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The Influence of Intravenous Lipopolysaccharide Injection on TLR4 Transcription Levels in Duck (*Anas domestica*) Liver, Spleen and Bursa of Fabricius

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ABSTRACT

TLR4 is a member of the toll-like receptor family and can recognize lipopolysaccharide (LPS), the main component of gram-negative bacteria. To investigate how TLR4 responds to LPS, the *TLR4* transcription levels in duck liver, spleen and bursa of Fabricius were examined after a model for immune stress was implemented through LPS injection in Peking ducks. The results from this assay suggested that LPS injection significantly increased the concentration of tumor necrosis factor-α (TNF-α) in bursa of Fabricius ($P<0.05$), along with an increase in the concentrations of immunoglobulin-G (IgG) and interleukin-12 (IL-12) in the serum of ducks at 1 day and 7 days after LPS injection ($P<0.05$). However, there were no apparent changes in organ index or the relative expression of TLR4 and IgM ($P>0.05$). Furthermore, correlation analysis showed a positive relationship between the mRNA expression of *TLR4* and concentrations of TNF-α and IL-12. These results revealed that although there were no significant effects of LPS injection on *TLR4* mRNA expression in lymphoid organs, the response to LPS may not mainly rely on the transcription of *TLR4*, and the downstream molecular response in the immune system may contribute more to the TLR4 signaling cascade.

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JYL, YMZ and JK conducted all experiments. JYL, HHL, JWW, LL and XHD designed the experiments and drafted manuscript.

Key words
LPS, Toll-like receptor 4, Immunity index, Peking duck, *Tumor necrosis factor* -α, Interleukin-12, Immunoglobulin-G

INTRODUCTION

The innate immune system is the first response in protecting the body from foreign intruders. The initiation of innate immune responses is characterized by a recognition of microbial pathogens, such as the inflammatory response, which is mediated by pattern recognition receptors (PRRs) (Kawai and Akira, 2011). The PRRs mainly include two types of receptors: toll-like receptors (TLRs), which are membrane-bound receptors and can recognize extracellular pathogens; and intracellular PRRs, which include the nucleotide binding oligomerization domain (NOD)-like receptors (NLRs) and the retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs). As important members of PRRs, TLRs mainly act as sensors for conserved microbial components, where these receptors play a critical role in activating and initiating innate immunity. It has been clarified that different TLRs recognize differential pathogen-associated molecular patterns (PAMP). TLR2 mainly mediates the response to cell-wall components of gram-positive bacteria (Takeuchi *et al*., 1999). TLR3 has been proven to recognize double-strand RNA and synthetic RNA. TLR9 can recognize the CpG motif of bacterial DNA (Armant and Fenton, 2002; Boyd *et al*., 2007). Lastly, TLR4 mainly recognizes lipopolysaccharide (LPS), which is released from the cell walls of dead gram-negative bacteria and, therefore, plays essential roles in initiating inflammatory responses.

When host species are infected by gram-negative bacteria, TLR4 embedded in the membrane of host cells will recognize LPS and release pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and IL-12, to initiate the inflammatory response (Nomura *et al*., 2000). LPS has been shown to cause multiple sickness symptoms in both avian and mammals, including changing body temperature, reducing growth performance and immune functions (Star *et al*., 2008; Jiang *et al*., 2011b). Musa *et al*. (2009) demonstrated the exposure of chicken peripheral blood mononuclear cells to avian pathogenic *E. coli* resulted in rapid changes in both inflammatory and anti-inflammatory cytokines, which are both important in regulating the immune response (Musa *et al*., 2009). In ducks, Zhu (2009) investigated the

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harmful effect of bacterial endotoxin on ducks' organs by observing the pathological sections of organs following LPS injection (Zhu *et al*., 2009). Therefore, LPS was often used as a model antigen to study an animals' susceptibility to pathogens by mimicking bacterial infection (Gray *et al*., 2012; Nord *et al*., 2013).

As the specificity recognition receptor of LPS, TLR4 has been implicated in signal transduction by its extracellular leucine-rich repeat (LRR) domain, which associates with the LPS-LPS binding protein (LBP)-CD14 compounds. Additionally, when TLR4 binds to its ligand, the Toll/interleukin-1 receptor (TIR) homology domain (a cytoplasmic conserved region of TLR4) begins to interact with its downstream protein kinases (Armant and Fenton, 2002; Underhill, 2002). Subsequently, nuclear factor (NF)-κB signaling is activated and the inflammatory cascade reactions are initiated (Chow *et al*., 1999; Visintin, 2005). Previous studies concentrated more on the mechanism of TLR4 responding to LPS with regards to the protein-protein interactions, whereas the effect of LPS on mRNA expression levels of *TLR4* was seldom documented. The effect of LPS challenge on *TLR4* mRNA expression has been documented in a broad range of cell populations. In humans, Song *et al*. (2001) confirmed that *TLR4* mRNA was expressed in corneal epithelial cells and expression levels increased with the presence of LPS (Song *et al*., 2001). It also has been confirmed that the mRNA expression of *TLR4* could be induced by LPS in the peritoneal macrophages of zebra finches and duck embryo fibroblasts (Vinkler *et al*., 2009; Zhao *et al*., 2013).

In avian, the spleen and bursa of Fabricius are the major lymphoid organs, both playing key roles in immune function. It has been proven that *TLR4* is expressed in the ceacal tonsil and the spleen in chickens (Iqbal *et al*., 2005). However, it was not clear whether the expression of *TLR4* could be influenced by LPS in these lymphoid. Therefore, the present study attempted to study the effects of LPS on the expression of *TLR4* in duck lymphoid organs (the spleen and bursa of Fabricius), as well as the levels of *IgM* and cytokines during the inflammatory process, all of which are typical immune molecular markers. These studies may expand our understanding to the mechanism of TLR4 response to LPS.

MATERIALS AND METHODS

Animals

Twelve Peking ducks from the Waterfowl Breeding Farm at Sichuan Agricultural University were used in this study, and each duck weighed, on average, 2.041±0.1801 kg. Food and water were available *ad libitum*. At 36 days of age, the ducks were randomly divided into two groups of six (control in=6; LPS n=6). The LPS group was injected with an LPS-PBS solution (LPS powder was dissolved with 1 mL sterile PBS) in vein at a dose concentration of 400μg/kg BW (body weight) (Jiang *et al*., 2011b). The control group was injected the same dose of sterile PBS. After 24 h, the above-mentioned process was repeated.

Blood collection and sampling

After fasting for 12 h, blood was sampled using a 5 ml syringe through a vein in the duck's right shank at 1 day after the final LPS injection (1 dpi) and 7 dpi. Blood samples were maintained for 30 minutes at room temperature and then centrifuged for 10 minutes at 3000 g. The serum was collected and stored at -20°C immediately. At 7 dpi, the twelve ducks from the two groups were weighed. Next, the ducks were sacrificed, and the liver, spleen and bursa of Fabricius were separated from adhering tissues and accurately weighed. The organ index was calculated by the relative weight (grams) of the spleen, bursa of Fabricius and liver to BW (kilogram). The organ samples were frozen in liquid N_2 and stored at -80°C until use. All procedures were approved by the Beijing Animal Welfare Committee.

ELISA assay for cytokines

The samples (liver, spleen and bursa of Fabricius) were made into 10% tissue homogenate through homogenizing in ice-cold phosphate buffer saline (PBS) according to a W/V at 1:10 and then centrifuged at 3000 rpm for 10 min at 4°C. The collected supernatants were stored at -20°C. The amounts of TNF-α, IL-12 in both serum and organ samples were measured using ELISA kits (Qisong, Beijing, China) according to the manufacturer's protocols.

RNA extraction and real-time PCR

Total RNA from all samples was extracted using Trizol (Invitrogen, USA), following the manufacturer's instructions. RNAs were reverse transcribed using PrimeScript® RT reagent Kit (TaKaRa, Japan) according to the manufacturer's instructions. Real-time PCR primers for each of the genes of interest were designed, and the primer sequences are listed in Table I. GAPDH (AY436595) and β-actin (EF667345) were used as the housekeeping genes. The SYBR®ExScript™ RT-PCR Kit (TaKaRa, Japan) was used for real-time PCR, which was carried out using an iCycler IQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, CA, USA). The real-time PCR procedures included 3 min of pre-denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 30 s and 60°C for 40 s. Real-time PCR analysis of each sample was repeated in triplicate. The data were calculated by the normalized relative quantification method followed by $2^{-\Delta\Delta Ct}$ (Livak and Schmittgen, 2001).

Table I.- The primer sequences for real-time PCR.

Gene name	Primers sequences	Product size(bp)	GenBank ID
TLR4	F:GCTTGCTGCATTGGTGTTCATA	131	JN618073
	R:AGTGGATAACAAAGGCATCATAGGT		
IgM	F:ACCTCTTCATCCGTTGGCTCC	103	APU27213
	R:GTGAAGTAAGAGCGCGCCAGT		
GAPDH*	F:AAGGCTGAGAATGGGAAAC	254	AY436595
	R:TTCAGGGACTTGTCATATTC		
β-actin*	F:CAACGAGCGGTTCAGGTGT	92	EF667345
	R:TGGAGTTGAAGGTGGTCTCG		

The genes marked with"*" are housekeeping genes.

Data analysis

The results are expressed as the mean ± SEM and analyzed statistically using GLM procedures for a one-way analysis of variance. The Spearman's correlation analysis was used to determine whether the expressions of TLR4 mRNA in tissues correlated with organ index, cytokines production and parameters in serum. All of the statistical analyses were performed using SAS V.8.0 (SAS Institute Inc., Cary, NC, USA). A value for P<0.05 was considered significant.

RESULTS

Organ index

The results of the organ index were not significantly affected after ducks were injected with LPS (Table II). The liver index and bursa of Fabricius index decreased by 11.37% and 12.41%, respectively, compared with the control group (P>0.05). However, the spleen index increased by 13.92% after LPS injection compared with the control group (*P*>0.05).

Table II.- Organ indices.

	Liver (g/kg)	Spleen (g/kg)	Bursa of Fabricius (g/kg)
Control	26.65±0.87	0.79±0.06	1.45±0.09
Treatment	23.62±2.28	0.90±0.08	1.27±0.18

The label "*" on the each item indicates a significant difference between the different groups (P<0.05), n=6.

Concentrations of TNF-α, IL-12, and IgG and T-SOD activity

As shown in Figures 1A and B, the LPS-treated ducks secreted a larger amount of TNF-α in the bursa of Fabricius at 7 dpi (*P*<0.05).However, IL-12 levels in the liver and spleen were reduced significantly after the ducks were injected by LPS for 7 days (*P*<0.05, Fig. 1C). LPS injection had a significant influence on the concentrations of IgG and IL-12 in the serum from the ducks at 1 dpi and 7 dpi, respectively (*P*<0.05, Fig. 1D, E). In addition, there were no significant effects on T-SOD activity when the ducks were injected with LPS at 1 dpi and 7 dpi (P>0.05).

TLR4 and IgM mRNA expression levels

It has been shown that the stimulation of bacterial LPS slightly down-regulated the relative expression of TLR4 mRNA (Fig. 2A, *P*>0.05). The expression of IgM had a trend in variation that was similar to TLR4 in spleen, bursa of Fabricius and liver, with the mRNA levels of both reduced after LPS injection compared with the control group (Fig. 2B, *P*>0.05).

Relative expression of TLR4 mRNA and immunity indices

In the liver, the mRNA expression of TLR4 had a positive relationship with the production of TNF-α and the organ index respectively following injection of LPS (*P*<0.01). In the spleen, there was a strong positive relationship between the mRNA expression of TLR4 and the concentration of TNF-α in the treatment group (*P*<0.01) (Table III). The correlation between these two values in the treatment group was greater than in the control group. Additionally, in the bursa of Fabricius, the correlation between the TLR4 mRNA expression and IL-12 production also increased after LPS injection. In addition, there was a significant positive correlation between TLR4 mRNA expression and the organ index in the bursa of Fabricius under LPS challenge (*P*<0.01) compared with the negative correlation of these values before LPS injection. However, in lymphoid organs, bacterial LPS had no significant effect on the relationship between TLR4 mRNA expression and T-SOD activity or IgG concentration in the serum overall.

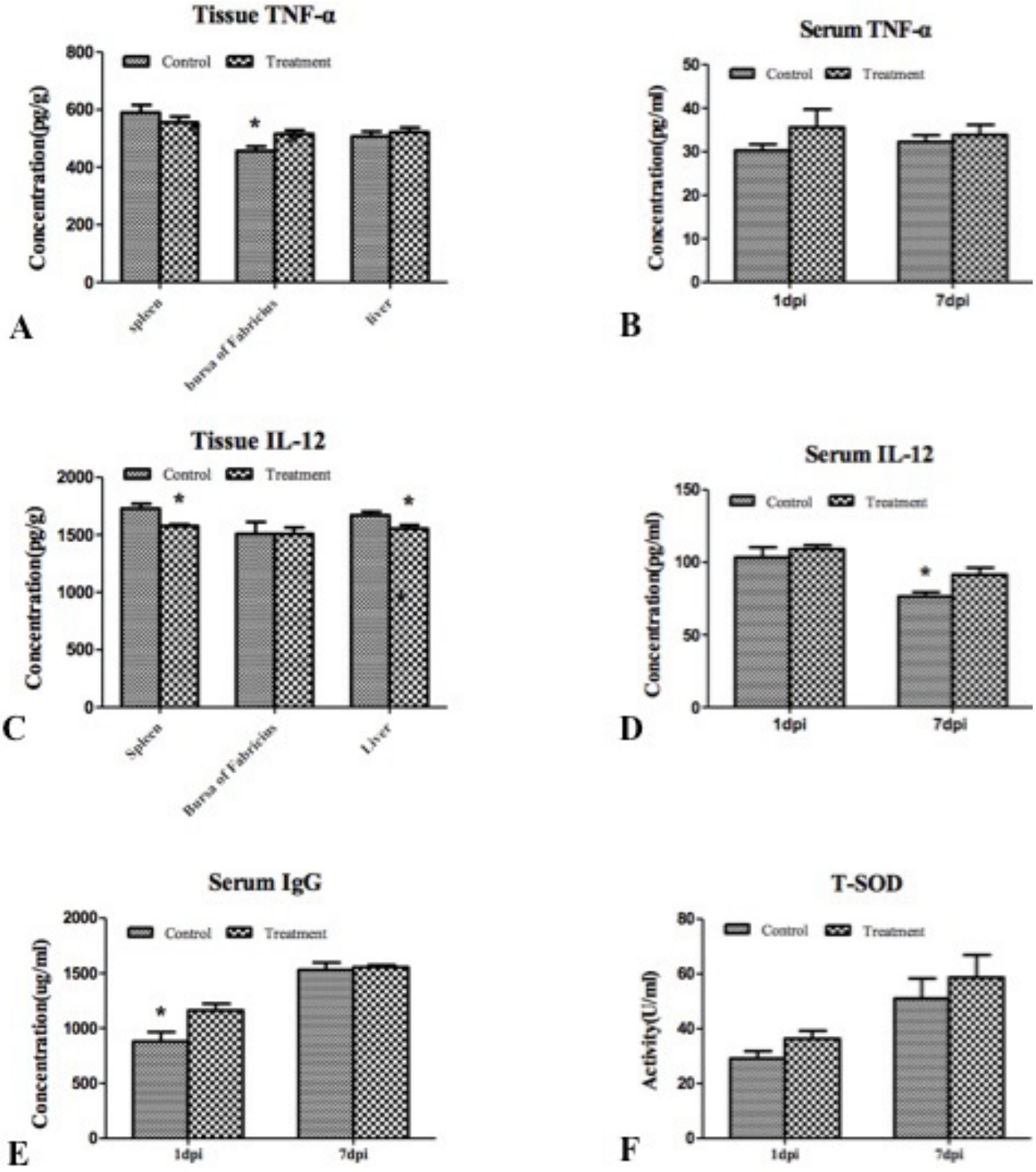

Fig. 1. Changes in TNF-α, IL-12, and IgG concentrations and T-SOD activity**.** A, the tissue concentrations of TNF-α in control and treatment groups; B, the serum TNF-α levels in control and treatment groups; C, the tissue concentrations of IL-12 in control and treatment; D, the serum IL-12 concentration in control and treatment; E, the serum concentrations of IgG in control and treatment groups; F, the T-SOD activity of serums in control and treatment groups. The label "*" on each item indicates a significant difference between the different groups ($P<0.05$), n=6. "dpi" represents the days post injection of LPS.

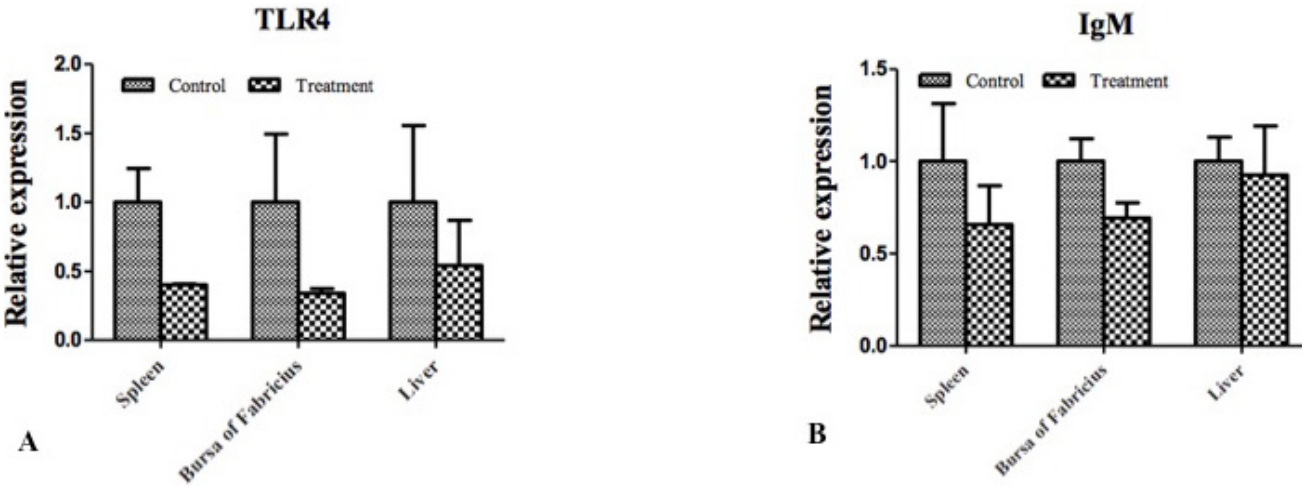

Fig. 2. Changes in TLR4 and IgM mRNA expression in tissues**.** Panel A & B show the relative expression of TLR4 and IgM, respectively, in liver, spleen and bursa of Fabricius between control and treatment groups. The label "*" on each item indicates a significant difference between the different groups (P<0.05), n=4

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Table III.- Association of the TLR4 gene expression and immunity indexes

	Liver TLR4(mRNA)		Spleen TLR4(mRNA)		Bursa of Fabricius TLR4(mRNA)	
	Control	Treatment	Control	Treatment	Control	Treatment
TNF-α	0.3	1.0**	0.5	1.0**	0.5	-0.5
IL-12	0.1	-0.5	0.5	-0.5	0.5	1.0**
Organ index	-0.8	1.0**	-0.5	0.5	-0.5	1.0**
IgG Serum(1dpi)	-0.3	-0.5	-0.5	0.5	-1.0**	-0.5
T-SOD Serum(1dpi)	-0.6	-0.5	-1.0**	0.5	-0.5	-0.5

The label "**" on the each data indicates a highly significant difference between the different groups (P<0.01), n=4. "dpi" indicates days post injection.

DISCUSSION

Colibacillosis, which is primarily caused by the gram-negative bacteria *Escherichia coli,* is one of the major bacterial diseases in ducks. The growth of *E. coli* could lead to LPS contamination in water and in animals' blood (Jiang *et al*., 2011a; Liu and Wang, 2012; Shi zhendan, 2011). In the present study, ducks were injected with LPS, and the subsequent phenotypic and molecular changes were measured and compared between control and treatment ducks. Our results showed that the organ index was not significantly changed following LPS injection, but there was a numerical reduction in the organ index, which was similar to the results of Chen's studies in piglets where the spleen and thymus index were significantly reduced in the LPS injection group after 14 days (Chen *et al*., 2010). These phenomena suggest that LPS might have a repression effect on the development of lymphoid organs. This discrepancy in whether the changes of immune indexes were significant after LPS injection is most likely due to the difference in the anti-infection response between pigs and ducks. In addition, the mRNA expression of IgM, an efficient anti-microbial molecule, was not significantly changed in the LPS group compared with the control. This result was contrary to Dresser's research demonstrating that LPS-induced B lymphocytes make IgM antibodies increase after intravenous injection of LPS in mice (Dresser, 1978). On the whole, the influences of LPS on lymphoid organs and duck immunity were not obvious, and these effects may be connected to the concentration of the dose of LPS and be time-dependent.

As the specific receptor for LPS in pathogen recognition, the importance of TLR4 in initiating a rapid innate immune response was proven by the fact that TLR4-deficient mice are LPS hyporesponsive but respond normally to gram-positive bacteria (Takeuchi *et al*., 1999). The objective of our study was to test whether the transcription levels of TLR4 will be influenced when faced

with an inflammatory stimulus in duck. We found that LPS down-regulated the mRNA expression of TLR4 slightly. Tu *et al*. (2007) detected that in chickens, TLR4 mRNA levels reduced 24 h after LPS treatment, and these results were similar to ours. However, Wu *et al*. (2008) reported that the mRNA expression of TLR4 increased after the injection of LPS in rats. The reduction of TLR4 mRNA levels suggested that the anti-inflammatory response occurred to prevent the amplification of the inflammatory response and to alleviate the injury of infection. Thus, this down-regulation of TLR4 protected the body from an excessive inflammatory response to some extent (Swank and Deitch, 1996; Tu *et al*., 2007; Wu *et al*., 2008). Based on our data, there were no significant differences in TLR4 mRNA expression between the control and treatment groups. Changes in mRNA expression may depend on the concentration of the dose of LPS as well as the time, which may decide the effects of the inflammatory responses (Chow *et al*., 1999). Furthermore, the suppression of TLR4 mRNA levels was thought to be involved in the LPS-tolerance caused by the repeated injection of LPS (Nomura *et al*., 2000). Overall, we proposed that the influences of LPS on TLR4 may not primarily rely on mRNA levels, but might be achieved through mediating the cascade of TLR4 downstream signaling.

The expression of TLR4 is relatively close to the secretion of inflammatory cytokines (Li *et al*., 2011). Cytokine production is a functional effect of LPS, and plays an important role in regulating inflammatory responses (Kogut *et al*., 2005). TNF-α and IL-12 induced by LPS are the main pro-inflammatory cytokines involved in inflammatory responses (Ozmen *et al*., 1994). In Blanque's *et al*. (1996) report, the sampling points were selected between 1 and 8 h after injection of LPS in mice. This group found that the peak levels of TNF-α expression were detected at 1-2 h and that these levels declined rapidly to control levels (Blanque *et al*., 1996). Kobayashi *et al*. (2013) found that compared with wild type mice, LPS-induced TNF-α and IL-12 production in dendritic cells and macrophages was significantly enhanced in BTLA$^{-/-}$ (B and T lymphocyte attenuator) mice after treatment for one and four hours, respectively (Kobayashi *et al*., 2013). As the typical indicator of acquired immunity, the production of serum IgG suggested the capacity of animals to overcome the invasion of pathogen. Additionally, SOD can combine with nitric oxide, which is released by activation macrophages and the hepatic cell, to resist liver injury (Josephs *et al*., 2000). From our results, LPS injection increased the concentration of TNF-α both in bursa of Fabricius, as well as the concentrations of IL-12 in serum. There was also a significant increase in IgG production between the control and treatment groups at 1 dpi. Thus, these results suggest that animals would initiate the defense mechanism by releasing a large amount of cytokines to resist challenges from bacteria.

Furthermore, correlation analysis between TLR4 expression and immunity indexes showed that there were strong positive relationships between TLR4 mRNA expression and the concentrations of TNF-α and IL-12 after LPS injection. These data suggest that the effects of the inflammatory response induced by LPS had led to a broad range of cytokine production. At the same time, a significant positive correlation was found between TLR4 mRNA expression and organ index in the treatment group, which was contrary to the correlation before the LPS challenge. In the present experiment, we found that bacterial infections would influence the production of TNF-α and IL-12 in liver, lymphoid organs and serum. This was especially noticeable when considering the concentration of TNF-α, as it increased when ducks encountered the invading LPS. Additionally, LPS injection was also found to increase IgG concentration in serum at 1dpi. Along with this, there were significant correlations between the mRNA expression of TLR4 and the concentration of TNF-α and IL-12 after LPS injection. Thus, even though LPS had no significant influence on the expression of TLR4 mRNA in lymphoid organs, the effects of LPS during the inflammatory response were not always dependent on the transcription of TLR4 mRNA. However, LPS may be involved in the cascade amplification of TLR4, which activates the signaling of LPS/TLR4 and influences a large range of other downstream genes and the release of cytokines.

Statement of conflict of interest

The authors declare no conflict of interest.

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