REVIEW

Role of protease-activated receptors 2 (PAR2) in ocular infections and inflammation

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Protease-activated receptors (PARs) belong to a unique family of G protein-coupled receptors (GPCRs) that are cleaved at an activation site within the N-terminal exodomain by a variety of proteinases, essentially of the serine (Ser) proteinase family. After cleavage, the new N-terminal sequence functions as a tethered ligand, which binds intramolecularly to activate the receptor and initiate signaling. Cell signals induced through the activation of PARs appear to play a significant role in innate and adoptive immune responses of the cornea, which is constantly exposed to proteinases under physiological or pathophysiological conditions. Activation of PARs interferes with all aspects of the corneal physiology such as barrier function, transports, innate and adoptive immune responses, and functions of corneal nerves. It is not known whether the proteinase released from the microorganism can activate PARs and triggers the inflammatory responses. The role of PAR2 expressed by the corneal epithelial cells and activation by serine protease released from microorganism is discussed here. Recent evidences suggest that activation of PAR2, by the serine proteinases, play an important role in innate and inflammatory responses of the corneal infection.

Keywords: Protease-activated receptors; PAR1, PAR2; aPA; *Acanthamoeba*; Keratitis; Inflammation; Corneal epithelial cells

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Introduction

Protease-activated receptors (PARs) belong to a distinctive family of the seven transmembrane G protein-coupled receptors (GPCRs) that are cleaved at an activation site within the N-terminal exodomain by the serine (Ser) proteinase (or serine endopeptidases) family [1-5]. The cleaved new N-terminal sequence acts as a tethered ligand which interacts with the receptor within extracellular loop-2 to activate the receptor and initiates signaling [1-5]. To date, four members of the PARs family have been recognized: PAR1-PAR4 [1-5]. PAR1, PAR3, and PAR4 can be stimulated by thrombin and PAR2 can be activated by trypsin, tissue factor/factor VIIa/factor Xa, neutrophil proteinase 3, mast cell tryptase, and membrane-tethered serine proteinase-1 [1-5]. Activation mechanism of PAR1 through PAR4 by serine proteases, coupling to intracellular signaling cascades, and their important physiological and pathophysiological functions in chemotaxis, inflammation, platelet aggregation, cell

growth and division, endothelial barrier dysfunction, neuronal cell survival, gastrointestinal, cardiovascular responses, regulation of skin function, thrombosis and vascular remodeling, genetic disorders, cancer, inflammatory diseases, and neurological disorders, have been extensively reviewed [6]. Lang *et al*. [7] first time demonstrated the PAR1 and PAR2 expression and their functional activity in proinflammatory cytokines secretion in human corneal epithelial (HCE) cells. Expression of PAR1 and PAR2 in HCE cells is a first step to investigate corneal biology by targeting PARs signaling in corneal inflammatory diseases (*Acanthamoeba* keratitis, bacterial keratitis, and fungal keratitis). Recently, we have demonstrated that *Acanthamoeba castellanii* trophozoites' secreted serine protease, *Acanthamoeba* plasminogen activator (aPA) [8], induces proinflammatory cytokine IL-8 by the activation of PAR2 signaling in HCE cells [9]. This signaling is the first illustration of PAR2 activation by microbial serine proteinase in corneal epithelial cells that triggers the inflammatory response; also this might be a novel mechanistic approach of aPA-induced pathogenesis of *Acanthamoeba* keratitis. This review briefly focuses the role of *Acanthamoeba* trophozoites' secreted serine protease, aPA, in the production of inflammatory mediator by the activation of PAR2 pathway in HCE cells.

Acanthamoeba Keratitis and Pathogenesis

Acanthamoeba keratitis is known as a rare but potentially sight-threatening and painful infectious corneal disease worldwide [10, 11]. It is caused by the ubiquitous free-living pathogenic species of *Acanthamoeba* [10, 12-15]. *Acanthamoeba* can be found commonly in soil, air, water, cooling towers, sewage systems, and heating, ventilating, air conditioning (HVAC) systems [16, 17]. Since the first case of *Acanthamoeba* keratitis reported by Naginton *et al*. [18] in United Kingdom and shortly thereafter by Jones *et al*. [19] in United States, the incidence of this disease has been augmented with increasing the number of contact lens (CL) wearers [20] (Table). Recently, the preliminary investigations of Centers for Disease Control and Prevention (CDC) of a national *Acanthamoeba* keratitis outbreak of 105 patients from 30 states of United States, 2005-2007, showed that enrolled patients had percent corneal symptoms of pain 74, redness 74, sensitivity to light 72, sensation of foreign body 68, increased tearing 56, blurred vision 54, and discharge from eye 19[33]. Thus, severe uneven ocular pain to the clinical signs has long been known as one hallmark of *Acanthamoeba* keratitis.

Many studies have been conducted on the pathogenesis of *Acanthamoeba* keratitis [9, 34-62]; however, biology and pathogenic mechanisms of *Acanthamoeba* are still beginning to emerge. The national outbreak of *Acanthamoeba* keratitis reported that *Acanthamoeba* primarily affects contact lens users [33]; however, other risk factors and environmental exposures associated with *Acanthamoeba* keratitis should be considered. Moreover, an increased incidence of *Acanthamoeba* keratitis is known as an important cause of amoebic keratitis in non-contact lens wearers. A three-year clinical study conducted between 1999 to 2002 in South India diagnosed thirty three patients with positive *Acanthamoeba* infection (approximate 1%) out of 3183 enrolled patients with corneal infections [63]. They observed that twenty six out of thirty three patients diagnosed with *Acanthamoeba* infection were peasants from countryside areas and got corneal injury from mud [63]. Treatments of *Acanthamoeba* keratitis exist with hourly applications of brolene, polyhexamethylene biguanide (PHMB), and chlorhexidine for several weeks. Even with such therapies, *Acanthamoeba* species can cause severe damage to the corneal epithelium and stroma, resulting in the need for corneal grafting [12]. Topical steroids are often used to control corneal inflammation and uveitis or is administered after surgery, to prevent the rejection of corneal transplant; however, in vitro exposure of *Acanthamoeba* cysts to dexamethasone increased trophozoite's number through excystment and growth [64]. Thus, reactivation of *Acanthamoeba* keratitis may occur after corneal grafting if residual *Acanthamoeba* cysts in the host peripheral cornea are exposed to steroids after surgery. Although the biology of *Acanthamoeba* is not fully explored, *Acanthamoeba* trophozoites' secreted serine proteases, mannose-induced protein (MIP-133) by contact-dependent mechanism [14, 15, 52, 65] and *Acanthamoeba* plasminogen activator (aPA) by contact-independent mechanism [8]. (a) Contact-dependent mechanism of the *Acanthamoeba* keratitis pathogenesis begins when *Acanthamoeba* trophozoites interact to the corneal surface by mannose binding protein (MBP) [11, 56]. This interaction releases the MIP-133 from *A. castellanii* trophozoites [15], which interacts with membrane phospholipids on corneal epithelium and triggers arachidonic acid production, pro-inflammatory cytokines (IL-8, IL-6, IL-1β, IFNγ, and CXCL2), apoptosis, and polymorphonuclear neutrophils (PMNs) infiltration that leads to corneal lesion by the activation of cytosolic phospholipase $A_{2\alpha}$ ($cPLA_{2\alpha}$) pathway; $cPLA_{2\alpha}$ inhibitors (AACOCF3, CAY10650, and MAFP) therapeutically in vitro and in vivo mitigate inflammation and resolved the *Acanthamoeba* keratitis [60, 61]. (b) Contact-independent mechanism of the *Acanthamoeba* keratitis pathogenesis involves in the secretion of the *Acanthamoeba* plasminogen activator (aPA) which has been characterized a serine protease [8]. *Acanthamoeba* plasminogen activator recently has been revealed to be involved in stimulation of pro-inflammatory cytokine in HCE cells via PAR2 pathway [9].

Table 1. Worldwide incidence of *Acanthamoeba* keratitis

Geographic Area	Years of Data Collection	*Acanthamoeba* keratitis cases (For depth review refer to reference[20])
USA	1981-1984	1.36 per million contact lens wearer (CLW)[21]
	1985-1987	1.65-2.01 per million CLW[22]
England	1992-1996	1.4 per million individual[23] 19.5 per million CLW[23]
	1997-1998	1.26 per million individual[24] 31 per million CLW[25]
	1998-1999	1.13 per million individual[24] 27 per million CLW[25]
Japan	1988-1993	89.7% CLW[26]
New-Zealand	1990-1996	7 cases[27]
Australia	1984-1994	130 cases[27,28]
Scotland	1994-1995	149 per million CLW[29]
Hong Kong	1997-1998	33 per million CLW[30]
Africa	1990-1995	22 cases[31]
Netherlands	1996	3.06 per million CLW[32]

Acanthamoeba Plasminogen Activator (aPA)

Pathogenic species of *Acanthamoeba* elaborate proteolytic enzymes, especially plasminogen activator (aPA) in the PYG medium [8, 65] (Figure 1A). aPA characterized as a 40 kDa protease which causes a single band of lysis on fibrinogen-agarose zymographs [8]. Activity of aPA is completely blocked by the diisopropylfluorophosphate (DFP or DIFP) treatment [8], phenylmethanesulfonyl fluoride (PMSF), and 1, 10-phenanthroline (1, 10-ph) [65], indicating that it is a serine protease (Figure 1B); however, aPA activity is not blocked by amiloride, which is a robust inhibitor of urokinase-type plasminogen activator (uPA) [8]. Additionally, the activity of this enzyme is not blocked by plasminogen activator inhibitor-1 (PAI-1) which is the primary physiological blocker of both uPA and tissue-type plasminogen activator (tPA) [8] (Figure 2). aPA does not cross-react with antibodies specific for uPA or tPA[8]. aPA stimulates plasminogen from a number of mammalian species, including human, cow, and pig [8].

Activation of PARs and Role of Pathogen-Derived Proteases in Inflammatory Response by PAR2 Pathway

Proteases cleave PARs' molecules at a specific activation site on the extracellular N-terminal domain. The released new N-terminal domain of the receptor acts as a tethered ligand which binds to the second extracellular loop of the receptor and triggers intracellular signaling [5]. Stimulation of PAR2 leads to a variety of responses such as activating the secretion of cytokines, prostanoids, and metalloproteases which involves to play critical pathophysiological roles in the inflammatory response in joints, skin and kidney, allergic inflammation of airways, and leukocyte migration [2]. Importantly, *P. aeruginosa* secrets elastase B (EPa) which potentially silences the role of PAR2 in the respiratory tract and alters the host's innate defense mechanisms and respiratory tract functions, thus EPa by PAR2 signaling contributes a significant role in the pathogenesis of a disease like cystic fibrosis [66]. Other microorganisms such as *Serratia marcescens* (gram-negative enteric bacterium), and *Porphyromonas gingivalis* and *Aggregatibacter actinomycetemcomitans* (gram-negative bacterium causes periodontitis), activate PAR2 and induce proinflammatory cytokines secretion [67-69] and stimulate inflammatory responses in vitro and in vivo [70, 71].

Role of Plasminogen Activator System in Corneal Biology

Fibrinolytic activity is caused by plasminogen activators. Pandolfi and Astrup [72] demonstrated histochemically that corneal epithelial cells of human and several mammals contain plasminogen activator (PA) which appears firmly bound to cellular structures so that it is only available for interaction when cells have been

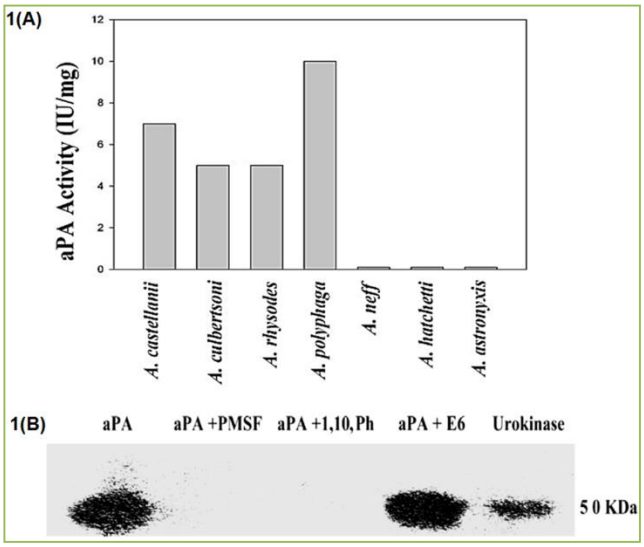

Figure 1. (A) Levels of aPA activity produced by pathogenic (*A. castellanii*, *A. polyphaga*, *A. rhysodes*, and *A. culbertsoni*) and nonpathogenic (*A. hatchetti*, *A. astronyxis*, and *A. castellanii neff*) strains of *Acanthamoeba*. aPA activity of the purified aPA (1 µg) from *Acanthamoeba* trophozoites was measured by radial diffusion in the fibrin agarose (1%) clot. Clots lacking plasminogen were included in all experiments for plasminogen-independent fibrinolysis. aPA activity was expressed in international units per milligram relative to human tPA standard. **(B)** Fibrin plasminogen zymography of purified aPA from *A. castellanii*. Purified protein (10 µg) was electrophoresed in 4% to 15% SDS-PAGE-ready gels and then was washed three times in PBS to remove SDS. Gels were then overlaid onto fibrinogen containing plasminogen in agarose clots for 2 hours. Lane 1: untreated aPA protein; lanes 2 and 3: pretreatment with serine proteinase inhibitors 1 mM PMSF and 1 mM 1, 10-phenanthroline (1,10-ph); lane 4: pretreatment with 10 µM cysteine proteinase inhibitor E6; lane 5: human standard urokinase (UK). kDa, molecular mass standard. Reprinted with permission [65]. Copyright held by The Association for Research in Vision and Ophthalmology (ARVO).

injured or have undergone degeneration. Furthermore, Pandolfi and Lantz [73] isolated a 55 kDa keratokinase (KK), a fibrinolytic enzyme, from in vitro cultures of cornea and characterized the fibrinolysis activity of KK similar to uPA and tPA. They investigated that KK exhibits a biphasic activity to increase the concentration of epsilon aminocaproic acid (EACA) and is scarcely adhered to fibrin during clotting. Berman *et al.* [74] observed the important roles of the PA-plasmin system in corneal ulceration. They demonstrated that epithelium, keratocytes, and PMNs of ulcerating corneas are all capable of releasing PA which can initiate plasmin-dependent processes for mediating corneal ulceration.

In addition, Wang *et al.* [75] observed that normal corneal epithelial cells' cultures and non-ulcerating corneas have PA molecular weight (MW) species of 72 kDa and 46 kDa, and ulcer corneas, species of 72 kDa, 46kDa, and 35 kDa MW. They showed that rabbit epithelial cells, fibroblasts, and ulcer corneas release uPA-like PA; and human cornea extracts and tears also have PA immunoreactivity with anti-uPA antibodies. Noteworthy, the tPA expressed by the corneal epithelium, endothelium, stroma, and the lens, as well as the aqueous and vitreous humors, dog, calf, and monkey eyes. It suggests that tPA plays an important role in non-fibrinolytic processes, mitosis and cell migration, as well as in the destructive remodeling of the extracellular matrix such as the turnover of collagen and glycoproteins in the corneal stroma and vitreous body [76]. However, Tervo *et al.* [77] observed a robust immunoreaction for uPA, a weaker reaction for PAI-1, and a very weak tPA-like immunoreaction in the anterior stroma of the wounded cornea. Mirshahi *et al.* [78] support the findings of Tervo *et al.* [77] and showed that bovine corneal epithelial cells secrete tPA, uPA, and their inhibitor (PAI-1). This study first time demonstrated the role of PAI-1 in the regulation of active protease which is plasmin, may be involved in the destructive processes or tissue remodeling such as the normal turnover of stromal collagens and glycoproteins.

Berk *et al* .[79] observed the overexpression of PA system (uPA, the receptor of uPA (uPAR), tPA, and both inhibitors, PAI-1 and PAI-2) in mice corneas during the inflammatory response intracorneally infected with *P. aeruginosa*. Urokinase PA activity also investigated in the epithelial cell migration during corneal epithelial wound healing, which was significantly inhibited by antibodies to uPA. Thus, the activity of uPA in corneal epithelial wound healing may be facilitated in part by the expression of uPAR on epithelial cell surface [80]. Recently, Sugioka *et al.* [81] investigated the role of uPA in a murine model of lipopolysaccharide (LPS)-induced corneal inflammation. Their results suggest that uPA promotes LPS-induced leukocyte infiltration in cornea, and that uPA is an important component in LPS-induced corneal inflammatory responses. Thus, PA released from all layers of cornea may play an important role in corneal biology, as for example, in the regulation of vessel permeability, angiogenesis, and in the initiation of PMNs chemotaxis.

Interestingly, PARs are specific targets to initiate protease mediated inflammation [1-3, 82]. Zieske and Bukusoglu [83] demonstrated that protease inhibitors inhibit corneal epithelial migration. Moreover, Lang *et al.* [7] and Nickel *et al.* [84] demonstrated that human corneal and conjunctival epithelial cells express functional PAR1 and PAR2. Activation of PAR1 and PAR2 on HCE cells by thrombin and trypsin resulted in the production of proinflammatory cytokines such as IL-6, IL-8, and TNFα [7]. In this regard, PAR1 and PAR2 may be important in inflammatory eye diseases.

The Role of aPA in Recognition of PAR2, But not

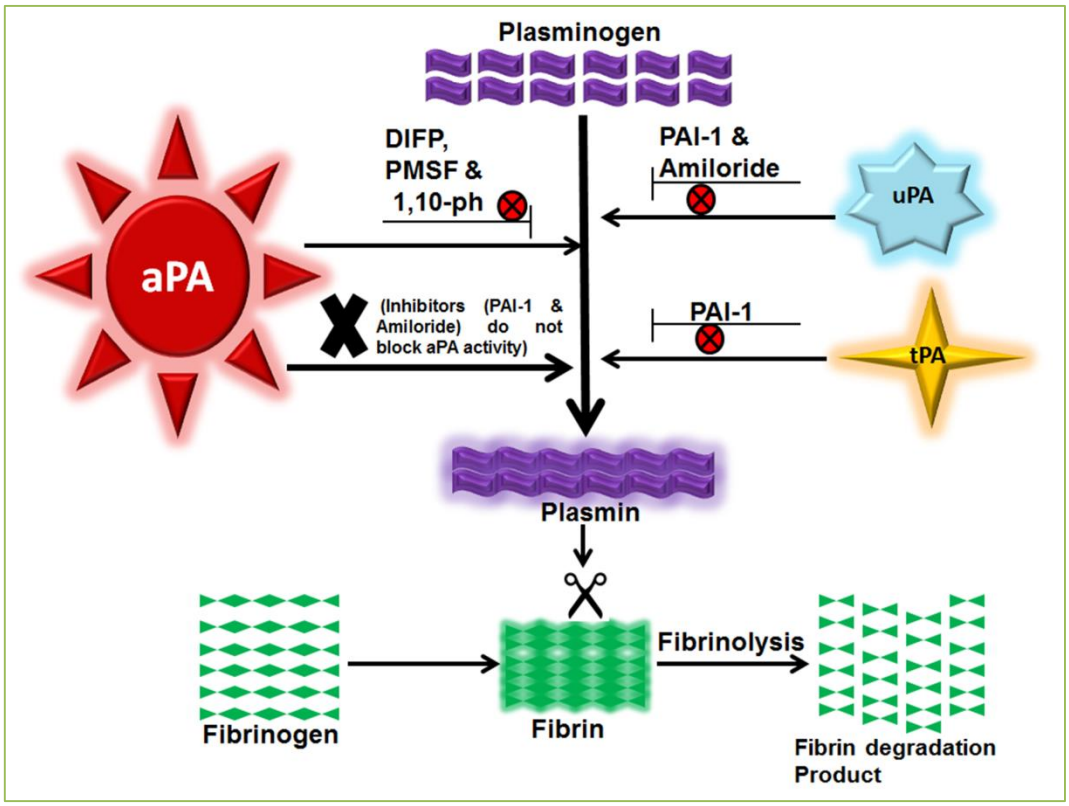

Figure 2. Schematic representation of *Acanthamoeba* plasminogen activator (aPA) characterization. aPA produces a single band of lysis on fibrinogen-agarose zymographs in the presence of plasminogen. Activity of aPA is completely inhibited by the treatment with diisopropylfluorophosphate (DIFP), phenylmethylsulfonyl fluoride (PMSF), and 1,10-phenanthroline (1,10-ph) indicating that it is a serine protease. aPA activity is not inhibited by amiloride (a strong inhibitor of uPA) and plasminogen activator inhibitor-I (PAI-1, an inhibitor of uPA and tPA).

PAR1 in HCE Cells

We have shown that PAR1 and PAR2 transcripts were constitutively expressed in HCE cells and aPA induced up regulation of PAR2, but not PAR1 transcript in the HCE cells. HCE cells stimulated with PAR2 agonists (SLIGRL-NH2 and AC 55541) and PAR1 agonists (thrombin and TRAP-6) upregulate PAR2 and PAR1 mRNA, respectively. PAR2 antagonist (FSLLRY-NH2), but not PAR1 antagonist (SCH 79797), effectively inhibits PAR2 mRNA expression induced by aPA, suggested that aPA activates PAR2 similar to PAR2 agonist. Likewise, upregulated transcripts of PAR1 and PAR2 by their specific agonists were inhibited by SCH 79797 and FSLLRY-NH2, respectively (Figure 3A and 3B). Furthermore, immunolocalization studies demonstrated that HCE cells stimulated with specific PAR1 and PAR2 agonist activate PAR1 and PAR2 on HCE cell surface. aPA specifically activates PAR2 expression on HCE cell surface, but not PAR1[9]. Thus, taken together, the flow cytometry and immunocytochemistry assessments demonstrated that aPA is a PAR2 activator, but not a PAR1 activator (Figure 4A and 4B) [9]. Immunolocalization findings of our study [9] agree with surface protein expression of PAR1 and PAR2 in whole human cornea [7]; however, in our study PAR1 and PAR2 cell surface expression observed upon stimulation by specific agonists [9] while whole human cornea expressed PAR1 and PAR2 immunoreactivity on the apical cell surface of the most superficial corneal epithelial cells in normal condition [7]. Gene expression and cell surface expression of PAR1 and PAR2, and specific activation of PAR2 by aPA seems to play a critical role in pathophysiology of *Acanthamoeba* keratitis and fascinates to use PAR2 blockers in vivo to determine therapeutic approach in management of *Acanthamoeba* keratitis.

The Role of aPA in Proinflammatory Cytokine Production by PAR2 Pathway in HCE Cells

Thrombin and trypsin are the multifunctional serine proteinase and stimulate proinflammatory cytokines by PAR1 and PAR2 pathways, respectively [7, 85-87]. We observed the functional activity of *Acanthamoeba* serine proteinase, aPA, in HCE cells. aPA upregulated proinflammatory cytokine IL-8 expression by PAR2 pathway, but not by PAR1 pathway. Treatments with aPA, PAR2 agonists (SLIGRL-NH2 and AC 55541), and PAR1

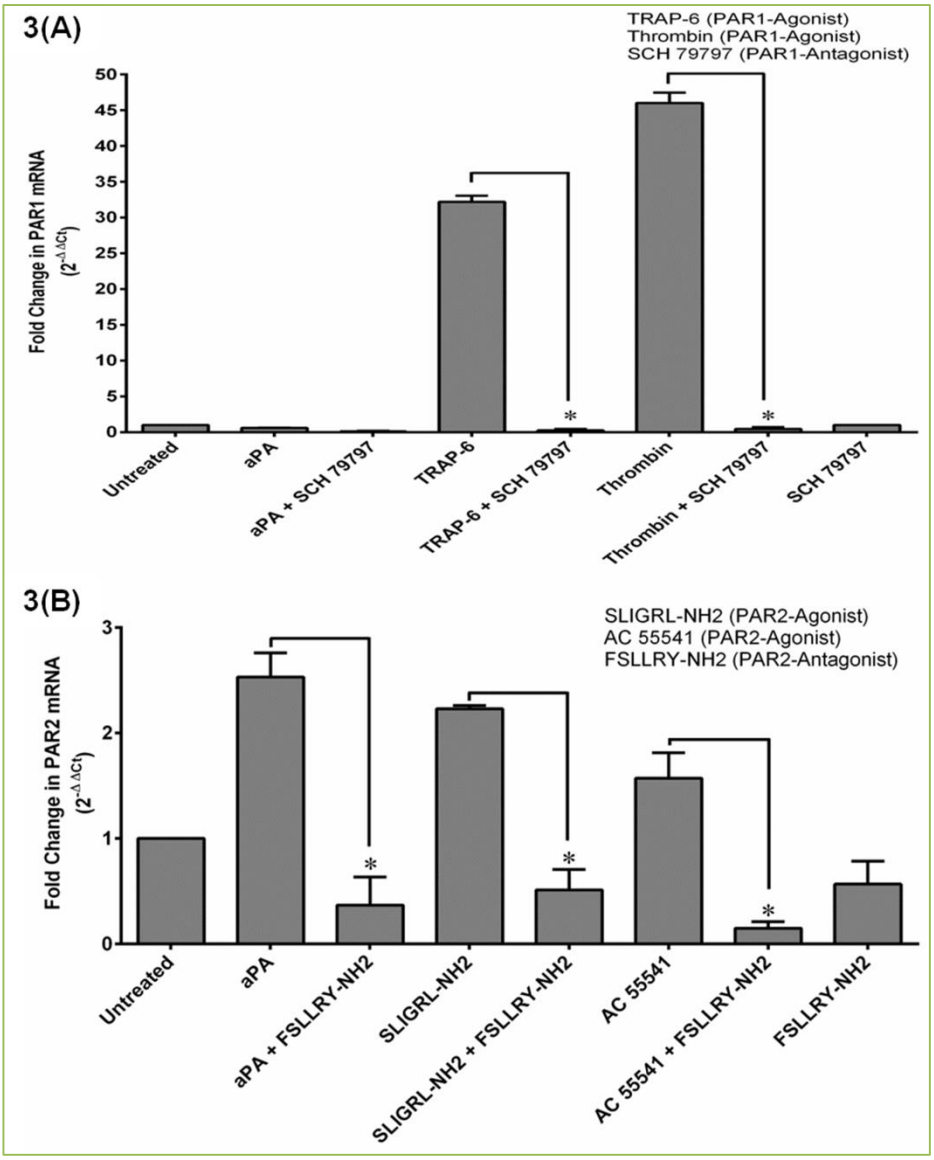

Figure 3. Protease-activated receptor 2 (PAR2) is upregulated by *Acanthamoeba* plasminogen activator (aPA) in HCE cells. HCE cells were incubated with aPA (100 µg/ml), PAR1 agonists (thrombin, 10 µM; TRAP-6, 10 µM), and PAR2 agonists (SLIGRL-NH2, 100 µM; AC 55541, 10 µM) for 24 hours. Inhibition of PAR1 and PAR2 involved pre-incubating the HCE cells for 1 hour with the antagonist of PAR1 (SCH 79797, 60 µM) and PAR2 (FSLLRY-NH2, 100 µM) and then incubated with or without aPA, PAR1 agonists, and PAR2 agonists for 24 hours. Total RNA was isolated and assessed using quantitative RT-PCR (qRT-PCR) for mRNA expression of PAR1 **(3A)** and PAR2 **(3B)**. Reprinted with permission [9]. Copyright held by The Association for Research in Vision and Ophthalmology (ARVO).

agonists (thrombin and TRAP-6) upregulated the IL-8 mRNA expression and protein production in HCE cells. While PAR2 antagonist, FSLLRY-NH2, inhibited IL-8 mRNA expression and protein production stimulated by aPA, SLIGRL-NH2, and AC 55541. In contrast, PAR1 antagonist (SCH 79797) blocked IL-8 mRNA expression and protein production induced by PAR1-agonists, but not by aPA. These results suggest that aPA activates expression and production of pro-inflammatory cytokine IL-8 in HCE cells by PAR2 pathway, but not by PAR1 pathway [9] (Figure 5). It is unlikely that the IL-8 protein production stimulated by aPA is due to activation of TLR2 and TLR4 receptors on HCE cells. We have shown that PAR2 specific antagonist, FSLLRY-NH2, inhibited IL-8 mRNA expression and protein production in HCE cells activated by aPA. Moreover, aPA stimulated IL-8 mRNA expression and protein production is not inhibited by PAR1-antagonist, SCH 79797, in HCE cells [9]. These findings are in agreement with Lang *et al*. [7] who demonstrated that trypsin (PAR1 agonist) and SLIGRL-

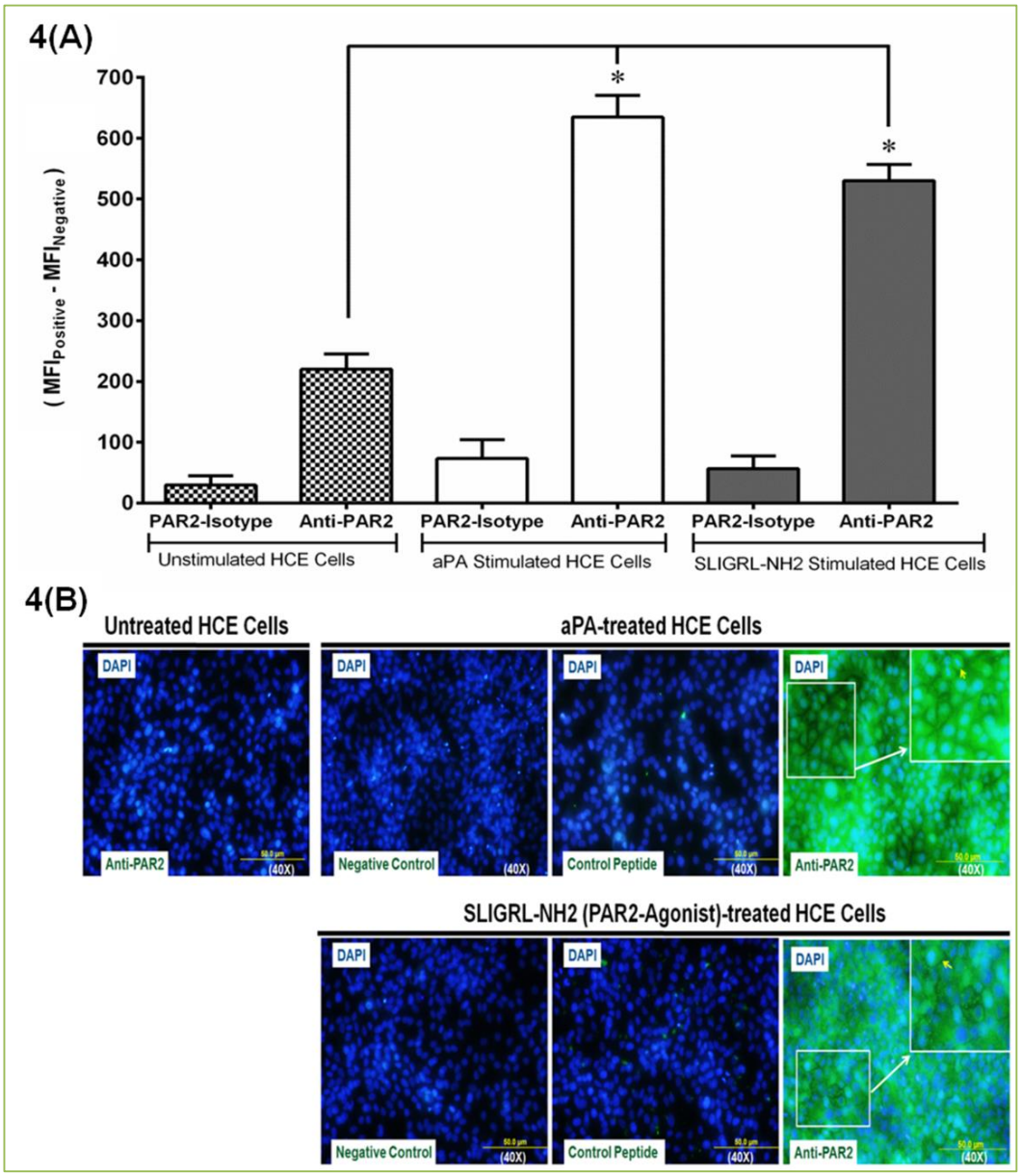

Figure 4. PAR2 surface protein expression is upregulated by aPA in HCE cells. HCE cells were incubated with or without aPA (100 µg/ml) and PAR2-agonist (SLIGRL-NH2, 100 µM) for 24 hours. PAR2 surface protein expression in HCE cells were examined by flow cytometry and immunocytochemistry. **(4A)** Briefly, to flow cytometry assessment cells were incubated with PE-labeled mouse IgG2a anti-human PAR2 and isotype control (PE-labeled mouse IgG2b) antibody. PAR2 expression in untreated HCE cells were compared with treated HCE cells. The results were expressed as normalized median fluorescence intensity (nMFI) units of positively stained HCE cells with PE-labeled antibody subtracted from MFI of unstained HCE cells as, ($MFI_{Positive}$ - $MFI_{Negative}$). The data are mean ± SEM of three independent experiments (*P < 0.05). P values were obtained by unpaired Student's t-test. **(4B)** To immunocytochemistry assessment, cells were stained with polyclonal rabbit anti-PAR2 antibody and Alexa Fluor 488-conjugated anti-rabbit antibody. Cells without primary antibody incubation were used as a negative control. Rat PAR2 (368-382) peptide were used as absorption control. 4, 6-diamidino-2-phenylindole (DAPI) counterstaining was used to visualize cell location and morphology. Three slides in each group were viewed using fluorescence microscopy. Images were captured with an Olympus AX70 Upright Compound Microscope. Reprinted with permission [9]. Copyright held by The Association for Research in Vision and Ophthalmology (ARVO).

NH2 (PAR2 agonist) specifically induced production of pro-inflammatory cytokines (IL-6, IL-8, and TNFα) protein in HCE cells. Our study suggests that aPA is an inflammatory protein which plays a critical role in pathogenesis of *Acanthamoeba* keratitis through PAR2 pathway [9], and suggests further in vivo studies to

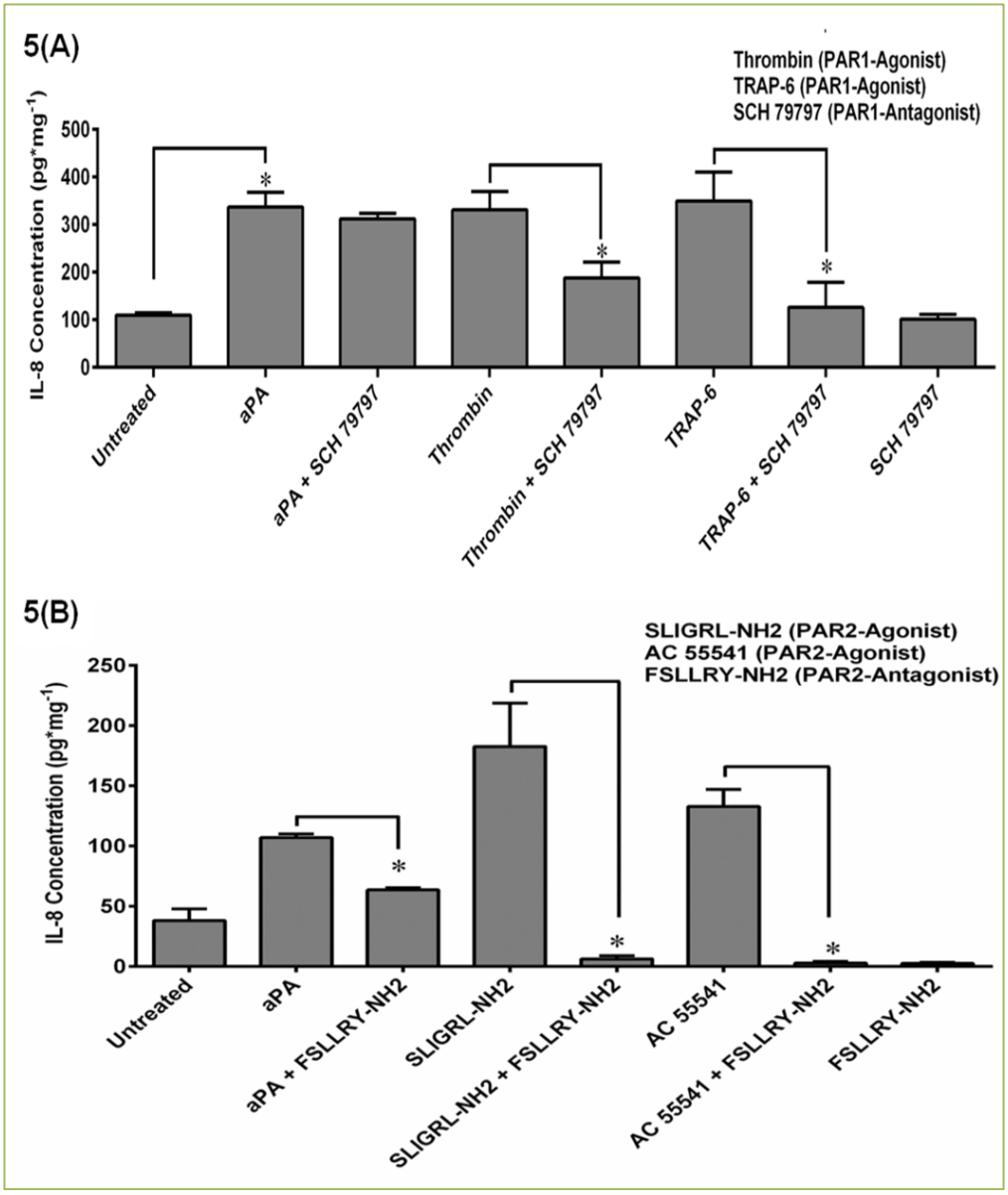

Figure 5. *Acanthamoeba* plasminogen activator (aPA) upregulated IL-8 is diminished by PAR2 antagonist, but not by PAR1 antagonist. HCE cells were incubated with aPA (100 µg/ml) and PAR1 agonists (thrombin, 10 µM; TRAP-6, 10 µM) for 48 hours. Inhibition of PAR1 involved pre-incubating the HCE cells for 1 hour with the PAR1 antagonist (SCH 79797, 60 µM) and then incubated with or without aPA, PAR1 agonists for 48 hours **(5A)**. HCE cells were incubated with aPA (100 µg/ml) and PAR2 agonists (SLIGRL-NH2, 100 µM; AC 55541, 10 µM) for 24 hours. Inhibition of PAR2 involved pre-incubating the HCE cells for 1 hour with the PAR2 antagonist (FSLLRY-NH2, 100 µM) and then incubated with or without aPA and PAR2 agonists for 24 hours **(5B)**. Supernatants were collected from harvested cells and subjected to IL-8 ELISA. The data are mean ± SEM of three independent experiments (**P* < 0.05). *P* values were obtained by unpaired Student's *t*-test. Reprinted with permission [9]. Copyright held by The Association for Research in Vision and Ophthalmology (ARVO).

delineate the PAR2 pathway as a specific target to approach *Acanthamoeba* keratitis related therapy.

Summary: Potential Role of PAR2 Antagonists to Attenuate aPA Induced Proinflammatory Mediators

The potential role of PAR2 antagonist in attenuating proinflammatory cytokine IL-8 induced by *Acanthamoeba* plasminogen activator, aPA, in human corneal epithelial (HCE) cells, is summarized in figure 6. It is possible that other chemokines/cytokines are involved in inflammatory responses in *Acanthamoeba* keratitis. We have shown that CXCL2 (IL-8 equivalent in rodents) is the major chemokines that plays a major role in attracting

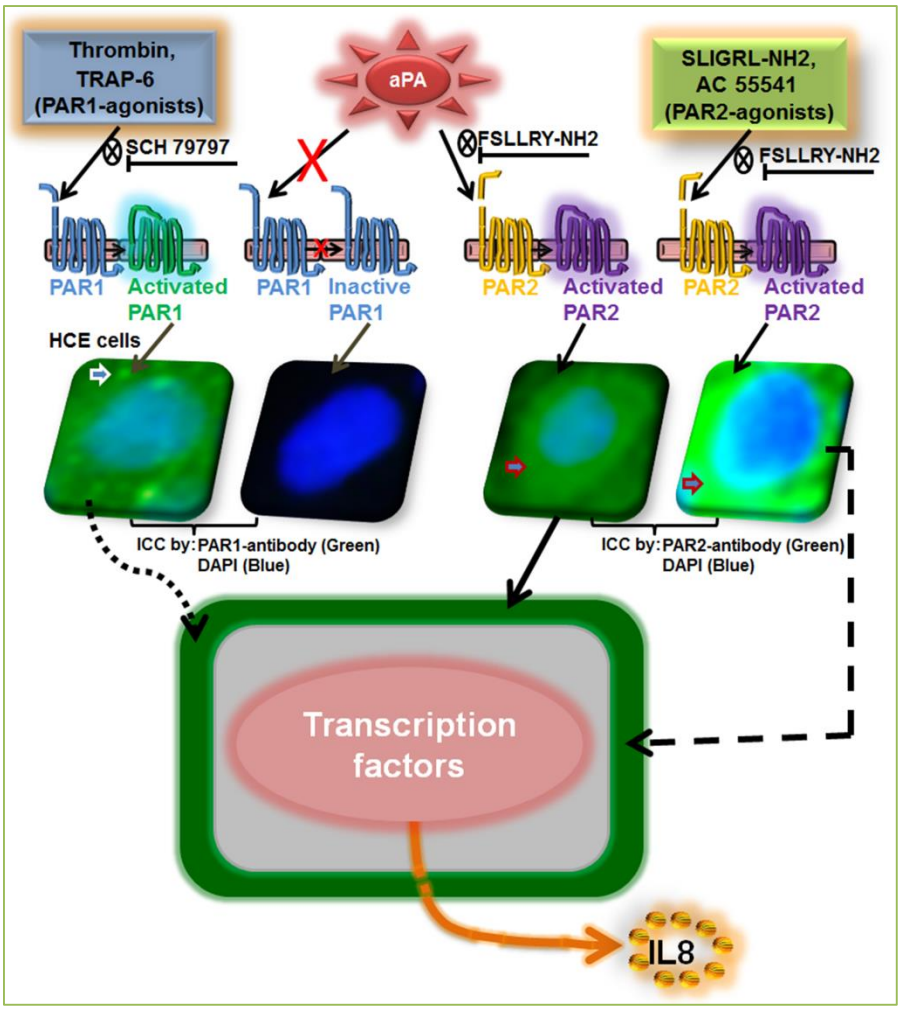

Figure 6. Schematic representation of aPA role in IL-8 production by PAR2 signaling, but not by PAR1 signaling, in human corneal epithelial (HCE) cells. PAR1 agonist (thrombin) and PAR2 agonist (SLIGRL-NH2) specifically activate the tethered receptors, PAR1 and PAR2, respectively, and induce the IL-8 production which specifically blocked by PAR1 antagonist (SCH 79797) and PAR2 antagonist (FSLLRY-NH2), respectively. However, aPA specifically activates PAR2, but not PAR1, and upregulates IL-8 production which is attenuated by FSLLRY-NH2 in HCE cells. Thus, *Acanthamoeba* plasminogen activator activates PAR2 similar to PAR2 agonist. PAR2 antagonists may be an important therapeutic target in *Acanthamoeba* keratitis. (Abbreviations: aPA, *Acanthamoeba* plasminogen activator; IL-8, Interleukin 8; PAR1, Protease activated receptor 1; PAR2, Protease activated receptor 2; ICC, Immunocytochemistry assay; DAPI, 4, 6-diamidino-2-phenylindole).

inflammatory cells such as polymorphonuclear neutrophils (PMNs) at the site of infection in Chinese hamster model of *Acanthamoeba* keratitis [61, 62]. Therefore, to determine PAR2 implication in *Acanthamoeba* keratitis using in vitro model of human corneal epithelial cells, we focused on IL-8 production to explore the functional activity of aPA which effectively inhibited by the use of PAR2 antagonist [9]. Likewise the agonists of PAR2 including trypsin and SLIGRL-NH2, modulate IL-6, IL-8, TNFα in HCE cells [7], aPA can induce other chemokines/cytokines in corneal epithelial cells, warranting further study.

In conclusion, increased expression and activation of PAR2 in HCE cells by aPA exposure is closely associated with inflammatory conditions, suggesting that PAR2 is relatively novel receptor to contribute critical role in inflammatory process during contact-independent mechanism of *Acanthamoeba* infection. *Acanthamoeba* plasminogen activator induced PAR2 expression and IL-8 modulation at gene and protein levels is specifically inhibited by PAR2 antagonist [9]. Thus, PAR2 antagonists may be therapeutic target to cure *Acanthamoeba* keratitis.

Future Work

In order to further evaluate the potential roles of PAR2

in *Acanthamoeba* keratitis, the following experiments should be considered: (i) investigating further expression and production of various inflammatory mediators mediated by aPA through PAR2 pathway; (ii) investigating the effects of various antagonists of PAR2 associated G protein and anti-aPA antibody on expression and function of inflammatory mediators during aPA exposures; (iii) investigating PAR2 associated downstream signaling pathway induced by aPA exposures. More importantly, future studies are warranted to investigate pharmacological approach using the selective PAR2 antagonists and proteinase inhibitors to determine the role of PAR2 in corneal infections and inflammation induced by *Acanthamoeba* infection.

Conflicting interests

The authors have declared that no competing interests exist.

Acknowledgments

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References

1. Hollenberg MD, Compton SJ. International Union of Pharmacology. XXVIII. Proteinase-activated receptors. Pharmacol Rev 2002; 54:203-217.
2. Ossovskaya VS, Bunnett NW. Protease-activated receptors: contribution to physiology and disease. Physiol Rev 2004; 84:579-621.
3. Steinhoff M, Buddenkotte J, Shpacovitch V, Rattenholl A, Moormann C, Vergnolle N, *et al*. Proteinase-activated receptors: transducers of proteinase-mediated signaling in inflammation and immune response. Endocrine Rev 2005; 26:1-43.
4. Holzhausen M, Spolidorio LC, Vergnolle N. Role of protease-activated receptor-2 in inflammation, and its possible implications as a putative mediator of periodontitis. Mem Inst Oswaldo Cruz, Rio de Janeiro 2005; 100(Suppl. I): 177-180.
5. Vergnolle N. Protease-activated receptors as drug targets in inflammation and pain. Pharmacol Ther 2009; 123:292-309.
6. Macfarlane SR, Seatter MJ, Kanke T, Hunter GD, Plevin R. Proteinase-activated receptors. Pharmacol Rev 2001; 53(2):245-282.
7. Lang R, Song PI, Legat FJ, Lavker RM, Harten B, Kalden H, *et al*. Human corneal epithelial cells express functional PAR-1 and PAR-2. Invest Ophthalmol Vis Sci 2003; 44:99-105.
8. Mitra MM, Alizadeh H, Gerard RD, Niederkorn JY. Characterization of a plasminogen activator produced by *Acanthamoeba castellanii*. Mol Biochem Parasitol 1995; 73:157–164.
9. Tripathi T, Abdi M, Alizadeh H. Protease-Activated Receptor 2 (PAR2) Is Upregulated by *Acanthamoeba* Plasminogen Activator (aPA) and Induces Proinflammatory Cytokine in Human Corneal Epithelial Cells. Invest Ophthalmol Vis Sci 2014; 55:3912-3921.
10. Lorenzo-Morales J, Martín-Navarro CM, López-Arencibia A, Arnalich-Montiel F, Piñero JE, Valladares B. *Acanthamoeba* keratitis: an emerging disease gathering importance worldwide? Trends Parasitol 2013; 29:181-187.
11. Clarke DW, Niederkorn JY. The pathophysiology of *Acanthamoeba* keratitis. Trends Parasitol 2006; 22:175-180.
12. Alizadeh H, Niederkorn JY, McCulley JP. *Acanthamoeba* keratitis. In: Ocular Infection and Immunity. Edited by Pepose JS, Holland GN, Wilhelmus KR. St. Louis, Missouri: Mosby; 1996:1062–1071.
13. Khan NA, Jarroll EL, Panjwani N, Cao Z, Paget TA. Proteases as markers for differentiation of pathogenic and nonpathogenic species of *Acanthamoeba*. J Clin Microbiol 2000; 38:2858-2861.
14. Alizadeh H, Neelam S, Hurt M, Niederkorn JY. Role of contact lens wear, bacterial flora, and mannose-induced pathogenic protease in the pathogenesis of amoebic keratitis. Infect Immun 2005; 73:1061-1068.
15. Hurt M, Neelam S, Niederkorn J, Alizadeh H. Pathogenic *Acanthamoeba* spp secrete a mannose-induced cytolytic protein that correlates with the ability to cause disease. Infect Immun 2003; 71:6243-6255.
16. Centers for Disease Control and Prevention (CDC). *Acanthamoeba* keratitis multiple states, 2005–2007. MMWR Morb Mortal Wkly Rep 2007; 56:532-534. http://www.cdc.gov/mmwr/preview/mmwrhtml/mm562la4.htm
17. Kettesy B, Modis L Jr., Berta A, Kemeny-Beke A. Keratoplasty in contact lens related *Acanthamoeba* keratitis. In: Keratoplasties-Surgical techniques and complications. Edited by Mosca L. Rijeka, Croatia: InTech Europe 2012:31-52.
18. Naginton J, Watson PG, Playfair TJ, McGill J, Jones BR, Steele AD. Amoebic infection of the eye. Lancet 1974; 2:1537-1540.
19. Jones BR, Visvesvara GS, Robinson NM. *Acanthamoeba polyphaga* keratitis and *Acanthamoeba* uveitis associated with fatal meningoencephalitis. Trans Ophthalmol Soc UK 1975; 95:221-232.
20. Ibrahim YW, Boase DL, Cree IA. Factors affecting the epidemiology of *Acanthamoeba* keratitis. Ophthalmic Epidemiol 2007;14:53-60.
21. Stehr-Green JK, Bailey TM, Visvesvara GS. The epidemiology of *Acanthamoeba* keratitis in the United States. Am J Ophthalmol 1989; 107:331-336.
22. Schaumberg DA, Snow KK, Dana MR. The epidemic of *Acanthamoeba* keratitis: where do we stand? Cornea 1998; 17:3-10.
23. Radford CF, Lehmann OJ, Dart JKG. *Acanthamoeba* keratitis:multi-centre survey in England 1992–1996. Br J Ophthalmol 1998; 82:1387-1392.
24. Radford CF, Minassian DC, Dart JKG. Acanthamoeba keratitis in England and Wales: incidence, outcome, and risk factors. Br J Ophthalmol 2002; 86:536-542.
25. Seal DV. *Acanthamoeba* keratitis update-incidence, molecular epidemiology and new drugs for treatment. Eye 2003; 17:893-905.

26. Tachikawa T, Ishibashi Y, Fujisawa S, Takazawa S, Nyunt AK, Miyanaga Y. A nation-wide survey on the occurrence of amoebic keratitis in Japan. Nippon Ganka Gakkai Zasshi 1995; 99:68-75.
27. Murdoch D, Gray TB, Cursons R, Parr D. *Acanthamoeba* keratitis in New Zealand, including two cases with in vivo resistance to polyhexamethylene biguanide. Aust N Z J Ophthalmol 1998; 26:231-236.
28. Jackson TN, Heinze JB, Tuxen J, Weiner JM. Successful medical treatment of a corneal ulcer due to *Acanthamoeba polyphaga*. Aust N Z J Ophthalmol 1986; 14:139-142.
29. Seal DV, Kirkness CM, Bennett HGB. Population-based cohort study of microbial keratitis in Scotland: incidence and features. Contact Lens Anterior Eye 1999; 22:49-57.
30. Lam DS, Houang E, Fan DS, Lyon D, Seal D, Wong E. Incidence and risk factors for microbial keratitis in Hong Kong: comparison with Europe and North America. Eye 2002; 16:608-618.
31. Quinio D, Le Flohic AM, Moalic E, Resnikoff S. *Acanthamoeba* keratitis: search for a healthy carrier in Mali. Med Trop 2000; 60:61-63.
32. Cheng KH, Leung SL, Hoekman HW, Beekhuis WH, Mulder PG, Geerards AJ, *et al*. Incidence of contact-lens-associated microbial keratitis and its related morbidity. Lancet 1999;354:181-185.
33. Verani JR, Lorick SA, Yoder JS, Beach MJ, Braden CR, Roberts JM, *et al*. National outbreak of *Acanthamoeba* keratitis associated with use of a contact lens solution, United States. Emerg Infect Dis 2009; 15:1236-1242.
34. Ferrante A, Bates EJ. Elastase in the pathogenic free-living amoebae, Naegleria and *Acanthamoeba* spp. Infect Immun 1988, 56:3320-3321.
35. Pellegrin JL, Ortega-Barria E, Prioli RP, Meijia JS, Pereira ME. The neuraminidases of *Trypanosoma cruzi* and *Acanthamoeba castellanii* are immunologically related. Trop Med Parasitol 1992; 43:33-37.
36. van Klink F, Alizadeh H, He Y, Mellon JA, Silvany RE, McCulley JP, *et al*. The role of contact lenses, trauma, and Langerhans cells in a Chinese hamster model of *Acanthamoeba* keratitis. Invest Ophthalmol Vis Sci 1993; 34:1937-1944.
37. Alizadeh H, Pidherney MS, McCulley JP, Niederkorn JY. Apoptosis as a mechanism of cytolysis of tumor cells by a pathogenic free-living amoeba. Infect Immun 1994; 62:1298-1303.
38. Yang Z, Cao Z, Panjwani N. Pathogenesis of *Acanthamoeba* keratitis: carbohydrate-mediated host-parasite interactions. Infect Immun 1997; 65:439-445.
39. Toney DM, Marciano-Cabral F. Resistance of *Acanthamoeba* species to complement lysis. J Parasitol 1998; 84:338-344.
40. Niederkorn JY, Alizadeh H, Leher H, McCulley JP. The pathogenesis of *Acanthamoeba* keratitis. Microb Infect 1999; 1:437-443.
41. Vemuganti GK, Sharma S, Athmanathan S, Garg P. Keratocyte loss in *Acanthamoeba* keratitis: phagocytosis, necrosis or apoptosis? Indian J Ophthalmol 2000; 48:291-294.
42. Na BK, Kim JC, Song CY. Characterization and pathogenetic role of proteinase from *Acanthamoeba castellanii*. Microb Pathog 2001; 30:39-48.
43. Mattana A, Cappai V, Alberti L, Serra C, Fiori PL, Cappuccinelli P. ADP and other metabolites released from *Acanthamoeba castellanii* lead to human monocytic cell death through apoptosis and stimulate the secretion of proinflammatory cytokines. Infect Immun 2002; 70:4424-4432.
44. Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. Clin Microbiol Rev 2003; 16:273-307.
45. Sissons J, Alsam S, Jayasekera S, Khan NA. Ecto-ATPases of clinical and non-clinical isolates of *Acanthamoeba*. Microb Pathog 2004; 37:231-239.
46. Garate M, Cao Z, Bateman E, Panjwani N. Cloning and characterization of a novel mannose-binding protein of *Acanthamoeba*. J Biol Chem 2004; 279:29849-29856.
47. Hong YC, Lee WM, Kong HH, Jeong HJ, Chung DI. Molecular cloning and characterization of a cDNA encoding a laminin-binding protein (AhLBP) from *Acanthamoeba healyi*. Exp Parasitol 2004; 106:95-102.
48. Sissons J, Kim KS, Stins M, Jayasekera S, Alsam S, Khan NA. *Acanthamoeba castellanii* induces host cell death via a phosphatidylinositol 3-kinase dependent mechanism. Infect Immun 2005; 73:2704-2708.
49. Garate M, Alizadeh H, Neelam S, Niederkorn JY, Panjwani N. Oral immunization with *Acanthamoeba castellanii* mannose-binding protein ameliorates amoebic keratitis. Infect Immun 2006; 74:7032-7034.
50. Khan NA. *Acanthamoeba*: biology and increasing importance in human health. FEMS Microbiol Rev 2006; 30:564-595.
51. Garate M, Marchant J, Cubillos I, Cao Z, Kban AN, Panjwani N. In vitro pathogenicity of *Acanthamoeba* is associated with the expression of the mannose-binding protein. Invest Ophthalmol Vis Sci 2006; 47:1056-1062.
52. Alizadeh H, Li H, Neelam S, Niederkorn JY. Modulation of corneal and stromal matrix metalloproteinase by the mannose-induced *Acanthamoeba* cytolytic protein. Exp Eye Res 2008; 87:286-291.
53. Alsam S, Jeong SR, Dudley R, Khan NA. Role of human tear fluid in *Acanthamoeba* interactions with the human corneal epithelial cells. Int J Med Microbiol 2008; 298:329-336.
54. Rocha-Azevedo BD, Jamerson M, Cabral GA, Marciano-Cabral F. *Acanthamoeba culbertsoni*: Analysis of amoebic adhesion and invasion on extracellular matrix components collagen I and laminin-1. Exp Parasitol 2009; 126:79-84.
55. Chusattayanond AD, Boonsilp S, Kasisit J, Boonmee A, Warit S. Thai *Acanthamoeba* isolate (T4) induced apoptotic death in neuroblastoma cells via the Bax-mediated pathway. Parasitol Int 2010; 59:512-516.
56. Panjwani N. Pathogenesis of *Acanthamoeba* keratitis. Ocul Surf 2010; 8:70-79.
57. Ren MY, Wu XY. Toll-like receptor 4 signalling pathway activation in a rat model of *Acanthamoeba* keratitis. Parasite Immunol 2011; 33:25-33.
58. Mortazavi PN, Keisary E, Loh LN, Jung SY, Khan NA. Possible roles of phospholipase A_2 in the biological activities

of *Acanthamoeba castellanii* (T4 genotype). Protist 2011; 162:168-176.

59. Siddiqui R, Khan NA. Biology and pathogenesis of *Acanthamoeba*. Parasit Vectors 2012; 5:6.
60. Tripathi T, Smith AD, Abdi M, Alizadeh H. *Acanthamoeba*-cytopathic protein induces apoptosis and proinflammatory cytokines in human corneal epithelial cells by $cPLA_{2\alpha}$ activation. Invest Ophthalmol Vis Sci 2012; 53:7973-7982.
61. Tripathi T, Abdi M, Alizadeh H. Role of phospholipase A_2 (PLA_2) inhibitors in attenuating apoptosis of the corneal epithelial cells and mitigation of *Acanthamoeba* keratitis. Exp Eye Res 2013; 113:182-191.
62. Alizadeh H, Tripathi T, Abdi M, Smith AD. Pathogenic strains of *Acanthamoeba* are recognized by TLR4 and initiated inflammatory responses in the cornea. PLoS One 2014; 9:e92375.
63. Bharathi JM, Srinivasan M, Ramakrishnan R, Meenakshi R, Padmavathy S, Lalitha PN. A study of the spectrum of *Acanthamoeba* keratitis: a three-year study at a tertiary eye care referral center in South India. Indian J Ophthalmol 2007; 55:37-42.
64. McClellan K, Howard K, Niederkorn JY, Alizadeh H. Effect of steroids on *Acanthamoeba* cysts and trophozoites. Invest Ophthalmol Vis Sci 2001; 42:2885-2893.
65. Alizadeh H, Neelam S, Niederkorn JY. Effect of immunization with the mannose-induced *Acanthamoeba* protein and *Acanthamoeba* plasminogen activator in mitigating *Acanthamoeba* keratitis. Invest Ophthalmol Vis Sci 2007; 48:5597-5604.
66. Dulon S, Leduc D, Cottrell GS, D'Alayer J, Hansen KK, Bunnett NW, *et al*. *Pseudomonas aeruginosa* elastase disables proteinase-activated receptor 2 in respiratory epithelial cells. Am J Respir Cell Mol Biol 2005; 32:411-419.
67. Rohani MG, DiJulio DH, An JY, Hacker BM, Dale BA, Chung WO. PAR1- and PAR2-induced innate immune markers are negatively regulated by PI3K/Akt signaling pathway in oral keratinocytes. BMC Immunol 2010; 11:53.
68. Lourbakos A, Potempa J, Travis J, D'Andrea MR, Andrade-Gordon P, Santulli R, *et al*. Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. Infect Immun 2001; 69:5121-5130.
69. Euzebio Alves VT, Bueno da Silva HA, de França BN, Eichler RS, Saraiva L, de Carvalho MH, *et al*. Periodontal treatment downregulates protease-activated receptor 2 in human gingival crevicular fluid cells. Infect Immun 2013; 81:4399-4407.
70. Kida Y, Inoue H, Shimizu T, Kuwano K. *Serratia marcescens serralysin* induces inflammatory responses through protease activated receptor 2. Infect Immun 2007; 75:164-174.
71. Holzhausen M, Spolidorio LC, Ellen RP, Jobin MC, Steinhoff M, Andrade-Gordon P, *et al*. Protease-activated receptor-2 activation: a major role in the pathogenesis of *Porphyromonas gingivalis* infection. Am J Pathol 2006; 168:1189-1199.
72. Pandolfi M, Astrup T. A histochemical study of the fibrinolytic activity. Cornea, conjunctiva, and lacrimal gland. Arch Ophthalmol 1967; 77:258-264.
73. Pandolfi M, Lantz E. Partial purification and characterization of keratokinase, the fibrinolytic activator of the cornea. Exp Eye Res 1979; 29:563-571.
74. Berman M, Leary R, Gage J. Evidence for a role of the plasminogen activator--plasmin system in corneal ulceration. Invest Ophthalmol Vis Sci 1980; 19:1204-1221.
75. Wang HM, Berman M, Law M. Latent and active plasminogen activator in corneal ulceration. Invest Ophthalmol Vis Sci 1985; 26:511-524.
76. Geanon JD, Tripathi BJ, Tripathi RC, Barlow GH. Tissue plasminogen activator in avascular tissues of the eye: a quantitative study of its activity in the cornea, lens, and aqueous and vitreous humors of dog, calf, and monkey. Exp Eye Res 1987; 44:55-63.
77. Tervo T, Tervo K, van Setten GB, Virtanen I, Tarkkanen A. Plasminogen activator and its inhibitor in the experimental corneal wound. Exp Eye Res 1989; 48:445-449.
78. Mirshahi M, Mirshahi S, Soria C, Soria J, Thomaidis A, Pouliquen Y, *et al*. Production of proteases type plasminogen activator and their inhibitor in cornea. Biochem Biophys Res Commun 1989; 160:1021-1025.
79. Berk RS, Katar M, Dong Z, Day DE. Plasminogen Activators and Inhibitors in the Corneas of Mice Infected with *Pseudomonas aeruginosa*. Invest Ophthalmol Vis Sci 2001; 42:1561-1567.
80. Watanabe M, Yano W, Kondo S, Hattori Y, Yamada N, Yanai R, *et al*. Up-regulation of urokinase-type plasminogen activator in corneal epithelial cells induced by wounding. Invest Ophthalmol Vis Sci 2003; 44:3332-3338.
81. Sugioka K, Kodama A, Yoshida K, Okada K, Mishima H, Aomatsu K, *et al*. The Roles of Urokinase Plasminogen in Leukocyte Infiltration and Inflammatory Responses in Mice Corneas Treated with Lipopolysaccharide (LPS). Invest Ophthalmol Vis Sci 2014. pii: IOVS-14-14867. doi: 10.1167/iovs.14-14867.
82. Zhang H, Zeng X, He S. Evaluation on potential contributions of protease activated receptors related mediators in allergic inflammation. Mediators Inflamm 2014; 2014:829068.
83. Zieske JD, Bukusoglu G. Effect of protease inhibitors on corneal epithelial migration. Invest Ophthalmol Vis Sci 1991; 32:2073-2078.
84. Nickel TJ, Kabir MH, Talreja J, Stechschulte DJ, Dileepan KN. Constitutive expression of functionally active protease-activated receptors 1 and 2 in human conjunctival epithelial cells. Mediators Inflamm 2006; 2006:61359.
85. Scholz M, Vogel JU, Höver G, Kotchetkov R, Cinatl J, Doerr HW, *et al*. Thrombin stimulates IL-6 and IL-8 expression in cytomegalovirus-infected human retinal pigment epithelial cells. Int J Mol Med 2004; 13:327-331.
86. Yoshida N, Katada K, Handa O, Takagi T, Kokura S, Naito Y, *et al*. Interleukin-8 production via protease-activated receptor 2 in human esophageal epithelial cells. Int J Mol Med 2007; 19:335-340.
87. Ludwicka-Bradley A, Tourkina E, Suzuki S, Tyson E, Bonner M, Fenton JW 2nd, *et al*. Thrombin upregulates interleukin-8 in lung fibroblasts via cleavage of proteolytically activated receptor-I and protein kinase C-gamma activation. Am J Respir Cell Mol Biol 2000; 22:235-243.