RESEARCH ARTICLE

Identification of *Acanthamoeba* membrane protein that is recognized by TLR4 on corneal epithelial cells

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We have shown that *Acanthamoeba* spp. activate TLR4 on corneal epithelial cells and induce secretion of chemokines. However, the components of *Acanthamoeba* trophozoites that induce chemokines production remain unknown. We sought to identify the trophozoite molecules that interact with TLR4 on human corneal epithelial (HCE) cells and trigger IL-8 production. *Acanthamoeba* membrane protein (AcMP) was isolated by homogenization of trophozoites. The supernatants were collected, solubilized, and membrane fractions were separated by centrifugation using Mem-PER™ plus kit. To examine functional activity of AcMP, HCE and TLR4-expressing HEK293 cells were incubated with or without *A. castellanii* (1×10^5 cells/ml) and AcMP (10, 25, and 50 µg/ml) for 24 hours. AcMP was chromatographed by fast protein liquid chromatography (FPLC) and fractions were pooled into four peaks (AcMP-P1 - AcMP-P4). TLR4-ligand in AcMP-P1 - AcMP-P4 was determined by Western blotting. HEK293 and HCE cells were incubated with or without *A. castellanii*, lipopolysaccharide (LPS, 10 µg/ml), and AcMP-P1 - AcMP-P4 (20 µg/ml) for 24 hours. qRT-PCR and ELISA were used to examine the ability of AcMP-P1 - AcMP-P4 to stimulate IL-8 production in HEK293 and HCE cells. Inhibition of TLR4 involved preincubating HEK293 and HCE cells for 1 hour with neutralizing TLR4-antibody (10 µg/ml) or with the control antibody (10 µg/ml, goat serum) followed by incubation with or without *A. castellanii*, LPS, and AcMP-P2 for 24 hours. AcMP induced significant IL-8 production at doses of 10, 25, and 50 µg/ml in HEK293 cells while IL-8 mRNA expression and IL-8 secretion were significantly increased in HCE cells at the dose of 50 µg/ml. Treatments of HEK293 with FPLC chromatographed trophozoites' proteins, AcMP-P1 - AcMP-P4; only AcMP-P2 upregulated significant IL-8 production and mRNA expression. Western blotting of AcMP-P1 - AcMP-P4 showed TLR4-antigen in AcMP-P2 and was recognized an approximate 15-kDa protein band. Anti-TLR4 antibody attenuated IL-8 secretion that is stimulated by AcMP-P2 from HEK293 and HCE cells. These results suggest that *A. castellanii* trophozoites recognize TLR4 on HCE and HEK293 cells by an approximate 15-kDa molecular mass protein of AcMP and induce IL-8 secretion.

Keywords: *A. castellanii*; Trophozoites; TLR4; Ligand; IL-8; Corneal Epithelial Cells; HEK293

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Introduction

Acanthamoeba keratitis (AK), a rare but potentially vision-threatening and painful infectious corneal disease, is caused by the ubiquitous free-living species of pathogenic amoebae belonging to the genus *Acanthamoeba* [1]. A five-year study of corneal scrapings of clinically diagnosed infectious keratitis cases determined that *Acanthamoeba* spp.

are the second most-important microbial agent after bacteria in causing microbial keratitis [2]. Recently, a report by the Centers for Disease Control and Prevention (CDC) declared that the incidence of AK has augmented in several states in the United States of America and poor contact lens hygiene is considered the main risk factor of AK [3]. Although the worldwide increased prevalence of AK is predominantly caused by the improper use or care of contact lenses (CL), other risk factors and environmental exposures associated with AK should be considered. Moreover, non-contact lens wearers have been diagnosed with AK, leading to a need for more study [4].

A successful cure for AK is still evolving. Topical treatments (a combination of brolene, polyhexamethylene biguanide (PHMB), and chlorhexidine) are administered hourly for several weeks, but such therapies are not effective, and *Acanthamoeba* species can cause excruciating damage to the corneal epithelium and stroma, often resulting in practitioners' recommendations for corneal transplantation [5]. Study of AK pathogenesis raises the hope of solving difficulties in targeting therapeutic agents to treat AK; therefore, several studies have been orchestrated on the pathogenesis of AK [6-23]. More recently, we observed that *Acanthamoeba* trophozoites activate toll-like receptor 4 (TLR4) on human and Chinese hamster corneal epithelial cells *in vitro* and *in vivo* and stimulate cytokine secretion and progression of keratitis [24].

Many studies have shown that *Pseudomonas aeruginosa* infection upregulates several pathways of the innate immune system via activation of toll-like receptors (TLRs) on corneal epithelial cells and causes bacterial keratitis [25]. For example, TLRs recognize lipopolysaccharide (LPS) or flagella from *P. aeruginosa* and induce corneal epithelial cells to produce inflammatory mediators (cytokines and chemokines), which infiltrate polymorphonuclear neutrophils (PMNs) at the infection site to induce phagocytosis and kill *P. aeruginosa*; however, continued infiltration in the corneal tissue leads to destruction of corneal cells and corneal stroma and causes scarring and vision loss [25].

An investigation of the mechanism of the TLR4 signaling cascade that produces cytokines and chemokines discovered that the TLR4 receptor works in a complex of proteins that permit for the identification of its known specific ligand, LPS [26, 27]. LPS binding protein (LBP), cluster of differentiation 14 (CD14, a pattern recognition receptor), and myeloid differentiation protein 2 (MD-2) are all expressed in the eye. These all are integral components of the TLR4 recognition system [28, 29]. LBP binds to LPS and transfers the pathogen-associated molecular patterns (PAMPs) onto CD14. MD-2 is a co-receptor, which binds to TLR4 and to LPS, making it compulsory for response [28-32]. It has been shown that the filarial nematode *Acanthocheilonema viteae* produces a molecule (ES-62) that is recognized by TLR4 and induces proinflammatory responses in mouse macrophages, dendritic cells, and mast cells [33]. We sought to identify the components of the *Acanthamoeba* trophozoites that interact with TLR4 on the corneal epithelial cells, trigger chemokines production, and lead to AK pathogenesis.

In this study, we determined that *A. castellanii* membrane protein (AcMP) is recognized by TLR4 on human corneal epithelial (HCE) and TLR4-expressing HEK293 cells. Characterization of AcMP showed an approximately 15-kDa molecular mass protein in fraction #2 (AcMP-P2) out of four fractions (AcMP-P1 - AcMP-P4) isolated by FPLC. From AcMP-P1 - AcMP-P4, only AcMP-P2 induced IL-8 production, which was further diminished by the neutralizing TLR4 antibody. The present study is the first to isolate and identify *A. castellanii* reactive components that recognize TLR4 on HCE cells and its role in stimulating IL-8 production by HCE cells.

Materials and Methods

Amoebae and human cell lines

Acanthamoeba castellanii (ATCC 30868), isolated from a human cornea, was obtained from the American Type Culture Collection (ATCC), Manassas, VA. Amoebae were grown as axenic cultures in peptone-yeast extract-glucose at 35°C with constant agitation on a shaker incubator at 125 rpm [21, 34]. Human telomerase-immortalized corneal epithelial (HCE) cells, a gift from James Jester, PhD (University of California, Irvine), were cultured in keratinocyte medium (KBM-2 Bullet Kit; BioWhittaker, Lonza, Walkersville, MD) containing 10% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT), at 35°C with 5% CO_2 [35]. Human embryonic kidney 293 (HEK293) cells stably transfected with TLR4 were obtained from Eicke Latz, PhD (University of Massachusetts Medical School, Worcester, MA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/liter glucose with L-glutamine (BioWhittaker, Lonza, Walkersville, MD), 10% fetal bovine serum (FBS, HyClone, Logan, UT), and 10 mg/ml Cipro (Cellgro Media Tech, Inc., Manassas, VA) at 35°C with 5% CO_2 [36].

Isolation of *Acanthamoeba* trophozoites membrane protein (AcMP)

Acanthamoeba castellanii trophozoites were grown in peptone-yeast extract glucose for 3 days and trophozoites were collected and centrifuged. *Acanthamoeba* membrane

protein (AcMP) was isolated by homogenization of trophozoites (1×10^6/ml) using sterile glass beads in PBS, and the resultant homogenates were centrifuged at 1000×*g* for 20 minutes at 4ºC. The supernatants were collected and solubilized, and membrane fractions were separated by centrifugation using a Mem-PER™ plus kit (Thermo Fisher Scientific, Inc., Rockford, IL).

Isolation of AcMP fractions

AcMP was chromatographed by fast protein liquid chromatography (FPLC) as described previously [33]. FPLC analysis was carried out on an ÄKTApurifier™ UPC 10 equipped with fraction collector Frac-920 (GE Healthcare Bio-Science AB, Uppsala, Sweden). All FPLC runs were performed at 4°C. All of the elutions were observed at an absorbance of 280 nm. The size-exclusion chromatography was carried out on a single Superdex 200 10/300 GL column (GE Healthcare Bio-Science AB, Uppsala, Sweden). Elutions were performed in a 0.05M Na_2HPO_4, 0.05M NaH_2PO_4, 0.15M NaCl solution, pH 6.8, as recommended for gel filtration by Bio-Rad Laboratories, U.S.A. This eluent buffer was filter-sterilized (0.22 µM filter system; Corning Incorporated, Corning, NY) and then degassed for 30 minutes under vacuum on a stir plate. Prior to sample injection into the ÄKTApurifier™ UPC 10 system, 24 ml of eluent buffer, run at 0.2 ml/minutes, was used to confirm equilibration of the column. The column was washed with filtered and degassed 20% ethanol between runs. Immediately prior to injection, the protein sample (AcMP) was centrifuged at 10,000×*g* for 5 minutes. AcMP aliquots of 100 µL were used for each injection. Also, 100 µL of eluent buffer was added to each injection to completely fill the injection loop. Elutions were carried out at a flow rate of 0.2 ml/minutes with a maximum pressure of 1.5 MPa. The Frac-920 fraction collector was used to collect fractions of 1.0 ml throughout the analysis. Fractions were collected in polypropylene round-bottom tubes (BD Falcon™; BD Biosciences, Bedford, MA) siliconized with SurfaSil™ Siliconizing (Thermo Scientific, Pittsburgh, PA). Fractions corresponding to discrete elution peaks were pooled into four peaks (AcMP-P1 – AcMP-P4) and saved for subsequent analysis. The fraction sets were then concentrated using Microsep™ Advance Centrifugal Devices 1K Omega membrane filter (Pall Corporation, MI).

Cell cultures and treatment experiments

TLR4-expressing HEK293 and HCE cells were cultured in 24 well plates (Corning Incorporated, Corning, NY) until confluent. Once confluent, cells were stimulated with or without *A. castellanii* trophozoites (1×10^5/ml) and AcMP (10, 25, and 50 µg/ml) for 24 hours. HEK293 cells were further incubated with or without *A. castellanii*, lipopolysaccharide (LPS, 10 µg/ml), and (AcMP-P1 – AcMP-P4; 20 µg/ml) for 24 hours. Inhibition of TLR4 involved preincubating HEK293 and HCE cells for 1 hour with neutralizing TLR4 antibody (10 µg/ml of anti-hTLR4 affinity purified goat IgG; R&D Systems, Minneapolis, MN) or with the normal goat serum (10 µg/ml, goat serum; Vector Laboratories, Inc., Burlingame, CA), followed by stimulation with or without *A. castellanii*, LPS, and AcMP-P2 for 24 hours. Cells cultured without stimulation served as a control. Cells and supernatants were collected by centrifugation at 2000×*g* for 10 minutes at 4°C.

Isolation of RNA

TLR4-expressing HEK293 and HCE cells were collected from 24 well plates at the indicated times after treatment. The total cellular RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The quality of the extracted RNA and the RNA integrity was confirmed as described previously [23].

Real-Time qRT-PCR

cDNA was synthesized from 2 µg of total RNA using the RT^2 First Strand Kit (Qiagen, Valencia, CA, USA) by C1000™ Thermal Cycler RT-PCR system (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's protocol as previously described [23].

PCR was performed using the CFX Connect™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) with SYBR green fluorescent dye. The reactions were conducted in a total volume of 25 µl containing 12.5 µl 2X RT^2 SYBR Green Mastermix (Qiagen, Valencia, CA, USA), 2.5 µl sense and 2.5 µl antisense primers of 0.5 µM, 5 µl cDNA and 2.5 µl RNase-free water, all in Low Tube Strip, CLR (0.2 ml, Bio-Rad Laboratories, Hercules, CA, USA). After PCR, melting curves were acquired stepwise from 65 to 95°C to ensure that a single product was amplified in the reaction. Data are calculated using $2^{-\Delta\Delta CT}$ method [37]. The oligonucleotide primers for IL-8 and GAPDH were as follows: IL-8 (289 bp): 5'-ATGACTTCCAAGCTGGCCGTGGCT-3' (sense), 5'-TCTCAGCCCTCTTCAAAAACTTCTC-3' (antisense); GAPDH (450 bp): 5'-ACCACAGTCCATGCCATCAC-3' (sense), 5'-TCCACCACCCTGTTGCTGTA-3' (antisense). All primers were verified by BLAST (Basic Local Alignment Search Tool; in the public domain, http://blast.ncbi.nlm.nih.gov/Blast.cgi) search of the National Center for Biotechnology Information database for specificity to the human genes of interest.

ELISA

Chemokine IL-8 was quantified from cell supernatants using ELISA [21, 23, 24]. Briefly, cell culture supernatants were collected at the indicated times after treatments and centrifuged to remove cell debris. Total protein concentrations of supernatants were determined by BCA protein assay [38]. A level of IL-8 was assessed using a specific ELISA test kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Gen 5 1.10, BioTek Instruments Inc., Winooski, VT). The minimum detectable levels of IL-8 by ELISA were 3.5 pg/ml. The results were expressed in pg*mg^{-1} protein of IL-8.

Ponceau S staining and western blot analysis

The AcMP elution profile was divided into a total of four fraction (AcMP-P1 - AcMP-P4) sets based on their correspondence with either a discrete elution peak by FPLC. The fraction of four peaks were collected, pooled, and concentrated. The protein concentration of each fraction set was measured by BCA protein assay [38]. A total of 30 µg protein from each fraction set was boiled with 4×NuPAGE LDS loading sample buffer (without NuPAGE reducing agent) at 70°C for 10 minutes [39], separated on a 4-20% polyacrylamide gel (Mini-PROTEAN® TGXTM Gels, Bio-Rad, USA) at 200 V at 4°C and then proteins in polyacrylamide gel was transferred to a immun-blot PVDF membrane (Bio-Rad Laboratories, Inc., USA) at 25V overnight at 4°C. To determine the protein bands in four fractions, PVDF membrane was stained with Ponceau S staining (G-Biosciences, St. Louis, MO), according to the manufacturer's instructions. Membrane was further blocked overnight at 4°C in 5% BLOT-Quick Blocker (a modified milk protein; G-Biosciences, St. Louis, MO) diluted in 1X phosphate buffered saline (PBS) with 0.08% Tween 20 (Sigma-Aldrich, Co., St. Louis, MO). Membranes were incubated with primary antibody (Mouse mAb-TLR4; abcam, Cambridge, MA) at a dilution of 1:1000 in PBS-Tween with 1% BLOT-Quick Blocker for overnight at 4°C. The blots were then washed and incubated with goat-anti-mouse IgG (H+L)-HRP-conjugated secondary antibody (Southern Biotech, Alabama, USA) at a dilution of 1:4000 in PBS-Tween with 1% BLOT-Quick Blocker for 2 hours at room temperature. The membrane was then developed with Pierce ECL 2 Western Blotting substrate (Thermo Scientific, Rockford, IL). Immunoblot was imaged in a FOTODYNE FOTO/Analyst Luminary/FX Imaging (FOTODYNE Incorporated, Hartland, WI). Control consisted of membrane incubated with control serum (normal goat serum, 2.5%, Vector Laboratories, Inc., Burlingame, CA), no primary antibody or secondary antibody only.

Statistics

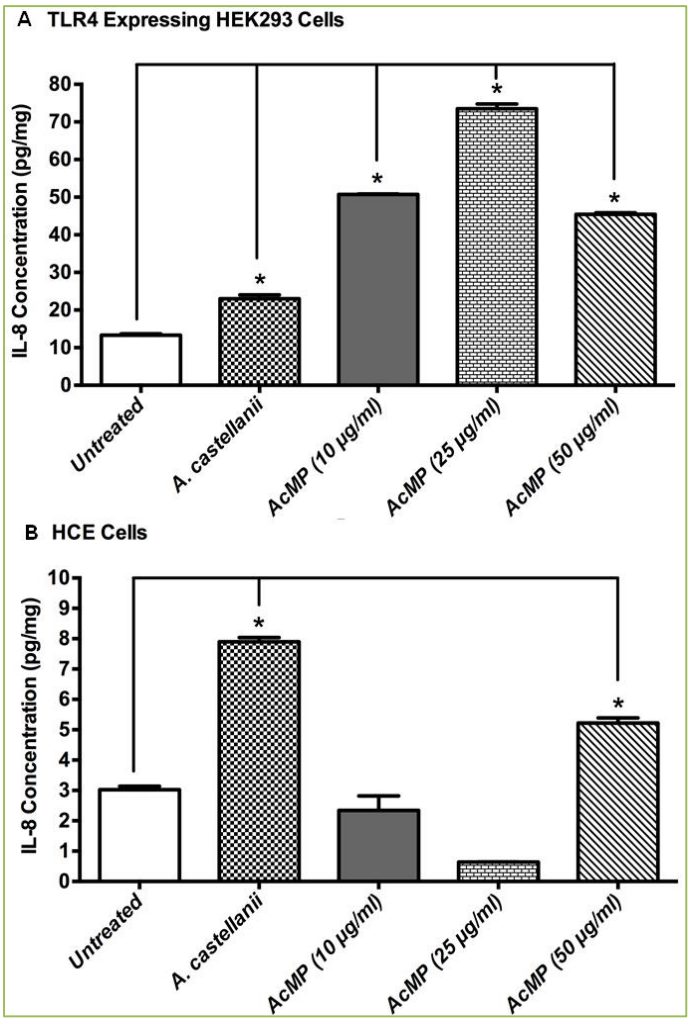

Figure 1. *Acanthamoeba*-membrane protein (AcMP) upregulates IL-8 secretion from TLR4-expressing HEK293 and HCE cells. TLR4-expressing HEK293 **(A)** and HCE cells **(B)** incubated with or without *A. castellanii* (1×10^5 cells/ml) or AcMP (10, 25, and 50 µg/ml) for 24 hours. Cells incubated without treatment served as control (untreated group). Supernatants were collected and IL-8 secretion was quantified by ELISA. The data are mean ± SEM of three independent experiments. *Asterisk (*)* indicates *P* value < 0.05 and was obtained by unpaired Student's *t*-test.

All experiments were performed in triplicate, and results are presented as mean ± SEM. Differences between two groups were determined by unpaired Student's *t*-test. In all analysis, $P<0.05$ was considered statistically significant.

Results

***Acanthamoeba*-membrane protein (AcMP) isolated from *A. castellanii* trophozoites upregulates IL-8 secretion in TLR4-expressing HEK293 and HCE cells**

We have shown that *Acanthamoeba* trophozoites of pathogenic spp. recognize TLR4 on corneal epithelial cells *in vitro* and *in vivo* and induce chemokines production [24]. To

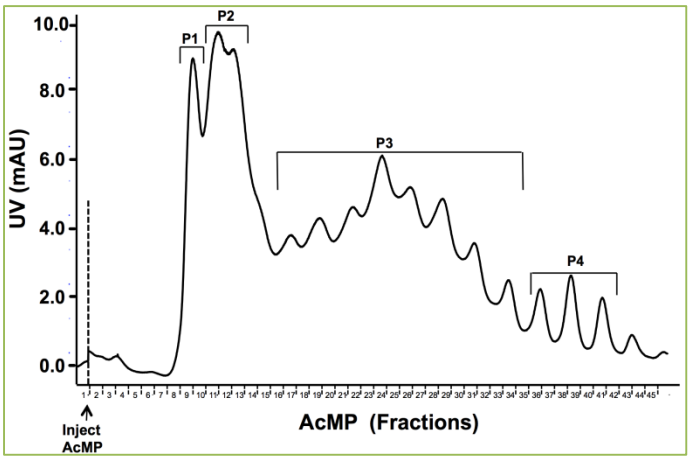

Figure 2. AcMP chromatographed by fast protein liquid chromatography (FPLC). AcMP (10 mg/ml protein) was chromatographed 100 µL with 0.2 ml/min flow rate on a gel filtration (Superdex 200 10/300 GL column). Fractions of 1 ml were collected and effluent was monitored by reading each fraction at 280 nm. Fractions comprising peaks were pooled into four major peaks (AcMP-P1 - AcMP-P4).

determine the components of *Acanthamoeba* trophozoites that interact with TLR4 and stimulate chemokine IL-8 secretion from TLR4-expressing HEK293 and HCE cells, cells were treated with different doses of AcMP and IL-8 production was assessed by ELISA. AcMP stimulated significant upregulation (approximate 3.2- to 5.2-fold change) of IL-8 secretion from HEK293 cells at doses of 10, 25, and 50 µg/ml after 24 hours incubation ($P< 0.05$). AcMP at the dose of 25 µg/ml induced 5.2-fold increase in IL-8 production by HEK293 cells (**Fig. 1A**). However, IL-8 secretion significantly increased only at the dose of 50 µg/ml of AcMP in HCE cells after 24 hours stimulation ($P< 0.05$) (**Fig. 1B**). HEK293 and HCE cells stimulated with *A. castellanii* were used as a positive control. *A. castellanii* induced significant upregulation of IL-8 secretion in HEK293 cells (approximate 1.8-fold change) and from HCE cells (approximate 2.6-fold change) ($P< 0.05$) (**Figs. 1A and 1B**). Cells without treatments served as control. These results indicate that AcMP induced upregulation of IL-8 secretion from TLR4-expressing HEK293 and HCE cells.

Fast protein liquid chromatography (FPLC) chromatographed AcMP comprised four major Peaks

We aimed to isolate *Acanthamoeba* trophozoites' membrane fractions from AcMP. Therefore, AcMP was chromatographed on a Superdex 200 10/300 GL column gel filtration. Four fractions comprising many peaks were collected and pooled into four major peaks: AcMP-P1, AcMP-P2, AcMP-P3, and AcMP-P4 (**Fig. 2**).

FPLC isolated AcMP fractions of peak 2 (AcMP-P2) upregulate IL-8 expression in TLR4-expressing HEK293 cells

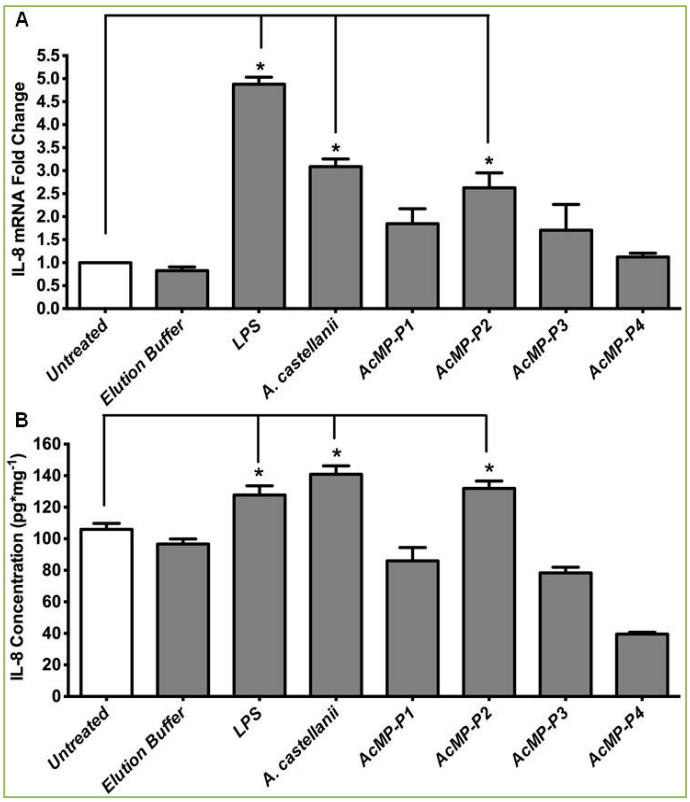

Figure 3. FPLC fractionated AcMP-P2 upregulates IL-8 expression in TLR4-expressing HEK293 cells. TLR4-expressing HEK293 cells incubated with or without *A. castellanii* (1×10^5 cells/ml), LPS (10 µg/ml), and AcMP-P1 – AcMP-P4 (20 µg/ml) for 24 hours. TLR4-expressing HEK293 cells incubated without treatment and elution buffer served as control group. **(A)** Cells were collected for isolation of total RNA, and IL-8 mRNA expression was analyzed by qRT-PCR. **(B)** Supernatants were collected, and IL-8 secretion was quantified by ELISA. The data are mean ± SEM of three independent experiments. *Asterisk (*)* indicates *P* value < 0.05 and was obtained by unpaired Student's *t*-test.

cells

To determine the functional activity of AcMP fractions (AcMP-P1 – AcMP-P4) to induce proinflammatory cytokine IL-8 production, TLR4-expressing HEK293 cells were incubated with or without *A. castellanii*, LPS, and AcMP-P1 – AcMP-P4. Expression of IL-8 mRNA and protein production were detected by qRT-PCR and ELISA. HEK293 cells stimulated with either elution buffer or medium constitutively expressed low levels of IL-8 mRNA and protein production (**Figs. 3A and 3B**). AcMP-P1 – AcMP-P4 induced upregulation of IL-8 mRNA; however, only AcMP-P2 upregulated significant IL-8 mRNA (an approximately 1.6-fold change) in HEK293 cells ($P< 0.05$). Cells stimulated with LPS and *A. castellanii* served as a positive control. IL-8 mRNA expression was significantly increased in HEK293 cells stimulated with either LPS or *A. castellanii* trophozoites (approximately 4- and 2-fold change of IL-8 mRNA, respectively). Treatment with elution buffer

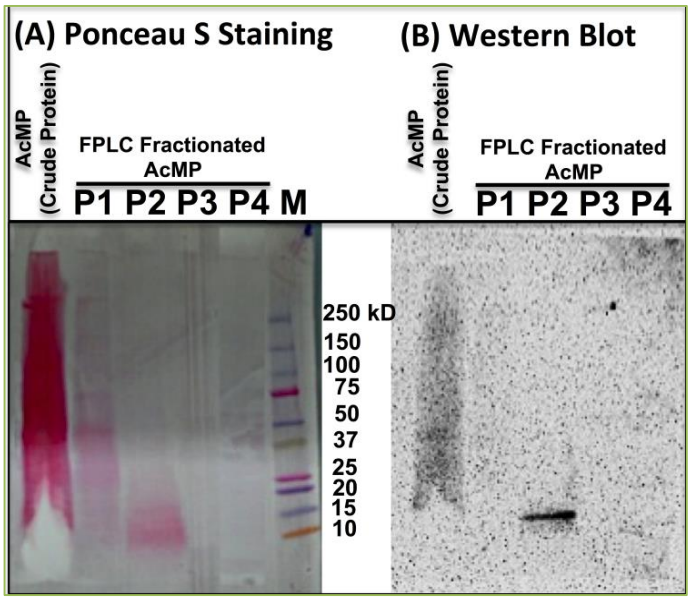

Figure 4. AcMP-P2, but not AcMP-P1, AcMP-P3, or AcMP-P4, has TLR4-reactive protein. 30 µg protein eluted in various AcMP peaks (P1-P4) was analyzed by Ponceau S staining **(A)** and Western Blot **(B)** in non-reducing condition. M = Molecular marker (Precision Plus Protein™ Kaleidoscope™ Standards #161-0375, Bio-Rad).

alone had no significant effect on IL-8 expression in HEK293 cells when compared with controls, which were untreated cells (**Fig. 3A**).

AcMP-P2 was the only fraction that induced significant IL-8 protein production ($P< 0.05$), whereas AcMP-P1, AcMP-P3, and AcMP-P4 did not stimulate IL-8 protein production in HEK293 cells. Nevertheless, these fractions may have an inhibitory effect on IL-8 production. Positive controls, LPS, and *A. castellanii* induced significant IL-8 protein production by HEK293 cells (**Fig. 3B**). These results indicate that AcMP-P2 but not AcMP-P1, AcMP-P3, and AcMP-P4 upregulated IL-8 transcription and protein production by TLR4-expressing HEK293 cells.

AcMP-P2, but not AcMP-P1, AcMP-P3, and AcMP-P4 recognizes TLR4 reactive molecule

To determine TLR4 ligand in *Acanthamoeba* trophozoites membrane protein (AcMP), FPLC fractionated AcMP-P1 - AcMP-P4 were electrophoresed in 4-20% polyacrylamide gels in non-reducing conditions. After electrophoresis, the proteins were analyzed by Ponceau S staining and Western blot analysis. Ponceau S staining on PVDF membrane visualized many protein bands of different molecular mass in AcMP-P1 and AcMP-P2 peaks of FPLC fractioned AcMP; however, Ponceau S staining on PVDF membrane could not detect protein bands in AcMP-P3 and AcMP-P4 peaks of AcMP. While in Western blotting, an approximate 15-kDa

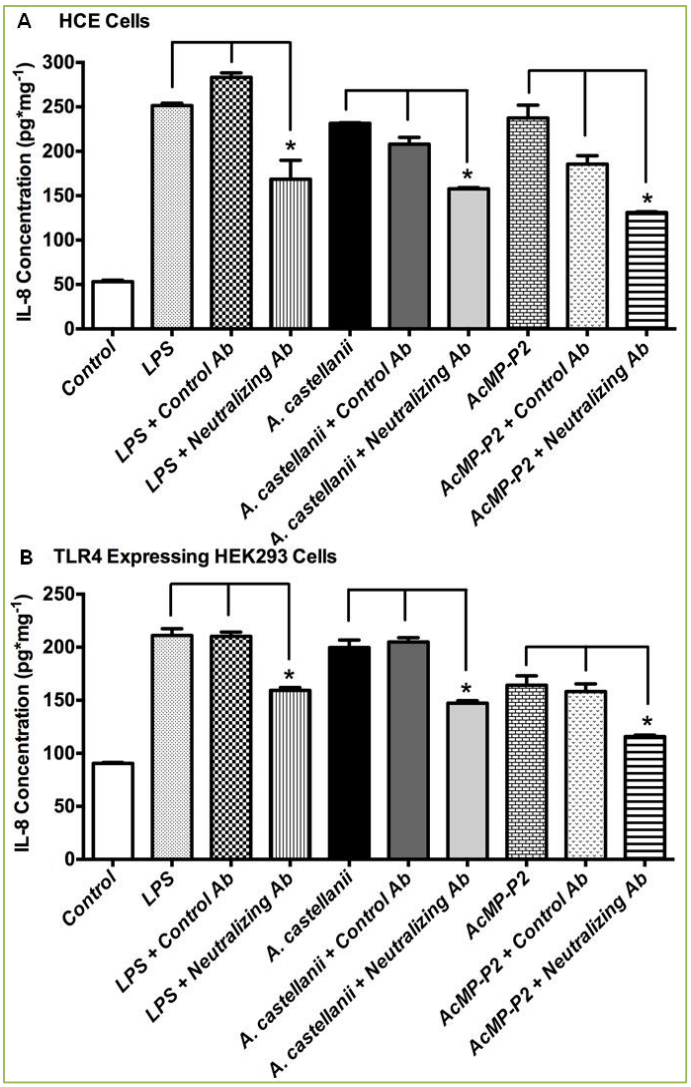

Figure 5. Neutralizing TLR4 antibody diminishes AcMP-P2–induced IL-8 secretion from HCE and TLR4-expressing HEK293 cells. HCE **(A)** and TLR4-expressing HEK293 cells **(B)** were pre-incubated for 1 hour with neutralizing TLR4 antibody (10 µg/ml) of anti-TLR4 affinity purified goat IgG, or with the normal goat serum (10 µg/ml), followed by incubation with or without *A. castellanii* (1×10^5 cells/ml), LPS (10 µg/ml), and AcMP-P2 (10 µg/ml) for 24 hours. Cells incubated without treatment served as control untreated group. Supernatants were collected and IL-8 secretion was quantified by ELISA. Data are mean ± SEM of IL-8 production of three independent experiments. *Asterisk (*)* indicates *P* value<0.05 by unpaired Student's *t*-test.

anti-TLR4 reactive component was detected in AcMP-P2 fraction (**Fig 4**). Control consisted of membrane incubated with control serum, or secondary only (data not shown). These results indicate that AcMP in P2 peak contains an approximate 15-kDa protein that interacts with anti-TLR4 antibody.

Neutralizing TLR4-antibody inhibits AcMP-P2 stimulated IL-8 secretion from TLR4-expressing HEK293 and HCE cells

To determine whether TLR4 is involved in AcMP-P2-induced chemokine release from HCE and TLR4-expressing HEK293 cells, cells were pre-incubated with neutralizing TLR4-monoclonal antibody (anti-TLR4 mAb) and then incubated with or without LPS, *A. castellanii*, and AcMP-P2 fraction for 24 hours. IL-8 production was measured by ELISA. The anti-TLR4 mAb significantly attenuated AcMP-P2 induced IL-8 secretion from both HCE and HEK293 cells ($P< 0.05$) (**Figs. 5A and 5B**). IL-8 secretion was significantly diminished by anti-TLR4 mAb in HCE and HEK293 cells treated with *A. castellanii* and LPS (positive control; $P< 0.05$) (**Figs. 5A and 5B**). Control antibody (Goat serum) was ineffective to diminished AcMP-P2, *A. castellanii*, and LPS induced IL-8 secretion from the cells (**Figs. 5A and 5B**). Taken together, our findings reveal that AcMP-P2 fraction isolated from *Acanthamoeba* trophozoites recognizes TLR4 on HCE and HEK-293 cells. Interaction of TLR4 by AcMP-P2 induces activation of proinflammatory signaling in HCE and TLR4-expressing HEK293 cells.

Discussion

TLRs are pattern recognition receptors (PRRs) that identify specific pathogen-associated molecular patterns (PAMPs) [27]. The host's immune response activates through the recognition of a foreign pathogen by the key receptors of the innate immune system (i.e. TLRs) and induces downstream inflammatory signaling cascade producing proinflammatory mediators to combat the infection [27]. Several groups have shown that TLRs recognize pathogens during ocular infection [26, 27]. Recently, others and we have shown that TLR4 is expressed by HCE cells and is responsible for the recognition of *Acanthamoeba* trophozoites [24, 40, 41]. In this study, we aimed to determine the components of *Acanthamoeba* trophozoites that interact with TLR4 on corneal epithelial cells and trigger chemokines production. The present study is the first to identify and partially characterize *A. castellanii* TLR4 reactive molecule and the involvement of this molecule in activation of proinflammatory chemokine in corneal epithelial cells.

Previous findings showed that while several TLRs, including TLR1, TLR2, TLR3, TLR4, TLR5, and TLR9, are expressed constitutively, only TLR4 is involved in *Acanthamoeba* recognition on corneal epithelial cells [24, 40, 41]. *Acanthamoeba* spp. are extracellular pathogens that attach to corneal epithelial cells [24]. *Acanthamoeba* spp. do not become intracellular and do not have flagella, which makes it impossible for the amoebae to be recognized by TLR3 and TLR5. It has been demonstrated that double-stranded RNA and bacterial flagellin activate TLR3 and TLR5, respectively [42-46]. Attachment of *Acanthamoeba* to the corneal epithelial cells is the first step in pathogenesis of AK. Recognition of TLR4 by *Acanthamoeba* trophozoites prompted us to determine the components of membrane molecules of *Acanthamoeba* trophozoites that recognize TLR4. We isolated *Acanthamoeba* membrane proteins (AcMP) from *Acanthamoeba* trophozoites and showed that *Acanthamoeba* membrane proteins contain molecules that recognize TLR4 and induce IL-8 secretion from TLR4-expressing HEK293 cells and HCE cells. These results are in agreement with our previous studies showing that interaction of *Acanthamoeba* with the corneal epithelial cells persuades a rapid immune response by IL-8 secretion that can initiate efficient host inflammatory responses to combat corneal infections [24].

Furthermore, isolation of TLR4 reactive molecule is achieved by size exclusion chromatography. Four fractions were identified by chromatography. Functional analysis of AcMP-P1, AcMP-P2, AcMP-P3, and AcMP-P4 revealed that only AcMP-P2 stimulates IL-8 secretion from TLR4-expressing HEK293 cells. AcMP-P2 stimulation of IL-8 secretion was similar to the stimulation of LPS and *A. castellanii* trophozoites. In addition, analysis of four peaks by Western blot for the presence of TLR4 reactive molecule showed that AcMP-P2 contains an approximate 15-kDa anti-TLR4 reactive component that induced IL-8 production in TLR4-expressing HEK293 cells. In contrast, the AcMP-P1, AcMP-P3, and AcMP-P4 fractions did not contain detectable levels of TLR4 reactive molecule. There were several peaks in these fractions; however, immunoreaction to TLR4 antigen was not detected by Western blot in these fractions. The lack of TLR4 reactive molecule in these three peaks was subsequently confirmed through their deficiency in inducing IL-8 transcript and secretion in TLR4-expressing HEK293 cells.

Previous studies showed that neutralizing TLR4 antibody inhibits the interaction of *Acanthamoeba* trophozoites in corneal epithelial cells and diminishes IL-8 secretion [24, 40]. In the present study, we determined whether the neutralizing TLR4 antibody has the capacity to inhibit AcMP-P2-stimulated IL-8 secretion from HCE and TLR4-expressing HEK293 cells. Pretreatment of HCE and TLR4-expressing HEK293 cells with neutralizing TLR4 antibody diminished IL-8 secretion induced after stimulation with AcMP-P2. Neutralizing TLR4 antibody did not bring the IL-8 secretion level down to a basal level in HCE and TLR4-expressing HEK293 cells stimulated with AcMP-P2. These findings indicate that either other TLRs are involved in AcMP-P2 recognition and stimulation of IL-8 secretion, or there is an insufficient antibody to neutralize TLR4. Given that neutralizing TLR4 antibody did not fully inhibit IL-8

secretion in the TLR4 transfected HEK293 cell line, we believe the latter to be the correct rationale for our outcomes.

These findings are in agreement with those of Ren *et al*. [40] who found TLR4 was the main receptor that upregulated in HCE cells and induced upregulation of IL-8 and other cytokines after *Acanthamoeba* challenge. It is also possible that AcMP-P2 induced proinflammatory chemokines production by HCE cells through other mechanisms. More recently, we have shown that *Acanthamoeba* plasminogen activator stimulates proinflammatory cytokine in HCE cells via the protease-activated receptors (PARs) pathway [23]. Experiments are under way to fully characterize AcMP-P2 fraction, analyze for amino acid composition of AcMP-P2, and sequence the AcMP-P2 molecule. Moreover, our data indicate that AcMP-P2 is not an LPS-like molecule, as it has an approximate 15-kDa molecular mass, which is much smaller than the standard LPS molecular weight (when the LPS is treated with sodium dodecyl sulfate (SDS) and heat, the molecular mass is approximately 50-100 kDa) [47]. However, LPS has not been reported in *Acanthamoeba* cell membrane components. It has been shown that the filarial nematode *Acanthocheilonema viteae* produces a molecule containing glycoprotein (ES-62) that is recognized by TLR4 and induces proinflammatory responses in mouse macrophages, dendritic cells, and mast cells [33, 48].

TLRs-mediated innate immune responses are essential for reducing *Acanthamoeba* infection in the eye. However, TLRs-mediated host inflammatory responses may also contribute to corneal destruction. Thus, an over-exuberant inflammatory response might damage the cornea even as it clears the trophozoites. In this context, it is interesting to find a mechanism for limiting the TLRs-triggered excessive inflammation, which might be a way to develop potential new therapies to treat sepsis and other inflammatory disorders. The ability of AcMP-P2 to modulate TLR4 may have its therapeutic potential in septic shock caused by bacteria.

In summary, our data indicate that *A. castellanii* trophozoites contain a molecule with approximately 15-kDa that recognizes TLR4 on corneal epithelial cells and induces TLR4-mediated downstream signaling of proinflammatory chemokines production from corneal epithelial cells. AcMP-P2 may be a potential target in the development of novel treatment strategies in *Acanthamoeba* and other microbial infection, which trigger TLR4 in corneal epithelial cells.

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Conflicts of Interest

The authors have no conflicts of interest to disclose.

Author Contributions

Conceived and designed the experiments: HA TT. Performed the experiments: TT MA. Analyzed the data: TT HA. Contributed reagents/ materials/analysis tools: HA. Wrote the paper: TT HA.

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