

# 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- $\kappa$ B 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- $\kappa$ B activation in HEK-Blue™-hTLR2 cells in dose-dependent manner. *IL-6* and *IL-8*, but not *IL-1 $\beta$* , were upregulated in HCECs after stimulation with *P. insidiosum*. Blockade of TLR2 on HCECs altered neither *IL-6* nor *IL-8* expressions. In contrast, the 3 cytokines were upregulated in the stimulated MDMs and the expression levels of *IL-1 $\beta$*  and *IL-8* but not *IL-6* were attenuated in TLR2 blockade MDMs.

**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

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## 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  $\beta$ -glucan but not chitin. *P. insidiosum* is the causative agent of pythiosis, which mainly occurs in tropical and subtropical areas.<sup>1</sup> 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.<sup>1</sup> Non-ocular pythiosis usually occurs in patient with blood dyscrasias such as thalassemia.<sup>1,2</sup> Ocular pythiosis, *P. insidiosum* keratitis, can occur in healthy people resulting in

visual loss in approximately 80% of ocular infection cases.<sup>2-4</sup>

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 respond to pathogen associated molecular patterns (PAMPs).<sup>5</sup> 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.<sup>5,6</sup> 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.<sup>7</sup> 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. Angka-na 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.<sup>8</sup> 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 zoospore were observed under light microscopy. Zoospores were harvested by filtrating through sterilized gauze and centrifuged. For preparation of hyphae, zoospores at 10<sup>6</sup> 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 × 10<sup>4</sup> 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-κB 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-κB activation

For determination of NF-κB 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-Blue™ 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.<sup>32</sup>

### 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% CO<sub>2</sub> for at least 2 h.

### PHCEC and HCEC stimulations

PHCECs and HCECs at  $2.5 \times 10^4$  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  $1 \times 10^8$  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.<sup>31</sup> MDMs at day 9 were used for stimulation experiment.  $3 \times 10^4$  MDMs in 96-well plate were stimulated with live zoospore at m.o.i. of 0.005. Control zoospore and  $1 \times 10^8$  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.<sup>33</sup>

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 ddC<sub>T</sub>. 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  $\pm$  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

**Table 1. Primer sequences used for qRT-PCR analysis, their corresponding melting temperatures (T<sub>m</sub>) and sizes of PCR products (base pairs).**

Gene	Sequence (5' $\rightarrow$ 3')	T <sub>m</sub> (°C)	PCR product (bp)
<i>IL-8_F</i>	GCCAACACAGAAATTATTGTAAAGCTT	56	112
<i>IL-8_R</i>	AATTCTCAGCCCTCTTCAAAAACTT	55	
<i>IL-1<math>\beta</math>_F</i>	TGGAGCAACAAGTGGTGT	56	157
<i>IL-1<math>\beta</math>_R</i>	TTGGGATCTACTCTCCAGC	58	
<i>IL-6_F</i>	CGGGAACGAAAGAGAAGCTCTA	59	68
<i>IL-6_R</i>	GGCGCTTGTGGAGAAGGAG	60	
<i>TNF-<math>\alpha</math>_F</i>	CCCAGGCAGTCAGATCATCTTC	60	75
<i>TNF-<math>\alpha</math>_R</i>	CAGCTTGAGGGTTTGCTACAAC	60	
<i>GAPDH_F</i>	ACAGCCTCAAGATCATCAGCA	59	119
<i>GAPDH_R</i>	GATGGCATGGACTGTGGTCA	60	

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- $\kappa$ B activation on HEK-Blue™-hTLR2 cells

Hyphae and zoospores of *P. insidiosum* significantly induced NF- $\kappa$ B 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- $\kappa$ B 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**). Thus both hypha and zoospore 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-1 $\beta$* ) 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-1 $\beta$*  mRNA expression in PHCECs at both 3 h and 6 h (**Figure 2**). However, stimulation of PHCECs

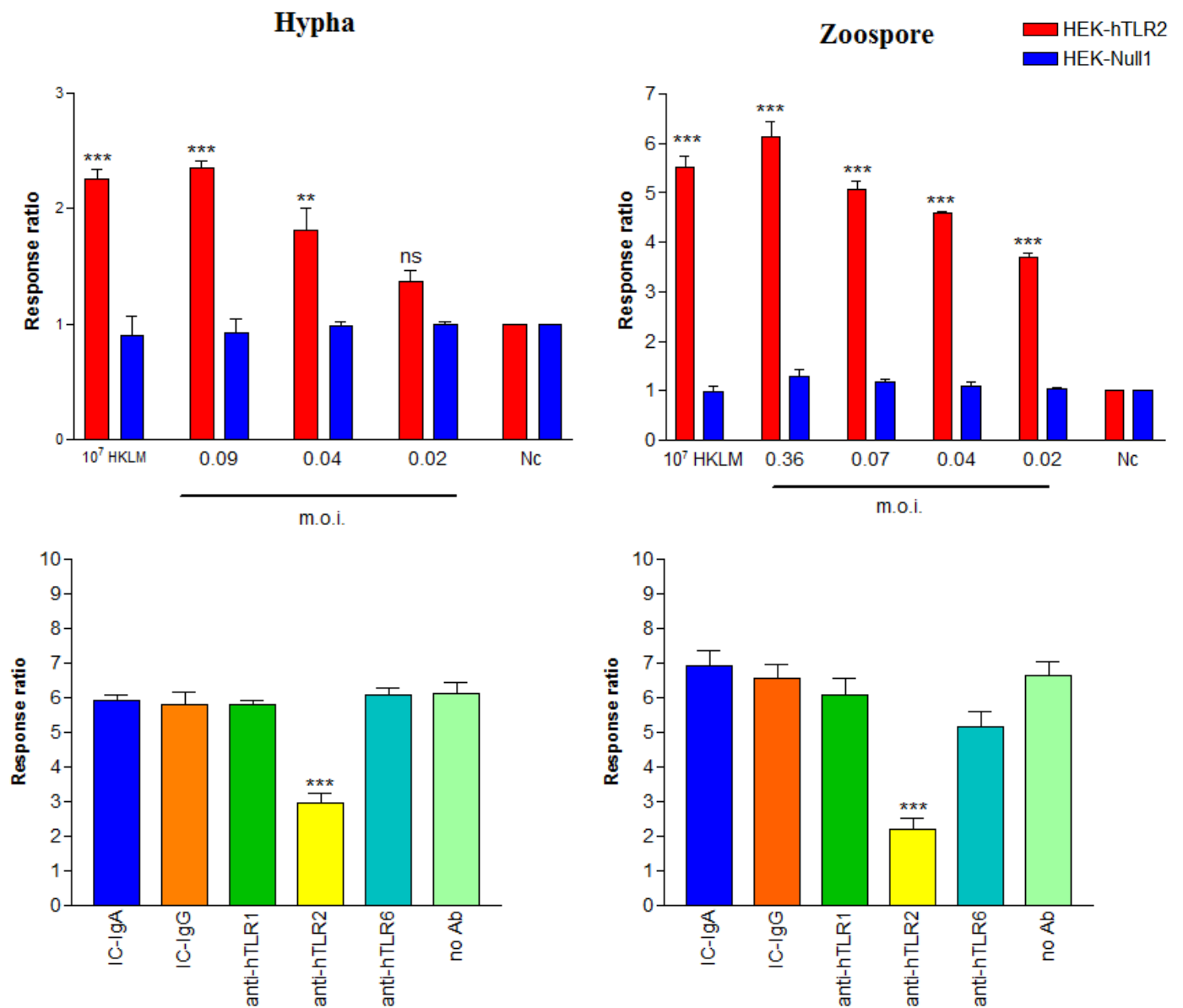


Figure 1. Response ratios of HEK-Blue™-hTLR2 cells to hypha and zoospore at various m.o.i. The HEK-Null1 was used as negative control. Statistical significant difference between the stimulated HEK-Blue™-hTLR2 cells and HEK-Blue™-hTLR2 cells stimulated with its negative control hypha or zoospore (Nc) 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.

Sample No.	TLR expressions									
	1	2	3	4	5	6	7	8	9	10
1	+	+	+	-	+	+	+	+	+	+
2	+	+	+	+	+	+	+	-	+	+
3	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	-	+	+
5	-	+	+	-	+	+	+	+	+	+
6	-	+	+	-	+	+	+	+	+	+
7	-	-	+	-	+	+	+	+	+	+
8	+	+	+	-	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+
10	+	+	+	-	+	+	+	-	+	+
11	+	+	+	-	+	+	+	+	+	+
12	-	+	+	-	+	+	+	+	+	+
13	+	+	+	+	+	+	+	-	+	+
14	+	+	+	+	+	+	-	-	+	+
15	+	+	+	+	+	+	+	-	+	+

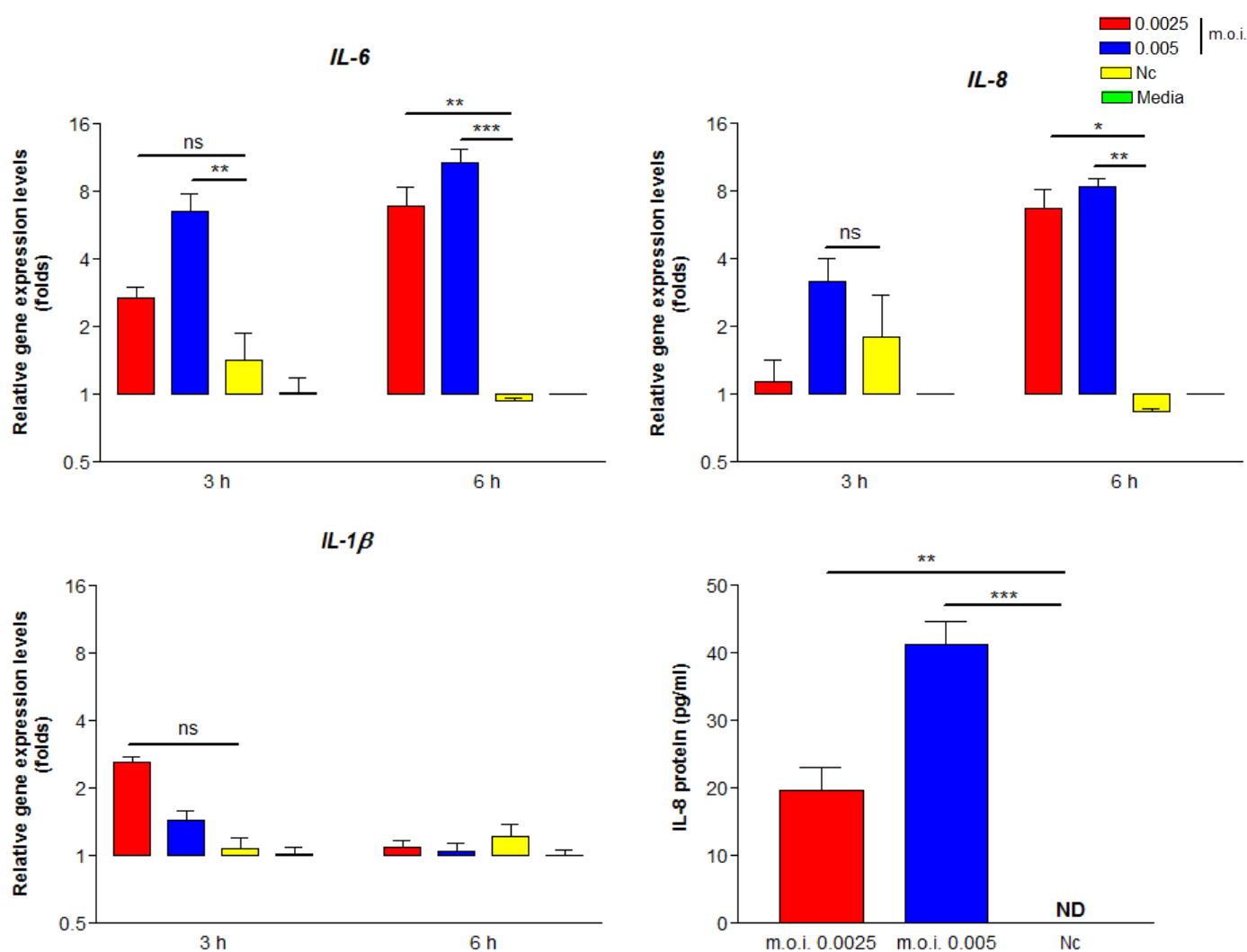


Figure 2. The early inflammatory cytokine production of PHCECs stimulated with live zoospore. The gene expressions of *IL-6*, *IL-8*, and *IL-1β* were determined at m.o.i. of 0.0025 and 0.005 for 3 and 6 h using qRT-PCR. The *IL-8* protein secretion was determined using ELISA at 6 h after stimulation. Statistical significant difference of relative gene expression levels ( $ddC_T$ ) 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 *P. insidiosum* inoculation.



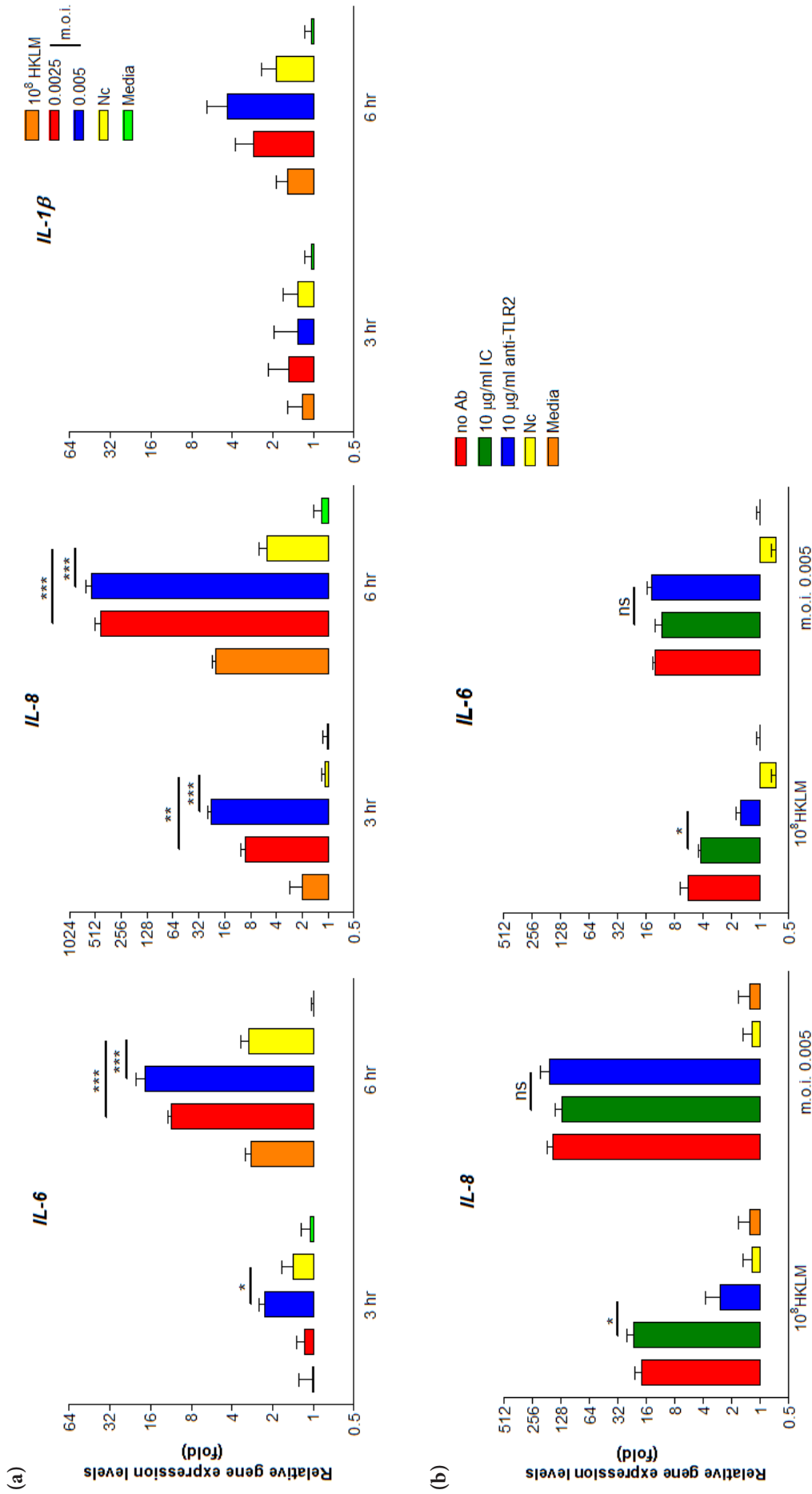


Figure 3. The inflammatory cytokine response of HCEC stimulated with live zoospore (a) at m.o.i. of 0.0025 and 0.005 at 3 h and 6 h. Statistical significant difference of relative gene expression levels (ddC<sub>1</sub>) between the zoospore and the negative control zoospore (Nc) stimulated cells were 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<sub>1</sub>) 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.

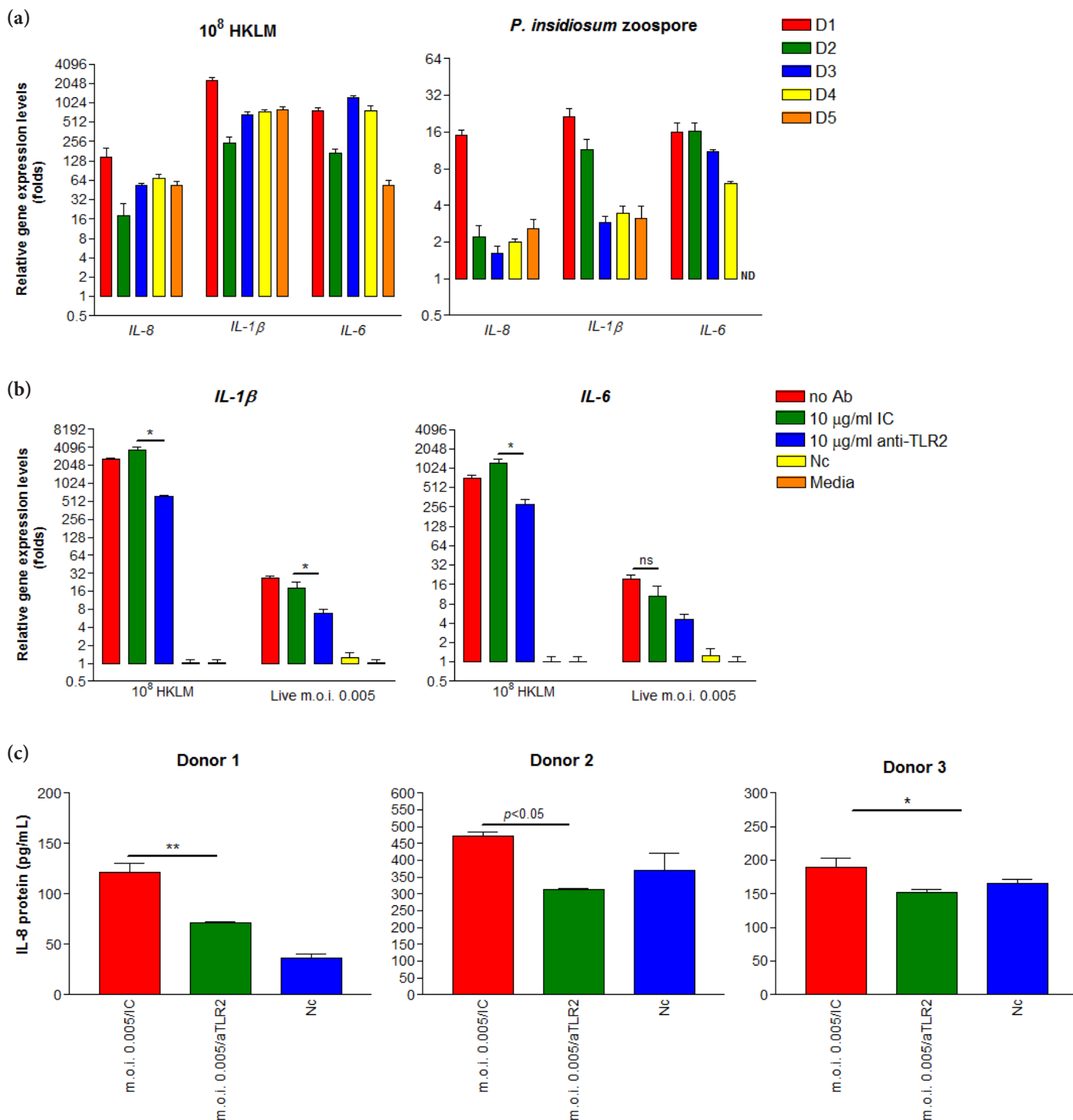


Figure 4. The cytokine mRNA expressions of MDMs from 5 healthy donors stimulated with  $10^8$  HKLM or live zoospores at m.o.i. of 0.005 for 6 h. The data showed the difference of relative gene expression levels ( $ddC_T$ ) (a). Relative gene expression level is the gene expression of stimulated MDMs which subtracted with unstimulated MDMs. The *IL-1 $\beta$*  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  $10 \mu\text{g/ml}$  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 ( $ddC_T$ ) 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-1 $\beta$*  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-1 $\beta$*  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-1 $\beta$*  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-1 $\beta$*  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-1 $\beta$*  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-1 $\beta$*  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.<sup>1,4,9-11</sup> 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.<sup>5,6</sup> TLR is an important receptor in the innate immune response to fungal infection and initiation of adaptive immunity.<sup>12,13</sup> TLR1-10 are expressed in human corneal epithelial cells.<sup>5,6</sup> 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.<sup>14,15</sup>

In the present study, we used HEK-Blue™-hTLR2 cells and found that both forms of *P. insidiosum*, zoospore and hypha, induced NF- $\kappa$ B 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. fumigates*.<sup>16</sup>

Fungal cell wall components including mannan, phopolipomannan and glucuroxylomannan are ligands of TLR2.<sup>17</sup> To date, the cell wall compositions of *P. insidiosum* have not been addressed. In the genus of *Pythium*, the hypha wall is composed of 80-90% of polysaccharide mainly  $\beta$ -glucans and cellulose. The cell wall of the mycelium of *P. aphanidermatum* comprise 18% of cellulose and 82% of (1  $\rightarrow$  3), (1  $\rightarrow$  6)- $\beta$ -d-glucans.<sup>18</sup> The  $\beta$ -1, 3-linked,  $\beta$ -1, 4-linked and  $\beta$ -1, 6-linked branching glucan are the cell wall component of *P. debaryanum*.<sup>19</sup> In 1993, Cherif M. *et al* found the presence of chitin in *P. ultimum* cell wall.<sup>20</sup> Therefore, the  $\beta$ -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,<sup>20-23</sup> 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 epithelium 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.<sup>24-26</sup> 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.<sup>6</sup> Jin *et al*, found that HCECs respond to inactive hyphae of *F. solani* by upregulation of TLR2, 3, 4 and 6 as well as release of *IL-6* and *IL-8*.<sup>27</sup> 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



*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.<sup>22</sup> The inhibition of TLR2 can attenuate corneal inflammation and prevent *A. fumigatus* invasion.<sup>20</sup>

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-1 $\beta$*  mRNA and *IL-8* protein productions in MDMs at the early stage of infection. The blockade of TLR2 on MDMs significantly decreased *IL-1 $\beta$*  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-1 $\beta$*  mRNA, and *IL-8* protein. Other PRRs may be involved in cytokine productions.<sup>25,28</sup> 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-containing protein (NOD)-like receptors; NOD1 and C-type lectin receptor,<sup>16,29,30</sup> 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-hTLR2 cell and the cytokine reduction in TLR2-blockade MDM indicating that *P. insidiosum* contains TLR2 ligand. Unrecognizing TLR2-mediated cytokine expression in corneal epithelium may due to the different and specificity of each cell type in distinguished the cellular response during infection. The knowledge of prominent factors of innate immune and inflammatory response in human corneal epithelium against *P. insidiosum*

might be a potential novel therapeutic target for the treatment of the fungal keratitis.

## Conclusion

This study focuses on the production of pro-inflammatory cytokines at early stage of infection *in vitro*. It provides better understanding of innate immune response of human corneal epithelial cell against *P. insidiosum* infection. The pathogen associated molecular patterns (PAMPs) or virulence factors found only in live *P. insidiosum* affect the induction of pro-inflammatory cytokine expression and may cause pathological effects.

The symptoms of *P. insidiosum* keratitis include corneal ulcer, dense stromal infiltration, increased stromal necrosis, endothelial plaque and high intraocular pressure. In present study, we found that both zoospore and hypha form of *P. insidiosum* can be recognized by TLR2/2 homodimer in HEK-Blue™-hTLRs cells leading to NF- $\kappa$ B activation. The induction of TLR2-dependent NF- $\kappa$ B 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-1 $\beta$*  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.

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