

# Toll-like receptor 2-mediated induction of human beta-defensin 2 expression by *Leptospira interrogans* in human kidney cells

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## Abstract

**Background:** Leptospirosis is a zoonotic disease caused by *Leptospira interrogans*. Severe leptospirosis is often accompanied by kidney dysfunction caused by chronic infection. The kidney pathology involves bacterial invasion and inflammation caused by pro-inflammatory cytokines. Human beta defensins (hBDs) are antimicrobial peptides induced by microbial infection and/or pro-inflammatory cytokines. One function of hBDs is the recruitment of immune cells that leads to inflammation. However, the expression of hBDs by kidney epithelium in response to pathogenic *Leptospira* has never been investigated.

**Objective:** To determine the expression of hBDs in human kidney epithelium responses to *Leptospira*.

**Methods:** Human kidney cells were infected with *Leptospira interrogans* serovar Autumnalis in the presence or absence of anti-TLR2 neutralizing antibody (Ab) for 6 hours. TLR2, hBDs and pro-inflammatory cytokines mRNA expressions were analyzed by quantitative polymerase chain reaction (qPCR).

**Results:** Pathogenic *Leptospira* upregulated the expressions of pro-inflammatory cytokines and *hBD2*, but not TLR2, *hBD1* and *hBD3* in kidney cells. The expressions of *hBD2* and pro-inflammatory cytokines were inhibited in the presence of anti-hTLR2 neutralizing Ab.

**Conclusions:** Our results provide the first evidence that pathogenic *Leptospira* induces *hBD2* expression in kidney cells. The expressions of pro-inflammatory cytokines and *hBD2* in the cells in response to pathogenic *Leptospira* are regulated by TLR2. Pro-inflammatory cytokines and *hBD2* might be play role in recruitment of immune cells to the kidney and contribute to the development of inflammation-mediated tissue damage in the kidney. However, further study is needed to improve the understanding of the role of these molecules in immune response activation.

**Key words:** TLR2, leptospirosis, kidney epithelium, human beta-defensins, *Leptospira*

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## Introduction

The innate immune response plays an important role in recognizing pathogens and stimulating the production of pro-inflammatory cytokines. It also affects host defense via the induction of antimicrobial peptides. Defensins, which belong to a family of antimicrobial peptides, provide protection against a broad spectrum of pathogens, viruses, bacteria, and fungi.<sup>1-4</sup> In humans, defensins are classified into two groups:  $\alpha$ - and  $\beta$ -defensins.<sup>5</sup> Human  $\beta$ -defensin-2 (*hBD2*) is detected in the epithelium of almost the entire human body, including renal epithelium.<sup>6-14</sup> Toll-like receptor 2 (TLR2) is the major pattern recognition receptor (PRR) that recognizes and monitors the host for the presence of *Leptospira*.<sup>15-20</sup> Our research group previously demonstrated that human TLR2 regulates *hBD2* expression in oral mucosal epithelium induced by *L. interrogans*.<sup>21</sup> The expression of *hBD2* at the portal of entry acts to neutralize the virulence or prevent the invasion of the microbes.<sup>22</sup> However, overexpression of hBDs can result in diseases such as psoriasis and kidney disease.<sup>23-26</sup> The kidney is a target organ of leptospiral infection; however, a connection between *Leptospira*, TLR2 recognition/activation, and *hBD2* production has not been established in human kidney cells. Understanding the mechanisms that regulate the pro-inflammatory cytokine and hBD response are important for controlling the disease. Accordingly, the aim of this study was to investigate the role of TLR2 in mediating the production of cytokines and hBDs in the kidney epithelium response to a leptospiral infection.

## Methods

### Bacterial culture and preparation

*L. interrogans* serovar Autumnalis was isolated from clinical specimens obtained from a patient admitted to Siriraj Hospital, 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 (COA no 639/2551[EC2]) of the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. The bacteria were cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium supplemented with 10% of leptospiral enrichment (HiMedia Laboratories, Mumbai, India) at 30°C. On the day of infection, the bacteria were washed by centrifugation at  $10,000 \times g$  for 10 minutes in sterile phosphate-buffered saline (PBS). After being washed twice, the pellet was resuspended in cell culture medium and infected into cultivated kidney epithelium.

### Human kidney epithelial cell cultivation and stimulation

A human kidney epithelium (HK2) cell line cultured in Dulbecco's Modified Eagle Medium (DMEM; Life Technology, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; GE Healthcare, IL, USA), 50 U/ml of penicillin, and 50  $\mu$ g/ml of streptomycin was incubated in a humidified incubator at 37°C with 5% CO<sub>2</sub>. One hundred microliters of the cells at quantity of  $2 \times 10^4$  were plated onto each well of a 96-well plate. After incubation overnight, the culture medium was removed and replaced with 100 MOI of *Leptospira* or with 40 MOI of heat killed *Listeria monocytogenes* (HKLM; a TLR2 ligand) suspended in DMEM without antibiotics. In some experiments, the cells were pretreated with anti-hTLR2 neutralizing antibody (Ab) or its isotype control Ab at the same concentration (final concentration of 5  $\mu$ g/ml; InvivoGen, CA, USA) for 1 hour at 37°C with 5% CO<sub>2</sub> before adding the *Leptospira*. After a 6-hour stimulation with the bacteria at 37°C with 5% CO<sub>2</sub>, the supernatant was removed and the infected cell was collected by adding 350  $\mu$ l of lysis buffer (Qiagen, Hilden, Germany). The experiment was performed in duplicate at least three times independent experiments. The statistical different at *p*-value < 0.05 of mean of three biological replicated were analyzed together by paired *t*-test.

### RNA extraction and total RNA quantification

Total RNA was extracted from the infected cells using RNeasy Mini Kit (Qiagen, Hilden, Germany). The genomic DNA was removed by the DNA removing column included in the kit. Then, 50–100 ng/ $\mu$ l of extracted RNA was reversed to cDNA by Superscript III<sup>®</sup> reverse transcriptase enzyme (Life Technologies, St Paul, MN, USA), following the manufacturer's protocol.

### Analysis of mRNA expression by RT-qPCR

The PCR master mix consisted of 1X iTaq SYBR Green Master Mix (Bio-Rad Laboratories, CA, USA), 0.5  $\mu$ M of forward and reverse primers,<sup>21</sup> and 5  $\mu$ l of cDNA. The primer sequences used in the RT-qPCR analysis are detailed in **Table 1**. The PCR was performed under the following conditions: enzyme activation at 95°C for 10 minutes and 40 cycles of denaturation at 95°C for 10 seconds; annealing at 59°C for 15 seconds; and extension at 72°C for 5 seconds with fluorescence detection. The quantification cycle (Cq) of *hBD1-3*, pro-inflammatory cytokines (*IL-1 $\beta$* , *IL-6*, *IL-8*, and *TNF- $\alpha$* ) and hTLR2 mRNA were quantified and normalized with the reference gene (*GAPDH*). The delta Cq of the stimulated cells were then normalized with the delta Cq of the unstimulated cells. The relative fold change of mRNA expression was calculated with the  $2^{-\Delta\Delta Cq}$  formula.

**Table 1.** The primer sequences used for RT-qPCR analysis, their corresponding melting temperatures, and the sizes of PCR products.

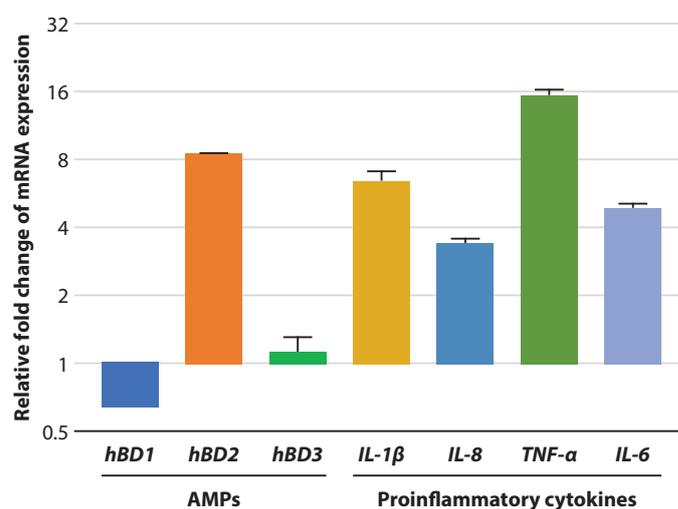
Gene	Sequence (5'→3')	T <sub>m</sub> (°C)	PCR product (bp)	Accession Number
IL-1β_F	TGGAGCAACAAGTGGTGT	56	157	NM_000576.3
IL-1β_R	TTGGGATCTACACTCTCCAGC	58		
IL-6_F	CGGGAACGAAAGAGAAGCTCTA	60	68	NM_000600.5
IL-6_R	GGCGCTTGTGGAGAAGGAG	61		
IL-8_F	GCCAACACAGAAATTATTGTAAAGCTT	56	112	NM_000584
IL-8_R	AATTCTCAGCCCTCTCAAAAAGCTT	55		
TNF-α_F	CCCAGGCAGTCAGATCATCTTC	60	75	NM_000594.4
TNF-α_R	CAGCTTGAGGGTTTGCTACAAC	60		
hBD1_F	CTGCTGTTACTCTCTGCTTACTTTT	62	107	NM_005218
hBD1_R	CCTCCACTGCTGACGCA	50		
hBD2_F	GATCCTGTTACCTGCCTTAAGAGT	62	83	NM_001205266
hBD2_R	CCACAGGTGCCAATTGTTTATACC	60		
hBD3_F	GTCATGGAGGAATCATAAACACATTACAG	56	100	NM_018661
hBD3_R	CCGATCTGTTCTCCTTTGGA	62		
TLR2_F	GTACCTGTGGGGCTCATTGT	57	178	NM_003264
TLR2_R	TACCATTGCGGTCACAAGAC	55		
GAPDH_F	ACAGCCTCAAGATCATCAGCA	59	119	NM_002046
GAPDH_R	GATGGCATGGACTGTGGTCA	60		

**Abbreviations:** RT-qPCR, reverse transcription quantitative polymerase chain reaction; T<sub>m</sub>, melting temperature; bp, base pair

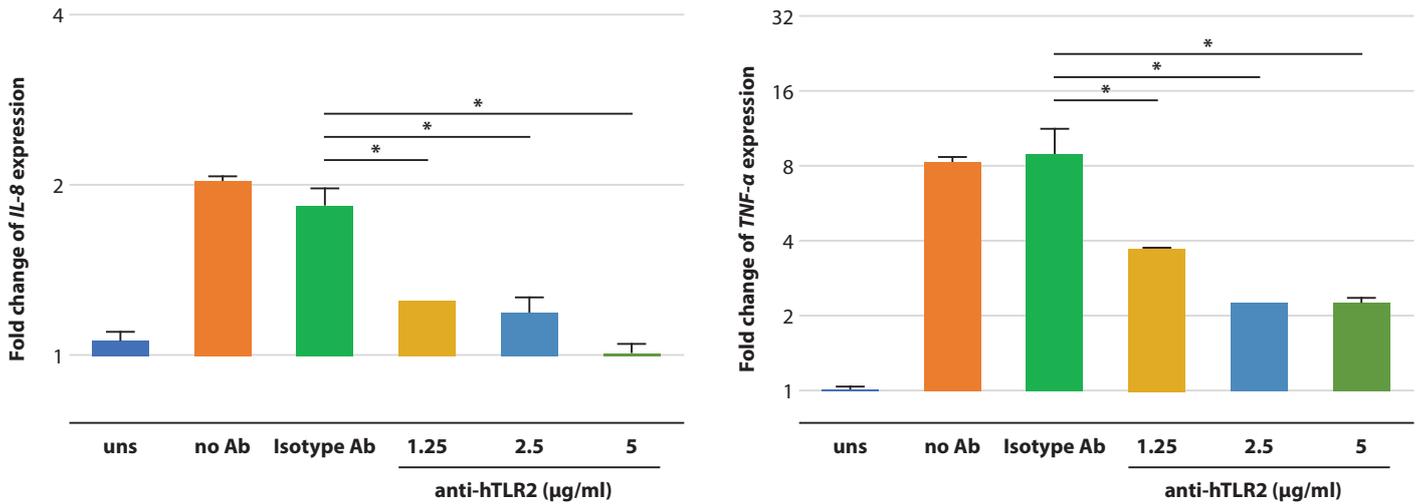
## Results

### Induction of hBD and pro-inflammatory cytokine expressions in response to *Leptospira interrogans* in HK2 cells

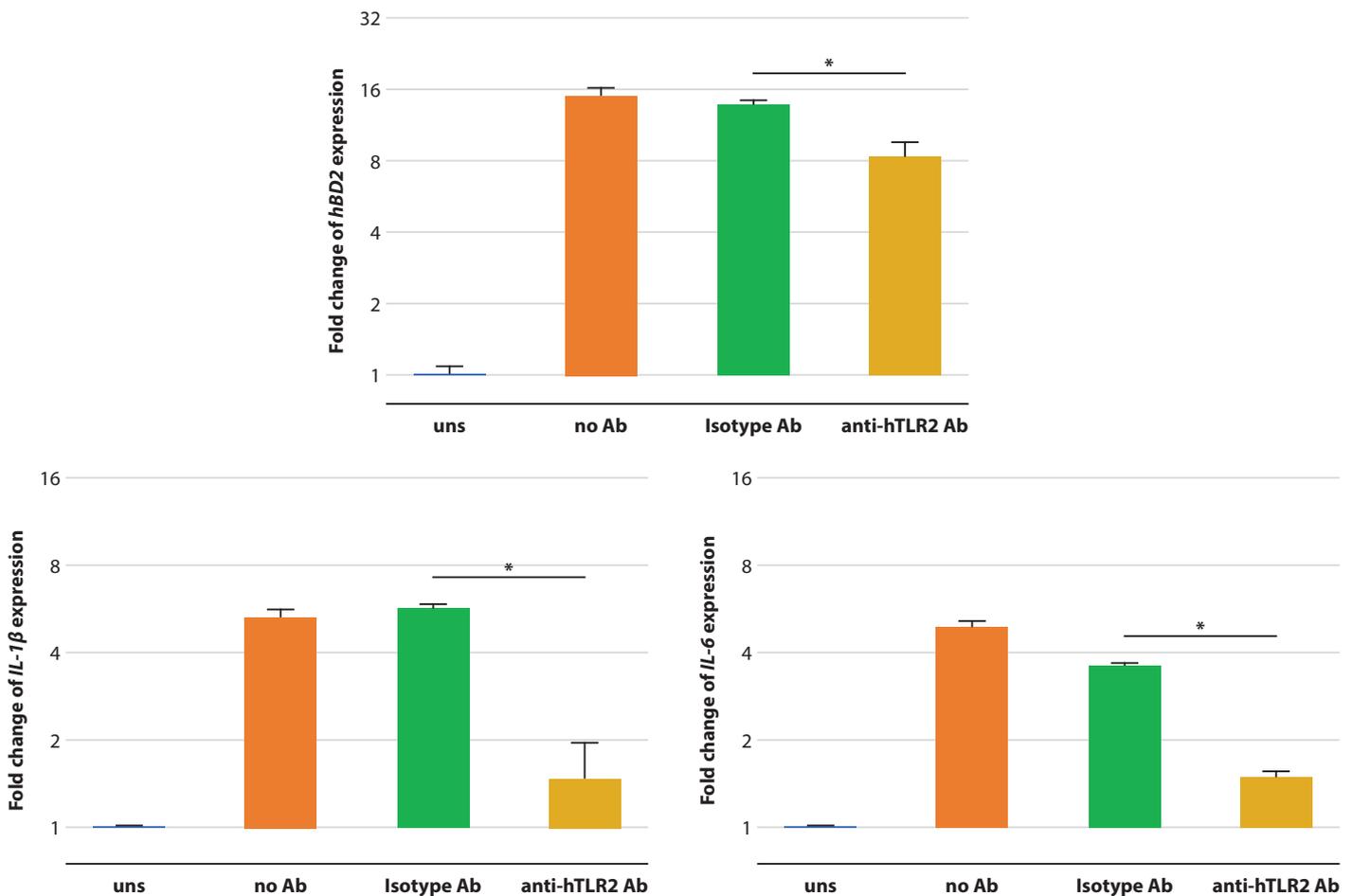
Pro-inflammatory cytokines including *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* were reported to be involved in kidney pathology and severe leptospirosis.<sup>23-25</sup> Therefore, in this study we determined whether these pro-inflammatory cytokines in the cultivated kidney epithelium responded to the leptospiral infection. HK2 cells were infected with *L. interrogans* for 6 h. The infected cells were collected and evaluated for hBD and pro-inflammatory cytokine expressions by RT-qPCR. We found that *L. interrogans* induced the increasing of the pro-inflammatory cytokines *IL-1β*, *IL-6*, *IL-8*, and *TNF-α* and of *hBD2*, but not of *hBD1* and *hBD3* (Figure 1). The mean fold change of mRNA expression of three replicated experiments of *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, and *hBD2* were  $5.48 \pm 0.5$ ,  $6.53 \pm 2.34$ ,  $4.69 \pm 1.16$ ,  $13.04 \pm 2.53$ , and  $12.78 \pm 3.745$ , respectively. This supports that cultivated kidney epithelium has the ability to respond to a leptospiral infection by increasing the expression of *hBD2* and pro-inflammatory cytokines.



**Figure 1.** Human beta-defensin (hBD) and pro-inflammatory cytokine expressions in cultivated kidney epithelium response to 100 multiplicity of infection (MOI) of *Leptospira*. The relative fold change of mRNA expression was normalized with *GAPDH* gene and unstimulated cell by  $2^{-\Delta\Delta C_q}$  method. The graph shows mean  $\pm$  SEM of one representative from three biological replicated experiments. Unstimulated cell is the cultivated kidney epithelium response to the cell culture medium.



**Figure 2. The neutralizing activity of anti-hTLR2 Ab to *IL-8* and *TNF-α* mRNA expressions from HKLM-stimulated HK2 cells.** HK2 cells were pre-incubated with various concentrations of the anti-hTLR2 Ab (1.25, 2.5, 5 µg/ml) or isotype control Ab (5 µg/ml) for 1 h before stimulation with the TLR2 ligand (HKLM). The relative fold change of mRNA expression was normalized with *GAPDH* gene and unstimulated cell by  $2^{-\Delta\Delta Cq}$  method. The graph shows mean  $\pm$  SEM of one representative experiment that was performed in duplicate. Unstimulated cell is the HK2 cell response to the cell culture medium.



**Figure 3. Effect of anti-hTLR2 neutralizing Ab on *L. interrogans* stimulated pro-inflammatory cytokine and *hBD2* expressions.** Cultivated kidney epithelium was pre-incubated with or without anti-hTLR2 neutralizing or isotype control Ab for 1 hour before infection, and then stimulated with *L. interrogans* at MOI 100. The relative fold change of mRNA expression was normalized with *GAPDH* gene and unstimulated cell by  $2^{-\Delta\Delta Cq}$  method. The graph shows mean  $\pm$  SEM of one representative experiment that was performed in duplicate. The statistical different of mean between isotype Ab and anti-hTLR2 Ab of three biological replicated were analyzed together by paired *t*-test. (\*statistically significant difference at *p*-value < 0.05). Unstimulated cell is the HK2 cell response to the cell culture medium.

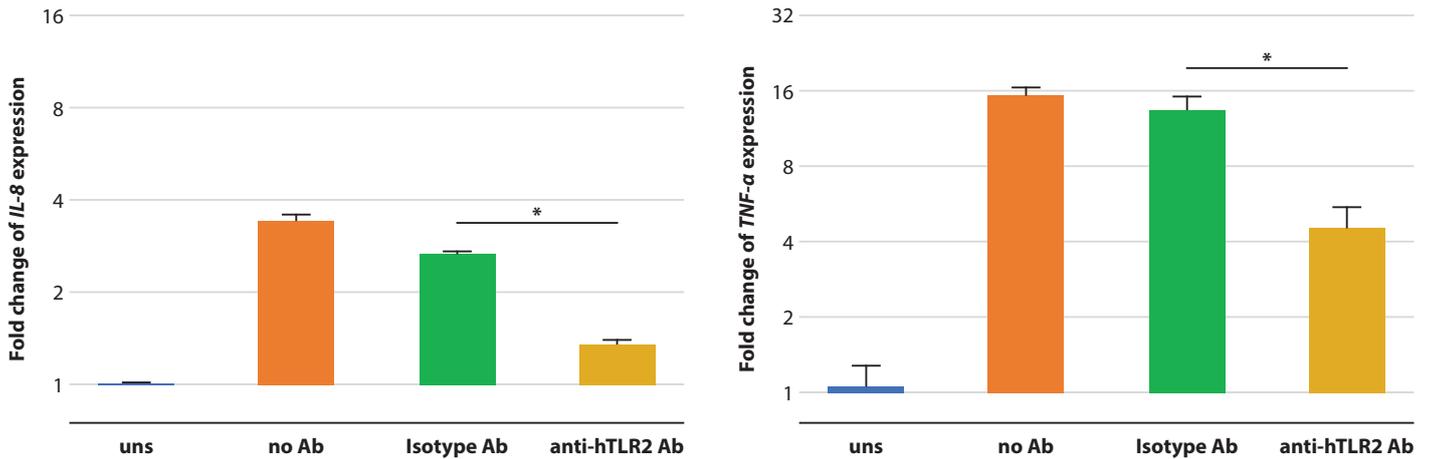


Figure 3. (Continued)

#### *hTLR2 mediated pro-inflammatory cytokine and hBD2 expressions in HK2 cells*

We first examined the optimal concentration of the anti-hTLR2 neutralizing Ab and found that the Ab at the final concentration of 5  $\mu\text{g/ml}$  was sufficient to inhibit IL-8 mRNA expression from HKLM-stimulated HK2 cells (Figure 2). Therefore, we used the final concentration of 5  $\mu\text{g/ml}$  Ab in the next experiment.

As TLR2 is a major human PRR that recognizes *Leptospira*, we investigated whether the expressions of hBD2 and pro-inflammatory cytokines in HK2 cells are mediated by hTLR2. The data in figure 3 indicate that

hTLR2 was involved in the expressions of hBD2 and pro-inflammatory cytokines (IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ). Since, the expressions of these mRNA were significantly reduced in the Ab pre-treated HK2 cells before being infected with *Leptospira*. This experiment indicated that hTLR2 was involved in the expressions of hBD2 and pro-inflammatory cytokines in *Leptospira*-infected HK2 cells.

We further investigated the expression of hTLR2 in HK2 cells and found that the fold of TLR2 mRNA expression in *Leptospira*-infected HK2 cells did not differ from unstimulated HK2 cells (Figure 4).

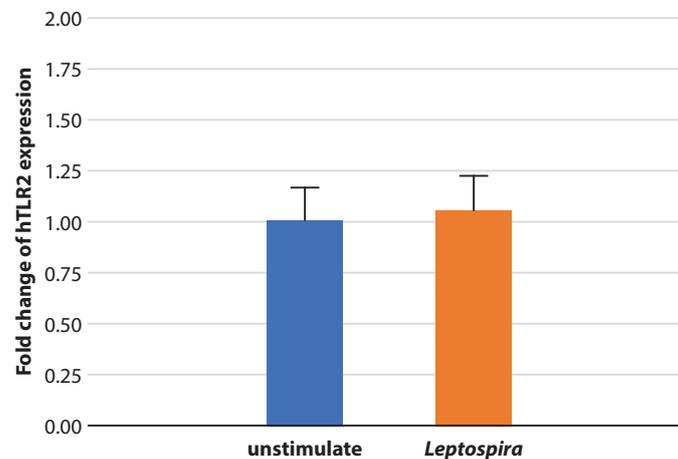


Figure 4. Human TLR2 expression in unstimulated and *Leptospira*-infected HK2 cells. The relative fold change of TLR2 mRNA expression was normalized with GAPDH gene and unstimulated HK2 cell by  $\Delta\Delta\text{Cq}$  method. Unstimulated cell is the HK2 cell response to the cell culture medium.

## Discussion

We focused on the innate immune response in the kidneys which are a major target organ of leptospirosis. The pathology of kidney leptospirosis caused by bacterial invasion and chronic inflammation has been well described.<sup>26-28</sup> The pro-inflammatory cytokines and chemokine which cause inflammation are induced to express in kidneys by pathogenic leptospires.<sup>29</sup> However, the induction of antimicrobial peptide (AMP) expression in response to *Leptospira* infection in kidneys has never been studied.

AMP directly kill microbes or function as a chemotactic factor that results in the recruitment of immune cells and inflammation. The human beta defensins (hBDs) were studied because they can be detected in the epithelium of almost the entire human body, including renal epithelium. Overexpression of hBDs can result in diseases that include psoriasis and kidney disease.<sup>22,30</sup> In an *in vivo* study in hamsters, a susceptible animal,<sup>31</sup> there was significantly upregulated expressions of cytokine *IL-1β*, and chemokines MIP-1α/CCL3 and IP-10/CXCL-10 in response to virulent *Leptospira borgpetersenii* serogroup Ballum isolate B3-13S in the kidneys. However, that study did not examine *hBD2* expression. This AMP may act as chemotactic factor or antimicrobial host defense in kidneys.<sup>32</sup> Therefore, further study is needed to improve our understanding of the role of *hBD2* in immune response activation.

TLR2 is the most important TLR in response to *Leptospira* infection; however, a connection between *Leptospira*, TLR2 recognition/activation, pro-inflammatory cytokine, and hBD productions has not been established in human kidney cells.

We tested the responsiveness of kidney epithelial cells that were found to produce increased amounts of *IL-1β*, *IL-6*, *IL-8*, *TNF-α*, and *hBD2* (but not *hBD1* and *hBD3*) in response to leptospiral infection via a TLR2-dependent mechanism. While anti-TLR2 neutralizing Ab could significantly reduce the expression of hBD-2 and pro-inflammatory cytokines, there may be other PRRs that also play this role. This is plausible because anti-TLR2 neutralizing Ab could not completely inhibit the expression of hBD-2 and pro-inflammatory cytokines.

Renal-associated TLR2 is an important initiator of the inflammatory response.<sup>20,32,33</sup> TLR2 is mainly expressed by tubular cells, and is enhanced upon infection.<sup>34</sup> In this study, TLR2 expression at the transcriptional level from HK2 cells at an acute phase of *Leptospira* infection was not significantly different from unstimulated cells. Human TLR2 is the main PRR recognizing *Leptospira* lipopolysaccharide (LPS) and other outer membrane proteins like LipL32, which is the major lipoprotein of pathogenic leptospires.<sup>15-19</sup> Our findings link leptospiral infection, TLR2 activation, and the induction of both pro-inflammatory cytokines and *hBD2* using human kidney epithelial cells.

Although *hBD2* was initially considered to be an antimicrobial peptide, an advanced study revealed that *hBD2* has multiple functions.<sup>39</sup> However, its role in the pathology of kidney disease caused by leptospirosis is unclear. During the infection processes of the kidney epithelium, *hBD2* is expressed by the effect of the infection mediated to TLR2 activation. This might be associated with the disease severity. There are some limitations in this study. Only one kidney cell line was used; therefore, the results may vary in other human kidney cell lines, primary cells or *in vivo* study. In addition, we determined hBD-2 and pro-inflammatory cytokines only at the transcription level. The transcription results may not be the same as protein expression. An improved understanding of the role of TLR-mediated antimicrobial expression would provide greater insight on the mechanisms of inflammation-mediated kidney tissue damage caused by leptospiral infection. Further study is needed on the role of these molecules in immune response activation.

## Conflicts of interest declaration

All authors declare that they have no personal or professional conflicts of interest related to this research, and they have not received financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

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## Author contributions

All authors contributed to the data analysis and the writing of this paper, gave final approval of the version to be published, and agreed to be accountable for all aspects of the work.

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