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—Full Paper—

Changes in the Expression of Toll-Like Receptor mRNAs During Follicular Growth and in Response to Lipopolysaccharide in the Ovarian Follicles of Laying Hens

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Abstract. The aim of this study was to determine the changes in the mRNA expression of Toll-like receptors (*TLR*s) in hen ovarian follicles during follicular growth and in response to lipopolysaccharide (LPS). White follicles and the fifth largest to largest follicles (WF and F_5–F_1, respectively) were collected from laying hens. To examine the effects of LPS, the laying hens were treated intravenously with LPS (1 mg/kg BW) 0, 3, 6, 12 and 24 h before examination. Expressions of *TLR*s and *IL-1β* in the theca and granulosa layers were examined by semi-quantitative RT-PCR. Immunocytochemistry was performed to identify immunoreactive TLR-4. The theca layer expressed *TLR-2*, *TLR-4*, *TLR-5* and *TLR-7*, whereas the granulosa layer expressed only *TLR-4* and *TLR-5*. The expression of *TLR-4* and *TLR-5* in the theca layer increased significantly during follicular growth. In the granulosa layer, the expression of *TLR-5* increased, but that of *TLR-4* was unchanged. Expression of *TLR-4* increased significantly during the period of 6 to 12 h of LPS treatment in the theca layer and during the period of 12 to 24 h in the granulosa layer of F_3. Immunoreaction products for TLR-4 were observed in theca interna and granulosa layers of WF and F_5-F_1, with the greater amount observed in the theca interna. LPS treatment significantly increased expression of *IL-1β* in the theca layer after 3 h and in the granulosa layer during the period of 12 to 24 h. These results suggest that TLRs are expressed in ovarian follicles and that *TLR-4* and *TLR-5* expression increase with the growth of follicles. Enhanced expression of *TLR-4* and *IL-1β* by LPS in the theca and granulosa layers suggests possible roles of TLR in recognition of microorganisms.

Key words: Laying hen, Lipopolysaccharide, mRNA expression, Ovarian follicles, Toll-like receptors

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The ovary of the laying hen contains numerous prehierarchical follicles and several hierarchical preovulatory yellow follicles protruding from the ovarian stroma [1]. The follicular wall consists of a loose connective tissue coat, the theca and granulosa layers. *Salmonella enteritidis* (SE) has been isolated from the ovary and the theca and granulosa layers following oral, intraperitoneal or intravenous inoculation and in culture [2–8] and it may infect hen ovarian tissues and subsequently be transmitted to the eggs [3]. Therefore, ovarian immune function is essential for the protection of ovarian tissue from pathogenic microorganisms. Toll-like receptors (TLRs), a class of pattern recognition receptors, recognize pathogens or their products and initiate an intracellular signaling cascade that leads to activation of innate host defense [9].

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In the chicken, 6 TLR genes (*TLR*)s, including *TLR-1* (*type 1* and *2*), *TLR-2*, *TLR-3*, *TLR-4*, *TLR-5* and *TLR-7* have been identified in heterophils and other tissues [10–12]. TLR-1 and TLR-2 recognize the bacterial lipoproteins and peptidoglycans of Gram-positive bacteria, whereas TLR-4 recognizes the lipopolysaccharides (LPS) of Gram-negative bacteria, such as *E. coli* and *Salmonella* [10, 13]. TLR-2 also recognizes LPS to a lesser extent than TLR-4 [14]. TLR-3, TLR-5 and TLR-7 recognize double-stranded RNA, bacterial flagellin and single-stranded RNA, respectively [10]. LPS is usually used to mimic bacterial infections [15, 16]. There are reports that *TLR*s induce cytokines, antimicrobial peptides and immunocompetent cells, such as antigen presenting cells expressing major histocompatibility complex (MHC) [11, 17–20]. Interleukin -1β (IL-1β), a cytokine that functions in mediating inflammatory response, is also induced by LPS via TLR-4 [11, 21]. Despite recent information regarding the expressions of chicken *TLR*s in different tissues, neither their expression in ovarian follicles during follicular growth nor their induction during infection in ovarian tissue has been reported until now. If TLRs mediate the immune response in hen ovarian follicles, the expressions of the IL-1β gene (*IL-1β*) may be enhanced in response to LPS in association with expression of *TLR*s. Furthermore, the expression of *IL-1β* in the ovarian follicle in response to LPS has not been studied to date.

Ovarian follicles undergo changes in structure and function during their growth that may affect the expression of immune cells. Furthermore, the expressions of *TLR*s may be affected by follicular growth. Therefore, the aim of this study was to determine the changes in the expression of *TLR*s in ovarian follicles during follicular growth and in response to LPS *in vivo*. The effects of LPS on *TLR*s were examined using *TLR-2* and *TLR-4* only because these two TLRs have been reported to be sensitive to LPS. The mRNA expression of *IL-1β* in response to LPS was also examined to confirm the possibility that TLRs play roles in cytokine induction.

Materials and Methods

Experimental birds

White Leghorn laying hens approximately 400-d-old that laid 5 or more eggs in sequence were kept in individual cages under a 14 h light:10 h dark regime and provided with free access to feed and water. Ovarian follicles were collected after euthanization of the birds under anesthesia with sodium pentobarbital. Handling of the chickens was conducted in accordance with the regulations of Hiroshima University for animal experiments.

Tissue collection

To determine the *TLR*s expressed in follicles, white follicles (WF) and the third largest follicles (F_3) were collected. For analysis of the changes in the expression of *TLR*s with follicular growth, WF, the fifth largest follicles (F_5), F_3 and the largest follicles (F_1) were collected (n=5) 18–20 h before the estimated time of ovulation (5 h after oviposition). The theca and granulosa layers were isolated separately from these follicles by the method of Gilbert *et al*. [22], in which the granulosa layer is separated from the theca layer without contamination of either.

For the analysis of the effects of LPS on *TLR-2*, *TLR-4* and *IL-1β* expressions, laying hens were treated intravenously with LPS at a dose of 1 mg/kg body weight (BW) using 4 mg/ml stock solution (n=4 each). This dose was sufficient to induce an immune response in our previous study [23]. The stock solution of LPS was prepared by dissolving LPS from *E. coli* 0111:B4 (Wako Pure Chemical, Osaka, Japan) in Dulbecco's phosphate buffer (Nissui Pharmaceutical, Tokyo, Japan) at 4 mg/ml concentration. The LPS treatment was performed for 0, 3, 6, 12 and 24 h before collection of follicles. Kidneys and livers were also collected at 0 and 12 h of LPS treatment as a control tissue (n=4).

RNA extraction

RNA extraction was performed using Sepasol RNA I super (Nacalai Tesque, Kyoto, Japan) as described previously [24]. The total RNA samples were dried and dissolved in TE buffer (10 mM Tris, pH 8.0, with 1 mM EDTA). The RNA samples were treated with 2 U of DNase I (Takara Bio, Ohtsu, Japan) in a Programmable Thermal Controller, PTC-100 (MJ Research, Waltham, MA, USA), programmed at 37 C for 1 h and 80 C for 30 min and were then stored at 4 C. The concentration of RNA in the sample was measured with Gene Quant Pro (Amersham Pharmacia Biotech, Cambridge, England).

Table 1. The primer sequences of the *TLR*s, *IL-1β* and *β-actin* used for PCR amplification

Targets	Primer sequence 5′–3′		Accession numbers
TLR-1 type 1	F-AGGTTGGACTTCTTATTGAGGCATAC	R-AGATGAATCCCAAACTAGCAGAAAAA	AY633574
TLR-1 type 2	F-TGGCCTGGCTAAGAAAAATGA	R-GCTCATGTCCAGATACCTCAGTGA	AY633573
TLR-2	F-TGCCTGAAGCCACAGACATTCCTA	R-TTTCCACCCAGTTGGAGTCGTTCT	AB046119
TLR-3	F-CCACTCTGGAAGAAAATGAGC	R-TCATTCTCACCGCTTTTCAG	BI066273
TLR-4	F-AGTCTGAAATTGCTGAGCTCAAAT	R-GCGACGTTAAGCCATGGAAG	AY064697
TLR-5	F-TGCACATGTTTTCTCCTAGGT	R-CCACATCTGACTTCTGCCTTT	AJ626848
TLR-7	F-GCCTCAAGGAAGTCCCCAGA	R-AAGAAACATTGCATGGATTACGG	AJ632302
IL-1β	F-GGGCATCAAGGGCTACAA	R-CTGTCCAGGCGGTAGAAGAT	NM_204524
β-actin	F-TTCCAGCCATCTTTCTTG	R-TCCTTCTGCATCCTGTCA	X00182

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT- PCR)

Semi-quantitative RT-PCR was conducted as described previously [23]. The RNA samples were reverse transcribed using reaction mixture (10 μl) consisting of 1 μg of total RNA, 1 × RT buffer, 1 mM of each dNTP, 20 U RNase inhibitor, 0.5 μg of oligo $(dT)_{20}$ and 50 U of ReverTra Ace in a PTC-100 programmed at 42 C for 30 min, and this was followed by heat inactivation for 10 min at 99 C. The PCR was performed in a PTC-100 in a volume of 25 μl of reaction mixture containing cDNA corresponding to 1 μg of the initial total RNA, 1 × PCR buffer, 0.2 mM each dNTP, 0.4 μM each primer and 0.625 U Takara Taq (Takara Bio). The cycle parameters were 94 C initial denaturation for 2 min, 35 or 40 cycles of 94 C denaturation for 30 sec, 58 C (for *TLR*s) or 60 C (for *IL-1β*) annealing for 1 min and 72 C extension for 2 min followed by a final extension at 72 C for 10 min and storage at 4 C. The forward and reverse primers used in this study are shown in Table 1. *β-actin* was used for standardization. Different cycles of PCR, namely 30, 35, 40 and 45 were tested using cDNA samples of thecal F_3 to determine the optimal cycle, and 35 cycles (for *TLR-2* and *TLR-4*) or 40 cycles (for *TLR-5*, *TLR-7* and *IL-1β*) were considered to be optimal. PCR products were separated by electrophoresis using 2% (w/v) agarose gels containing ethidium bromide (0.5 mg/mL) and photographed under UV illumination. Densitometry was performed using UN-SCAN-IT gel (ver. 6.1, Silk Scientific Corporation, Orem, UT, USA), and the ratios of the *TLR-2*, *TLR -4* and *IL-1β* mRNAs to *β-actin* mRNAs were obtained.

The PCR products of *TLR -2*, *TLR -4*, *TLR -5*, *TLR-7* and *IL-1β* that were found in the theca layer were sequenced using a Big Dye Terminator Sequence Kit (ver. 3.1, Applied Biosystems, Foster City, CA, USA) with an ABI 3100 automated sequencer (Applied Biosystems), as described previously [25]. The sequences of these targets corresponded to the sequences in GenBank.

Immunocytochemistry for TLR-4

To localize immunoreactive TLR-4, WF, F_3, F_5 and F_1 were collected from healthy laying hens. They were fixed in formalin, washed and dehydrated with a graded series of alcohols, cleared with xylene and embedded in paraffin. Paraffin sections (6 μm in thickness) were prepared and air-dried on aminpropyltriethoxysilane treated slides. After deparafinization, antigen retrieval was performed using 10 μg/ml proteinase K (Merck, Darmstadt, Germany) at 37 C for 40 min in a humidified chamber. After washing with phosphate buffered saline (PBS), sections were incubated with 1% casein milk in PBS for 15 min. The sections were then incubated overnight with goat polyclonal antibody raised against a peptide near the C-terminus of human TLR-4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted to 1:50 in PBS containing 1% BSA and washed with PBS for 15 min (3 × 5 min). This TLR-4 antibody has previously been used to identify TLR-4 in chickens [26]. The sections were then incubated with mouse anti-goat biotinylated IgG (Santa Cruz Biotechnology) and avidin-biotin HRP complex (Vector Laboratories, Burlingame, CA, USA) for 1 hr each and were washed with PBS for 15 min (3 × 5 min) after each step. Immunoprecipitates were visualized by incubation with 0.02% (w/v) 3′, 3′-diaminobenzidine tetrahydrochloride and 0.005% (v/v) H_2O_2 in 0.05 M Tris-HCl buffer (pH 7.6). The sections were dehydrated with a graded series of alcohols and mounted. They were examined under a light microscope (Eclipse E600; Nikon,Tokyo,

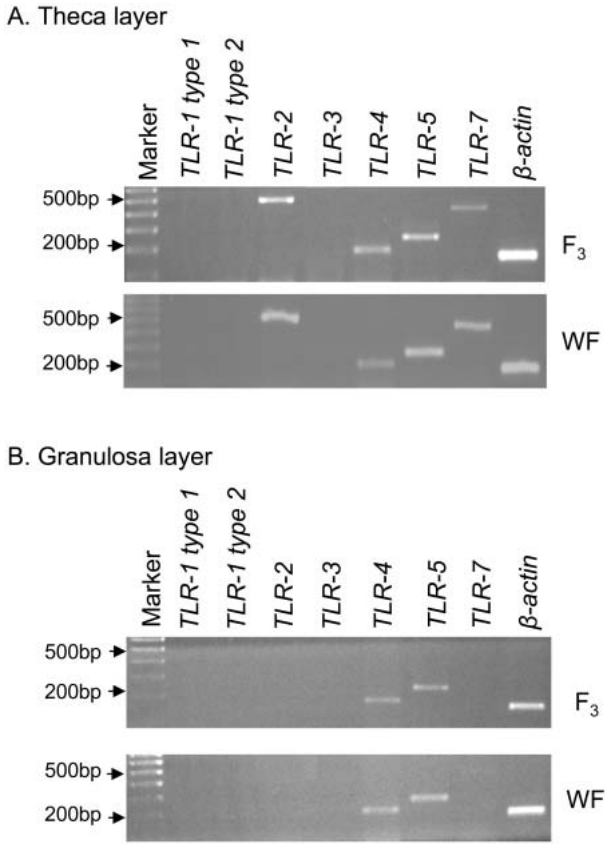

Fig. 1. Expressions of Toll-like receptors (*TLR*s) in the theca and granulosa layers. (A) Theca layer; (B) Granulosa layer of the white and third largest follicles (WF and F_3, respectively) of laying hens.

Japan) using a Normarsky filter. Control staining was carried out simultaneously in which the first antibody was replaced with normal goat IgG (R & D Systems, Minneapolis, MN, USA), and no specific staining was found in control slides.

Statistical analysis

The results were expressed as the mean ± SEM of the ratio of the *TLR*s or *IL-1β* to *β-actin* mRNA. Prior to analysis, the data were first analyzed by Bartlett's test to ensure homogeneity of variance. Square root transformation was performed in the case of unequal variances. The significance of differences among the follicles or LPS treated groups were examined using one-way ANOVA followed by Duncan's multiple range test. The expression levels of *TLR-4* and *IL-1β* in the liver and kidney were compared for the control and treated groups using the Student's t-test. Differences with a P value of <0.05 were considered statistically significant.

Results

Figure 1 shows the expression of various *TLR*s in the theca and granulosa layers of WF and F_3. The theca layer expressed *TLR-2*, *TLR-4*, *TLR-5* and *TLR-7* (Fig. 1A), whereas the granulosa layer only expressed *TLR-4* and *TLR-5* (Fig. 1B).

The changes in the expression level of *TLR*s during follicular growth are shown in Fig. 2. In the theca layer, the expression levels of *TLR-2* and *TLR-7* did not differ among WF, F_5, F_3 and F_1. The expression levels of *TLR-4* and *TLR-5* exhibited a tendency to increase with follicular growth in yellow follicles, namely it was higher in the theca layer of F_1 compared with WF and F_5 (Fig. 2A). In the granulosa layer, the *TLR-4* expression level was similar among follicles. However, the *TLR-5* expression level in the granulosa layer increased during follicular growth and was higher in F_1 than in WF, F_5 and F_3 (Fig. 2B).

The changes in the expression levels of *TLR-2* and *TLR-4* in the theca and granulosa layers of F_3 in response to LPS during different time periods are shown in Fig. 3. The theca expression level of *TLR-2* in response to LPS did not change significantly during any time period. In contrast, that of *TLR-4* in the theca layer increased significantly during the 6 to 12 h period before declining at 24 h (Fig. 3A). The *TLR-4* expression level in the granulosa layer also increased during the 12 to 24 h period of the LPS treatment (Fig. 3B).

The changes in the expression of *IL-1β* in the theca and granulosa layers of F_3 in response to LPS during different time periods are shown in Fig. 4. The expression level of *IL-1β* in the theca layer increased significantly during the 3 to 24 h period of the LPS treatment (Fig. 4A). However, its expression level in the granulosa layer increased during the 12 to 24 h period of the LPS treatment (Fig. 4B).

Unlike in the theca and granulosa layers, the expression levels of *TLR-4* and *IL-1β* in the kidney and liver were unaffected by 12 h after treatment with LPS (data not shown).

Figure 5 shows the sections of WF, F_5, F_3 and F_1 from the healthy laying hens that were immunostained for TLR-4. Dense immunoreaction

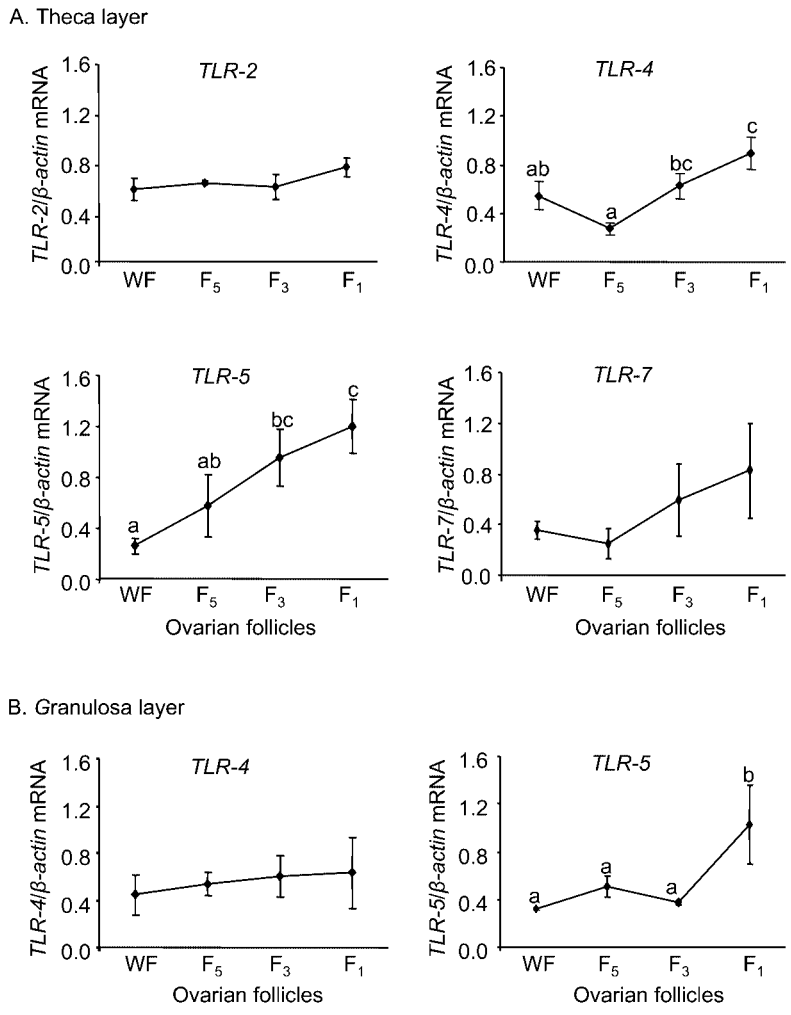

Fig. 2. Changes in the expression of *TLR*s in the theca and granulosa layers of ovarian follicles during follicular growth. (A) Theca layer; (B) Granulosa layer. Values are means ± SEM of the ratio of target mRNA to *β-actin* mRNA (n=5). WF: white follicle. F_5: the fifth largest follicle. F_3: the third largest follicle. F_1: the largest follicle. Values with different letters differ significantly (P<0.05).

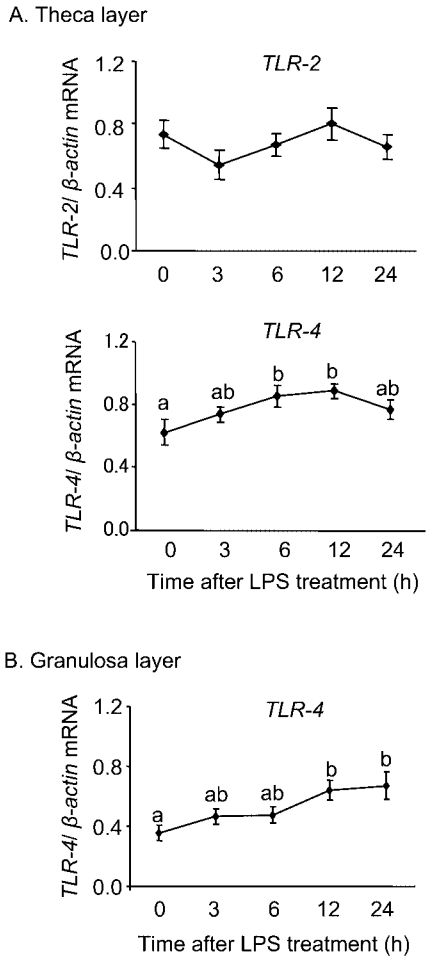

Fig. 3. Changes in the expression of *TLR -2* and *TLR-4* in the theca and granulosa layers of F_3 during different time periods after LPS treatment. (A) Theca layer; (B) Granulosa layer. Values are means ± SEM of the ratio of *TLR-2* or *TLR-4* mRNA to *β-actin* mRNA (n=4). Values with different letters differ significantly (P<0.05).

products were observed in the theca interna of WF, F_5, F_3 and F_1 (Fig. 5A, B, C and D, respectively). The positive cells were localized in the cells associated with capillaries, and the inner layer of the theca interna faced the basal lamina. Small amounts of immunoreaction products were observed in the granulosa layers of WF, F_5, F_3 and F_1.

Discussion

To our knowledge, we are the first to examine the expressions of *TLR*s in hen ovarian follicles and their changes with follicular growth and in response to LPS. Noteworthy findings of the present study are as follows. 1) Of the reported 6 *TLR*s, 4 were expressed in the theca layer, whereas only 2 were expressed in the granulosa layer 2) During follicular growth, expression of *TLR-4* and *TLR-5* increased in the theca layer, and that of *TLR-*

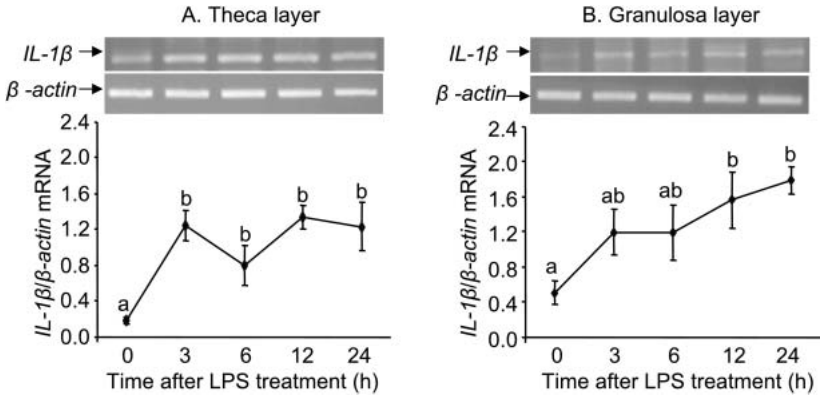

Fig. 4. Changes in the expression of *IL-1β* in the theca and granulosa layers of F_3 during different time periods after LPS treatment. (A) Theca layer; (B) Granulosa layer. The corresponding PCR products of *IL-1β* and *β-actin* during different time periods are also shown. Values are means ± SEM of the ratio of *IL-1β* mRNA to *β-actin* mRNA (n=4). Values with different letters differ significantly (P<0.05).

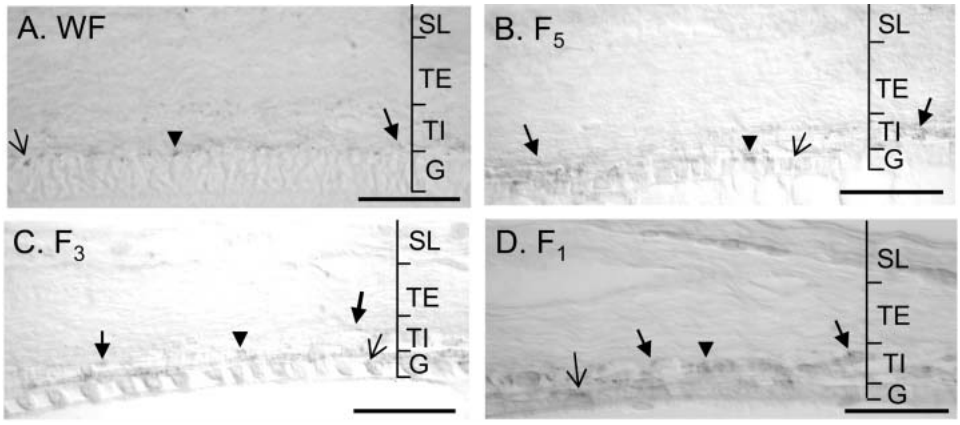

Fig. 5. Sections of ovarian follicles immunostained for TLR-4. (A) White follicle (WF), (B) the fifth largest follicle (F_5), (C) the third largest follicle (F_3) and (D) the largest follicle (F_1) of a healthy laying hen. Thick arrows, blunt arrows and thin arrows indicate examples of positive cells in cells associated with capillaries and the inner layers of the theca interna and granulosa layers, respectively. G: granulosa layer. TI: theca interna. TE: theca externa. SL: superficial layer. Scale bars represent 40 μm.

5 increased in the granulosa layer 3) The expressions of *TLR-4* and *IL-1β* were enhanced in response to LPS in both the theca and granulosa layers.

RT-PCR analysis showed that *TLR-2*, *TLR-4*, *TLR-5* and *TLR-7* were expressed in the theca layer, whereas only *TLR-4* and *TLR-5* were expressed in the granulosa layer of the white and yellow follicles of laying hens. The immunoreaction products for TLR-4 were identified in the theca interna and granulosa cells, with a greater amount found in the theca interna of WF and yellow follicles. Thus, the theca layer likely recognizes more types of microorganism components compared with the granulosa layer. Based on the expression of *TLR*s, both the theca and granulosa layers may recognize the LPS of Gram-negative cells and flagellin via TLR-4 and TLR-5, respectively. In addition, the theca layer may recognize the peptidoglycan of Gram-positive cells and single-stranded RNA via TLR-2 and TLR-

7, respectively. The theca interna is the site where the capillary network develops, and thus microorganisms reach it before the granulosa layer is infected. Theca interna cell phagocytosed foreign agents [27] and expression of MHC class II in the theca layer is enhanced by SE stimuli [28, 29]. Thus, theca TLRs may play roles as one of the first defense systems to induce innate immunity.

The expressions of *TLR-4* and *TLR-5* in the theca layer and *TLR-5* in the granulosa layer increased with the growth of follicles. Thus, this suggests that the ability to recognize microorganism components of these TLRs increases with follicular growth. Immunoreactive TLR-4 was localized in the cells associated with capillaries in the theca interna. Blood flow increases with follicular growth [30, 31], which may result in accumulation of larger numbers of circulating foreign antigens to larger rather than smaller follicles. The increased *TLR*s expression in the larger follicles may be required to defend against accumulating foreign antigens, including microorganisms. Larger follicles also have increased phagocytosis activity in theca interna cells for defense [27]. The theca and granulosa cells are the targets of sex steroids because receptors for progesterone, androgen and estrogen are expressed in these tissues [32–34]. We have previously shown that estrogen increases the number of immunocompetent cells, including macrophages, T lymphocytes and Ig-containing B cells, in follicles [35–37]. We assume that expression of *TLR*s may be locally regulated by endocrine factors in follicles as in the case of other immunocompetent cells. Although the expressions of *TLR-2* and *TLR-7* in the theca layer and *TLR-4* in the granulosa layer do not change during follicular growth, they may be expressed constantly in all follicles as a potential ability to recognize Gram-positive, Gram-negative and viral components.

The expression of *TLR-4* was enhanced by LPS treatment in both the theca and granulosa layers. It is likely that LPS is recognized by the TLR-4 of both theca and granulosa cells, the expression of which in turn is enhanced in those cells. The theca wall is a highly vascularized tissue with vessels [38], and this anatomical structure facilitates the transport of microorganisms from blood to developing follicles [39]. Circulating agents may be deposited near the basement membrane because of the termination of vessels near it [38]. They may then pass through the basement membrane and enter the yolk after invading the granulosa cells [4]. SE has been identified in both the theca and granulosa layers of birds [4, 6–8]. The increased TLR-4 in the theca interna may recognize the LPS of microorganisms as the first defense mechanism, and then the granulosa TLR-4 may recognize the LPS that enters the granulosa layer. Although there are reports that TLR-2 may recognize LPS in some cases [40], the expression of theca *TLR-2* was not increased by LPS, suggesting that it is less sensitive to LPS than theca TLR-4.

We identified *IL-1β* mRNA expression in the theca and granulosa layers. There are reports that induction of *IL-1β* expression by LPS is mediated by TLR-4 [11, 21, 41]. The current study is the first to show expression of *IL-1β* in the theca and granulosa layers of hens, which was enhanced by LPS. Expression of *TLR-4* mRNA was also found in the theca layer. The TLR-4 identified in the theca layer may mediate enhancement of *IL-1β* expression in tissues by LPS. The granulosa cells of the follicles expressed *TLR-4* before and after LPS treatment. It is likely that granulosa cells express *TLR-4* and that this expression was enhanced by LPS. An increased level of IL-1β stimulates infiltration of T cells and macrophages [42]. Treatment of birds with SE causes infiltration of these immunocompetent cells in ovarian follicles [5, 7, 43]. We assume that TLR-4 plays a significant role in recognition of SE to enhance *IL-1β* expression, leading to influx of immunocompetent cells. TLRs also mediate induction of antimicrobial peptides [19]. Expression of gallinacins (*GAL*s), a class of antimicrobial peptide of chicken, in the theca layer is enhanced by LPS [23]. In mammals, expression of antimicrobial peptide β-defensin-2 is regulated by TLR-4 in response to LPS in intestinal epithelial cells [44]. As in mammals, TLR-4 may also mediate expression of *GAL*s in chickens. Therefore, the TLR-4 in the theca and granulosa layers may recognize LPS and enhance IL-1β and antimicrobial peptides synthesis.

Expression of *TLR-4* and *IL-1β* in the kidney and liver did not increase until 12 h of LPS treatment. The expressions of these tissues may be late to respond or less sensitive to LPS compared with ovarian cells.

In conclusion, we showed that 4 (*TLR-2*, *TLR-4*, *TLR-5* and *TLR-7*) and 2 (*TLR-4* and *TLR-5*) TLRs are expressed in the theca and granulosa layers of laying hens, respectively. Expression of *TLR-4* and *TLR-5* in the theca layer and *TLR-5* in the granulosa

layer increased with follicular growth, and TLR-4 may mediate induction of *IL-1β* expression by LPS. We suggest that a TLRs-mediated immune system exists in the ovary and that it probably plays a role in recognition of pathogens and initiation of immune response to protect ovarian tissues.

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