at 15°C, washed once and resuspended in sterile normal saline for use as indicated below.

Experimental Animal Protocol

Female, 8-week-old C57BL/6J (B6) mice (Jackson Laboratory, Bar Harbor, ME) were anesthetized, and the left central cornea was scarified as previously described.[14] An aliquot (5 μL) of bacterial suspension containing 10^6 colony-forming units (CFU)/μL of *P. aeruginosa* was topically applied to the wounded corneal surface. Mice were infected with the following strains of *P. aeruginosa*: cytotoxic strain ATCC 19660, invasive strain PAO1 (ATCC strain 15692), and a clinical isolate, KEI 1025 (Kresge Eye Institute, Detroit, MI, USA). Eyes were monitored daily and disease response was graded using an established grading scale[15] for statistical comparison at 1, 3 and 5 days post injection (p.i.). Disease response was further illustrated photographically at 3 and 5 days p.i. For all in vivo experiments, $n = 5$ animals/group/time point were used unless specified otherwise. Analysis of wounded (debrided), uninfected corneas with or without VIP treatment has been previously published[8] and therefore, was not included as an additional control in the current study in order to minimize animal usage. All animals were treated in a manner approved by Wayne State University Institutional Animal Care and Use committee and conformed to the Association for Research in Vision and Ophthalmology statement on the Use of Animals in Ophthalmic and Vision Research.

VIP Treatment

Mice received daily intraperitoneal (IP) injections of VIP (5 nM in 100 μL; Bachem, Torrance, CA, USA) beginning 1 day prior to infection (day −1) through a maximum of 5 days p.i. Control mice were similarly injected with sterile PBS.

Bacterial Load

Corneas from VIP- and PBS-treated B6 mice were collected at 3 and 5 days p.i., and the number of viable bacteria was quantitated. Individual corneas were homogenized in sterile 0.9% saline containing 0.25% BSA. Serial 10-fold dilutions of samples were plated on *Pseudomonas* isolation agar (BD Biosciences) in triplicate, and plates were incubated overnight at 37°C. Results are reported as CFU/cornea ± SEM.

Myeloperoxidase (MPO) Assay

An MPO assay was performed to estimate the number of PMN in corneas of VIP- and PBS-treated B6 mice. Corneas were removed at 1, 3, and 5 days p.i. and homogenized in 1.0 mL of 50 mM phosphate buffer (pH 6.0) containing 0.5% hexadecyltrimethyl-ammonium. Samples were freeze-thawed, and then after centrifugation, 100 μL of the supernatant was added to 2.9 mL of 50 mM phosphate buffer containing *o*-dianisidine dihydrochloride (16.7 mg/100 mL) and hydrogen peroxide (0.0005%). Changes in absorbancy (460 nm) were monitored for 5 minutes at 30 second intervals. The slope of the line was determined for each sample and used to calculate units of MPO/cornea. One unit of MPO activity is equivalent to $\sim 2 \times 10^5$ PMN.[16]

Real-time RT-PCR

Total RNA was isolated from individual whole corneas for in vivo analysis (RNA STAT-60; Tel-Test, Friendswood, TX, USA) according to the manufacturer's instruction, and quantitated by spectrophotometric determination (260 nm). Total RNA (100 ng) was reverse transcribed and used to produce a cDNA template as previously described.[7] cDNA products were diluted 1:20 with DEPC-treated water, and 2 μL cDNA (10-μL total reaction volume) was used for semi-quantitative real-time RT-PCR analysis (CFX Connect Real-Time RT-PCR Detection System; BioRad, Hercules, CA, USA). All mouse primer pair sequences designed in the laboratory (PrimerQuest, Integrated DNA Technologies, Coralville, IA, USA) are listed in the Table. SOCS3, COX-2 and ALOX-12 were purchased commercially (Qiagen, Venlo, The Netherlands). PCR amplification conditions were determined using routine methods.[17] Relative transcript levels were calculated using the relative standard curve method comparing the amount of target normalized to an endogenous reference, β-actin. Data are shown as the mean ± SD for relative transcript levels and represent at least two individual experiments.

Total RNA was similarly processed for detection of ExoS and ExoU expression by PA 19660, PAO1 and KEI 1025 grown in culture. Results obtained by real-time RT-PCR were confirmed by agarose gel electrophoresis. Amplified products were run on a 1% agarose gel for 40 minutes at 80V. No reverse transcriptase (NRT) controls were included for all PCR analyses to ensure PCR amplicon specificity to cDNA and not chromosomal DNA.

ELISA

Interleukin-1 β (IL-1β), IL-10, TNF-α, TGF-β, and MIP-2 protein levels were selectively tested by enzyme-linked immunosorbent assay (ELISA) at 3 and 5 day p.i. Whole corneas were individually homogenized in 500 μL of PBS containing 0.1% Tween-20, with a glass micro tissue grinder and centrifuged at 13,000*g* for 10 minutes. Supernatants were run in triplicate per the manufacturer's instruction (R&D Systems, St. Paul, MN, USA). The reported sensitivity of this assay was 4.8 pg/mL for IL-1β, 5.22 pg/mL for IL-10, 7.21 pg/mL for TNF-α, 15.4 pg/mL for TGF-β and 1.5 pg/mL for MIP-2. Results are expressed as average picogram of each cytokine/mL ± SEM.

Cytotoxicity and Invasion Assays

HT-29 cells, a human colorectal adenocarcinoma cell line with epithelial morphology (HTB-38; ATCC), were seeded into tissue culture plates rinsed with Hanks balanced salt solution and grown to 80 to 90% confluency (1–2 days) in Dulbecco modified Eagle medium (DMEM) (Gibco, Gaithersburg, MD,

Table. Nucleotide Sequences of Specific Primers Used for PCR Amplification

Gene	Nucleotide Sequence	Primer
β-actin	5′- GAT TAC TGC TCT GGC TCC TAG C -3′	F
	5′- GAC TCA TCG TAC TCC TGC TTG C -3′	R
IL-1β	5′- CGC AGC AGC ACA TCA ACA AGA GC -3′	F
	5′- TGT CCT CAT CCT GGA AGG TCC ACG -3′	R
TNF-α	5′- ACC CTC ACA CTC AGA TCA TCT T -3′	F
	5′- GGT TGT CTT TGA GAT CCA TGC -3′	R
CXCL2/MIP-2	5′- TGT CAA TGC CTG AAG ACC CTG CC -3′	F
	5′- AAC TTT TTG ACC GCC CTT GAG AGT GG -3′	R
IL-10	5′- TGT CAA TGC CTG AAG ACC CTG CC -3′	F
	5′- AAC TTT TTG ACC GCC CTT GAG AGT GG -3′	R
TGF-β	5′- TCT CTG CTC TCT GCT GCT GAT ATG C -3′	F
	5′- AGG ACA AAT GGC TCT GAC ACA GTA CC -3′	R
exoS	5′- GGA GCT GGA TGC GGG GAC A -3′	F
	5′- GGC CGC CTC TTC GAG AAC -3′	R
exoU	5′- GCT AAG GCT TGG CGG AAT -3′	F
	5′- GGC CGC CTC TTC GAG AAC -3′	R

F, forward; R, reverse.

USA). The cells were cultured at 37°C and 5% CO_2. On the day of the experiment, the cells were rinsed with PBS, and then incubated in fresh culture medium containing a bacterial suspension (200 μL) of either PA 19660, PAO1, or KEI 1025 at a concentration of 10^7 CFU/μL (as prepared above) resulting in a multiplicity of infection (MOI) of 100. Cells were co-incubated with the bacteria for 3 hours (37°C, 5% CO_2), rinsed with PBS, treated with gentamicin (200 μg/mL) for an additional 1 hour to kill any extracellular bacteria, and washed with PBS (3×) to remove the antibiotic and dead bacteria. Cells were further processed for cytotoxicity and invasion assays as described below.

Cytotoxicity was determined using an enzymatic assay (Cytotox 96; Promega, Madison, WI, USA) per the manufacturer's instruction. Briefly, supernatants from infected HT-29 cells were collected from which levels of lactate dehydrogenase (LDH), a cytoplasmic enzyme released upon cell death, were determined. HT-29 cells exposed to a lysis solution served as a control for maximum LDH release. Results are reported as % of cytotoxicity ± SEM.

Regarding bacterial invasion, cells were lysed by incubation with Triton X-100 (0.5%) for 15 minutes. Cell lysates were diluted 1:100, and then plated in triplicate onto *Pseudomonas* isolation agar plates. Results are represented as CFU/mL of cellular lysate ± SEM.

Bactericidal Assay

Pseudomonal cultures were grown as described above and diluted to a concentration of 10^4 CFU/μL. A 1:100 dilution was made into 200 μL of PBS with or without various concentrations of VIP (10^{-8} to 10^{-10} M) and incubated for 2 hours at 37°C (5% CO_2). Aliquots containing 50 μL were plated on *Pseudomonas* isolation agar (BD Biosciences) in triplicate, and plates were incubated overnight at 37°C. Results are reported as total ± SEM CFU.

Statistical Analysis

Differences in clinical scores between two groups at each time point were tested by the Mann-Whitney *U* test. For all other experiments, an unpaired Student's *t*-test (for comparison between two groups) was used. Data were considered significant at a *P* value of <0.05. All experiments were repeated at least twice and representative data from a typical experiment are shown unless otherwise noted.

Results

Ocular Response to Multiple *P. aeruginosa* Strains

To investigate the efficacy of VIP as a treatment against multiple strains of *P. aeruginosa*, mice were infected with three different strains of *P. aeruginosa* (including both invasive and cytotoxic) and disease outcome compared between VIP-treated experimental groups and PBS-treated controls. As represented in Figure 1, infected corneas consistently demonstrated central corneal opacity (+1) at 1 day p.i. for all bacterial strains. PA 19660-infected controls progressed to dense opacity covering the entire anterior segment (+3) by 3 days p.i. with 60% of corneas perforating by 5 days p.i. Disease response was less severe in PAO1 and KEI 1025 infected controls, which resulted in dense opacity, partially or fully covering the pupil (+2) at 3 days p.i. and only 30% and 10% corneal perforation, respectively, by 5 days after infection.

Ocular disease response of infected mice after VIP injection was improved over time compared to respective controls for all three bacterial strains. In fact, median clinical score of VIP-treated animals indicated significantly less disease at both 3 and 5 days p.i. after infection with PA 19660 and KEI 1025, while only 5 days p.i. was significant for PAO1. Representative photographs taken at 3 and 5 days p.i. for each bacterial strain and treatment group are provided in Figure 2 and demonstrate that the early disease response appears to be more severe for the cytotoxic strain PA 19660 compared to PAO1 and KEI 1025. At 5 days p.i., PAO1 and PA 19660 exhibit corneal perforation with severe corneal thinning and increased vasculature observed in KEI 1025. A marked decrease in corneal opacity, however, can be seen in all experimental mice compared to the respective control corneas at both time points suggesting that VIP treatment improves the disease response regardless of the invasive/cytotoxic phenotype.

Bacterial Load

Direct plate counts were used to determine the number of viable bacteria in infected corneas at 3 and 5 days p.i. and the results are shown in Figure 3. Overall, bacterial loads detected in PBS-treated controls were similar over time across the three bacterial strains, with significantly elevated bacteria at 5 days p.i. in PA 19660 versus PAO1 ($P = 0.047$) only. VIP treatment of PA 19660-infected corneas revealed significantly less bacteria at both 3 and 5 days p.i. when compared to respective controls.

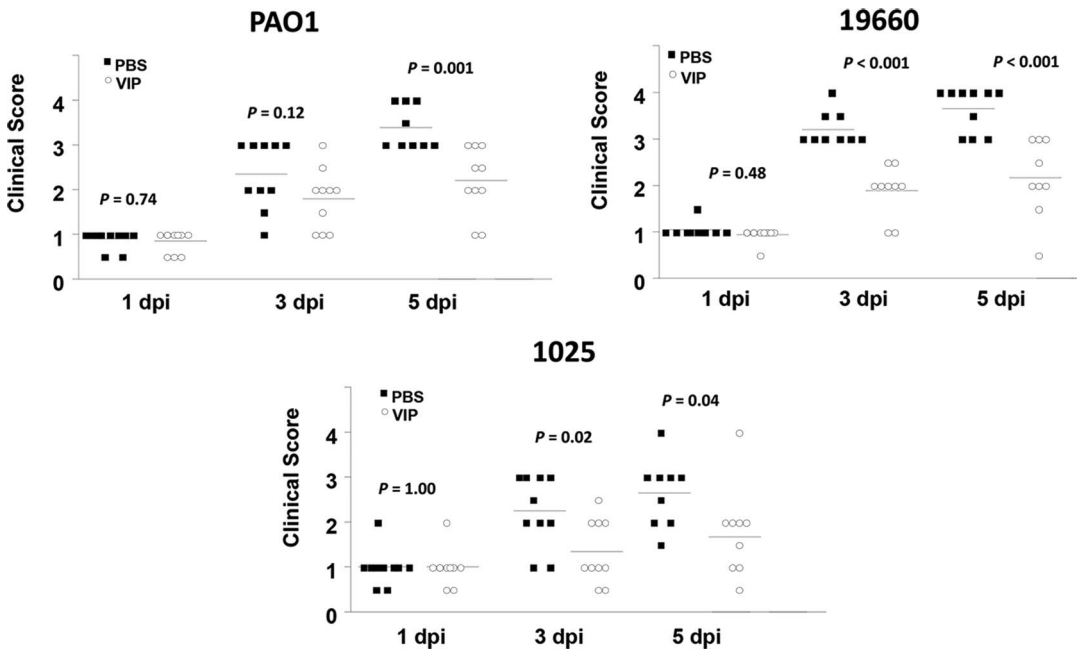

Figure 1. Ocular disease response of infected B6 mice after injection of VIP or PBS. Results are median clinical scores ± SEM. Significant differences between the two groups were observed at both 3 and 5 days after infection with PA 19660 and KEI 1025. Although disease response was improved at 3 days, as well, only 5 days were significant after PAO1 infection.

PAO1 showed a similar trend albeit only 3 days p.i. was significantly reduced. Regarding KEI 1025-infected animals, there was no difference in bacterial load at 3 days after VIP treatment, though it was significantly decreased at 5 days p.i.

MPO Assay

As the most abundant protein in neutrophils, the amount of MPO was quantitated to assess PMN numbers over time following infection. MPO levels (Fig. 4) were up-regulated in PBS-treated controls after infection with PAO1, PA 19660, and KEI 1025 at all time points. VIP treatment resulted in a significant reduction in the amount of MPO detected compared to controls for all three bacterial strains tested, except for PAO1 at 1 day p.i.

Cytokine/Chemokine Expression

At 5 days p.i., mRNA levels of select pro- and anti-inflammatory cytokines/chemokines, known to be differentially expressed in PA 19660-infected corneas,[8] were analyzed in both PAO1- and KEI 1025-infected mice, as well (Fig. 5). Regarding PBS-treated control mice, results indicated that corneal expression levels of IL-1β, TNF-α, and MIP-2 were similarly up-regulated, while IL-10 and TGF-β were down-regulated during PAO1- and KEI 1025-induced infection, similar to that observed after PA 19660 infection. Furthermore, the aforementioned pro-inflammatory molecules were effectively down-regulated in VIP- versus PBS-treated mice infected with PA 19660, PAO1 and KEI 1025. In contrast, VIP treatment significantly enhanced levels of anti-inflammatory mediators, IL-10 and TGF-β, compared to PBS-treated animals after infection with all three bacterial strains. Moreover, three additional molecules were examined, including a member of the suppressor of cytokine signaling (SOCS) family, SOCS3, was similarly examined, as this molecule has been implicated as a cytokine-inducible negative regulator of cytokine signaling.[18] SOCS3 was up-regulated after VIP treatment against PA 19660, PAO1, and KEI 1025 when compared to PBS-treated controls, as shown in Figure 5. Cyclooxygenase (COX)-2, rapidly induced in response to pro-inflammatory cytokines, was enhanced after VIP treatment over PBS-treated controls for all three strains. Additionally the

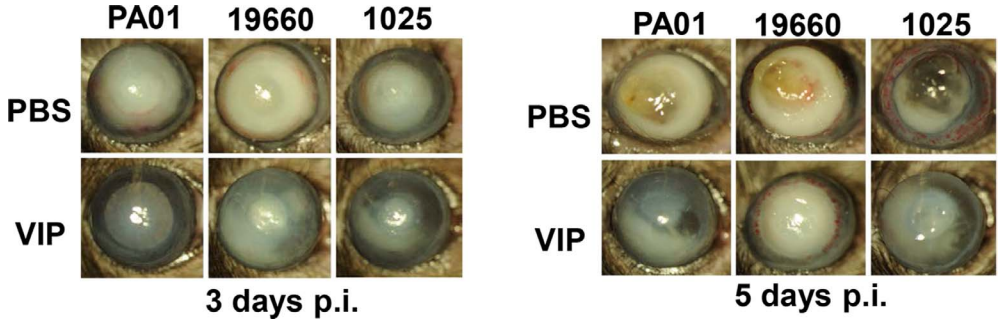

Figure 2. Slit-lamp views of *P. aeruginosa* bacteria-infected B6 mice. Representative eyes from PBS-treated (*top row*) and VIP-treated (*bottom row*) mice were photographed at 3 (*left*) and 5 (*right*) days following infection with PAO1, PA 19660, and KEI 1025. Control eyes illustrate the typical disease response that ultimately results in corneal perforation, whereas VIP treatment demonstrated improved disease outcome. *Magnification*: ×31.

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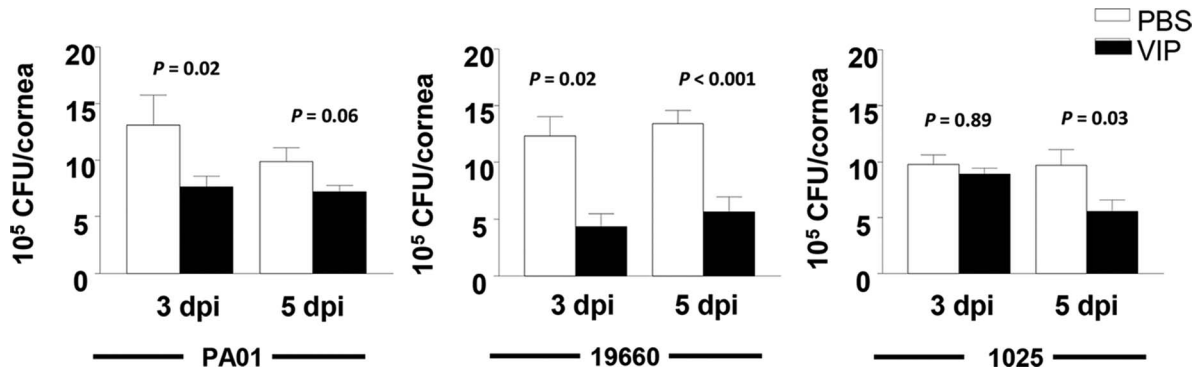

FIGURE 3. Quantitation of viable bacteria. Bacterial counts were detected from corneas of VIP- treated versus PBS-treated animals after *P. aeruginosa* ocular infection with PAO1 (*left*), PA 19660 (*middle*), and KEI 1025 (*right*). Results showed a decrease in viable bacteria after VIP treatment for all three strains tested at both 3 and 5 days p.i. Statistical significance was observed at 3 days p.i. for PAO1 and at 5 days for KEI 1025, whereas PA 19660 was significant at both time points after VIP treatment.

lipoxygenase, ALOX12—known to convert leukotriene A_4 (LTA_4) into the pro-resolving bioactive lipoxin A_4 (LXA_4) and LXB_4—was up-regulated in corneas of VIP-treated mice compared to PBS-treated controls after infection with all three strains as illustrated in Figure 5.

Next, protein levels of selected molecules IL-1β, TNF-α, MIP-2, IL-10, and TGF-β were confirmed at 3 and 5 days p.i., and results are shown in Figure 6. Results at the protein level corroborated observations at the transcript level, whereby proinflammatory mediator (IL-1β, TNF-α and MIP-2) expression was significantly reduced, and anti-inflammatory molecules (IL-10 and TGF-β) were significantly augmented in VIP-treated versus PBS-treated mice infected with PA 19660, PAO1, and KEI 1025 at both time points.

Characterization of Clinical Isolate KEI 1025

We next sought to characterize the clinical isolate KEI 1025 by its mode of pathogenicity as compared with PA 19660 (cytotoxic) and PAO1 (invasive). Cytotoxicity was determined via detection of LDH released by bacterially infected HT-29 cells in vitro (Fig. 7A). Results indicated that KEI 1025 was the least cytotoxic strain at only 0.5% ± 0.1% cell lysis after 4 hours of co-incubation. PAO1 was slightly higher with 3% ± 0.1% lysis, while PA 19660 demonstrated cytotoxic activity as high as 89% ± 0.9%.

Evaluation of invasive capacity was carried out using bacterial plate counts and the gentamicin exclusion test (Fig. 7B). Results revealed that both PAO1 and KEI 1025 exhibit invasive behavior with 11.7×10^4 and 8.3×10^4 CFU/mL detected intracellularly, respectively. In contrast, PA 19660 resulted in significantly less intracellular bacteria at 0.4×10^4 CFU/mL.

Cytotoxic and invasive capacities were confirmed by the presence of the cytotoxin, ExoU, which has been correlated with acute cytotoxicity,[19,20] or ExoS, expressed only by invasive strains.[21] As shown in Figure 7C, PA 19660 was $ExoU^+/ExoS^-$ (Fig. 7C, lanes 1 and 4) as expected for the cytotoxic strain; while both KEI 1025 (Fig. 7C, lanes 2 and 5) and PAO1 (Fig. 7C, lanes 3 and 6) were $ExoU^-/ExoS^+$, corresponding to the invasive phenotype. C_q values obtained from PCR analyses for $ExoU^-$ and $ExoS^-$ samples and NRT controls were >35 (data not shown).

Bactericidal Activity of VIP

Given that VIP was more effective at reducing bacterial load following ocular infection with the cytotoxic strain 19660 compared to invasive strains KEI 1025 and PAO1, we next examined the bactericidal activity of this neuropeptide. VIP demonstrated direct antibacterial activity against all three strains of *P. aeruginosa* as indicated by decreased plate counts for the three tested concentrations shown in Figure 8. While the effects observed in PAO1 appeared to be dose-dependent, this was not the case for PA 19660 or KEI 1025.

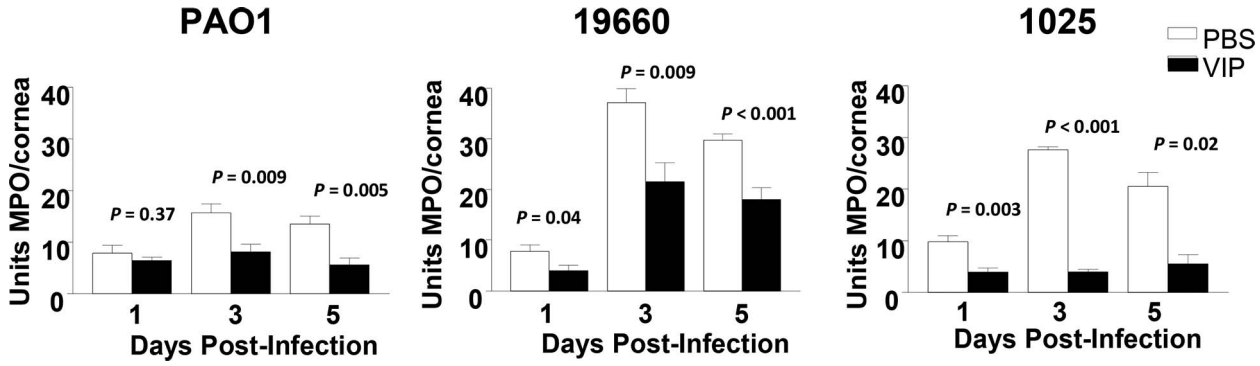

FIGURE 4. MPO levels as detected from corneas of VIP-treated versus PBS-treated B6 mice following ocular infection with the *P. aeruginosa* strains PAO1 (*left*), PA 19660 (*middle*), and KEI 1025 (*right*). Levels of MPO were significantly lower at 1, 3, and 5 days p.i. for PA 19660 and KEI 1025, indicating fewer infiltrating PMN after infection. MPO levels after PAO1 infection were similar at 1 day p.i. but significantly decreased at 3 and 5 days.

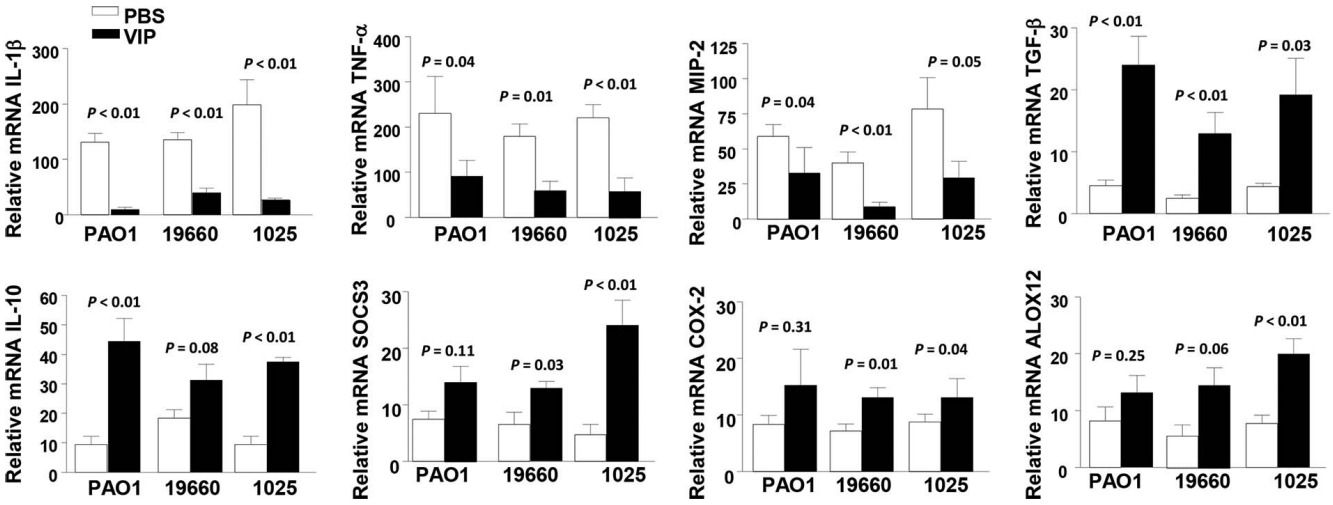

Figure 5. Select cytokine, chemokine, and lipid mediator-related transcript levels were assessed after VIP treatment in B6 mice infected with the *P. aeruginosa* strains PAO1, PA 19660, and KEI 1025 at 5 days p.i. Proinflammatory molecules (IL-1β, TNF-α, MIP-2) were reduced, whereas anti-inflammatory molecules, TGF-β and IL-10, were enhanced after VIP treatment. SOCS3, COX-2, and ALOX12 (12-LOX) were also up-regulated compared to PBS treatment for all three bacterial strains tested.

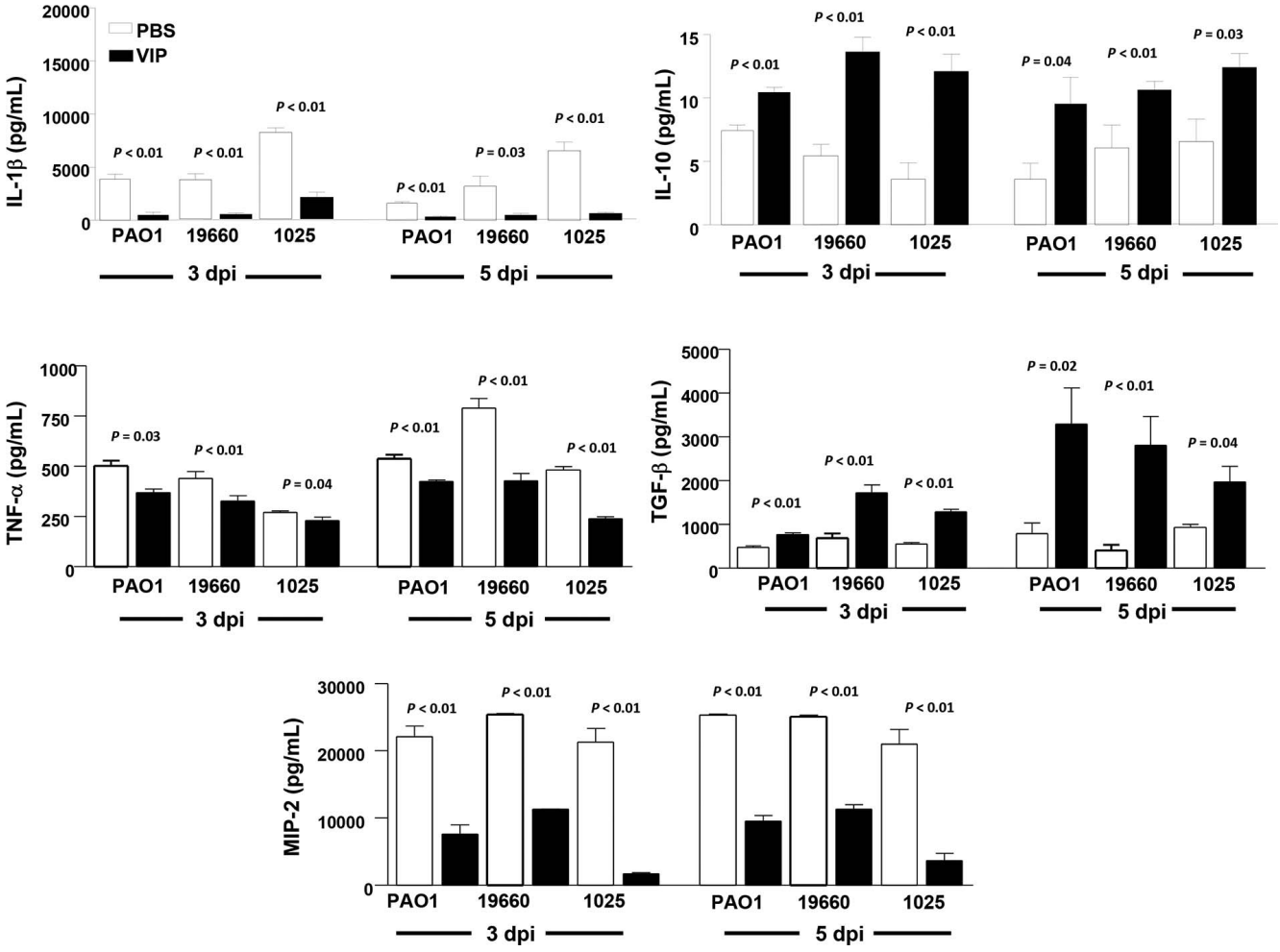

Figure 6. Protein expression of select cytokines and chemokines in VIP-treated versus PBS-treated B6 mice. IL-1β, TNF-α, MIP-2, IL-10, and TGF-β protein levels are shown as detected by ELISA at 3 and 5 days following infection with PAO1, PA 19660, and KEI 1025. Significance was observed between VIP treatment and PBS controls at all time points tested for all three *P. aeruginosa* strains. Pro-inflammatory molecules were consistently reduced, and anti-inflammatory cytokines were elevated with VIP treatment.

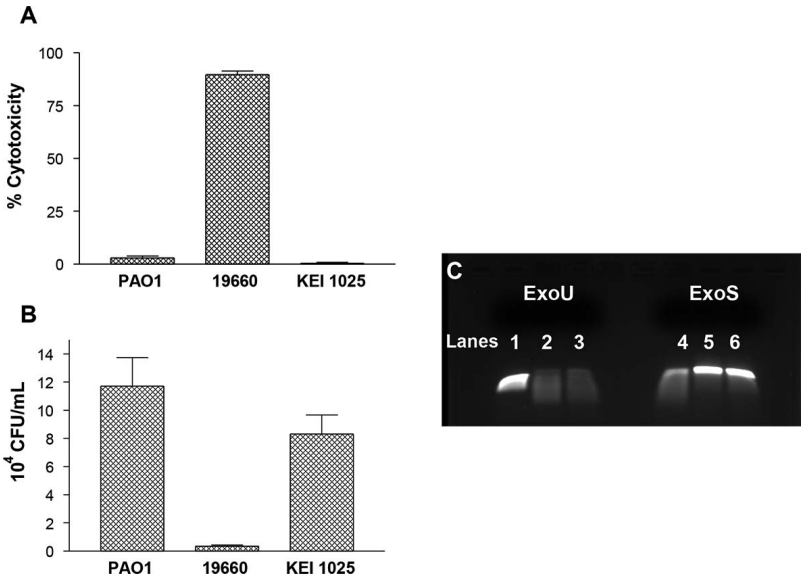

Figure 7. (**A**) Representative results from a cytotoxicity assay detecting LDH release from HT-29 cells after infection with PAO1, PA 19660, and KEI 1025. Results are expressed as the percent of cytotoxicity compared to that of positive control (maximum LDH release). PA 19660 demonstrated higher levels of cytotoxicity than PAO1 and KEI 1025. (**B**) Invasion assay as detected by bacterial plate counts after infection of HT-29 cells with PAO1, PA 19660, and KEI 1025. Results are expressed as CFU/mL of cell lysate. PAO1 and KEI 1025 indicated greater invasive capacity compared to PA 19660. (**C**) Amplified PCR products for ExoU (*left*) and ExoS (*right*) expression by PA 19660, PAO1, and KEI 1025, as confirmed by agarose gel electrophoresis.

Discussion

Previous studies examining the immunoregulatory effects of VIP have supported its role as a potent anti-inflammatory molecule during pseudomonal keratitis. To this end, VIP has been shown to modulate cytokine production, adhesion molecules, components of the extracellular matrix (ECM), growth factors, angiogenic molecules, defensins, TLRs and TLR-related molecules such as ST2 and SIGIRR, ultimately enhancing disease resolution after infection with PA 19660, a cytotoxic strain.[8–12] In light of these studies and the implication for VIP in the clinical setting, it is imperative to assess whether this neuropeptide is equally efficacious against other strains of *P. aeruginosa* including PAO1, an invasive strain, and KEI 1025, a previously uncharacterized clinical isolate. Therefore, in the current study, we examined the protective effects of VIP against both cytotoxic and invasive strains.

Overall, VIP treatment resulted in reduced disease pathogenesis for all three *P. aeruginosa* strains tested as indicated by mean clinical score, bacterial load, cytokine/chemokine expression and host inflammatory cell infiltrate. Although mean clinical score was improved after VIP treatment for all strains, it was evident that the clinical isolate KEI 1025 caused significantly less disease than both PAO1 ($P = 0.02$) and PA 19660 ($P = 0.003$). On the other hand, it is noteworthy that PA 19660 resulted in most severe disease outcome as supported by a higher rate of corneal perforation, increased bacterial load and increased PMN infiltrate. This more aggressive infection serves well for the efficacy of VIP, which has been consistently used in our past studies. This study is also the first to characterize the clinical isolate, KEI 1025 as an $ExoU^{-}$ /$ExoS^{+}$ invasive strain of *P. aeruginosa*. Although administering VIP post infection would be more clinically relevant, injections for the current study were started one day prior in order to stay in alignment with previously published studies investigating PA 19660 corneal infection.[8–12] Furthermore, studies examining both post-infection and topical treatments are currently ongoing and preliminarily appear to be efficacious against *P. aeruginosa*-induced keratitis (unpublished data).

In addition, it appears as though VIP is more effective at reducing bacterial load when corneas are infected with the cytotoxic strain (PA 19660) versus an invasive strain (KEI 1025/PAO1). This finding suggests extracellular bactericidal activity by VIP, which was confirmed in vitro. VIP was found to possess direct antimicrobial activities against the three tested

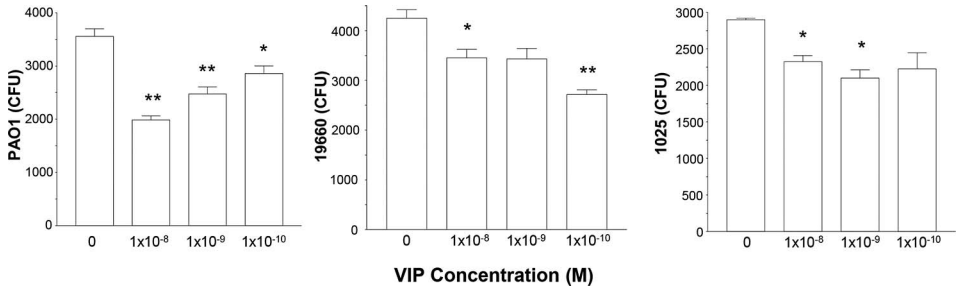

Figure 8. VIP-mediated antimicrobial activity against *P. aeruginosa* strains 19660, PAO1, and KEI 1025. Bactericidal assay results as determined by bacterial plate counts after incubation with VIP (10^{-10} to 10^{-8} M) for 2 hours at 37°C. Results are total CFUs ± SEM. Reduced bacterial viability was observed for both the cytotoxic strain (19660) and the invasive strains (PAO1 and KEI 1025). *$P < 0.05$; **$P < 0.01$.

strains as revealed by decreased bacterial viability. These results correlate well with previous publications indicating a direct antimicrobial effect on a variety of pathogens, including *Pseudomonas*.[22,23] In fact, generation of two synthetic VIP analogs (VIP51 and VIP51 [6-30]) by Campos-Salinas et al.[23] has resulted in increased metabolic stability as well as enhanced antimicrobial features.

Because it is clear that VIP functions to alter the cytokine/chemokine expression profile during inflammation/infection, our preliminary findings indicating for the first time that SOCS3, COX-2, and 12-LOX are affected by VIP treatment provide potential mechanisms by which the neuropeptide exerts its effects. SOCS3, in particular, is known to convey resistance against *Mycobacterium tuberculosis* infection[24] by regulating IL-6/STAT3 signaling. Although it is unclear whether VIP influences this signaling pathway directly (or through other SOCS genes), it is a provocative lead that would expand upon the immunoregulatory capacity of this neuropeptide.

Given that VIP has been found to have pleiotropic immune functions (including anti-inflammatory and antimicrobial), we began investigating potential pro-resolving effects related to lipid mediator pathways. Resolution of inflammation is an active process by which SPMs are generated, subsequently leading to restoration of immune (and tissue) homeostasis.[25] Once arachidonic acid is released from the cell membrane, it can be metabolized by three families of enzymes: (1) cyclooxygenases (COXs), (2) lipoxygenases (LOXs), and (3) cytochrome P450s to form lipid autocoids, prostaglandins (PGs), leukotrienes (LTs), and LOX-derived lipoxins. Although proinflammatory mediators such as PGs and LTs are essential components for the initiation of inflammation, endogenous specialized pro-resolving lipid mediators (SPMs) such as lipoxins act as potent agonists of resolution. Previous studies have indicated that both COX-derived PGs and LOX-derived LTs are present in the eye.[26–29] Furthermore, endogenous LXA4, which can be formed by 12-LOX, has been detected in both healthy and injured corneas and appears to play an important role in corneal wound healing.[26] As such, COX-2 and 12-LOX mRNA expression was examined and VIP treatment resulted in an up-regulation of both enzymes after infection. These data further support an immunoregulatory versus anti-inflammatory role for VIP, where inflammatory-induced release of PGs is required for lipid mediator switch toward resolution. In other words, VIP does not simply reduce all pro-inflammatory mediators, but regulates their expression so that resolution can be properly and effectively carried out. Moreover, it provides yet another avenue for investigation regarding the immunoregulatory effects of this neuropeptide. Although Ciabattoni et al.[30] found that VIP inhibited the synthesis and release of thromboxane A_2 in guinea-pig lung during an anaphylactic reaction; this is the first study to describe a role for endogenous VIP and lipid mediators in a bacterial keratitis model.

In conclusion, results from the current study suggest that VIP improves disease outcome not only against infection with the cytotoxic strain, PA 19660, but also against the invasive strain, PAO1, and a clinical isolate, KEI 1025; thus, reinforcing its clinical applicability for bacterial keratitis. In addition, VIP treatment immunoregulates the host response on multiple levels; including cytokine/chemokine expression, inflammatory cell activation/persistence and lipid mediator pathways.

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