Vasoactive Intestinal Peptide Downregulates Proinflammatory TLRs While Upregulating Anti-Inflammatory TLRs in the Infected Cornea

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TLRs recognize microbial pathogens and trigger an immune response, but their regulation by neuropeptides, such as vasoactive intestinal peptide (VIP), during Pseudomonas aeruginosa corneal infection remains unexplored. Therefore, C57BL/6 (B6) mice were injected i.p. with VIP, and mRNA, protein, and immunostaining assays were performed. After VIP treatment, PCR array and real-time RT-PCR demonstrated that proinflammatory TLRs (conserved helix-loop-helix ubiquitous kinase, IRAK1, TLR1, TLR4, TLR6, TLR8, TLR9, and TNFR-associated factor 6) were downregulated, whereas anti-inflammatory TLRs (single Ig IL-1–related receptor [SIGIRR] and ST2) were upregulated. ELISA showed that VIP modestly downregulated phosphorylated inhibitor of NF-κB kinase subunit α but upregulated ST2 –2-fold. SIGIRR was also upregulated, whereas TLR4 immunostaining was reduced in cornea; all confirmed the mRNA data. To determine whether VIP effects were cAMP dependent, mice were injected with small interfering RNA for type 7 adenylate cyclase (AC7), with or without VIP treatment. After silencing AC7, changes in mRNA levels of TLR1, TNFR-associated factor 6, and ST2 were seen and unchanged with addition of VIP, indicating that their regulation was cAMP independent. In contrast, changes were seen in mRNA levels of conserved helix-loop-helix ubiquitous kinase, IRAK1, 2, TLR4, 9 and SIGIRR following AC7 silencing alone; these were modified by VIP addition, indicating their cAMP independence. In vitro studies assessed the effects of VIP on TLR regulation in macrophages and Langerhans cells. VIP downregulated mRNA expression of proinflammatory TLRs while upregulating anti-inflammatory TLRs in both cell types. Collectively, the data provide evidence that VIP downregulates proinflammatory TLRs and upregulates anti-inflammatory TLRs and that this regulation is both cAMP dependent and independent and involves immune cell types found in the infected cornea. The Journal of Immunology, 2012, 189: 000–000.

Keratitis induced by Pseudomonas aeruginosa progresses rapidly and is characterized by a supplicative stromal infiltrate with a marked mucopurulent exudate. Other untoward characteristics include inflammatory epithelial edema, ocular pain and redness, stromal ulceration, and often, decreased vision (1). Experimentally, infection with P. aeruginosa leads to corneal perforation in strains of mice, such as C57BL/6 (B6; susceptible), whereas less severe disease is seen in similarly challenged BALB/c (resistant) animals (2). In contrast, treatment of B6 mice with vasoactive intestinal peptide (VIP) promotes better disease outcome postinfection with P. aeruginosa, mainly through regulation of cytokine production and subsequent alteration of the host inflammatory cell response (3). Recent studies from this laboratory provided further information regarding the anti-inflammatory effects of VIP and showed that it modulates keratitis through regulation of growth factors, angiogenic molecules, and β defensins in the infected cornea, contributing to healing (4). However, the role of VIP in the regulation of TLRs has not been well-defined, despite the fact that other studies demonstrated that TLRs play an important role in ocular immune defense (5). They direct the ocular immune response by differentially regulating a variety of events, including bacterial killing, polymorphonuclear leukocyte infiltration, and cytokine expression at both mRNA and protein levels (6–9). In particular, TLR4, a specific receptor for LPS contained in the outer membrane of various Gram-negative bacteria, is required for host resistance against P. aeruginosa in the infected cornea (8). In fact, upon activation by P. aeruginosa, both TLR4 and TLR5 on corneal macrophages were shown to regulate P. aeruginosa keratitis through MyD88-dependent and -independent pathways (10). Therefore, it is important to understand which TLRs are regulated by VIP, as well as the mechanisms involved during microbial keratitis. In this regard, studies suggested that VIP can signal through cAMP-dependent or -independent pathways (11), but it is not known whether this is important in VIP regulation of TLRs in other diseases (12) or bacterial keratitis.

Thus, the studies described in this article investigated the expression of TLR-signaling pathways in P. aeruginosa-infected corneas, with or without VIP treatment, to determine whether it regulates their expression to favor disease resolution. Our data provide evidence that VIP treatment downregulates proinflammatory TLRs while upregulating anti-inflammatory TLRs at both the mRNA and protein levels in the cornea after P. aeruginosa infection and that, mechanistically, two transduction path-
ways, cAMP dependent and independent, are involved. Furthermore, in vitro studies suggest that at least two cell types in the cornea may be responsive, because VIP reduced proinflammatory TLRs and increased anti-inflammatory TLRs in both LPS-stimulated elicited peritoneal macrophages and XS52 (Langerhans) cells.

Materials and Methods

Infection

Eight-week-old female B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were anesthetized with ethyl ether and placed beneath a stereoscopic microscope at ×40 magnification. The cornea of the left eye was wounded (13), and a 5-μl aliquot containing 1.0×10^6 CFU *P. aeruginosa* (strain 19660; American Type Culture Collection, Manassas, VA) was topically delivered. Animals were treated humanely and in compliance with the Association for Research in Vision and Ophthalmology Statement on the Use of Animals in Ophthalmic and Vision Research.

VIP treatment

Treatment with synthetic VIP was described previously (3) at a dosage reported to be efficacious in a lethal endotoxemia model (14). In brief, using that dosage, B6 mice received daily i.p. injections of VIP (5 nM in PBS (100 μl) sterile PBS) at 1 d before infection (day −1) through a maximum of 7 d postinfection (p.i.). Control mice were injected similarly with sterile PBS (100 μl).

RNA interference

Use of small interfering RNA (siRNA) was described in previous work from this laboratory (15). For the current studies, siRNA for type 7 adenylate cyclase (AC7) or an appropriate scrambled control (Santa Cruz Biotechnology, Santa Cruz, CA) was injected subconjunctivally (5 μl/mouse at a concentration of 8 μM) into the left eye of B6 mice (n = 5/group/time) 1 d before infection, with or without VIP treatment (described above). AC7 (4 μM) was applied topically to the infected corneas (5 μl/mouse/time) once on the day of infection and twice on days 1 and 3 p.i. Corneas from scrambled control, silenced AC7, or silenced AC7 plus VIP-treated (n = 5/group/time) mice were individually collected at 5 p.d. and processed for RT-PCR (described below). Using a similar assay, the efficacy of silencing AC7 in cornea (n = 5/group/time) was confirmed by real-time RT-PCR (5 d p.i.).

Real-time RT-PCR

Total RNA was isolated from individual corneas (n = 5/group/time) or cells using RNA-Stat 60 (Invitrogen, Carlsbad, CA), per the manufacturer’s recommendations, and quantitated spectrophotometrically (260 nm). One microgram of total RNA was reverse transcribed using Moloney murine leukemia virus (MMLV) reverse transcriptase. The 20-μl reaction mixture contained 200 U MMLV-reverse transcriptase, 10 μM MMLV reverse transcriptase, 10 U Rnasin, 500 ng oligo(dT)12-18 primers, 10 mM deoxyribonucleotide triphosphate, 500 ng oligo(dT)12-18 primers, 10 mM deoxyribonucleotide triphosphate, and 1 μl cDNA. The reaction mixture was amplified using SYBR Green Master Mix (SA Biosciences, Frederick, MD), per the manufacturer’s recommendation. Briefly, the reaction system contained 1 μl SYBR Green PCR Master Mix, 0.5 μM primers, and 2 μl cDNA (diluted 1/10) in diethyl pyrocarbonate water. All primer sets for the PCR array were purchased as a 96-well plate (RT2 Profiler TLR Signaling Pathway PCR Array; SA Biosciences). The individual primer sets were designed using PrimerQuest (Integrated DNA Technologies, Cambridge, MA). Sequences of primers for TRAF (6), single Ig IL-1-related receptor (SIGIRR), and ST2 are shown (Table I). Quantitative real-time RT-PCR was performed using the myQ Single-Color Real-Time RT-PCR detection system (Bio-Rad). Optimal conditions for PCR amplification of cDNA were established using routine methods (16). Relative mRNA levels were calculated after normalization to β-actin.

ELISA

Protein levels of TLR-associated molecules were tested by ELISA. Corneas from PBS- and VIP-treated B6 mice were individually collected (n = 5/group/time) from normal uninfected and infected mice at 1 and 7 d p.i. For total Chuk (IKKα) and p-IKKα (Cell Signaling Technology, Danvers, MA), corneas were homogenized in 0.5 ml lysis buffer (Cell Signaling Technology) with 1 mM PMSE (Sigma, St. Louis, MO) and protease inhibitory (1 tablet/10 ml; Roche, Indianapolis, IN). For ST2 (R&D Systems, Minneapolis, MN), corneas were homogenized in 0.5 ml PBS with 0.1% Tween 20 and protease inhibitor (as above). All samples were centrifuged at 13,000 rpm for 5 min, and an aliquot of each supernatant was assayed in duplicate for total IKKα, p-IKKα, and ST2, per the manufacturer’s instructions.

Western blot

Corneas (n = 5/group/time) were collected from PBS- and VIP-treated normal uninfected and infected B6 mice at 1 and 5 d p.i. Pooled corneas (n = 5/group) were lysed in 250 μl radioimmunoprecipitation assay lysis buffer (Santa Cruz Biotechnology) and homogenized for 15 min. Tissue debris was pelleted by centrifugation for 10 min at 12,000 rpm, and protein concentration of the supernatant was determined by bicinecinonic acid (Bio-Rad) protein assay. Supernatants were separated on 10% SDS-PAGE, after loading an 80-μg sample in each lane; as a control, recombinant murine SIGIRr (15 ng; R&D Systems) was similarly loaded. The electrophoretically separated material was transferred to a supported nitrocellulose membrane (Bio-Rad) and blocked in a 5% solution of nonfat dry milk prepared in 1× PBS and 0.05% Tween 20. Blots were incubated with primary goat anti-mouse SIGIrr Ab (R&D Systems) diluted in PBS containing 10% goat serum for 1 h. After washing the blots (4×, 10 min each) with TBST, detection was performed with HRP-conjugated secondary Ab (R&D Systems) diluted 1/1000 in PBS containing 5% nonfat milk, and developed using the ECL method (PerkinElmer, Waltham, MA), per the manufacturer’s protocol. The blot was scanned on a FluorChem E imaging system (Cell Biosciences, Santa Clara, CA), and band relative integrated density value was analyzed by AlphaView software (Cell Biosciences).

Immunoﬂuorescent staining

Normal, uninfected and infected eyes were enucleated from VIP- or PBS-treated B6 mice (n = 5/group/time) at 1 and 5 d p.i. Tissue was immersed in 1× Dulbecco’s PBS (Mediatech, Herndon, VA), embedded in Tissue-Tek OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Ten-micrometer-thick sections were cut, mounted on polylysine-coated glass slides, and incubated in a moist chamber at 37˚C overnight. After a 2-min acetone fixation, nonspecific staining was blocked with 10 mM sodium periodate for 10 min, and buffer containing 1% BSA and donkey IgG (1:100) for 30 min at room temperature. For immunostaining, sections were incubated for 1 h each with goat anti-mouse TLR4 (Santa Cruz Biotechnology), followed by an Alexa Fluor 594-conjugated donkey anti-goat secondary Ab (1:1,500, Invitrogen). Sections were incubated for 2 min with SYTOX Green nuclear acid stain (1:20,000; Lonza, Walkersville, MD). Controls were treated identically but the primary Ab was replaced with the secondary Ab. The sections were visualized (TLR4* staining = red), and digital images were captured with a Leica TCS SP2 confocal laser-scanning microscope (Leica Microsystems, Bannockburn, IL).

Cell isolation, culture, and treatment

Peritoneal macrophages were elicited and isolated from B6 mice, as described before (3). Briefly, cells were induced into the peritoneal cavity by i.p. injection of 1.0 ml 5% Brewer’s thioglycollate medium (Becton Dickinson, Sparks, MD) 5 d before harvest (n = 12 mice). Cells were collected by peritoneal lavage with DMEM and stained with trypan blue (1:1); viable cells (>95%) were counted using a hemacytometer. After a differential cell count, cells were collected and pooled, and 3×10^6 macrophages/well (five wells/treatment) were seeded into six-well plates. Nonadherent cells were removed 24 h later, and isolated macrophages were used for the in vitro-stimulation assays described below. Mouse XS52 (Langerhans) cells were cultivated in complete RPMI 1640 with 10% FCS (both from Life Technologies, Grand Island, NY) supplemented with 0.5 ng/ml murine rGM-CSF (Life Technologies), as described previously (6). These cells (capable of presenting protein Ag to primed CD4+ T cells) were derived from newborn BALB/c mouse epidermis as stable long-term cell lines (dendritic shape, CD45+/E-cadherin+ and B7-1+) (17). After differential cell count, cells were seeded into six-well plates. Nonadherent cells were removed 24 h later, and isolated macrophages were used for the in vitro-stimulation assays described below. Mouse XS52 (Langerhans) cells were cultivated in complete RPMI 1640 with 10% FCS (both from Life Technologies, Grand Island, NY) supplemented with 0.5 ng/ml murine rGM-CSF (Life Technologies), as described previously (6). These cells (capable of presenting protein Ag to primed CD4+ T cells) were derived from newborn BALB/c mouse epidermis as stable long-term cell lines (dendritic shape, CD45+/E-cadherin+ and B7-1+) (17). After differential cell count, cells were seeded into six-well plates. Nonadherent cells were removed 24 h later, and isolated macrophages were used for the in vitro-stimulation assays described below. Mouse XS52 (Langerhans) cells were cultivated in complete RPMI 1640 with 10% FCS (both from Life Technologies, Grand Island, NY) supplemented with 0.5 ng/ml murine rGM-CSF (Life Technologies), as described previously (6). These cells (capable of presenting protein Ag to primed CD4+ T cells) were derived from newborn BALB/c mouse epidermis as stable long-term cell lines (dendritic shape, CD45+/E-cadherin+ and B7-1+) (17). After differential cell count, cells were seeded into six-well plates. Nonadherent cells were removed 24 h later, and isolated macrophages were used for the in vitro-stimulation assays described below.
Langerhans cell staining and quantitation

Normal, uninjured corneas were harvested at 1 and 3 d.p.i. and placed in 0.02 mol/l EDTA (pH 7.2) for 1 h at 37°C for epithelial removal and Langerhans cell staining. Epithelial sheets were fixed in cacodylate-buffered formaldehyde for 20 min at 4°C, washed four times with cold 0.1 mol/l cacodylate buffer, and incubated in ADPase buffer, 2% lead nitrate, and ADP (5 mg/ml; Sigma) for 15 min at 37°C. Sheets were washed four times with trismaleate buffer (pH 7.2), developed for 5 min in a 1:10 ammonium sulfide solution, washed three times with buffer, mounted onto slides with glycerol, flattened, and coverslipped (18). Epithelial sheets were observed and photographed with a Zeiss Axioshot microscope with Axioim digital imaging (Carl Zeiss, Moring Instruments, Cincinnati, OH) at ×375, and the number of cells was quantitated/field (n = 12; field size = 270 μm²) by two of the authors who were blinded to the treatment. Staining of Langerhans cells for ADPase activity was done previously and was shown to provide data similar to immunostaining these cells with surface markers, such as DEC205 (18).

Statistics
Two-way ANOVA, followed by the Bonferroni posttest, was used for analysis of the data shown in Figs. 1, 2, and 7 (Prism 3.0; GraphPad Software, La Jolla, CA). One-way ANOVA, followed by the Tukey posttest, was used for the data shown in Figs. 4–6 (SPSS, Chicago, IL). For each test, differences were considered significant at p < 0.05, and data shown are mean ± SEM. Each experiment was performed at least twice, and combined data are shown, with the exception of Figs. 5 and 6, in which representational data from a single similar experiment are provided. Infection of mice, confocal microscopy, and Langerhans cell quantitative studies were done in a blinded fashion.

Results

PCR array and real-time RT-PCR
To determine the effects of VIP treatment on the regulation of TLRs in cornea during bacterial keratitis, mRNA levels were tested by RT-PCR (Table I) and 84 TLR-related genes were profiled by PCR array at 3 d.p.i. VIP treatment decreased the expression (>3-fold) of 21 genes compared with PBS-treated controls (Table II). RT-PCR was used to confirm the array data and to test TLR4 (8) and IRAK1 (19, 20), which are important in Pseudomonas-induced infections, but were not changed on the PCR array greater >3-fold (TLR4, −1.11 and IRAK1, −2.9), in contrast to the PCR array that were shown previously to be resistant to P. aeruginosa keratitis (7, 9) also were tested.

VIP significantly downregulated mRNA expression for TLR1 only at 7 d.p.i. (Fig. 1A, p < 0.01). However, compared with PBS treatment, VIP decreased mRNA levels of TLR4 only at 1 d postinfection (Fig. 1B, p < 0.001). VIP also downregulated mRNA levels of TLR6 at 5 and 7 d.p.i. (Fig. 1C, p < 0.05 and p < 0.001, respectively), for TLR8 only at 7 d.p.i. (Fig. 1D, p < 0.001), and for TLR9 only at 5 d.p.i. (Fig. 1E, p < 0.001). Compared with PBS treatment, VIP treatment also decreased mRNA expression of TLR adaptors IRAK1 at 1 and 5 d.p.i. (Fig. 1F, p < 0.001 and p < 0.001, respectively), but no differences were seen between the groups for IRAK2 (Fig. 1G). TRAF6, which interacts with IRAK1 (19), also was reduced at the mRNA level by VIP treatment compared with PBS treatment at 5 and 7 d.p.i. (Fig. 1H, p < 0.05 and p < 0.001, respectively). mRNA levels of Chuk, a downstream molecule of the IRAK-TRAF6-signaling pathway, which regulates NF-κB expression (19), also was downregulated by VIP at 1 and 7 d.p.i. (Fig. 1I, p < 0.01 and p < 0.001, respectively). In contrast, VIP treatment increased SIGIRR mRNA levels at 5 and 7 d.p.i. (Fig. 1J, p < 0.001 for both). However, levels in both groups decreased at 1 d.p.i. ST2 mRNA levels also were increased by VIP treatment at all times tested, but it was significant only at 7 d.p.i. (Fig. 1K, p < 0.05).

Table I. Nucleotide sequence of the primers used in PCR amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence Sense</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-actin</td>
<td>5′-GATTATCTGCTTGCTCGTCAGC-3′</td>
<td>Forward</td>
<td>9′-GATTATCTGCTTGCTCGTCAGC-3′</td>
</tr>
<tr>
<td>AC7</td>
<td>5′-TGGCTGTTGCTTCACTGATCTA-3′</td>
<td>Reverse</td>
<td>9′-TGGCTGTTGCTTCACTGATCTA-3′</td>
</tr>
<tr>
<td>Chuk</td>
<td>5′-GCTTTCCTTTGATTTGTGGTACGGC-3′</td>
<td>Forward</td>
<td>9′-GCTTTCCTTTGATTTGTGGTACGGC-3′</td>
</tr>
<tr>
<td>IRAK1</td>
<td>5′-CAGACCAAACCAAGATACATCATAC-3′</td>
<td>Forward</td>
<td>9′-CAGACCAAACCAAGATACATCATAC-3′</td>
</tr>
<tr>
<td>IRAK2</td>
<td>5′-GGGATGTACGGACAGATCTGGTCAACGC-3′</td>
<td>Forward</td>
<td>9′-GGGATGTACGGACAGATCTGGTCAACGC-3′</td>
</tr>
<tr>
<td>TLR1</td>
<td>5′-TCTCCTGCGAGTCTGTCATGCTG-3′</td>
<td>Forward</td>
<td>9′-TCTCCTGCGAGTCTGTCATGCTG-3′</td>
</tr>
<tr>
<td>TLR4</td>
<td>5′-CGGTTTACTTGTACCTGTCACGAC-3′</td>
<td>Forward</td>
<td>9′-CGGTTTACTTGTACCTGTCACGAC-3′</td>
</tr>
<tr>
<td>TLR6</td>
<td>5′-CAACAGTTTACTTGTACCTGTCACGAC-3′</td>
<td>Forward</td>
<td>9′-CAACAGTTTACTTGTACCTGTCACGAC-3′</td>
</tr>
<tr>
<td>TLR8</td>
<td>5′-CAACAGTTTACTTGTACCTGTCACGAC-3′</td>
<td>Forward</td>
<td>9′-CAACAGTTTACTTGTACCTGTCACGAC-3′</td>
</tr>
<tr>
<td>TLR9</td>
<td>5′-ACGGATGTTTACTTGTACCTGTCACGAC-3′</td>
<td>Forward</td>
<td>9′-ACGGATGTTTACTTGTACCTGTCACGAC-3′</td>
</tr>
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</table>

Table II. Selected TLRs from RT² Profiler PCR array

<table>
<thead>
<tr>
<th>Genes</th>
<th>VIP/PBS Treatment (Fold Difference)</th>
</tr>
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<tbody>
<tr>
<td>Casp8</td>
<td>−3.45</td>
</tr>
<tr>
<td>Chuk</td>
<td>−11.78</td>
</tr>
<tr>
<td>Csf2</td>
<td>−8.27</td>
</tr>
<tr>
<td>Csf3</td>
<td>−5.42</td>
</tr>
<tr>
<td>Fos</td>
<td>−18.74</td>
</tr>
<tr>
<td>Hmgbl</td>
<td>−13.72</td>
</tr>
<tr>
<td>Hspd1</td>
<td>−8.10</td>
</tr>
<tr>
<td>Il1a</td>
<td>−3.70</td>
</tr>
<tr>
<td>Il6</td>
<td>−3.01</td>
</tr>
<tr>
<td>Irak2</td>
<td>−5.27</td>
</tr>
<tr>
<td>Myd88</td>
<td>−3.58</td>
</tr>
<tr>
<td>Ripk2</td>
<td>−3.31</td>
</tr>
<tr>
<td>Tcam2</td>
<td>−3.05</td>
</tr>
<tr>
<td>Tlr1</td>
<td>−11.78</td>
</tr>
<tr>
<td>Tlr6</td>
<td>−6.36</td>
</tr>
<tr>
<td>Tlr8</td>
<td>−7.77</td>
</tr>
<tr>
<td>Tlr9</td>
<td>−4.40</td>
</tr>
<tr>
<td>Tnfaip3</td>
<td>−14.20</td>
</tr>
<tr>
<td>Tnfrsf1a</td>
<td>−3.24</td>
</tr>
<tr>
<td>Traf6</td>
<td>−7.61</td>
</tr>
<tr>
<td>Ube2n</td>
<td>−5.49</td>
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</table>

ELISA and Western blot

ELISA and/or Western blot was used to selectively confirm the mRNA data for total IKKα (Chuk), p-IKKα, ST2, and SIGIRR. For total IKKα (Fig. 2A), there was no significant difference between PBS and VIP treatment at any time tested. However, compared with PBS treatment, VIP treatment slightly downregulated p-IKKα protein expression only at 7 d.p.i. (Fig. 2B, p < 0.05). In contrast, protein levels of ST2 (Fig. 2C) were significantly enhanced by VIP treatment compared with PBS treatment at 7 d.p.i. (p < 0.001), not different at 1 d.p.i., and not detectable in either normal sample. SIGIRR protein (Fig. 2D, 2E) was constitutively expressed at similar levels in normal PBS- and VIP-treated mouse cornea. At 1 d.p.i., greater levels of SIGIRR (p <
0.001) were observed after VIP treatment, with no difference between the two groups at 5 d p.i.

**TLR4 immunostaining**

Immunohistochemistry was used to spatially localize TLR4 (red staining) in the cornea of VIP- versus PBS-treated B6 mice at 1 and 5 d p.i. At 1 d p.i., reduced TLR4 staining was seen after VIP treatment (Fig. 3B), compared with PBS treatment (Fig. 3A), in corneal epithelium and stroma. At 5 d p.i., compared with PBS treatment, VIP treatment further reduced TLR4 epithelial and stromal staining (compare Fig. 3D with 3C). Controls in which primary Ab was substituted with species-specific IgG were negative after PBS (Fig. 3E) or VIP (Fig. 3F) treatment at 1 or 5 d p.i. (data not shown).

**Silencing AC7**

To determine whether the anti-inflammatory effect of VIP on TLRs was cAMP dependent, siRNA was used in vivo to knock down AC7; it was selected because, among the 10 mammalian adenyl cyclase isotypes, AC7 is highly expressed in the immune system (21). When tested at 5 d p.i., RT-PCR confirmed that silencing AC7 versus scrambled control treatment significantly decreased AC7 mRNA levels (Fig. 4A, \( p < 0.01 \)) and that silencing plus VIP treatment did not reverse the effect. Furthermore, at this time, silencing AC7 increased mRNA levels of TLR1 (Fig. 4B), whereas TRAF6 (Fig. 4C) and ST2 (Fig. 4D) were decreased (\( p < 0.05 \), \( p = 0.001 \), and \( p < 0.01 \), respectively). Silencing plus VIP injection did not change the effect, indicating that VIP regulation of these TLRs is cAMP independent. mRNA levels of proinflammatory TLRs, including Chuk (Fig. 4E), IRAK1 (Fig. 4F), TLR4 (Fig. 4G), TLR9 (Fig. 4H), and IRAK2 (Fig. 4I), were increased after silencing compared with scrambled control treatment (\( p < 0.01 \), \( p < 0.01 \), \( p = 0.001 \), \( p = 0.01 \), and \( p < 0.05 \), respectively); levels of the anti-inflammatory TLR SIGIRR were decreased (Fig. 4J, \( p = 0.001 \)). Silencing plus VIP injection changed the effect (Chuk: \( p < 0.001 \); IRAK1: \( p < 0.05 \); TLR4: \( p = 0.001 \); TLR9: \( p < 0.01 \); IRAK2: \( p < 0.05 \); SIGIRR: \( p < 0.05 \)), indicating that VIP regulation of these molecules is cAMP independent. No significant changes in mRNA levels of TLR6 (Fig. 4K) were seen with AC7 siRNA treatment, but VIP plus silencing decreased mRNA levels of TLR6 (\( p < 0.05 \)), suggesting the possibility of its regulation by another adenyl cyclase isoform (22). No effects were seen for TLR8 (Fig. 4L), suggesting that it is independent of AC7 and VIP regulation.

**In vitro studies: macrophages**

To provide some indication of which cells may be involved in VIP regulation of TLRs, peritoneal exudate macrophages and XS52 (Langerhans) cells were tested in an in vitro assay. Compared with media control, ultrapure LPS (only activates the TLR4 pathway) significantly increased TLR1 (Fig. 5A, \( p < 0.001 \)), TLR4 (Fig. 5B, \( p < 0.01 \)), MyD88 (Fig. 5C, \( p < 0.001 \)), IRAK1 (Fig. 5D, \( p < 0.01 \)), and TRAF6 (Fig. 5E, \( p < 0.001 \)) mRNA, with no effect on ST2 mRNA (Fig. 5G). However, SIGIRR mRNA (Fig. 5F, \( p < 0.001 \)) was decreased after LPS stimulation compared with media control stimulation. Compared with LPS alone, LPS plus VIP significantly decreased mRNA levels of TLR1 (Fig. 5A, \( p < 0.01 \)), TLR4 (Fig. 5B, \( p < 0.01 \)), MyD88 (Fig. 5C, \( p < 0.001 \)), IRAK1 (Fig. 5D, \( p < 0.01 \)), and TRAF6 (Fig. 5E, \( p < 0.001 \)) mRNA, with no effect on ST2 mRNA (Fig. 5G). However, SIGIRR mRNA (Fig. 5F, \( p < 0.001 \)) was decreased after LPS stimulation compared with media control stimulation. Compared with LPS alone, LPS plus VIP significantly decreased mRNA levels of TLR1 (Fig. 5A, \( p < 0.05 \)), TLR4 (Fig. 5B, \( p < 0.01 \)), MyD88 (Fig. 5C, \( p < 0.001 \)), IRAK1 (Fig. 5D, \( p < 0.01 \)), and TRAF6 (Fig. 5E, \( p < 0.001 \)) but increased ST2 (Fig. 5G, \( p < 0.05 \)); no difference was seen for SIGIRR (Fig. 5F). Cultured cells treated with VIP alone showed no significant difference compared with media-treated controls for any of the molecules tested.
**In vitro studies: XS52 (Langerhans) cells**

LPS versus media control treatment of XS52 (Langerhans) cells significantly increased mRNA levels of TLR1 (Fig. 6A, \(p < 0.001\)), TLR4 (Fig. 6B, \(p < 0.001\)), MyD88 (Fig. 6C, \(p < 0.001\)), and TRAF6 (Fig. 6E, \(p < 0.001\)) but decreased ST2 (Fig. 6G, \(p < 0.001\)). IRAK1 (Fig. 6D) and SIGIRR (Fig. 6F) mRNA levels were unchanged after LPS stimulation compared with media control stimulation. Compared with LPS alone, LPS plus VIP significantly decreased mRNA levels of MyD88 (Fig. 6C, \(p = 0.05\)) and TRAF6 (Fig. 6E, \(p < 0.001\)) but increased SIGIRR mRNA expression (Fig. 6F, \(p < 0.001\)). Compared with LPS alone, LPS plus VIP did not significantly change mRNA levels of TLR1 (Fig. 6A), TLR4 (Fig. 6B), IRAK1 (Fig. 6D), or ST2 (Fig. 6G). Cultured cells treated with VIP alone did not differ from media-treated controls for any of the molecules tested.

**Langerhans cell detection and quantitation**

In normal, uninfected eyes, dendritic-shaped Langerhans cells were detected in the peripheral cornea/conjunctiva of PBS-treated (Fig. 7A) and VIP-treated (Fig. 7B) mice; quantitatively, they were slightly decreased (not significantly) after VIP treatment (Fig. 7G). At 1 d p.i., rounded, motile Langerhans cells appeared to be decreased in the conjunctiva and peripheral cornea of VIP-treated mice (Fig. 7D) compared with PBS-treated mice (Fig. 7C), and quantitation showed that the difference was significant (\(p < 0.05\), Fig. 7G). By 3 d p.i., the cell number appeared similar in PBS-treated (Fig. 7E) and VIP-treated (Fig. 7F) mice; when quantitated, no difference was seen between the two groups (Fig. 7G).

**Discussion**

VIP regulates a wide variety of immune reactions; among its numerous anti-inflammatory functions, it participates in the maintenance of immune homeostasis (23, 24). Previously, this laboratory showed that, in the cornea of B6 mice infected with \textit{P. aeruginosa}, VIP treatment regulates cytokine production through upregulation of VIP receptor 1 on inflammatory cells, leading to less stromal destruction and prevention of corneal perforation (3). Recent work provided further evidence that similar VIP treatment of B6 mice also has an indirect effect: it modulates growth factors, angiogenic molecules, and defensins in the infected cornea, which, in turn, promote healing (4). In addition, other studies showed that VIP downregulates proinflammatory TLRs and re-
lated molecules in several nonocular disease models (12). TLRs, important in innate immunity, are critical for recognition of conserved pathogen-associated molecular patterns, through TLR/IL-1R expressed on the surface of various cell types, to trigger cytokine/chemokine production (25, 26). In this regard, previous studies from this laboratory and other investigators showed that TLR1 (27), TLR3 (28), TLR4 (8), and TLR9 (6) play important roles in the pathogenesis of experimentally induced keratitis. However, VIP regulation of TLRs during these diseases has not been characterized. Thus, the current study, using a PCR array to

**FIGURE 4.** (A) When tested at 5 d p.i., RT-PCR confirmed that, compared with scrambled control treatment, silencing AC7 significantly decreased AC7 mRNA levels; silencing plus VIP treatment did not reverse the effect. When tested at 5 d p.i., silencing AC7 increased mRNA levels of TLR1 (B), whereas TRAF6 (C) and ST2 (D) were decreased. Silencing plus VIP injection did not change the effect of silencing for any of the above-mentioned molecules. In contrast, compared with scrambled control treatment, Chuk (E), IRAK1 (F), TLR4 (G), TLR9 (H), and IRAK2 (I) mRNA levels were increased after silencing AC7. Silencing plus VIP injection decreased mRNA expression of each of these molecules significantly. (J) SIGIRR mRNA levels were decreased after silencing AC7; however, silencing plus VIP treatment resulted in increased mRNA levels over silencing alone. No significant changes in mRNA levels of TLR6 (K) or TLR8 (L) were seen with AC7 siRNA treatment; however, silencing plus VIP treatment resulted in reduced mRNA levels of TLR6.
profile 84 TLR-related genes, provided an overview of the ability of VIP to provide master regulation of TLRs and signaling-pathway molecules in bacterial keratitis. Twenty-one genes were downregulated \( \geq 3 \)-fold by VIP, and several of them were selected for further confirmation by RT-PCR. The results indicated that VIP reduced proinflammatory TLRs and related molecules (TLR1, TLR4, TLR6, TLR8, TLR9, IRAK1, TRAF6, and Chuk), which agreed well with the array data. The array did not contain two anti-inflammatory TLRs important in promoting the resistance response of BALB/c mice to \( P. \) aeruginosa: SIGIRR (9) and ST2 (7); however, when tested by RT-PCR, these genes, in general, were upregulated by VIP treatment. Immunohistochemistry data further confirmed that VIP decreased TLR4 protein expression in both epithelial and stromal layers of the cornea. These results also are consistent with previous studies showing that VIP inhibits or even reverses TLR2 (29), TLR3 (30), and TLR4 (31, 32) expression or their stimulated signaling pathways in other nonocular disease models.

On ligand binding, TLRs recruit adaptor molecules, such as IRAK and TRAF6, to their intracellular signaling domain, leading to the activation of several kinases, as well as the transcription factor, NF-\( \kappa \)B, thereby directly upregulating immune-response genes (33). In the current study, VIP treatment in vivo downregulated IRAK1 and TRAF6 mRNA levels, which is consistent with other studies using synovial fibroblasts isolated from patients with rheumatoid arthritis: compared with LPS alone, LPS stimulation plus VIP treatment inhibited IRAK1 and TRAF6 mRNA and phosphorylated protein expression (31), thereby acting as a negative regulator of TLR4 signaling.

Another important signaling complex, the IKK complex, is found downstream of IRAK–TRAF6 signaling and is composed of IKK\( \alpha \), IKK\( \beta \), and IKK\( \gamma \) (34). The current in vivo study showed that VIP inhibited Chuk/IKK\( \alpha \) mRNA expression, but only modestly decreased its activity, compatible with previous in vitro studies showing that VIP directly inhibited IKK\( \alpha \) activity in human monocytes (35) and microglia cells (36). In microglial cells,
VIP inhibition of IKKα reduced Aβ-induced neurodegeneration by indirectly inhibiting the production of numerous inflammatory and neurotoxic molecules (36). Furthermore, loss or decreased IKKα also was suggested to negatively regulate NF-κB and subsequent gene expression by inhibiting its translocation to the nucleus and, thus, downregulating inflammation (37).

In contrast to other TLRs, SIGIRR (38) and ST2 (39), members of the TLR–IL-1R superfamily, act as negative regulators of IL-1 and LPS signaling. Previous studies from this laboratory found that SIGIRR (9) and ST2 (7) promote natural resistance of BALB/c versus B6 mice against *P. aeruginosa* keratitis and that Ab depletion of SIGIRR increased disease in BALB/c mice. However, it was not tested whether VIP treatment regulates expression of either molecule in susceptible B6 mice. The current study showed that in vivo treatment of infected B6 mice with VIP increased (but not significantly) SIGIRR mRNA levels over PBS-treated controls in normal uninfected eyes; at 1 d p.i., the levels decreased in both groups but remained higher with VIP treatment. In previous work from this laboratory (9) comparing the expression of SIGIRR in BALB/c versus B6 mice after *P. aeruginosa* infection, we also observed a decrease in mRNA expression in both groups at 12 h p.i. (and in B6 mice at 1 d p.i., similar to the current study), which increased in both mouse strains later in disease. SIGIRR protein levels decreased in the two mouse strains at 1 d p.i. and increased later in disease, most significantly in BALB/c mice (9). After in vivo LPS challenge, similar consumption of SIGIRR mRNA (protein not tested) was shown by Wald et al. (38); they suggested that this may reflect its functional involvement in decreasing inflammation. We cited this possibility as well but did not prove it functionally (9). The current study suggests that a decrease in SIGIRR protein is not required for functionality, because we did not observe SIGIRR consumption at the protein level in B6 mice treated with VIP; rather, protein levels were increased at 1 d p.i. These data are supported by other studies in which overexpression of SIGIRR (adenoviral vector expressing murine SIGIRR) was shown to ameliorate LPS-induced acute lung injury in mice (40);
ADPase+ Langerhans cells appeared similar in number after VIP (mal, uninfected eye (conjunctiva/peripheral cornea), dendritic-shaped treatment did not change the effects of silencing alone. These data are consistent with in vitro studies showing that VIP can function to regulate molecules, such as NF-kB, in a cAMP-independent manner (35). Unexpectedly, compared with scrambled control treatment, AC7 siRNA did not induce significant changes in TLR6; however, when silencing was combined with VIP treatment, TLR6 levels were decreased, suggesting the possibility that other adenyl cyclase isoforms (22) participate in TLR regulation by VIP.

Previous studies from this laboratory showed the importance of both macrophages (44) and Langerhans cells (18) in Pseudomonas keratitis. In this regard, VIP functions as a macrophage-deactivating factor and was shown to downregulate proinflammatory cytokine expression (IL-1β and MIP-2) after LPS (not ultrapure) stimulation in murine peritoneal macrophages (3). It also downregulates proinflammatory cytokine production in epidermal Langerhans cells (45), but its role in TLR regulation has not been characterized in these types of cells. Data from the current study showed that, in peritoneal macrophages and XS52 (Langerhans) cells, ultrapure LPS stimulation upregulated TLR1 and TLR4, but VIP plus LPS downregulated both TLRs only in macrophages. These data agree with those of other investigators who showed that, compared with LPS alone, stimulation of murine macrophages with LPS (not ultrapure) and VIP resulted in less TLR4 expression at the transcriptional level (32); however, no similar data are available for XS52 (Langerhans) cells or why they respond dissimilarly to macrophages. With regard to TLR1, because other investigators showed that cytokines, such as IFN-γ, can increase TLR1 expression in murine monocytes (46), we similarly tested for IFN-γ after ultrapure LPS stimulation of both cell types and found elevated levels, which also were decreased by treatment with LPS plus VIP (data not shown). With similar treatment, TLR4 downstream-signaling molecules, including MyD88, IRAK1, and TRAF6, also were downregulated in macrophages, whereas only MyD88 and TRAF6 were downregulated in XS52 (Langerhans) cells. The current study also showed that, compared with LPS treatment alone, LPS stimulation plus VIP increased ST2 mRNA expression in macrophages, but only SIGIRR was increased in XS52 (Langerhans) cells. These data suggest that VIP disparately regulates TLR on these cells and may reflect the kinetics of pathogen encounter (ocular surface versus stroma) p.i. in the eye. Despite the differential regulation of these cells, it is clear that VIP plus LPS reduces proinflammatory TLR expression while promoting anti-inflammatory TLR expression. VIP treatment in vivo also reduced Langerhans cell centripetal migration and, thus, decreased the number of cells in the infected cornea at 1 d p.i. Other investigators also showed that VIP treatment inhibits mouse dendritic cell migration to adjacent lymph nodes (47), limiting infection. All of this is consistent with the anti-inflammatory effects of VIP and with previous studies from this laboratory that showed that the presence of Langerhans cells in the cornea (before infection) exacerbates subsequent disease (18).

Overall, this study provides evidence that in vivo treatment with VIP downregulates proinflammatory TLRs and increases anti-inflammatory TLRs and related signaling molecule expression via cAMP-dependent and -independent pathways, as well as regulates Langerhans cell number and migration in the infected cornea. In vitro studies using ultrapure LPS, which only activates the TLR4

FIGURE 7. Staining and quantitation of Langerhans cells. In the normal, uninfected eye (conjunctiva/peripheral cornea), dendritic-shaped ADPase+ Langerhans cells appeared similar in number after VIP (B) or PBS (A) treatment. At 1 d.p.i., rounded ADPase+ cells in the peripheral cornea appeared to be decreased in number after VIP treatment (D) compared with PBS treatment (C). At 3 d.p.i., cells appeared qualitatively similar after PBS (E) or VIP (F) treatment. Insets show examples of cells. (G) Quantitation confirmed these findings. (A–F) Original magnification ×375; field size, 270 μm².

its overexpression in human macrophages and dendritic cells downregulated TLR-induced cytokines, whereas its knock down increased cytokine production following TLR stimulation (41). In contrast, ST2 protein is increased later (7 d.p.i.) in disease, suggesting that, after VIP treatment, the molecules could act disparate in time to ameliorate disease or participate in corneal healing.

Previous studies on the immunomodulatory properties of VIP led to the identification of two pathways: one cAMP dependent and the other cAMP independent (11). However, whether VIP regulation of TLRs is cAMP dependent or independent had not been tested. To address this issue, we silenced AC7 using siRNA, because among the 10 adenyl cyclases it is highly expressed in the immune system (21). Data showed that VIP regulation of TLR1, TRAF6, and ST2 was cAMP dependent, because silencing plus VIP treatment did not change the effects of silencing alone. These data agree with previous, similarly designed in vitro studies in which a PKA inhibitor was used to block upstream cAMP pathways (42, 43). Data from those studies concluded that VIP regulation of both a proinflammatory cytokine TNF-α (42) and NO (43) are cAMP dependent. In contrast, the current study showed that regulation of Chuk, IRAK1, IRAK2, TLR4, TLR9, and SIGIRR are cAMP independent, because silencing plus VIP treatment changed the effects of silencing AC7 alone. These data are consistent with in vitro studies showing that VIP can function to regulate molecules, such as NF-kB, in a cAMP-independent manner (35). Unexpectedly, compared with scrambled control treatment, AC7 siRNA did not induce significant changes in TLR6; however, when silencing was combined with VIP treatment, TLR6 levels were decreased, suggesting the possibility that other adenyl cyclase isoforms (22) participate in TLR regulation by VIP.
pathway, confirm the in vivo data and provide evidence that TLR expression in macrophages and X552 (Langerhans) cells is differentially regulated by VIP to improve disease outcome.

**Disclosures**

The authors have no financial conflicts of interest.

**References**