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ORIGINAL ARTICLE

Role of Toll-like receptor 2 in mediating the production of cytokines and human beta-defensins in oral mucosal epithelial cell response to Leptospiral infection

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Abstract

Background: Pathogenic *Leptospira spp.* is the causative agent of leptospirosis. Oral mucosal cavity is one of portal entry for this bacterium. Oral mucosal epithelium provides a physical barrier and secretes cytokines, chemokines and antimicrobial peptides (AMPs) in response to microbial infection. Human β-defensins (hBDs); hBD1, hBD2, and hBD3 are predominantly AMPs expressed in the oral cavity. Toll-like receptors (TLRs) have been reported in hBD regulation. TLR2 recognizes leptospiral lipopolysaccharide, and plays a key role in the early control of leptospirosis.

Objective: The aim of this study is to investigate the role of TLR2 in mediating the production of cytokines and hBDs in oral mucosal epithelial cell response to leptospiral infection.

Methods: Cultivated oral mucosal epithelial cells were prepared, characterized, and compared with oral mucosal tissues. The *TLR1-10* and *hBD* mRNA expressions were examined. Pro-inflammatory cytokine and *hBD1-3* expressions in response to leptospires were determined by quantitative (q) RT-PCR.

Results: The cultivated oral epithelium expressed *TLR2* and *hBD1-3*. The induction of *IL-1β*, *IL-8*, *TNF-α*, and *hBD2* were increased in response to *Leptospira* via TLR2 recognition.

Conclusion: The characteristics of primary epithelial cells and tissue were similar in terms of *TLR* expression. All primary epithelial cells expressed *TLR2* and *hBD1-3*. We used primary epithelial cells to study response to *L. interrogans*. Our results yielded the first evidence that human TLR2 regulates *hBD2* expression in oral mucosa epithelial responded to *L. interrogans*. Expression of *hBD2* may act to neutralize the virulence or prevent the invasion of *L. interrogans* at the portal of entry.

Key word: TLR2, leptospirosis, oral mucosal epithelium, human beta-defensins, *Leptospira*

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Introduction

Pathogenic *Leptospira* spp. is the causative agent of leptospirosis.[1] Portals of entry include cuts and abrasions, and mucous membranes, such as the conjunctival, genital, and oral mucous membranes. Disease transmission is usually associated with water, including flood and drinking or swimming in contaminated water. It was reported that swallowing of water while swimming increases the risk of developing leptospirosis.[2] Leptospires agglutinate when mixed with human or hamster saliva.[3-5] A high dose of leptospires is required for oral mucosal infection in hamster.[5] These studies suggest that oral mucosal immunity plays an important role in the defense against leptospiral infection.

Human β-defensins (hBDs) are cationic antimicrobial peptides (AMPs) that are secreted from epithelium and salivary glands to maintain balance between commensal structures and to prevent or act against infection.[6-8] The mechanism of hBDs is to induce bacterial lysis by targeting negatively charged molecules in the cell wall of bacteria, such as lipopolysaccharide

(LPS) and lipoteichoic acid. In addition to bactericidal activity, hBDs play a role in leukocyte recruitment to initiate adaptive immune response. The oral cavity expresses hBD1, hBD2, and hBD3. hBD1 is constitutively expressed, whereas hBD2 and hBD3 are induced by microbial infection or inflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ.[6, 8-10]

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) in the innate immune system that are specifically activated by their cognate ligands. The activation of TLRs initiates host inflammatory response to defend against infection. TLR2 is a major PRR in the oral cavity that induces the upregulation of hBDs. Several periodontal bacteria, including *Fusobacterium nucleatum*, *Treponema denticola*, and *Porphyromonas gingivalis*, induce TLR2-mediated hBD2 and/or hBD3 expression.[8,10,11] In addition to TLR2, other TLRs, including TLR3, TLR4, TLR5, and TLR9, have been reported to be associated with hBD regulation.[10-12]

TLR2 plays a significant role in human leptospirosis.[13-15] Due to the presence of leptospiral LPS, a previous study reported that leptospires are more susceptible to AMPs than are other spirochete bacterium.[16] Leptospiral LPS is recognized by human TLR2, whereas the LPS of Gram-negative bacteria are recognized by human TLR4.[17] Based on our review of the literature, the role of TLR as a mediator of hBD production in host response to leptospires has not been investigated. Accordingly, the aim of this study was to investigate the role of TLR2 in mediating the production of cytokines and hBDs in oral mucosal epithelium response to leptospiral infection. We found that the cultivated oral mucosal epithelium responded to *Leptospira* infection by increase the levels of pro-inflammatory cytokine; *IL-1β*, *IL-8*, *TNF-α*, and *hBD2* expressions. The *hBD2* expression, a mechanism against and neutralize bacterial invasion at the route of entry, was induced via TLR2 recognition.

Methods

Bacterial culture and preparation

L. interrogans serovar Autumnalis was isolated from clinical specimens obtained from patients admitted to Siriraj Hospital, which is located in Bangkok, Thailand. Siriraj Hospital is Thailand's largest university-based national tertiary referral center. The protocol for this study was approved by the Siriraj Institutional Review Board, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (IEC no 639/2551(EC2)). The bacteria were cultured in Ellinghausen -McCullough-Johnson-Harris (EMJH) medium supplemented with leptospiral enrichment (HiMedia Laboratories, Mumbai, India) at 30°C. On the day of infection, the bacteria were washed by centrifugation at 10,000-x g for 10 minutes in sterile PBS. After being washed two times, the pellet was re-suspended in cell culture medium and infected into cultivated oral mucosal epithelium. The cytokine expressions of the infected cell were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR).

Oral mucosal epithelium cultivation and isolation

Oral tissues were harvested from 8 healthy adults (5 females and 3 males; age range: 22-73 years) after obtaining written informed consent and performing a pre-surgical evaluation. Oral hygiene was optimized with preoperative 1% Betadine® mouthwash for 3 days prior to biopsy. On the day of operation, an oral mucosal biopsy of 5 mm x 5 mm was harvested from the buccal surface of the lower lip under local anesthesia.

Oral mucosal tissue specimens were digested using 2 IU/ml of Dispase II neutral protease (Thermo Fisher Scientific, Waltham, MA, USA) at 4°C overnight after being washed three times in Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific, Waltham, MA, USA). After incubation, the epithelial sheet was separated, and washed three times, cut into small pieces, and separated into single cells by trypsin digestion method. Cells were plated at 10^4 cells/cm^2 in a cell culture dish containing keratinocyte growth medium (KGM) (Lonza, Basel, Switzerland) and incubated for two to three weeks in a humidified incubator at 37°C with 5% CO_2. The medium was changed every other day. The cells of early passage were preserved in liquid nitrogen until use.

RNA extraction and total RNA quantification

RNA from oral mucosal epithelial sheet, cultivated oral mucosal epithelium, and infected cells was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. RNA concentration was determined by spectrophotometric measurement at 260 nm (Nanodrop, Thermo Scientific, Wilmington, DE, USA). A_{260}/A_{280} ratio was calculated to determine the purity of RNA. The allowable range of RNA purity was 1.9-2.1.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted and subsequently reverse-transcribed using the Superscript™ III First Strand Synthesis System (Life Technologies, Carlsbad, CA, USA). The cDNA preparations were stored at -20°C until PCR amplification. RT-PCR was used to determine the expression of *TLR1-10* and *hBD1-3* genes of oral epithelial tissue and cultivated cells. The optical density of PCR products was analyzed by agarose gel electrophoresis using ImageJ image analysis software. *GAPDH* was used as a housekeeping gene and as an internal control for normalization. Nucleotide sequences of primers used for PCR analysis are shown in **Table 1**.

Analysis of mRNA expression by qRT-PCR

The mRNA expression of *Leptospira*-infected oral mucosal epithelium was quantified at 4 hours after infection by qRT-PCR. For blocking of TLR2 activity, cells were pre-incubated with anti-human TLR2 (InvivoGen, San Diego, CA, USA) before infection. The culture media of infected cells was removed before adding the lysis buffer. Total RNA was extracted and the fifty to one-hundred ng/ml of RNA was reverse-transcribed to cDNA.

The PCR mixture consisted of iTaq SYBR Green Master Mix (Bio-Rad Laboratories, Hercules, CA, USA), 0.5 μM of forward and reverse primers, and 5 μl of 1/10 diluted cDNA. PCR was performed using the following conditions: enzyme activation at 95°C for 10 min and 40 cycles of denaturation at 95°C for 10 sec; annealing at 59°C for 15 sec; and, extension at 72°C for 5 sec with fluorescence detection. The expressions of pro-inflammatory cytokine, including *hBD1-3*, *IL-1β*, *IL-8*, and *TNF-α* were determined. The quantification cycle (Cq) of target mRNA was

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

Gene	Sequence (5' → 3')	Tm	Size	Accession no.
TLR1_F	CAGTGTCTGGTACACGCATGGT	59	105	NM_003263
TLR1_R	TTTCAAAAACCGTGTCTGTTAAGAGA	55		
TLR2_F	GTACCTGTGGGGCTCATTGT	57	178	NM_003264
TLR2_R	TACCATTGCGGTCACAAGAC	55		
TLR3_F	CCTCCAGCACAATGAGCTATC	55	197	NM_003265
TLR3_R	CCAGCTGAACCTGAGTTCCT	57		
TLR4_F	AACCAAGAACCTGGACCTGA	56	185	NM_138554
TLR4_R	AACTCTGGATGGGGTTTCCT	56		
TLR5_F	CCCTCTGCCCCTAGAATAAGA	55	150	NM_003268
TLR5_R	CTATTCGGCCATCAAAGGAG	53		
TLR6_F	CATGTTCCAAAAGACCTACCGC	56	234	NM_006068
TLR6_R	ACTCACAATAGGATGGCAGGATA	55		
TLR7_F	TGTTTCCAATGTGGACACTGAA	55	195	NM_016562
TLR7_R	TGTTCGTGGGAATACCTTCCAG	56		
TLR8_F	TTTCAAGGGCTGCAAAATCTCA	55	163	NM_138636
TLR8_R	ACTGGTTGTCTTCAAGCAGTAAC	55		
TLR9_F	CCCACCTGTCACTCAAGTACA	56	242	NM_017442
TLR9_R	GTGGCTGAAGGTATCGGGATG	58		
TLR10_F	GGCACAGGGTTAGGAAAACA	55	302	NM_001195108
TLR10_R	GAGATTGTGGTGGGCAAAGT	56		
hBD1_F	CTGCTGTTTACTCTCTGCTTACTTTT	62	107	NM_005218
hBD1_R	CCTCCACTGCTGACGCA	50		
hBD2_F	GATCCTGTTACCTGCCTTAAGAGT	62	83	NM_001205266
hBD2_R	CCACAGGTGCCAATTTGTTTATACC	60		
hBD3_F	GTCATGGAGGAATCATAAACACATTACAG	56	100	NM_018661
hBD3_R	CCGATCTGTTCCTCCTTTGGA	62		
IL-8_F	GCCAACACAGAAATTATTGTAAAGCTT	56	112	NM_000584
IL-8_R	AATTCTCAGCCCTCTTCAAAAACTT	55		
GAPDH_F	CCTGTTCGACAGTCAGCCG	58	101	NM_002046
GAPDH_R	CGACCAAATCCGTTGACTCC	56		

normalized with the *GAPDH* gene and an unstimulated cell using the ΔΔCq method.

Results

Characterization of TLR and hBD expressions in oral mucosal epithelium

To demonstrate that cultivated oral mucosal epithelium can be used as a model to study oral innate immunity, we compared *TLR1-10* and *hBD1-3* mRNA expressions of oral epithelium cell sheets with cultivated oral mucosal epithelium. We found that cultivated oral epithelium expressed *TLRs* similar to those expressed by the oral epithelium cell sheet (**Table 2, Figure 1**). Most patients expressed all of *TLR1-10*. However, differences in *TLR* expression were observed in some patients. One patient did not express both *TLR1* and *TLR2*, one patient did not express *TLR8*, and two of eight patients did not express *TLR7* on the oral epithelium sheet. After cultivation, one patient did not express *TLR1*, *TLR4*, or *TLR8*. Notably, *TLR2* was expressed in all of the cultivated cells. Regarding *TLR* expression, we found that all of *hBD1-3* were detectable by RT-PCR in all tissues and in all cultivated cells (**Table 2, Figure 1**). Therefore, the oral mucosal epithelium that was cultivated in our laboratory can be used as a model for studying the role of TLR2 as a mediator of induction of hBDs by *L. interrogans* in oral mucosal epithelial cells.

Induction of hBDs and pro-inflammatory cytokines in response to Leptospira interrogans

To determine whether cultivated oral mucosal epithelium responded to leptospiral infection, 2×10^4 cells was plated in a 96-well plate for 2 days before infection with 100 multiplicities of infection (MOI) of *L. interrogans*. After infection for duration of 4 hours, the infected cells were collected and evaluated for cytokine expression by qRT-PCR. We found that *L. interrogans* induced the up regulation of pro-inflammatory cytokine

Table 2. Eight human oral mucosal epithelial sheets (S) and their cultivated oral mucosal epithelium (C) expressed *TLR*- and *hBD*-specific mRNAs

Sample Number	TLR 1		TLR 2		TLR 3		TLR 4		TLR 5		TLR 6		TLR 7		TLR 8		TLR 9		TLR 10		hBD 1		hBD 2		hBD 3	
	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C
1	+	-	+	+	+	+	+	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

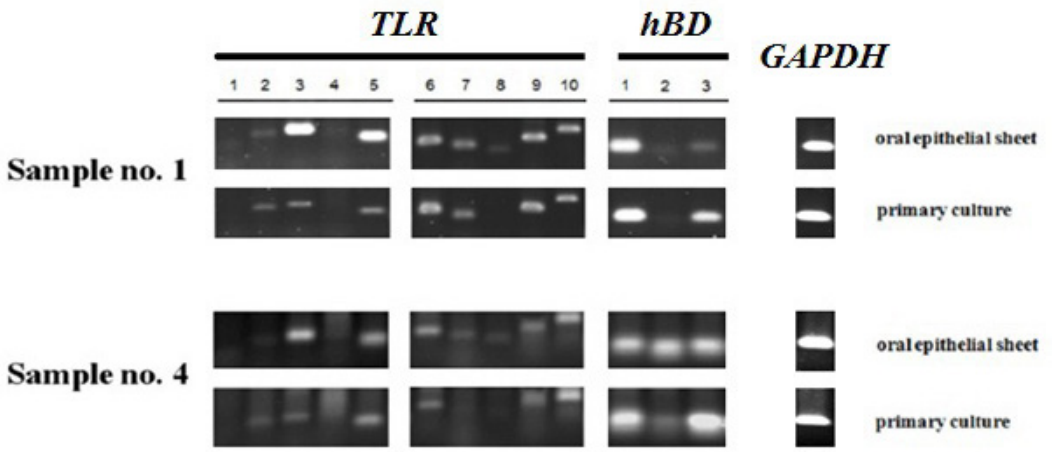

Figure 1. Expressions of *TLR1-10* and *hBD1-3* genes determined by RT-PCR in human oral mucosal epithelial sheets, and in corresponding cultured cells of 2 representative donors. The *GAPDH* gene was observed between samples as a loading control. The numbers of 1-10 and 1-3 represent *TLR1-10* and *hBD1-3*, respectively.

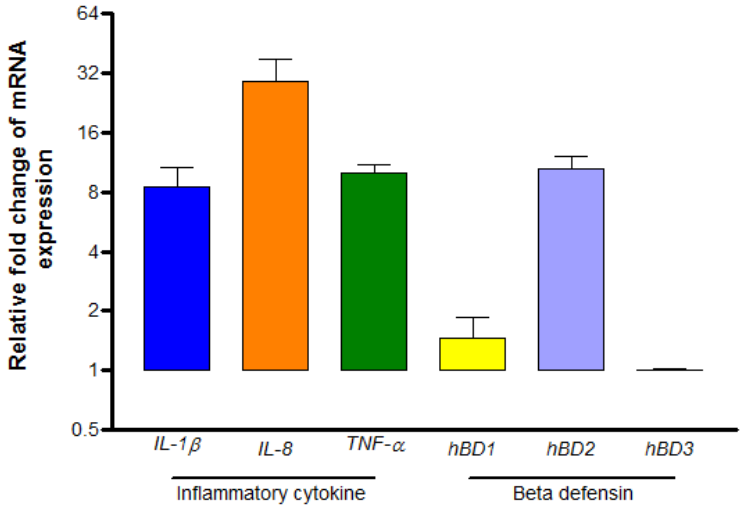

Figure 2. Pro-inflammatory cytokine and *hBD* expressions in cultivated oral mucosal epithelium response to 100 MOI *Leptospira*. The graph shows mean ± standard error of the mean (SEM) of one representative experiment which was performed in duplicate.

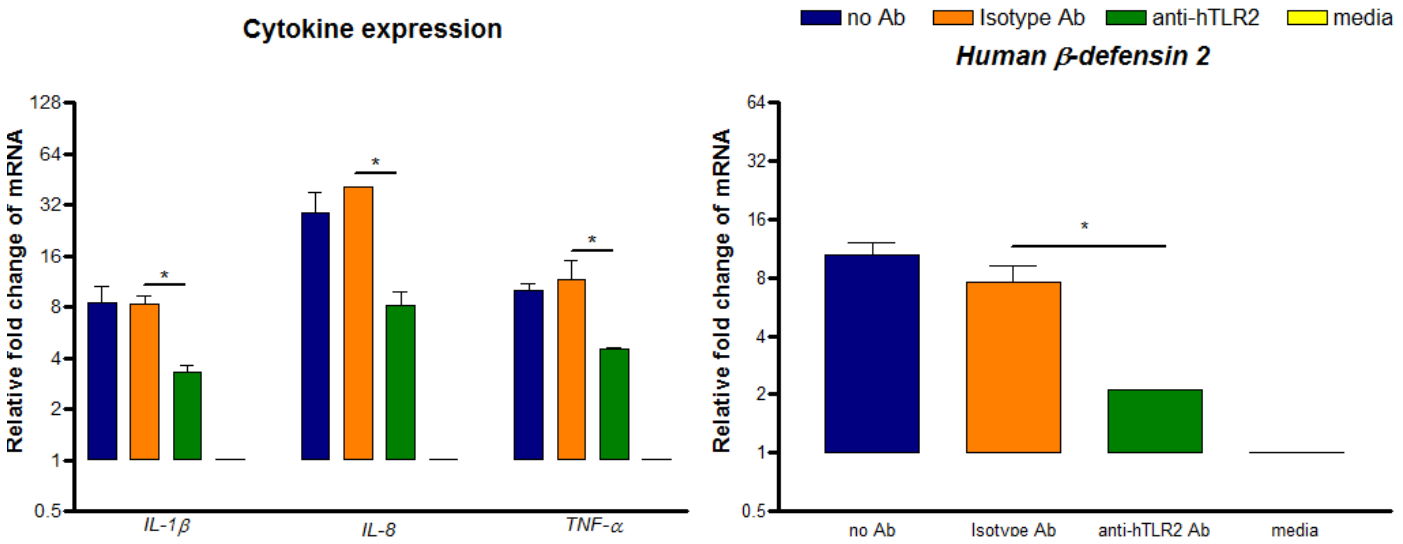

Figure 3. The effect of anti-hTLR2 neutralizing Ab on *L. interrogans* stimulated pro-inflammatory cytokine and *hBD2* expressions. Cultivated oral mucosal epithelium were pre-incubated with or without 10 µg/ml anti-hTLR2 neutralizing or isotype control Ab for 1 hour before infection, and were stimulated with *L. interrogans* at MOI 100. The graph shows mean ± standard error of the mean (SEM) of one representative experiment which was performed in duplicate. Statistical difference between anti-hTLR2 and isotype control Ab was analyzed by paired-t-test, *statistical difference at *p-value* < 0.05.

IL-1β, *IL-8*, and *TNF-α* (**Figure 1**). Expression of *hBD1-3* can be observed in both non-inflamed tissue and in cultured cells. However, only the expression of *hBD2* was increased after leptospiral infection (**Figure 2**). This result indicated that cultivated oral mucosal epithelium has the ability to respond to leptospiral infection by increasing the expression of pro -inflammatory cytokine and *hBD2*.

Role of TLR2 in oral mucosal response to leptospiral infection

To study the effect of human TLR2 on oral mucosal epithelium in response to leptospiral infection, we pre-incubated the cells with anti-hTLR2 Ab for 1 hour before infection. The expression of pro-inflammatory cytokine *IL-1β*, *IL-8*, *TNF-α*, and *hBD2* were significantly reduced in the presence of anti -hTLR2 Ab (**Figure 3**). From this result, we can conclude that TLR2 was the major receptor for induction of pro-inflammatory cytokine and *hBD2* expressions in oral mucosal epithelium response to leptospiral infection.

Discussion

The first evidence that *L. interrogans* induces the expression of *hBD2* at the route of infection is reported herein. Increases in *hBD2*, as well as *IL-1β*, *TNF-α*, and *IL-8*, in oral mucosal epithelium were dependent on TLR2 activation. TLR2 is recognized as an important TLR in human leptospirosis that initiates host inflammatory response.[13-15] However; the role of TLR2 in regulated hBD2 expression has never been reported. Therefore, this new finding adds to the existing body of knowledge about host innate immune response to *L. interrogans* at the site of infection.

Oral mucosal epithelium is a component of the innate immune system that plays a role as both a physical and chemical barrier in host defense. The secretion of hBD is one mechanism that helps to maintain balance in the oral cavity, and that helps to prevent the invasion of several different microbes. TLR, especially TLR2, is the most characteristic PRR that regulates hBD expression in the innate immune system.[8,11,12,18] TLR2 has a crucial role in mediating host inflammatory response in human leptospirosis.

First, we characterized TLR expressions in cultivated oral mucosal epithelium. We found that most cultivated oral mucosal epithelium had *TLR* and *hBD* mRNA expressions similar to those identified on the fresh isolated oral mucosal sheet. All donors expressed *TLR2* and had detectable levels of *hBD1-3* mRNA expression. This finding confirmed that the culture condition did not interfere with *TLR* expression, and that cultivated oral mucosal epithelium can be used as a model for studying innate immunity to *L. interrogans*.

Differences in constitutively expressed hBD1, hBD2 and hBD3 are induced by microbial or inflammatory stimuli.[8] Non-inflamed tissue and unstimulated cultured epithelium expressed a low amount of *hBD1-3* mRNA expression. However, *hBD3* mRNA expression increased after cultivation. This expression may have been caused by the culture condition, which contained epidermal growth factor, which has been reported to induce the expression of *hBD3*.[19] However, *hBD3* did not increase after leptospiral infection. The up regulation of *hBD2* in response to *L. interrogans* may be due to direct stimulation by *L. interrogans* and/or the indirect effect of pro-inflammatory cytokine IL-1β and TNF-α. Leptospiral LPS has been reported to be recognized by human TLR2.[13,17] Under the regulation of TLR, IL-8 is the key cytokine in activated epithelium. The induction of *hBD2* and *IL-8* by *L. interrogans* was mostly dependent upon TLR2 activation. The anti-hTLR2 neutralizing Ab blockade reduced the expression of *hBD2* by more than 70% when compared with the expression observed when no blockade was introduced. This finding may suggest that the expression of *hBD2* and *IL-8* were mostly induced by direct interaction between hTLR2 and leptospires.

In addition to the direct effect of the bacteria, the production of *IL-1β* and *TNF-α* in host response to *L. interrogans* enhanced *hBD2* expression. The reduction of these pro-inflammatory cytokine in the presence of anti-hTLR2 Ab could affect the expression of *hBD2*. Therefore, direct *L. interrogans* induction and host inflammation together synergistically promoted *hBD2* expression in oral mucosal response to *L. interrogans*.

In the present study, we demonstrated that the activation of hTLR2 in host response to *L. interrogans* not only promoted inflammation, but that it also induced the protective response of AMPs. Increased *hBD2* mRNA in response to *L. interrogans* might enhance host defense against the infection. The secretion of the cationic hBD2 peptide targets the negatively charged cell wall of bacteria. This mechanism may explain why infection via the oral cavity requires a high concentration of leptospiral infection.[5] The epithelial lining of the oral mucosa provides not only a physical barrier, but it also secretes hBD to neutralize the infection. In addition to this antibacterial activity, hBD promotes the recruitment of neutrophils to clear the microbes. The anti-inflammatory effect of hBD also reduces host ation, which causes pathology of leptospirosis.

In conclusion, our study provides the first evidence that the initiation of pro-inflammatory cytokine and *hBD2* were responsible to leptospiral mediated hTLR2 activation in oral innate immunity. The mechanism by which hBD2 affects *L. interrogans*, and host response at the other portals of entry will be investigated in future studies. The induction of AMPs may produce a protective effect that both prevents leptospiral invasion and that improves or initiates a systemic immune response that protects against leptospirosis. A recent study reported a fatal case of leptospirosis that expressed a higher level of inflammatory cytokine, but a lower level of AMPs than a comparative survival case.[20] The anti-inflammatory effect of AMPs may prevent excessive host inflammation and protect against fatal leptospirosis.

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Conflict of interest declaration

The authors hereby declare no personal or professional conflicts of interest regarding

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