Involvement of Toll-like receptor 2 on human corneal epithelium during an infection of Pythium insidiosum

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Abstract

Background: Pythium insidiosum, a pathogenic oomycete, is a common causative organism of infectious corneal ulcer. Studying the innate immune response at the ocular surface is important for better understanding of the underlying pathogenesis and host defense against P. insidiosum infection.

Objective: The present study aims to investigate the role of Toll-like receptor (TLR)2 on human corneal epithelial cells (HCECs) in P. insidiosum infection.

Methods: Human embryonic kidney (HEK) cells were stimulated with either P. insidiosum zoospores or hyphae. NF-κB activation was determined by spectrophotometric measurement of secreted embryonic alkaline phosphatase (SEAP) levels. The role of TLR2 in P. insidiosum infection was studied in HCECs and monocyte derived macrophages (MDMs) using anti-TLR2 neutralizing antibody. The expression levels of pro-inflammatory cytokines were determined.

Results: Both P. insidiosum hypha and zoospore stimulated TLR2-dependent NF-κB activation in HEK-Blue™-hTLR2 cells in dose-dependent manner. IL-6 and IL-8, but not IL-1β, were upregulated in HCECs and monocyte derived macrophages (MDMs) using anti-TLR2 neutralizing antibody. The expression levels of pro-inflammatory cytokines were determined.

Conclusion: P. insidiosum was recognized by human TLR2 on HEK cells. The mRNA expression levels of certain cytokines were dependent of TLR2 in P. insidiosum infected MDMs but not HCECs at early stage of infection.

Key words: Toll-like receptor 2, Pythium insidiosum, ocular pythiosis, human corneal epithelium, inflammatory cytokine

Introduction

Pythium insidiosum is an oomycetes fungus belonging to the kingdom Straminipila. This organism can present in two forms including non-septate mycelium and motile biflagellated zoospores. However, it is not a true fungus because its cell wall composed of cellulose and β-glucan but not chitin. P. insidiosum is the causative agent of pythiosis, which mainly occurs in tropical and subtropical areas. The fungus is ubiquitous in soils and aquatic environments. The sexual reproduction is oogamy and develops motile biflagellated zoospore in wet environment. The infection is by direct contact to water containing zoospore. Human pythiosis found mostly in Thailand. There are four different forms of pythiosis which are vascular (59%), ocular (33%), cutaneous/subcutaneous (5%) and disseminated (3%) forms. Non-ocular pythiosis usually occurs in patient with blood dyscrasias such as thalassemia.1,2 Ocular pythiosis, P. insidiosum keratitis, can occur in healthy people resulting in...
visual loss in approximately 80% of ocular infection cases.2,4 The corneal epithelium is outermost part of the eye and constant contact with potentially pathogenic microbes, especially opportunistic fungi that cause fungal keratitis. The role of an immune response at the corneal epithelium is mainly mediated by innate immunity because the cornea is avascular and has limited immune defenses. Pattern recognition receptors (PRRs) are expressed on the corneal epithelium to recognize and response to pathogen associated molecular patterns (PAMPs).3 The responsive mechanisms to invasive pathogens include production of pro-inflammatory cytokines, chemokines and antimicrobial peptides as well as recruitment of innate immune cells to the site of infection.5,6 However, the mechanism by which the host corneal epithelium can successfully defend against invasive P. insidiosum is unknown. Unfortunately, potential virulence factors of P. insidiosum as well as immune response against the fungal infection have not been fully elucidated. Thus, the knowledge of the innate immune response of corneal epithelium to P. insidiosum infection may provide the better understanding of the protection and pathogenesis of P. insidiosum keratitis.

Toll-like receptor (TLR)2 and TLR4 play important roles in fungal recognition and may cause chronic inflammation due to the recruitment of neutrophil to the corneal stoma and resulting in corneal destruction.7 However, in acute phase response, local inflammation may induce anti-fungal immune response. Thus, TLR2 and/or TLR4 may be involved in pro-inflammatory cytokine expressions of human corneal epithelium to P. insidiosum infection leads to neutrophil recruitment for fungal clearance and also promote pathogenesis.

The present study aims to study the involvement of TLR2 and TLR4 in recognition of P. insidiosum zoospore and hypha and to investigate the role of the TLR on human corneal epithelial cells in P. insidiosum infection.

**Methods**

**P. insidiosum strain and preparation of zoospore and hypha**

P. insidiosum was kindly provided by Assoc. Prof. Angkana Chaiprasert from the Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. It was isolated from left eye of a 14 years old female P. insidiosum keratitis patient. The isolate of P. insidiosum was grown on 2% water agar (BD, NJ, USA) plate and inoculated into sterilized water containing grass blades (Axonopus compressus) and further incubated at 37°C for 2 days. The induction of zoospore formation and preparation of hyphae were performed as previously described.8 Briefly, for induction of zoospore formation, water was removed, replaced with induction media and incubated at 37°C for approximately 3 h. The formation of sporangia and zoospores were observed under light microscope. Zoospores were harvested by filtering through sterilized gauze and centrifuged. For preparation of hyphae, zoospores at 106 cells were inoculated into Sabouraud’s dextrose broth (SDB) and incubated at 25°C for 24 h. The harvested zoospores and hyphae were washed with endotoxin-free phosphate buffer saline (PBS). Killed zoospores and hyphae were prepared by adding 0.01% of Sodium Hypochlorite (NaOCl) in endotoxin-free PBS and then washed. After washing, hyphae and zoospores were resuspended in the cell culture medium used in the stimulation experiments. Negative controlled zoospore and hypha (Nc) were also prepared by the same processes for preparation of zoospore and hypha without fungal inoculation. Enumeration of zoospores was performed using hemacytometer.

**HEK-Blue™-hTLRs cell cultivation and stimulation**

HEK-Blue™-hTLR2, hTLR4 and their controls (HEK-Blue™-Null1 and Null2, respectively) (InvivoGen, CA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Life technology, NY, USA) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS), 50 U/ml penicillin, 50 µg/ml streptomycin, and 100 µg/ml normocin™ (InvivoGen).

The HEK-Blue cells were seeded at a density of 2.8 × 104 cells/well of a flat-bottom 96-well plate. The cells were stimulated with hyphae at multiplicity of infection (m.o.i.) 0.02, 0.04, 0.09 and 0.17; zoospores at m.o.i. 0.02, 0.04, 0.07 and 0.36 for 20-24 h. After stimulation, supernatant from stimulated cells was collected for determination of NF-kB activation. Heat killed Listeria monocytogenes (HKLM) and E. coli lipopolysaccharide (LPS) are served as positive control for hTLR2 and hTLR4, respectively. Negative control (Nc) was performed using controlled hyphae or zoospore. For TLR blocking experiment, HEK-Blue cells were pre-incubated with neutralizing antibody or isotype control (InvivoGen) for 1 h before stimulation with the zoospores or hyphae.

**Determination of NF-kB activation**

For determination of NF-kB activation, 20 µl of supernatant collected from stimulated HEK-Blue cells was transferred to a well of 96-well plate containing 180 µl of QUANTI-BlueTM detection medium (InvivoGen). The reaction was incubated for 3 h and secreted embryonic alkaline phosphatase (SEAP) levels were determined using spectrophotometer at absorbance 630 nm.

**Primary human corneal epithelial cell (PHCEC) cultivation**

The limbal tissue was obtained from cadaveric donors provided by the Thai Red Cross Society. This study was approved by Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (Protocol number: 200/2555(EC3)). The PHCECs were cultured from limbal tissue as previously described.22

**Human corneal epithelial cell (HCEC) cultivation**

HCECs (ATCC CRL-11516) were cultured in Keratinocyte serum-free medium (K-SFM) supplemented with 5 ng/ml recombinant human epidermal growth factor, 0.05 mg/ml bovine pituitary extract, 0.005 mg/ml insulin, 500 ng/ml hydrocortisone, 50 U/ml penicillin and 50 µg/ml streptomycin (Invitrogen) in tissue culture flask pre-coated with 0.01 mg/ml bovine serum albumin (BSA) (SIGMA, St. Louis, MO), 0.01 mg/ml fibronectin (Roche, GM, Basel, Switzerland) and 0.03 mg/ml bovine collagen type I (SIGMA) at 37°C in 5% CO2 for at least 2 h.
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**PHCEC and HCEC stimulations**

PHCECs and HCECs at 2.5 × 10^4 cells were grown in supplement-free Keratinocyte Basal Medium-2 (LONZA, Basel, Switzerland) in 96-well plate for 2 days. The cells were stimulated with zoospores at m.o.i. 0.0025 and 0.005 for 3–6 h. Control zoospore and 1×10^6 HKLM were used as negative and positive controls for the cell stimulation, respectively. For TLR2 blocking experiment, HCECs were pre-incubated with neutralizing antibody or isotype control (InvivoGen) for 1 h before stimulation with the zoospores.

**Human monocyte-derived macrophage (MDM) cultivation and stimulation**

Peripheral blood mononuclear cells (PBMCs) were separated from the whole blood of anonymous donor by density gradient centrifugation method using Lymphocyte separation medium (LSM) (Biowest, KC, USA). The experiments were approved by Siriraj Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand (Protocol number: 200/2555(EC3)).

MDMs were prepared as previously describe.31 MDMs at day 9 were used for stimulation experiment. 3 × 10^4 MDMs in 96-well plate were stimulated with live zoospore at m.o.i. of 0.005. Control zoospore and 1 × 10^6 HKLM were used as negative and positive controls for the cell stimulation, respectively. For TLR2 blocking experiment, MDMs were pre-incubated with neutralizing antibody or isotype control (InvivoGen) for 1 h before stimulation with the zoospores.

**RNA extraction and cDNA synthesis**

Total RNA was extracted using the RNeasy-Plus Mini kit (QIAGEN Inc., California, USA). The extracted RNA was used as a template for cDNA synthesis using SuperScript™ III First-Strand Synthesis System kit (Invitrogen).

**Polymerase chain reaction (PCR)**

Reverse transcription PCR (RT-PCR) was used to determine the expression of TLR1-10 genes in PHCEC. The optical density of PCR products was analyzed by agarose gel electrophoresis.33 Quantitative Real time PCR (qRT-PCR) was performed and analyzed using Bio-Rad® CFX96™ (Applied Biosystem, CA, USA) with iTaq™ Universal SYBR® Green Supermix (Bio-Rad®, California, USA). PCR was performed following thermal cycling condition: initial denaturation at 95°C for 1 min, denaturation at 95°C for 10s, annealing at 59°C for 15s and extension at 72°C for 5s. Relative expression was normalized with GAPDH and calculated ddCt. Primers used for qRT-PCR analysis were listed in Table 1.

**Measurement of IL-8 protein expression**

The concentration of human IL-8 was measured using Human IL-8 ELISA MAX™ Standard Sets (BioLegend, San Diego, CA).

**Statistical analysis**

Data were expressed as mean ± SEM of triplicates from 3 independent experiments. Statistical significance of differences between two groups was analyzed by paired-sample t-test depending on the contextual format of information. Equality of means among multiple groups was analyzed by One-Way ANOVA using GraphPad Prism (version 3.00). Differences were considered statistically significant at P < 0.05.

**Results**

**P. insidiosum induced NF-κB activation on HEK-Blue™-hTLR2 cells**

Hyphae and zoospores of *P. insidiosum* significantly induced NF-κB activation on HEK-Blue™-hTLR2 in the dose dependent manner when compared with negative control (HEK-Null1 cell activation) (Figure 1). In contrast, the NF-κB activation was not induced in HEK-Blue™-hTLR4 by both hyphae and zoospores (data not shown).

To determine the requirement of TLR1 or TLR6 in TLR2 recognition, the blockade of TLR1 or TLR6 by specific neutralizing antibodies was performed. The response ratio of HEK-Blue™-hTLR2 cells pre-incubated with anti-TLR2, but not anti-TLR1 and TLR6 neutralizing antibodies, was significantly decreased when compared with the unblocked cells or isotype control (Figure 1). Both hypha and zoospores of *P. insidiosum* are mainly recognized by TLR2/2 homodimers.

**Characterization of TLR1-10 expressions in PHCEC**

TLR gene expression in PHCEC was shown in Table 2. Most patients expressed all of TLR1-10. However, differences in TLR expression were observed in some patients.

**Live *P. insidiosum* induced pro-inflammatory cytokine expression on PHCECs and HCECs at the early stage of infection**

In order to determine the innate immune response at the early stage of infection, zoospore, the infective stage, was used to stimulate PHCECs. The expression levels of pro-inflammatory cytokines (IL-6, IL-8 and IL-1β) were determined. Stimulation with live zoospores at the m.o.i. of 0.0025 significantly induced IL-6 and IL-8 expressions in PHCECs at 6 h. At m.o.i. of 0.005, the IL-6 and IL-8 expressions were significantly upregulated at both 3 and 6 h after stimulation (Figure 2). Live zoospores at m.o.i. of 0.0025 and 0.005 significantly increased IL-8 protein secretion (Figure 2) only at 6 h. In contrast, live zoospores did not significantly induce IL-1β mRNA expression in PHCECs at both 3 h and 6 h (Figure 2). However, stimulation of PHCECs

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**Table 1. Primer sequences used for qRT-PCR analysis, their corresponding melting temperatures (Tm) and sizes of PCR products (base pairs).**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’→3’)</th>
<th>Tm (°C)</th>
<th>PCR product (bp)</th>
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**Figure 1**

Involvement of Toll-like receptor 2 on human corneal epithelium during an infection of Pythium insidiosum
Figure 1. Response ratios of HEK-Blue™-hTLR2 cells to hypha and zoospore at various m.o.i. The HEK-Null1 was used as negative control. Statistical significant difference between the stimulated HEK-Blue™-hTLR2 cells and HEK-Blue™-hTLR2 cells stimulated with its negative control hypha or zoospore (Nc) was determined (upper panel). Blocking stimulated HEK-Blue™-hTLR2 cells with anti-hTLR1, 2, 6 or isotype control (IC) antibody (Ab) is showed in lower panel. HEK-Blue™-hTLR2 cells were pre-incubated with 2.5 μg/ml of neutralizing (anti-hTLR1-IgG, anti-hTLR2-IgA, anti-hTLR6-IgG) or IC (IgG or IgA) Ab for 1 h before stimulation with hypha at m.o.i. of 0.17 or zoospore at m.o.i. of 0.04. Statistical significant difference between TLR-blocking stimulated HEK-Blue™-hTLR2 cells and IC or without antibody was determined. Response ratio was calculated from subtraction of stimulated cell response with unstimulated cell response. These data are the average from three independent experiments (**p < 0.01, ***p < 0.001 and ns is no significant difference). Nc, process control, was prepared using the same process for fungal preparation without P. insidiosum inoculation.
Table 2. TLR gene expressions on PHCECs derived from corneal limbal tissue determined by RT-PCR.

<table>
<thead>
<tr>
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Figure 2. The early inflammatory cytokine production of PHCECs stimulated with live zoospore. The gene expressions of IL-6, IL-8, and IL-1β were determined at m.o.i. of 0.0025 and 0.005 for 3 and 6 h using qRT-PCR. The IL-8 protein secretion was determined using ELISA at 6 h after stimulation. Statistical significant difference of relative gene expression levels (ddC\textsubscript{T}) of the stimulated PHCECs and PHCECs with its negative control zoospore (Nc) was determined. Data are from three independent experiments (*\(p < 0.05\), **\(p < 0.01\), ***\(p < 0.001\), ns is no significant difference and ND is not detected). Nc, process control, was prepared using the same process for fungal preparation without \textit{P. insidiosum} inoculation.
Figure 3. The inflammatory cytokine response of HCEC stimulated with live zoospore (a) at m.o.i. of 0.0025 and 0.005 at 3 h and 6 h. Statistical significant difference of relative gene expression levels (ddC_T) between the zoospore and the negative control zoospore (Nc) stimulated cells were determined. TLR2 neutralized HCECs were stimulated with live zoospore at m.o.i. of 0.005 for 6 h. HCECs were pre-incubated with anti-TLR2 or IC Ab at concentration of 10 μg/ml for 1 h before stimulation with the zoospores (b). Statistical significant difference of relative gene expression levels (ddC_T) between HCECs pre-incubated with anti-TLR2 and IC Ab was determined. The gene expressions of IL-6, IL-8, and IL-1β were determined using qRT-PCR. Data are from three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001, ns is no significant difference). Nc, process control, was prepared using the same process for fungal preparation without P. insidiosum inoculation.
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Figure 4. The cytokine mRNA expressions of MDMs from 5 healthy donors stimulated with $10^8$ HKLM or live zoospores at m.o.i. of 0.005 for 6 h. The data showed the difference of relative gene expression levels ($\Delta\Delta CT$) (a). Relative gene expression level is the gene expression of stimulated MDMs which subtracted with unstimulated MDMs. The IL-1β and IL-6 mRNA (b) and IL-8 protein (c) expressions of the TLR2 neutralized MDMs were determined. MDMs were pre-incubated with anti-TLR2 or IC Ab at concentration of 10 μg/ml for 1 h before stimulation with $10^8$ HKLM or live zoospores at m.o.i. of 0.005 for 6 h. The cytokine mRNA expressions were determined using qRT-PCR. The IL-8 protein secretion from 3 healthy donors was determined using ELISA. Statistical significant difference of relative gene expression levels ($\Delta\Delta CT$) or protein concentration (pg/ml) between MDMs pre-incubated with anti-TLR2 and IC Ab were analyzed. The experiments were performed in three independent experiments (*p < 0.05, **p < 0.01, ns is no significant difference). Nc, process control, was prepared using the same process for fungal preparation without P. insidiosum inoculation.
with killed zoospores or killed hyphae cannot induce any cytokine expressions (data not shown). These results indicated that live zoospore of \( P. \) insidiosum induced IL-6 and IL-8 but not IL-1β mRNA expressions in PHCECs at early stage of infection.

Stimulation of HCECs with live zoospores at the m.o.i. of 0.0025 could significantly induced IL-6 and IL-8 mRNA expressions at 6 h. At m.o.i. of 0.005, IL-6 and IL-8 mRNA expressions were significantly upregulated at both 3 h and 6 h. (Figure 3a). Stimulation of HCECs with live zoospores did not significantly induce IL-1β mRNA expression at 3 h and 6 h (Figure 3b).

In conclusion, similar to PHCECs, live but not killed zoospores (data not shown) of \( P. \) insidiosum induced IL-6 and IL-8 but not IL-1β mRNA expressions on HCECs at early stage of infection. Because of the limitation of primary cell preparation, HCECs were used to determine the role of TLR2 in next experiment.

**Live \( P. \) insidiosum induced pro-inflammatory cytokine expression on HCECs through TLR2-independent pathway**

The blockade of TLR2 was used to determine the role of TLR2 to IL-8 and IL-6 mRNA expressions in HCECs. IL-8 and IL-6 expressions in the TLR2-blocked HCECs were compared with the cells blocked with the isotype control antibody and the unblocked cells. The results showed that the blockade of TLR2 on live zoospore-stimulated HCECs did not inhibit IL-8 and IL-6 mRNA expressions (Figure 3b). These results indicated that TLR2 did not play a major role in the induction of these cytokines at early stage of HCECs in response to live \( P. \) insidiosum infection.

**Live \( P. \) insidiosum induced pro-inflammatory cytokine expression on MDMs**

In order to determine the role of TLR2 in induction of pro-inflammatory cytokine expressions in other cell types, MDMs from 5 healthy individuals were used in this experiment. Stimulation of MDMs with live zoospores induced IL-6 mRNA expression in 4 of 5 donors and induced IL-1β mRNA expression in all donors after 6 h of stimulation (Figure 4c). However, mRNA expression of IL-8 was not upregulated in 4 of 5 donors. These results suggested that live zoospore of \( P. \) insidiosum induces pro-inflammatory cytokine expressions in MDMs at the early stage of infection. However, there are some variations in individual subjects.

**Live \( P. \) insidiosum induced pro-inflammatory cytokine expression on human MDMs through TLR2-dependent pathway**

The TLR2 blockade was done to confirm the role of TLR2 in induction of pro-inflammatory cytokine expressions in MDMs. The results showed that blockade of TLR2 significantly inhibit the expression of IL-1β mRNA after stimulated with live zoospores (Figure 4b). Furthermore, the secretion of IL-8 protein was significantly decreased in TLR2 blockade MDMs. These results indicated that TLR2 involved in the induction of IL-1β mRNA expression and secretion of IL-8 protein in MDMs at the early stage of live \( P. \) insidiosum infection.

**Discussion**

\( P. \) insidiosum is the causative agent of \( P. \) insidiosum keratitis or corneal ulcer.

The innate immunity at the ocular surface, especially in the corneal epithelium, plays an important role in defense against pathogen infection due to their lack of lymphatic drainage and presence of blood ocular barrier. Currently, the responsive mechanisms to invasive pathogens are unclear. However, the mechanisms including productions of inflammatory cytokines, chemokines, antimicrobial peptides as well as recruitment of innate immune cells to the site of infection have been reported. TLR is an important receptor in the innate immune response to fungal infection and initiation of adaptive immunity. TLR1-10 are expressed in human corneal epithelial cells. The TLR-mediated innate immune response of corneal epithelium involves protective immune response through induction of human beta defensin (hBD) expression and promotes local inflammation by induction of pro-inflammatory cytokine expression.

In the present study, we used HEK-Blue®-hTLR2 cells and found that both forms of \( P. \) insidiosum, zoospore and hypha, induced NF-κB activation through TLR2/2 homodimer but not TLR4 (data not shown). Our result correlates to several studies in the TLR2-mediated innate immune response to several fungal infections such as yeast zymosan, \( C. \) albicans, \( A. \) fumigatus.

Fungal cell wall components including mannan, chitin, lipomannan and glucuroxylomannan are ligands of TLR2. To date, the cell wall compositions of \( P. \) insidiosum have not been addressed. In the genus of \( P. \) fumigatus, the hypha wall is comprised of 80-90% of polysaccharide mainly β-glucans and cellulose. The cell wall of the mycelium of \( P. \) aphaniidermatum comprise 18% of cellulose and 82% of (1 → 3), (1 → 6)-β-d-glucans. The β-1, 3-linked, β-1, 4-linked and β-1, 6-linked branching glucan are the cell wall component of \( P. \) debaryanum. In 1993, Cherif M. et al found the presence of chitin in \( P. \) ultimum cell wall. Therefore, the β-glucans and cellulose may be the putative ligands of PRRs. However, the identification of the \( P. \) insidiosum cell wall component and potential PRR ligands should be further studied.

Similar pattern to other live fungi only live \( P. \) insidiosum, but not killed \( P. \) insidiosum (data not shown) significantly induce IL-6, and IL-8 mRNA and IL-8 protein secretion in both human primary and corneal epithelial cell line. Live and killed microorganisms can induce different immune response depending on their virulent factors. Live microorganisms can penetrate into the cell and secrete some effector molecules, which induce inflammation. The unresponsiveness of human corneal epithelium to killed hyphae of \( P. \) insidiosum might be caused by the loss of some virulent factors especially secretory components. The studies in dead bacteria also showed lower inflammatory stimuli compared to live pathogenic bacteria or T3SS expressing bacteria. Jin et al, found that HCECs respond to inactive hyphae of \( P. \) solani by upregulation of TLR2, 3, 4 and 6 as well as release of IL-6 and IL-8. We used killed hyphae at the m.o.i. of 0.1 and this m.o.i. of fungus could not induce pro-inflammatory cytokine expressions on HCECs. Thus, using live zoospores as a model to study the innate immune response to \( P. \) insidiosum is more suitable for understanding the immune response or pathogenesis of \( P. \) insidiosum infection. In the experiments, after live zoospores were inoculated, they generate germ tube and become hypha form. Therefore, the cytokine responses were induced by both zoospore and hypha forms of
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P. insidiosum. In addition, the study of the different components or PAMPs between live and killed P. insidiosum is still required.

The pathogenesis of P. insidiosum keratitis may be resulted from human corneal epithelium response at the early stage of infection. The expression of IL-6 transcript and production of IL-8 protein may involve in acute phase response, recruitment and activation of neutrophils. Neutrophils and other polymorphonuclear leukocytes (PMNs) migrate into infected cornea to eliminate fungal pathogen by releasing the toxic substance leading to corneal destruction. The study of A. fumigatus keratitis in rat model demonstrated that high severe score of cornea is resulted from influx of PMNs, upregulation of TLR2 and TLR4 expressions and over production of pro-inflammatory cytokines. The inhibition of TLR2 can attenuate corneal inflammation and prevent A. fumigatus invasion.

Human macrophage may participate in vascular and (sub) cutaneous pythiosis. The vascular pythiosis patients usually presented with fever, skin ulcer, cellulitis, and chronic arterial inflammation. Human macrophage may facilitate the recruitment of neutrophil or eosinophil and enhance the inflammation as well. In the present study, we provide the first evident that live zoospore of P. insidiosum induces IL-6, IL-1β mRNA and IL-8 protein productions in MDMs at the early stage of infection. The blockade of TLR2 on MDMs significantly decreased IL-1β mRNA expression and IL-8 protein secretion. This finding indicating that P. insidiosum contains TLR2 ligand on its surface and be recognized by MDMs mediated inflammatory cytokine induction.

The TLR2 blockade MDMs stimulated with live zoospore significantly decrease only in the productions of IL-1β mRNA, and IL-8 protein. Other PRRs may be involved in cytokine productions. Furthermore, blockade of TLR2 did not completely, but majorly, inhibit the expressions of those pro-inflammatory cytokines in response to P. insidiosum infections. Therefore, the further studies of P. insidiosum recognition via other PRRs are interested for better understanding in host response to P. insidiosum infection.

This study provides the understanding in human corneal epithelial and macrophage response to P. insidiosum infection by production of inflammatory cytokines at the early stage of infection. The PAMPs or virulence factor found only in live P. insidiosum have the effect on induction of pro-inflammatory cytokine expression in corneal epithelium. Other PRRs such as intracellular nucleotide-binding oligomerization domain (NOD)-like receptors; NOD1 and C-type lectin receptor, which had been reported in other fungal keratitis, may cooperate with TLR2 in the induction of pro-inflammatory cytokine expression and be involved in pathogenesis of P. insidiosum keratitis. In contrast, TLR2 is involved in the induction of cytokines in P. insidiosum infected MDM. The activation of TLR2 on HEK-hTLRs cells leading to NF-κB activation. The induction of TLR2-dependent NF-κB activation by P. insidiosum was dose-dependent. The human corneal epithelium responded to live P. insidiosum infection through the expressions of IL-6, IL-8, but not IL-1β during early stage of infection. However, stimulation of human corneal epithelium with killed P. insidiosum did not induce the expression of the pro-inflammatory cytokines. The expressions of IL-6 and IL-8 in P. insidiosum infected human corneal epithelium were not dependent only on TLR2 recognition. However, the expressions of pro-inflammatory cytokine genes during early stage of P. insidiosum infection may promote the recruitment of PMNs and facilitate local inflammation contributing to pathogenesis of P. insidiosum keratitis. This study is the first report of human corneal epithelium in response to P. insidiosum infection as well as the involvement of TLR2.

References