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RESEARCH ARTICLE

Leptospiral Lipopolysaccharide-Induced Cytokine Production is Dependent on Toll-Like Receptor 2 in Bovine Cells

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ABSTRACT

Bovine leptospirosis is a widespread zoonotic disease, leading to serious economic losses in beef production and causing potential hazards to human health. It has been known that *Leptospira* species possess lipopolysaccharides (LPS), which are responsible for the leptospirosis pathogenicity, but little is known about the bovine immune response to leptospiral LPS. In this study, we evaluated leptospiral LPS-induced cytokine production in bovine cells by ELISA, and quantitatively measured toll-like receptor 2 (TLR2) and toll-like receptor 4 (TLR4) using real-time PCR and western blotting. In addition, the major toll-like receptors (TLRs) were determined through using anti-TLRs antibodies. We used *Escherichia coli* LPS as a positive control. Leptospiral LPS showed the ability to induce pro-inflammatory cytokine production in bovine fibroblasts that was significantly suppressed by the anti-TLR2 antibody. These results indicate that TLR2 is the main receptor for leptospiral LPS, inducing pro-inflammatory cytokine production in bovine cells, while cytokine induction by *E.coli* LPS was dependent on both TLR2 and TLR4. This suggests that leptospiral LPS differs from *E. coli* LPS in the receptors by which it is recognized in bovine cells, and that the TLR2-dependent pathway might play a significant role in leptospiral LPS-induced bovine chronic inflammatory disease.

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INTRODUCTION

Leptospirosis is a common infection in dairy and beef herds of the world (Adler and de la Pena Moctezuma, 2010). Cattle can frequently become reservoirs for *Leptospira interrogans* serovar hardjo and *Leptospira borgpetersenii* serovar hardjo, with infections arising from direct or indirect contact with contaminated water, and from tissue or urine from infected cattle (Gamage *et al.*, 2014). The symptoms of infected cattle are not frequently developed into typical signs of acute leptospirosis. However, reproductive failure and abortion are most common clinical signs instead, resulting in severe economic losses in the cattle production industry worldwide (Andreoli *et al.*, 2014; Williams and Winden, 2014). Fundamental studies on cattle leptospirosis have been conducted since the 1930s (Ellis, 2015). Recent developments such as immunofluorescence (fluorescent antibody tests) and polymerase-chain-reaction (PCR) analysis methods help us to diagnose the *Leptospira* infection effectively (Ferreira *et al.*, 2014; Hamond *et al.*, 2014; Liu *et al.*, 2014). However, we still do not know how *Leptospira* strains expose their pathogenicity in cattle and how the bovine immune system reacts to the *Leptospira* infection. To better understanding of these is required for infallible prevention of the bovine leptospirosis.

Bacterial lipopolysaccharides (LPSs) are one of the critical factors that strongly stimulate the immune system to produce pro-inflammatory cytokines. In the recognition of bacterial LPS by the innate immune system of animals and humans, pattern recognition receptors such as toll-like receptors (TLRs) play an important role, which can initiate inflammation response to clear the infected bacteria (Akira and Takeda, 2004). Most LPSs of Gram-negative are mainly through TLR4 to stimulate the

production of pro-inflammatory cytokine, but leptospiral LPS showed a difference from most of Gram-negative LPS in structure and biology activities (Que-Gewirth *et al.*, 2004). Thus, leptospiral LPS might induce host immune reaction through different TLRs.

It has already showed that leptospiral LPS is one of the major outer membrane components in *Leptospira*, which can affect the virulence of pathogenic *Leptospira* (Murray *et al.*, 2010). In human cells, leptospiral LPS can be recognized by TLR2, while it is recognized by TLR2 and TLR4 in murine cells, causing species-specific immune responses and host sensitivity (Werts *et al.*, 2001). It still unknown how the bovine TLRs are involved in recognition of leptospiral LPS. In this study, we employed bovine cells to investigate the innate immune responses to leptospiral LPS by quantitatively measuring TLRs (TLR2 and TLR4), and pro-inflammatory cytokines expression.

MATERIALS AND METHODS

Preparation of leptospiral lipopolysaccharide: *L. interrogans* serovar hardjo was cultured in Ellinghausen-McCullough-Johnson-Harris liquid medium (BD Difco, NY, USA) with adding 10% normal rabbit serum (Gibco, NY, USA) at 28ºC for 7 days. The leptospiral LPS was extracted by the method of Isogai *et al.* (1989). *E. coli* serotype O111:B4 LPS (*E.coli* LPS; Sigma-Aldrich, St. Louis, MO, USA) was used as a positive control.

Cell culture and LPS stimulation: The bovine cell line was kindly given from Prof Fukuda (Donai *et al.* 2014). Cells were cultured in DMEM culture medium (Nacalai-tesque Inc., Kyoto, Japan) including 10% fetal bovine serum (Biowest SAS, Nuaillé, France), 1% antibiotic and antimycotic solution (Nacalai-tesque). The cells (1×10^6 cells/well) were suspended in a 6 well plates and cultured overnight at 37°C with 5% CO_2. Then the culture medium was changed into an antibiotic-free medium for 2 h before LPS stimulation. The unstimulated well was used as a control in the experiment.

RNA extraction, cDNA preparation and real time polymerase chain reaction: Total RNA was isolated from the bovine cells according to the protocol of NucleoSpin RNA II kit (Takara Bio Inc., Shiga, Japan). The total RNA was reverse transcript into cDNA using PrimeScript RT reagent kit (Takara Bio, Japan). One µg of cDNA was used to real-time polymerase chain reaction (qRT-PCR) performed on a Thermal Cycler Dice Real-time PCR System II (Takara Bio, Japan). SYBR Premix ExTaq II and primers in Table 1 was used in this study. The amplification condition of qRT-PCR was performed as follows: 3 min at 95ºC, 40 cycles of 95ºC for 15 sec and 60ºC for 1 min. Final results were exported in the relative expression level.

Western blotting analysis: Total proteins were obtained from bovine cells by lysine solution (50 mM Tris-HCl, 0.15 M NaCl, 1% Triton X-100, 2.5 mg/mL sodium deoxycholate, and a protease inhibitor cock-tail from Nacalai-tesque). The total protein concentrations were calibrated by a DC Protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA). Then 10 µg proteins was subjected to SDS-PAGE (ATTO Corporation, Tokyo, Japan) and blotted onto polyvinylidene fluoride membrane (Millipore Corporation, MA, USA) as described previously (Fukuda *et al.*, 2000). Proteins were detected using ECL chemilluminescence Western blot system for immunostaining. The expression of α-tubulin (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) was used as a loading control.

Enzyme-linked immunosorbent assay: The cytokine (TNF-α and IL-6) secretion of bovine cells were measured by following the introduction of bovine enzyme-linked immunosorbent assay Kits (ELISA; R&D Systems Inc., Minneapolis, MN, USA). The optical density values were observed at 450 nm wavelengths in an ELISA plate reader. The concentrations of samples were calculated by known standards in Microsoft Excel. Each sample was measured twice to ensure the reproducibility of the experiment.

Blocking tests using anti-TLRs antibodies: To determine the roles of TLR2 or TLR4 in LPS-induced cytokine production, the bovine cells were pretreated with 2 µg/mL anti-TLR2 or anti-TLR4 antibody (BioLegend Inc., San Diego, CA, USA) for 1 h before 10 µg/mL leptospiral LPS or *E. coli* LPS stimulation. After stimulation with LPS for 6 h, cell supernatants were collected and analyzed by ELISA. Supernatants from cells incubated with LPS, but without anti-TLRs were used as positive, and cells that had not been incubated with LPS or anti-TLRs were used as negative controls. All the experiments were done in triplicate.

Statistical analysis: Three independent experiments were performed in each assay. The mathematical results were displayed as mean±SD and evaluated by Dunnett's test. The values of $P<0.05$ (*) and $P<0.01$ (**) were considered as statistically significant.

RESULTS

Effect of LPS on cytokine production in bovine cells: We determined the ability of leptospiral LPS to increase the production of pro-inflammatory cytokines in bovine cells; then compared the different cytokine inducing abilities between leptospiral LPS and *E. coli* LPS. Bovine cells secreted low levels of IL-6 and TNF-α without LPS stimulation. When cells was stimulated for 6 h with 1 µg/mL leptospiral LPS, levels of IL-6 and TNF-α were markedly increased. In addition, IL-6 and TNF-α were significantly upregulated in a dose-dependent manner with increasing doses of leptospiral LPS (Fig.1A and 1B). Although levels of cytokines were slightly decreased at a 12 h and 24 h time point after 6 h stimulation, the cytokine expressions in stimulated cells was still higher than that in the unstimulated ones (Fig.1C and 1D). *E. coli* LPS was used as a positive control, and was found to induce cytokine up-regulation at concentrations over 10 µg/mL at each time point. Leptospiral LPS showed lower stimulation activity than *E. coli* LPS after 12 h incubation, but the maximum cytokine concentrations induced by leptospiral LPS were similar to those induced by *E. coli* LPS.

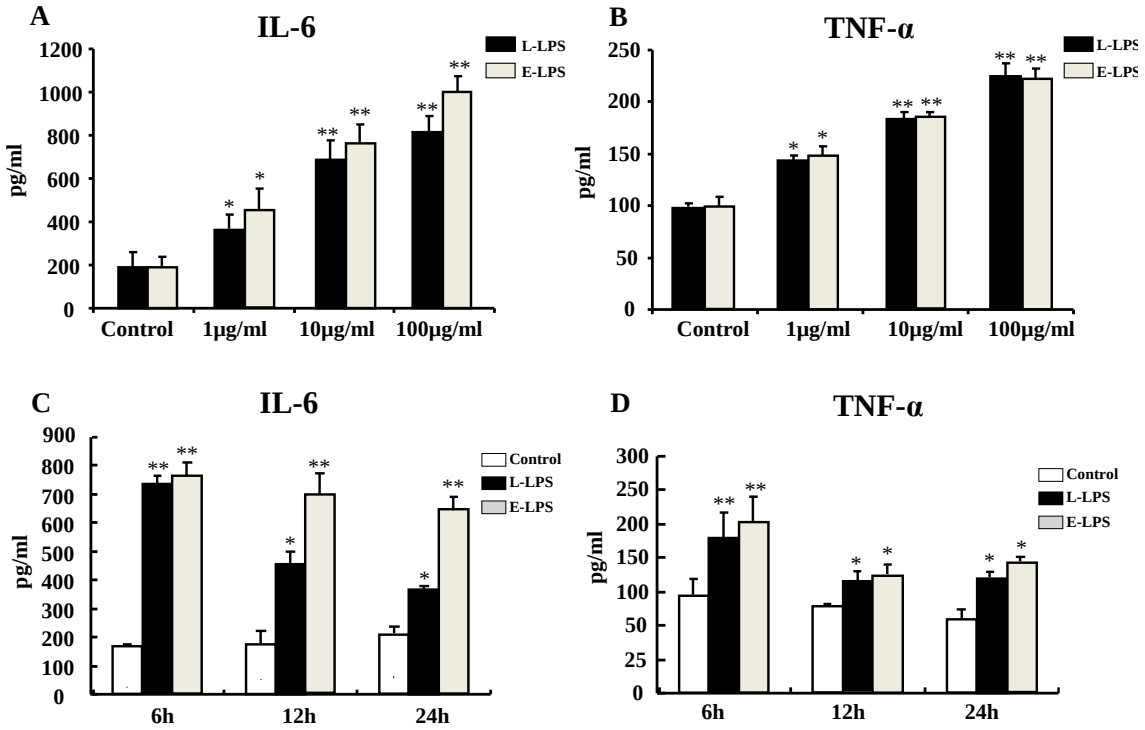

Fig. 1: Quantitative measurement of IL-6 and TNF-α protein secretion in bovine cells. (A) (B) Cells (1×10^6 cells/well) were isolated after a 6 h incubation with LPS at concentrations of 0, 1, 10 and 100 μg/mL. (C) (D) Cells were collected after a 6, 12 or 24 h incubation in the presence or absence of 10 μg/mL LPS (L-LPS and E-LPS represent leptospiral LPS and *Escherichia coli* LPS, respectively). Asterisks indicate significant differences (*P<0.05; **P<0.01).

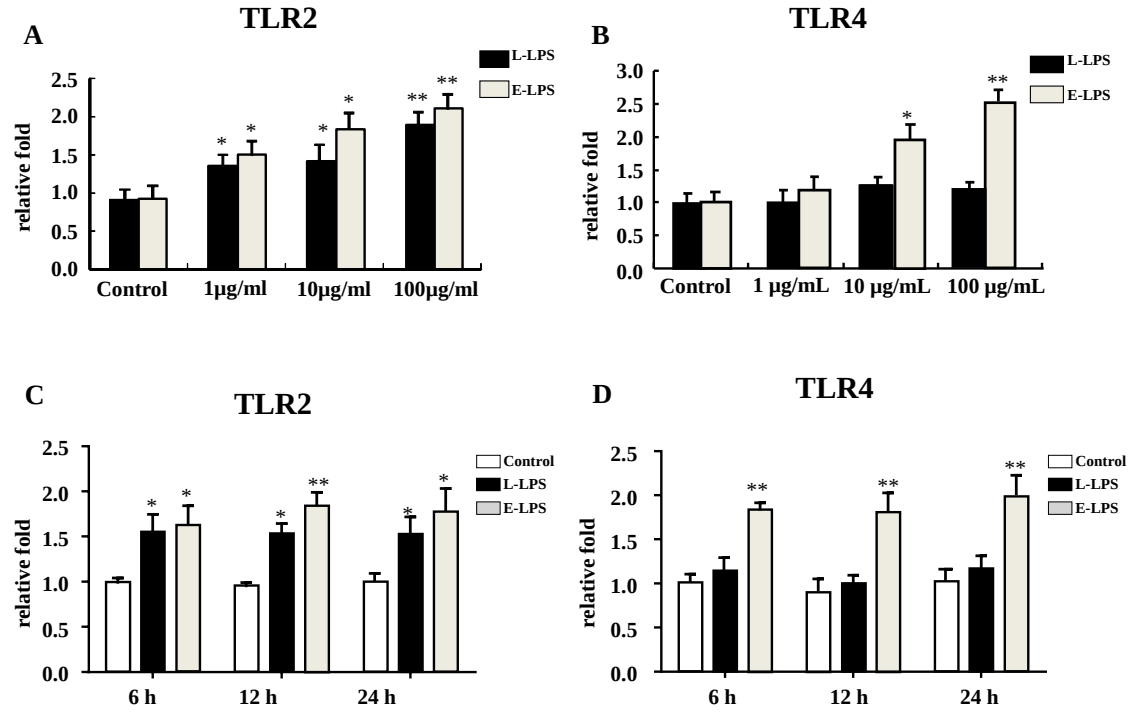

Fig. 2: Quantitative measurement of TLR2 and TLR4 mRNAs expression in bovine cells. (A) (B) Cells (1×10^6 cells/well) were isolated after a 6 h incubation with LPS at concentrations of 0, 1, 10 and 100 μg/mL. (C) (D) Cells were collected after a 6, 12 or 24 h incubation in the presence or absence of 10 μg/mL LPS. Asterisks indicate significant differences (*P<0.05; **P<0.01).

Table 1: Primer sequences used in this study

Gene	Forward	Reverse	Accession No.
TLR2	TCTGCTACGACGCCTTCGT	GCTCCTGGACCATGAGGTTCT	NM_174197.2
TLR4	AGCAGATGCAGAAACCAACC	TGGTACATGGCGGCATTTAC	NM_174198
IL-6	AGCGCATGGTCGACAAAATC	TCCTTGCTGCTTTCACACTC	NM_173923
TNF-α	ACCCAGCCAACAGAAGC	CCAGACGGGACACAGGA	NM_173966
β-action	TTTTTGGCGCTTGACTCAGG	TTGGGAATGCTCGATCAAC	AY141970.1

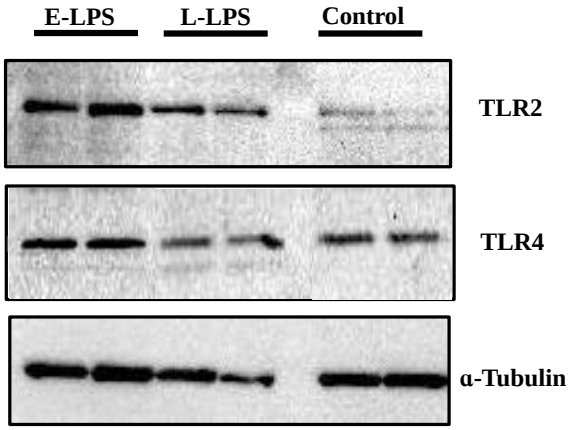

Fig. 3: Effect of LPS stimulation on TLR2 and TLR4 protein expression in bovine cells. Cells were stimulated with 10 µg/mL LPS for 6 h. Unstimulated cells were used as a control. Anti-α-tubulin polyclonal antibody was used to detect α-tubulin as an internal control.

Effects of LPS on TLR2 and TLR4 mRNA expression in bovine cells: To understand how the TLRs serve as the main mediators of bovine response to LPS, the bovine TLR2 and TLR4 mRNA expression levels were tested using real-time PCR. Figure 2A and 2B show that leptospiral LPS induces a significant dose-dependent increase in expression of TLR2 mRNA, but does not change expression of TLR4 mRNA at any concentration up to 100 µg/mL. Cell stimulated with *E. coli* LPS were used as a positive control, and showed a significant up-regulation in TLR2 and TLR4 mRNA when the concentration of *E. coli* LPS was greater than 10 µg/mL. Therefore, we used 10 µg/mL leptospiral LPS and *E. coli* LPS to investigate the time-dependent response on TLR2 and TLR4 mRNA expression in bovine cells. Incubation of bovine cells with 10 µg/mL *E. coli* LPS for 6 to 24 h strongly induced expression of both TLR2 and TLR4, and their levels were increased in comparison to those in unstimulated cells (Fig. 2 C and D). When cells were treated with 10 µg/mL leptospiral LPS, TLR2 mRNA expression was increased monotonically, and the relative value of the level of TLR2 mRNA expression to that of unstimulated cells at each time point after start of the incubation was greater than 1.5 ($P<0.05$) (Fig. 2 C). However, there was no significant increase in the expression level of TLR4 mRNA in the cells stimulated by leptospiral LPS (Fig. 2 D).

Estimation of expression level of the TLR2 and TLR4 protein in bovine cells: Western blot analysis showed that expression of the TLR2 protein was significantly increased by leptospiral LPS and *E. coli* LPS stimulation (Fig. 3), while there was no significant difference in levels of TLR4 protein expression between leptospiral LPS stimulated and unstimulated cells (Fig. 3). *E. coli* LPS-treated bovine cells showed a marked up-regulation of TLR4 compared with unstimulated cells, which is consistent with increase in TLR4 mRNA transcription (Fig. 2).

Functional role of TLR2 and TLR4: We sought to elucidate the functional involvement of bovine TLR2 and TLR4 in LPS-induced cytokine production. The cells were incubated with 2 µg/mL anti-TLR2/TLR4 polyclonal antibody before LPS stimulation. The anti-TLR2 antibody showed a significant inhibitory effect ($P<0.05$) on bovine IL-6 and TNF-α production in leptospiral LPS stimulated cells, but anti-TLR4 antibody showed no significant effect on IL-6 and TNF-α production (Fig. 4A and 4C). However, both anti-TLR2 and anti-TLR4 antibodies significantly inhibited the bovine IL-6 and TNF-α release of from *E. coli* LPS stimulated cells, and anti-TLR4 antibody inhibition had greater effect.

DISCUSSION

Most studies defining the mechanism of recognition and responses in bovine innate immune system were focused on Gram-positive and Gram-negative organisms, due to their importance as bovine pathogens (Rahman *et al.*, 2010). Nonetheless, spirochetes are one group of bacteria, which causes serious bovine disease (Klitgaard *et al.*, 2014). In this study, we studied one member of the spirochete family, *Leptospira*, which differs from all other invasive spirochetes by possessing outer membrane LPS (Murray *et al.*, 2010).

In this study, *E. coli* LPS was used as a positive control. It is recognized by TLR2 and TLR4 in bovine cells as shown in previous reports (Fu *et al.*, 2013; Ibeagha-Awemu *et al.*, 2008)). Bovine cells react to LPS of a pathogenic *Leptospira* strain causing significant up-regulation of TLR2, whereas it has no effect on TLR4 expression. Production of cytokines (IL-6 and TNF-α) was up-regulated by leptospiral LPS stimulation, and could be inhibited by anti-TLR2 antibody. These results indicate that the main response to leptospiral LPS is TLR2-dependent in bovine cells.

It is known that different pathogens can affect TLR-LPS interactions. For example, LPSs from some pathogenic bacterial species such as *Helicobacter pylori* (Smith *et al.*, 2011), *Porphyromonas gingivalis* (Taguchi *et al.*, 2015), and *Legionella pneumophila* (Fuse *et al.*, 2007) are recognized by TLR2 rather than TLR4 in humans, as is the case with *Leptospira*. Furthermore, these bacteria present atypical lipid A structure compared to those of enterobacterial species that are recognized by both TLR2 and TLR4. Leptospiral LPS showed a difference lipid A structure from *E. coli* LPS (Nahori *et al.*, 2005). Thus, variation in structural characteristics may lead to differences in recognition of the bacterial LPSs through TLRs in innate immune activity.

A previous report showed that variations in TLRs from different hosts could influence LPS recognition, leading to species-specific TLR-LPS responses (Werling *et al.*, 2009). For example, TLR4 activation by *E. coli* lipid A is a host species-independent, whereas the recognition of *Salmonella enterica* lipid A is host species-dependent (Hajjar *et al.*, 2012; Kong *et al.*, 2012). It is already known that the involvement of TLRs in recognition of leptospiral LPS in human and porcine cells was different from that in murine cells: TLR2 and TLR4 can recognize to leptospiral LPS in murine cells, but only TLR2 play a role in human and porcine cells (Werts *et al.*, 2001; Guo *et al.*, 2015). Different TLR expression patterns relate to differences susceptibility to *Leptospira* infection between humans and mice. Our experiment results showed that the bovine response to leptospiral LPS stimulation would be similar to that of human and porcine cells. Lack of bovine TLR4 recognition of leptospiral LPS may cause the subclinical symptoms mentioned above.

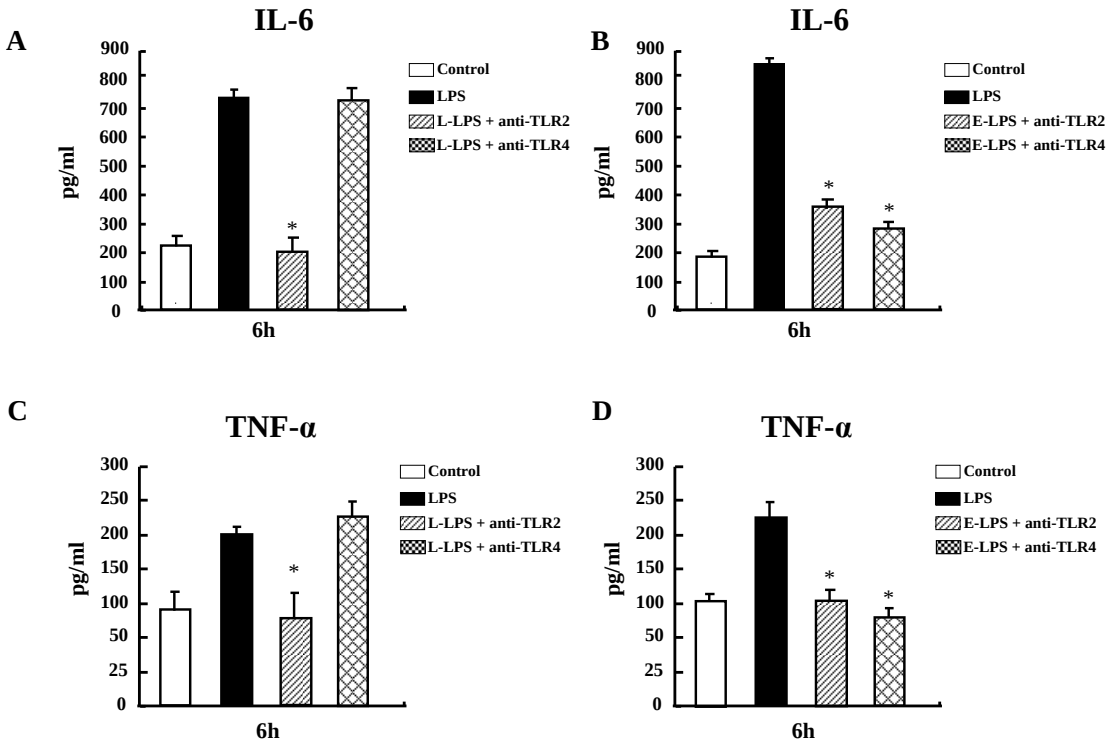

Fig. 4: Effects of anti-TLR antibodies on the production of IL-6 (A, B) and TNF-α (C, D) in bovine cells stimulated with LPS. Before the addition of leptospiral LPS (L-LPS) or *E. coli* LPS (E-LPS) (10 μg/mL), cells were pretreated for 1 h with anti-TLR2 and anti-TLR4 antibodies (2 μg/ml). Cell-free supernatants were collected at 6 h after 10 μg/mL LPS stimulation and assayed by ELISA. Asterisks indicate significant differences (*P<0.05).

Conclusions: The present study shows that the bovine TLR2 is the main receptor in the recognition of leptospiral LPS, which is the cause of bovine chronic inflammatory disease, and has biological relevance to human TLR2 in *Leptospira* infection. Through evaluating bovine cellular models for host-*Leptospira* interaction, it indicates that large animal models can offer unique experimental opportunities for design for a human vaccine against *Leptospira*.

Authors' contributions: YG and JX conducted the experimental procedures. LW and JX performed the data analysis. All authors interpreted the data, critically revised the manuscript for intellectual content, and approved the final version.

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