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Klebsiella pneumoniae induces an inflammatory response in human retinal-pigmented epithelial cells

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ABSTRACT

Purpose: The retinal pigment epithelium (RPE) is a barrier to *Klebsiella pneumoniae* infection in endogenous endophthalmitis. Nevertheless, the inflammatory response of RPE cells upon interaction with this pathogen has not been studied. Here we tested the hypothesis that *K. pneumoniae* induces an inflammatory response in human retinal epithelial cells.
Methods: In this study we set out to investigate the effects of whole *K. pneumoniae* and of its lipopolysaccharide on RPE cells *in vitro* using bacterial invasion and cytotoxicity assays, fluorescent microscopy and ELISA. For that, we utilized *K. pneumoniae* strain ATCC 43816 and the continuous human retinal-pigmented epithelial cell line ARPE-19.
Results: Stimulation of ARPE-19 with live *K. pneumoniae* for 24 h induced a 31.5-fold ($p = 0.0132$) increase in IL-6 and 6.5-fold ($p = 0.0004$) increase in MCP-1 levels compared to the non-infected control cells. Purified *K. pneumoniae* LPS (1 $\mu g\ ml^{-1}$) also induced cytokine levels, MCP-1 (1.7-fold upregulation; $p = 0.0006$) and IL-6 (1.3-fold upregulation, $p = 0.065$). The tested *K. pneumoniae* strain ATCC 43816 did not have a significant effect on the viability of ARPE-19 cells (11% decrease, $p = 0.096$) and showed a low ability to invade the cells.
Conclusions: Both whole live *K. pneumoniae* and *K. pneumoniae* LPS exert a strong pro-inflammatory effect on retinal pigmented epithelial cells, consistent with clinical manifestations of disease. Bacterial pro-inflammatory effects are not likely related to host cell invasion. This is the first investigation of the interactions of a major endogenous endophthalmitis pathogen, *K. pneumoniae* with human retinal pigmented epithelial cells.

1. Introduction

Bacterial endophthalmitis is an infection of the posterior segment of the eye. Despite aggressive therapeutic and surgical treatment endophthalmitis frequently results in partial or complete loss of vision [1]. There are three common ways for bacterial infectious agents to gain access into the eye: postoperative, posttraumatic or endogenous.

Endogenous endophthalmitis is a vital ocular emergency, as a result of a hematogenous spread from a remote primary site of infection. This form of endophthalmitis accounts for 2–8% of all endophthalmitis cases [2] and is often found in immunocompromised patients. Diabetes is the most common underlying condition, followed by chronic obstructive airway disease and end-stage renal disease [3].

Infections with Gram-negative bacteria are of imminent concern as they are more difficult to treat and visual outcome is poor. Gram-negative bacteria are the cause for 32–36% of bacterial endophthalmitis in North America and for 70% in East Asia. Among these cases, *Klebsiella* spp. is the most common etiologic agent followed by *Escherichia coli* [2,4]. K1 and K2 serotype are the 2 most common serotypes cultured from patients with *Klebsiella pneumoniae* endophthalmitis [5]. In addition, diabetes mellitus is a common risk factor for *K. pneumoniae* endophthalmitis patients [6].

The retinal pigment epithelium consists of a single layer of cells originating from neural ectoderm. It is embedded between the photoreceptors of the neural retina and the choroid, the tissue layer separating the retina and the sclera and containing blood vessels nourishing the retina. The main functions of RPE are phagocytosis of photoreceptor segments, transport of nutrients from the choroid into the retina and waste into the opposite direction and absorption of light, while serving as an adhesion surface for the

Abbreviations: RPE, retinal pigment epithelium; MCP-1, monocyte chemotactic protein-1; LPS, lipopolysaccharide.

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retina [7]. RPE cells also have an important role in different pathologic processes of the retina such as age-related macular degeneration, diabetic retinopathy or inherited pathologies [8–11]. Further, as a part of the blood-retinal barrier, RPE cells are also a target for hematogenous pathogens.

Gram-negative bacteria and their components such as lipopolysaccharide (LPS) carry pathogen-associated molecular patterns. Recognition of LPS leads to the rapid activation of intracellular signaling pathways, resulting in the release of pro-inflammatory mediators in mammalian phagocytic cells [12]. Recently, RPE cells have also been described as important cells in the immune system. They are able to secrete pro-inflammatory and anti-inflammatory cytokines and act as antigen-presenting cells [13].

Monocyte chemotactic protein-1 (MCP-1), a member of the C–C subfamily of chemokines is a potent chemoattractant inducing the infiltration of monocytes and macrophages into tissues [14]. IL-6 is a pro-inflammatory cytokine with a wide spectrum of activities activated during priming of the eye immune response to infection [15]. While IL-6 has been shown as a critical mediator of survival following *K. pneumoniae* infection and sepsis [16], the inflammatory response of retinal epithelium after interaction with this pathogen has not been studied.

Host-bacteria interactions are the key to understanding the pathology of endogenous endophthalmitis. In recognition of the importance of retinal pigmented epithelial cells as a barrier to endogenous infectious agents in human endophthalmitis, in this study we investigated the effects of *K. pneumoniae* and its lipopolysaccharide on RPE cells *in vitro*.

2. Materials and methods

2.1. Retinal-pigmented epithelium cell culture

Human retinal-pigmented epithelial cells (ARPE-19; CRL-2302) were purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM/F12 medium (ATCC) supplemented with 10% fetal bovine serum (FBS, Thermo Scientific HyClone, South Logan, UT). ARPE-19 cells are well characterized and have been used extensively in various studies to reduce heterogeneity that is often present in primary cultures. They are diploid non-transformed cells and show typical properties of differentiated RPE *in vivo* [17].

2.2. Preparation of K. pneumoniae

K. pneumoniae strain ATCC 43816 serotype 2 culture (ATCC, Matanzas, VA) was grown in Luria–Bertani (LB) medium (Difco, Sparks, MD) for 14 h at 37 °C. The culture was centrifuged at 3000× rpm for 10 min and the supernatant discarded. The bacterial pellet was washed with phosphate-buffered-saline (PBS, Gibco, Invitrogen, Carlsbad, CA) and serially diluted to the desired concentration. The density of bacteria was measured by enumerating the number of CFU on LB agar plates (Difco). As control, *E. coli* strain K12 (Invivogen, San Diego, CA) was used.

2.3. ARPE-19 infection with whole live K. pneumoniae

ARPE-19 cells were grown until confluency in 6-well plates (Corning, Corning, NY). FBS-containing medium was removed and serum-free medium added 4 h before infection with *K. pneumoniae* at a multiplicity of infection (MOI) of 1:100. Supernatants were removed 2 h after infection; cells were washed three times with PBS and re-incubated with DMEM/F12 without FBS for 24 h. *K. pneumoniae* – free ARPE-19 cells served as non-infected controls. Supernatants were collected, centrifuged for 10 min at 3000 rpm/4 °C, and stored at −20 °C until analyzed.

2.4. Determination of cytotoxicity

To determine the viability of ARPE-19 cells following infection with live *K. pneumoniae*, a colorimetric MTT assay (ATCC) was used [18]. Cells were cultured in 96-well plates at a density of 2×10^4 cells/well and infected with *K. pneumoniae* at a MOI of 1:100. After infection, cultures were incubated with 0.5 mg/ml MTT solution for 4 h at 37 °C and then solubilized with detergent reagent. The optical density of the samples was measured at 570 nm. Data are expressed as mean percentage of cell survival (±SD).

2.5. Stimulation of ARPE-19 with K. pneumoniae or E. coli lipopolysaccharide

Confluent ARPE-19 cultures were serum-starved for 4 h prior to stimulation and treated for 24 h with 2 different doses (1 and 0.01 μg ml^{-1}) of *K. pneumoniae* LPS (Sigma–Aldrich, St. Louis, MO) or control *E. coli* LPS (Invivogen) in serum-free DMEM/F12. Supernatants were tested for release of pro-inflammatory cytokines MCP-1 and IL-6.

2.6. Quantitation of MCP-1 and IL-6

Levels of MCP-1 and IL-6 were quantified in cell culture supernatants by enzyme-linked immunosorbent assay. Commercially available kits (MCP-1 and IL-6, Bender MedSystems, Vienna, Austria) were used according to the manufacturer's protocol.

2.7. Antibiotic protection assay

To explore whether *K. pneumoniae* invades RPE cells, a gentamicin protection assay was used. Bacterial suspension (10^7 cells) was co-incubated with cultured ARPE-19 cells for 2 h at MOI of 100, ARPE-19 monolayers were washed three times with PBS (Gibco, Invitrogen) and then incubated for another 2 h with DMEM/F12 medium containing gentamicin (200 μg/ml, Gibco, Invitrogen) to kill extracellular bacteria. Epithelial monolayers were washed three times with PBS, cells were lysed with sterile H_2O and lysates were plated on LB agar for enumeration of viable counts of intracellular bacteria. In control experiments, gentamicin kills all bacteria during a 2 h incubation in absence of host cells. For inhibition of invasion, ARPE-19 cells were pre-treated with 2 μM cytochalasin D (dimethyl sulfoxide solution), an established actin microfilament inhibitor. Results are expressed as colony forming units (CFU) per monolayer.

2.8. Fluorescent labeling and incubation of K. pneumoniae with ARPE-19 cells

To detect internalization of bacteria in ARPE-19, fluorescent microscopy was utilized. *K. pneumoniae* was stained with BacLight Green (Invitrogen, Carlsbad, CA) as described [19]. The infection was set up as in the antibiotic protection assay described above. After the treatment with gentamicin, the cells were fixed and permeabilized [19]. The nuclei were stained with 1 μg ml^{-1} DAPI (Invitrogen) for 15 min at room temperature and the slides were visualized on fluorescent microscope (Nikon Eclipse Ti) at 470 nm excitation wavelength.

2.9. Statistical analysis

All conditions in each experiment were repeated in triplicate wells, and each experiment was performed at least three times. Data were analyzed by Student's *t*-test and GrapPad Prism software (Version 5.0a, La Jolla, CA). Differences are considered statistically significant at a value of $p < 0.05$. Data are expressed as mean ± SD.

3. Results

3.1. Whole live K. pneumoniae induces release of pro-inflammatory IL-6 and MCP-1 by ARPE-19 cells

Stimulation of ARPE-19 with live *K. pneumoniae* for 24 h induced a 31.5-fold (control: 13.64 ± 10.07 pg ml^{-1}; *K. pneumoniae* live: 429.5 ± 169.5 pg ml^{-1}; *p*-value: 0.0132) increase in IL-6 (Fig. 1, panel A). In similar treatment, live *K. pneumoniae* induced a 6.5-fold increase (control: 818.7 ± 73.67 pg ml^{-1}; *K. pneumoniae* live: 5286 ± 721.3 pg ml^{-1}; *p*-value 0.0004) in MCP-1 levels compared to the non-infected controls (Fig. 1, panel B). In all cell culture supernatants of non-infected ARPE-19 cells a basal secretion of both cytokines could be detected (Fig. 1, A and B).

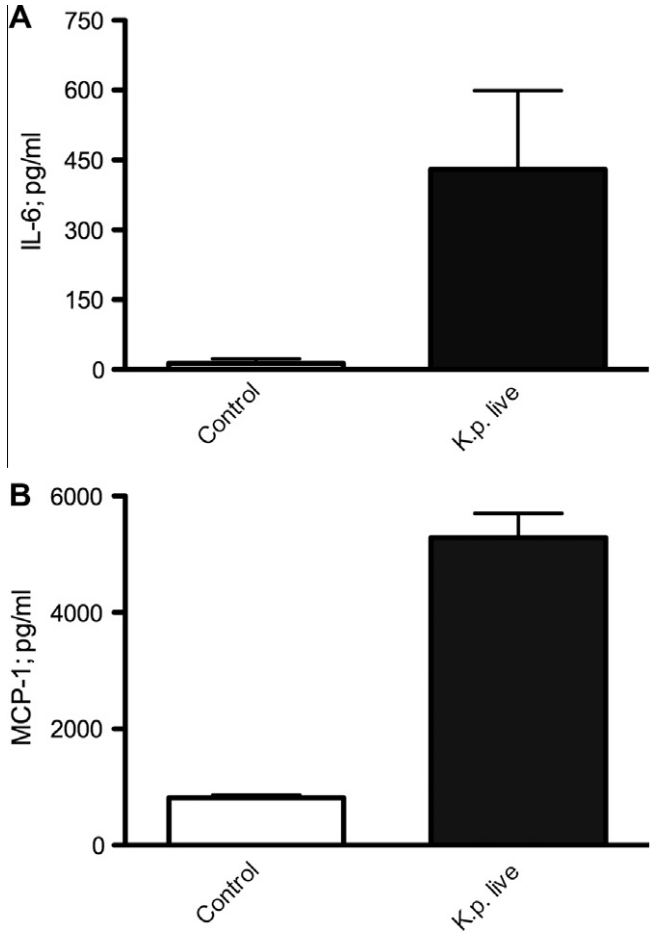

Fig. 1. *K. pneumoniae*-mediated cytokine induction in RPE cells. Proinflammatory cytokine expression in ARPE-19 cells induced upon treatment with live *K. pneumoniae*. Human retinal epithelial cells were exposed to bacteria at MOI of 1:100. Cytokine secretion in culture supernatants was quantified 24 h postinfection as described in the Methods section. Panel A: Effect of treatment on IL-6 secretion; panel B: effect of treatment on MCP-1 release in cell culture medium. Data represent the mean ± SD of cytokine levels.

3.2. K. pneumoniae lipopolysaccharide induces release of IL-6 and MCP-1 by ARPE-19 cells

K. pneumoniae LPS alone was also able to induce the release of IL-6 and MCP-1 in a dose-dependent manner.

MCP-1 secretion was statistically significantly upregulated (1.7-fold) by treatment with 1 μg ml^{-1} *K. pneumoniae* LPS (control: 751.8 ± 200.3 pg ml^{-1}; *K. pneumoniae* LPS: 1298 ± 102.1 pg ml^{-1}; $p = 0.0006$), while 0.01 μg.ml^{-1} *K. pneumoniae* LPS did not induce significant up-regulation (*K. pneumoniae* LPS: 863.2 ± 198.1 pg ml^{-1}; $p = 0.433$) (Fig. 2, panel A).

Control *E. coli* LPS significantly induced a 4.4-fold up-regulation of MCP-1 when compared to the untreated control at a concentration of 1 μg ml^{-1} (control: 893.3 ± 64.01 pg ml^{-1}; *E. coli* LPS: 3941 ± 660.7 pg ml^{-1}, $p = 0.0006$) and a 4.3-fold increase at a concentration of 0.01 μg ml^{-1} (*E. coli* LPS: 3854 ± 238.7 pg ml^{-1}), which also proved to be statistically significant ($p = 0.0002$) (Fig. 2, panel A).

Secretion of IL-6 was increased upon incubation with 1 μg ml^{-1} *K. pneumoniae* LPS (control: 20.72 ± 0.745 pg ml^{-1}; *K. pneumoniae*

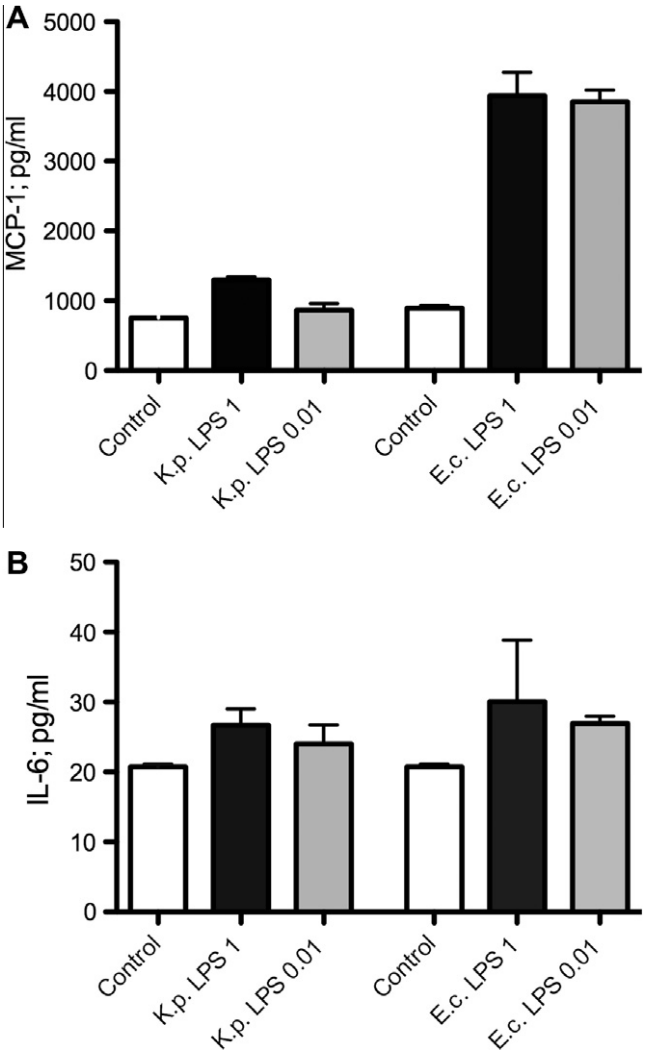

Fig. 2. *K. pneumoniae* LPS-mediated cytokine induction in RPE cells. Proinflammatory cytokine levels in ARPE-19 cells induced upon stimulation with *K. pneumoniae* and *E. coli* LPS. Cytokine secretion in cell culture supernatants was quantified 24 h later as described in the Methods section. Panel A: effect of treatment with *K. pneumoniae* and *E. coli* LPS on MCP-1 secretion; panel B: effect of treatment with *K. pneumoniae* and *E. coli* LPS on IL-6 release in the medium. Data are shown as mean ± SD of cytokine levels.

LPS: 26.69 ± 4.027 pg ml^{-1}; $p = 0.065$) and with 0.01 μg ml^{-1} *K. pneumoniae* LPS (*K. pneumoniae* LPS: 24.04 ± 4.694; $p = 0.293$) (Fig. 2, panel B).

Stimulation with control *E. coli* LPS also stimulated the secretion of IL-6. (control: 20.72 ± 0.745 pg ml^{-1}; *E. coli* LPS 1 μg ml^{-1}: 30.06 ± 12.42 pg ml^{-1}; $p = 0.25$; *E. coli* LPS 0.01 μg ml^{-1}: 26.94 ± 1.45 pg ml^{-1}; $p = 0.007$) (Fig. 2, panel B).

3.3. Invasion assays demonstrate internalization of 1.5×10^2 *K. pneumoniae ATCC 43816 per* 10^5 *RPE cells*

The results of the gentamicin protection assays showed that $1.5 \times 10^2 \pm 0.143 \times 10^2$ CFU per well of *K. pneumoniae* were internalized by ARPE-19 cells. ARPE-19 cells pretreated with cytochalasin D were invaded by $0.98 \times 10^2 \pm 0.095 \times 10^2$ CFU/well, a 35% decrease of the number of internalized bacteria, as expected ($p = 0.0033$). The assays were done in triplicate, in three independent experiments.

In addition, to detect internalization of bacteria in ARPE-19 cells, fluorescent microscopy was utilized. RPE cells were incubated with *K. pneumoniae*, followed by gentamicin treatment. Bacteria were stained with BacLight Green and nuclei were stained with DAPI. There were no visible internalized *K. pneumoniae* bacteria in ARPE-19 cells, judging from several images taken in this experiment (not shown) thus confirming the low invasive ability of *K. pneumoniae* ATCC 43816 in retinal pigment epithelial cells.

3.4. K. pneumoniae has low cytotoxicity towards ARPE-19

Using an MTT assay, we determined that *K. pneumoniae* did not have a significant adverse effect on the viability of ARPE-19. Specifically, MTT assay absorbance values (mean ± SD) were as follows: non-infected control cells: 0.21 ± 0.02; live *K. pneumoniae*-infected cells: 0.19 ± 0.02. 11% viability decrease, *p*-value: 0.096 (Fig. 3).

4. Discussion

K. pneumoniae is the most common Gram-negative bacterium causing endogenous endophthalmitis. The resulting inflammation may progress within days and lead to decreased vision, loss of light perception or enucleation despite systemic antibiotic treatment or surgical interventions such as intravitreal antibiotic injections or vitrectomy [2,20]. Recurrent endogenous endophthalmitis due to *K. pneumoniae* has also been reported [21]. In the present study, using an *in vitro* model of *K. pneumoniae* infection of retinal-pigmented epithelial cells, we tested the hypothesis that *K. pneumoniae* induces an inflammatory response in these barrier epithelial cells. Our goal was to investigate whether the interactions between *K. pneumoniae* and RPE cells contribute to the inflammation clinically observed in endogenous endophthalmitis.

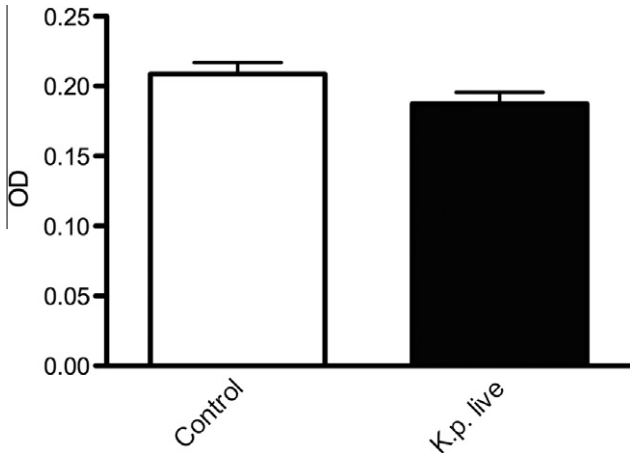

Fig. 3. *K. pneumoniae* cytotoxicity. Cells were cultured in 96-well plates and infected with *K. pneumoniae* at a MOI of 1:100. After infection, cultures were incubated with 0.5 mg ml^{-1} MTT solution for 4 h at 37 °C, solubilized and the optical density of the samples was measured at 570 nm. The figure shows decreased formazan absorbance at 570 nm (OD values) in cells infected with *K. pneumoniae* due to decreasing number of live ARPE-19.

Due to their location retinal epithelial cells are part of the defense against bacterial infection and potentially encounter hematogenous bacterial pathogens. One such pathogen, *K. pneumoniae* often originates from liver abscess [22]. There is only a limited body of evidence that bacterial pathogens elicit inflammatory responses at this specific retinal cell layer. In an experimental *in vivo* animal study *Bacillus cereus*-induced endophthalmitis caused increased production of pro-inflammatory cytokines such as IL-6 [15]. Petropoulos et al. showed in another *in vivo* animal study that *Staphylococcus epidermidis* induced-endophtalmitis resulted in an overexpression of TNF-α, IL-1β, and IFN-γ in the vitreous [23].

In this study, we found that both live *K. pneumoniae* and *K. pneumoniae* LPS induce IL-6 and MCP-1 in ARPE-19 cells. The level of induction of pro-inflammatory cytokines in RPE cells suggests a role of this cell type in the clinical presentation of bacterial endophthalmitis. Although it is challenging to directly compare a certain LPS concentration to a specific MOI of infection with live bacteria, it was interesting to see that the IL-6 and MCP-1 levels reached upon treatment with live bacteria are ~16-fold and ~4-fold higher than the levels induced by *K. pneumoniae* LPS, respectively. The higher level of induction by live bacteria in comparison to LPS suggests that there may be other pathogen-associated molecular patterns involved. These might be recognized by the pattern recognition receptors of retinal epithelial cells and lead to triggering of downstream inflammatory responses. Further, the observed superior efficiency of *E. coli* LPS in MCP-1 induction compared to *K. pneumoniae* LPS is likely pathogen-specific, as *E. coli* LPS has been shown to activate chemokine secretion in intestinal epithelial cells much stronger than LPS from other pathogens [24].

The observed cytokine production may contribute to the recruitment and activation of monocytes and other phagocytic cells to the site of infection, resulting in rapid inflammation within days and decreased vision [20]. It has been shown that the inflammatory response is important for *Klebsiella* infections [25].

Not surprisingly, the tested *K. pneumoniae* strain, ATCC 43816 displayed limited invasive ability in RPE cells as bacterial invasion varies among strains. Different bacterial phenotypes exist, with specific sets of virulence factors expressed by the organism that explain different clinical features of bacteremic infection with *K. pneumoniae* [26]. The limited invasion of strain 43816 in epithelial cells demonstrates that host cell invasion in high numbers is not necessarily a prerequisite for inflammation and pathogenicity. In *E. coli*, enterohemorrhagic (EHEC) strains associated with severe disease, such as the LEE locus career strain O157:H7, attach to the epithelial cell surface but also do not invade [27]. Similarly, enteropathogenic *E. coli* (EPEC), the causative agent of severe diarrhea that can lead to mortality in humans and animals, are non-invasive [28]. Testing clinical isolates, it has been observed that some *K. pneumoniae* strains possess higher invasive potential probably due to specific genes, in addition to wide difference in invasion depending on the host cell type [29]. As expected, treatment of the host cells with cytochalasin D, a microfilament disrupting drug, further reduced the invasion numbers by 35%, indicating that actin polymerization is involved in the limited bacterial internalization observed in the absence of inhibition. Similarly, cytotoxic effects have been reported to differ among *K. pneumoniae* strains [30].

Thus, this study sets the stage for future investigations of clinical isolates from endogenous endophthalmitis patients.

This is the first report to describe the responses of human retinal-pigmented epithelial cells to Gram-negative bacterial challenge. Taken together, our results suggest a pro-inflammatory effect of both *K. pneumoniae* and its LPS on human retinal-pigmented epithelium, which warrants further investigations of *K. pneumoniae*-induced endophthalmitis and bacteria-retinal epithelial cells interactions.

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