Toll-like receptor signaling in hen ovarian granulosa cells is dependent on stage of follicle maturation

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Abstract

The recent identification of toll-like receptor (TLR) signaling within ovarian granulosa cells has broad implications for ovarian physiology. Functions of TLRs within granulosa cells of the laying hen are of particular interest due to the method of transovarian transmission of *Salmonella enteritidis*, which results in egg contamination. This study utilized hen granulosa cells to evaluate the expression and function of *Gallus* TLR-signaling at distinct stages of follicular maturity. Data presented herein demonstrate the presence of *TLR2*, *TLR4*, and *TLR15* mRNAs in undifferentiated granulosa cells from prehierarchical follicles and differentiated granulosa cells from preovulatory follicles, together with mRNAs encoding adaptor proteins and signaling components required for TLR signaling gene. Treatment with lipopolysaccharide (LPS) or LH, *in vitro*, led to the differential regulation of TLRs based on the stage of follicle maturation, with the largest (F1) follicle granulosa cells having the most rapid response. Furthermore, treatment with LPS resulted in attenuation of agonist-induced progesterone synthesis in undifferentiated, but not differentiated, granulosa cells. Additionally, undifferentiated granulosa cells were significantly more sensitive to LPS-induced apoptosis than differentiated granulosa cells from the F1 follicle. Together, these data provide evidence for a complete and functional TLR signaling pathway in hen granulosa cells, with effects on steroidogenesis and cell viability dependent upon stage of maturation. These differences may reflect the susceptibility of granulosa cells at early stages of maturation to undergo apoptosis in response to select pathogenic stimuli, thus attenuating transovarian transmission, whereas granulosa cells from preovulatory follicles are comparably resistant to LPS-mediated apoptosis.

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Introduction

Toll-like receptor (TLR) pattern recognition receptors (PRRs) in vertebrate species are a key component in the distinction of self from non-self, and are indispensable for the innate immune response. Activation of TLR signaling occurs following recognition and interaction with specific conserved small molecular sequences found on the surface of invading pathogens, termed pathogen-associated molecular patterns (PAMPs). PAMPs include lipopolysaccharide (LPS) associated with the cell wall of gram-negative bacteria (including *Salmonella enteritidis*), lipoteichoic acid associated with the surface adhesion amphiphile from gram-positive bacteria, bacterial flagellin, peptidoglycan, and components associated with viruses such as double-stranded RNA. In addition to the recognition of pathogenic stimuli, TLRs (particularly TLR2 and -4) recognize endogenous ligands. Most notably, these include extracellular matrix components, such as hyaluronan fragments, heparan sulfate, fibrinogen, fibronectin, lung

surfactant protein A, and high-mobility group box 1 proteins (Zhang & Schluesener 2006). Following ligand binding, TLR signaling can result in up-regulation of pro-inflammatory cytokines (e.g. interleukin (IL)-1 β , tumor necrosis factor (TNF)), up-regulation, and facilitation of scavenger receptors, which result in the phagocytosis of pathogens, and presentation of antigen to naïve T-cells, providing a critical link to the acquired (i.e. adaptive) immune response (Medzhitov *et al.* 1997, Takeda & Akira 2005). Moreover, multiple reports of TLRs in non-immune tissues have begun to surface, bringing to light non-immune-related functions of these PRRs (Liu *et al.* 2008).

Specifically, recent reports have implied a role for TLR signaling in the regulation of vertebrate ovarian function (Shimada *et al.* 2006, 2008, Herath *et al.* 2007, Subdei *et al.* 2007, Liu *et al.* 2008). TLRs have been identified in ovarian cells from multiple species (murine, bovine, chicken), and have been shown in bovine and murine granulosa cells to functionally mediate steroidogenesis and pathogen phagocytosis respectively

(Herath *et al.* 2007, Shimada *et al.* 2006). Importantly, in bovine granulosa cells, TLR signaling has been shown to perturb steroidogenesis, a potential mechanism to curb ovarian follicle growth during bacterial infection (Herath *et al.* 2007).

Understanding the immune response in the ovary is of importance in the laying hen, as egg contamination by *S. enteritidis* represents a serious human health concern. Over 75% of *S. enteritidis* outbreaks are attributable to eggs or egg-based products (Braden 2006). The method of transmission to eggs occurs in the intra-ovarian environment through selective infection of the granulosa cell layer (Gast & Beard 1990, Thiagarajan *et al.* 1994). Subsequent transovarian transmission to offspring results in the spread of *S. enteritidis*, through the production of contaminated eggs and fecal matter. Given the pivotal role of the granulosa layer in maintaining follicle viability and promoting maturation, as well as in propagating transovarian *S. enteritidis* transmission in the hen, identification of key signaling pathways that can contribute to ovarian function, or curb infected follicle development, are of significant interest.

While much remains to be learned about the role for TLR signaling in the ovary, it is becoming clear that TLRs expressed on granulosa cells can and do respond to both exogenous (i.e. pathogenic) and endogenous (e.g. extracellular matrix components) stimuli (Liu *et al.* 2008). Additional evidence from mammals suggests a role for TLR signaling in granulosa or cumulus cells both pre- and post-ovulation (Herath *et al.* 2007, Shimada *et al.* 2007, 2008). Although the significance for TLR signaling during both of these distinct phases remains to be fully elucidated, hen granulosa cells from preovulatory follicles remain within the follicle after ovulation. Thus, the mechanisms by which TLR signaling may participate in reproduction or confer immunity to the ovulated oocyte are likely to differ between avian and mammalian species.

The studies presented herein utilize both differentiated and undifferentiated hen granulosa cells as a model system to evaluate the effects of TLR signaling on granulosa cell function and viability. Accordingly, we provide evidence for 1) expression of TLRs and related downstream signaling component mRNAs in both undifferentiated and differentiated granulosa cells; 2) differential regulation of TLRs in granulosa cells based upon stage of maturation; and 3) a role for TLR signaling in regulation of granulosa cell steroidogenesis and viability.

Results

PCR amplification of TLRs and signaling components in granulosa cells

PCR amplification revealed expression of *Gallus* orthologs to the mammalian TLR2 (type 2), TLR4, and TLR15, in granulosa cells from prehierarchical (3–8 mm diameter) and preovulatory (F1) follicles, plus bone marrow.

Additionally, critical downstream signaling components and adaptor proteins are expressed in both prehierarchical and preovulatory granulosa cells, with the notable exception of MAP3K7IP1, which was amplified only in bone marrow (Fig. 1). While IL-1 receptor-associated kinase (IRAK) 2 and 4 were amplified, a *Gallus* homolog to IRAK1 has not yet been identified experimentally or *in silico*.

TLR2 and TLR4 mRNA expression is increased following treatment with LH or LPS

Treatment with LPS or LH for 4 h led to an increase in *TLR2* and *TLR4* mRNAs in granulosa cells from the largest preovulatory (F1) follicle (Fig. 2A), while the same culture conditions in granulosa cells from the second largest F2 follicle failed to induce a significant increase (Fig. 2B). However, *TLR2* and *TLR4* mRNA levels were elevated in granulosa cells from F2 follicles following treatment with LPS for 20 h. Neither *TLR2* nor *TLR4* mRNA was increased in granulosa cells isolated from prehierarchical (3–8 mm diameter) follicles, although there was a significant decrease in *TLR4* mRNA following treatment with LPS for 20 h (Fig. 2C).

Effects of LPS on granulosa cell steroidogenesis are stage of development dependent

To investigate whether TLR signaling modulates steroidogenesis based on stage of development, progesterone production from cultured granulosa cells was measured following a 4- or 20-h culture in the presence or absence of gonadotropin or 8-bromoadenosine 3', 5'-cyclic monophosphate (8-br-cAMP), with or without LPS (10 µg/ml). Treatment with LPS attenuated FSH-induced progesterone in granulosa cells from prehierarchical (3–8 mm) follicles following 20 h in culture (Fig. 3A). The attenuation was reversed following co-culture with U0126, a pharmacologic inhibitor of the MAPK signaling pathway, and an additive effect of FSH + LPS with U0126 was observed. Notably, treatment with LPS had no measurable effect on LH-induced progesterone synthesis in granulosa cells from preovulatory (F2 and F1) follicles following 4-h incubation (Fig. 3B and C).

Sensitivity to LPS-induced cell death is dependent on stage of development

Treatment with LPS resulted in a significant decrease in cell density following 20 h in culture in granulosa cells from prehierarchical (3–8 mm diameter) and preovulatory (F1) follicles. This was mediated in part by LPS-induced apoptosis, and indicated qualitatively by the presence of low molecular weight oligonucleosome formation (Fig. 4A and B). The effect of LPS was related to follicle size.

Figure 1 PCR amplification of TLRs and downstream signaling component mRNAs from undifferentiated (U) and differentiated (D) granulosa cells. Bone marrow (M) was included as a reference tissue. —, negative control (no template). Based upon expression of requisite signaling proteins, the model depicts proposed *Gallus* TLR signaling in granulosa cells. PAMPs or endogenous ligands bind to a TLR complex (TLR4 depicted). TLR4 is unique among TLRs, as it forms a complex with the co-receptors CD14 and MD2 at the cell surface. Subsequent to ligand activation, toll-interacting protein, TIR-domain-containing adaptor protein, and myeloid differentiation primary-response protein 88 are recruited to the cytosolic TIR domain of the receptor complexes. Following recruitment, a signaling complex comprised of interleukin-1 receptor-associated kinase (IRAK) 2 and 4, as well as the cytoplasmic adaptor, TRAF6, is formed. TRAF6 then dissociates from the complex, activating MAP3K7 and MAP3K7IP2, resulting in the commencement of downstream signaling cascades, such as NFκB and MAPK1/MAPK, and subsequent transcription of target genes.

In prehierarchal follicle granulosa cells, a significant loss of viable cells was detected following treatment with 5 µg/ml LPS (Fig. 4A). However, in fully differentiated granulosa cells from F1 follicles, a significant reduction in granulosa cell viability occurred following treatment with 10 µg/ml LPS (Fig. 4B).

LPS-induced cell death is caspase-8 dependent

Following pre-treatment with the caspase-8 inhibitor, Z-IETD-FMK (10 µM), the decrease in cell viability promoted by LPS was significantly reduced in granulosa cells from prehierarchal follicles, and completely reversed in granulosa cells from preovulatory (F1) follicles (Fig. 5A and B). These data indicate the activation of an extrinsic mechanism for induction of cell death, as

caspase-8 activation is death-receptor mediated. This is likely due to an increase in cytokine production by granulosa cells following treatment with LPS. To investigate potential cytokines known to be responsive to LPS or to increase cell death in granulosa cells, gene expression analysis was used to monitor the levels of TNF apoptosis-inducing ligand (*TNFSF10*), tumor necrosis super-family member 15 (*TNFSF15*), and *IL1B* mRNA following stimulation with LPS for 4 h (F1 granulosa) or 20 h (3–8 mm granulosa). Both *TNFSF10* and *TNFSF15* belong to the TNF ligand superfamily, and have previously been shown to have cytotoxic effects on chicken granulosa cells, *in vitro* (Takimoto *et al.* 2005, Johnson *et al.* 2007). While levels of *IL1B* were significantly increased in granulosa cells from F1 follicles following a 4-h challenge with LPS, levels

Figure 2 Regulation of TLR mRNA in differentiated (F1 and F2) and undifferentiated (3–8 mm) follicles. (A) Treatment with LPS (10 µg/ml) or LH (100 ng/ml) in F1 granulosa for 4 h. $n=3$. (B) Treatment with LPS or LH in F2 granulosa cells for 4 or 20 h. $n=4$. (C) Treatment with LPS in undifferentiated granulosa cells for 20 h. $n=3$. * Indicates significance ($P<0.05$), not significant (ns; $P>0.05$) by independent t -tests, for the treatment group compared with control cultured cells.

were unchanged in granulosa cells from prehierarchical follicles (Fig. 6A and B). By comparison, *TNFSF10* and *TNFSF15* mRNA levels in granulosa cells were not altered by LPS treatment at either stage of differentiation (data not shown).

Discussion

Thus far, 10 *Gallus* TLRs have been identified, including: TLR1 and TLR2 (types 1 and 2), TLR3, TLR4, TLR5, TLR7, TLR15, and TLR21. With the exception of TLR 1 and 15, each *Gallus* TLR has a mammalian ortholog, with TLR1 sharing significant homology to mammalian TLR1, -6, and -10 (Lynn *et al.* 2003). Genes for the two types of chicken TLR1 are on chromosome 1, while the genes for the two types of TLR2 are located on chromosome 4, and are thought to be the products of gene duplication (Fukui *et al.* 2001, Temperly *et al.* 2008). Additionally, *in silico*

analysis has revealed *Gallus* homologs to downstream adaptor and signaling molecules, including myeloid differentiation primary-response protein 88 (MyD88), TIR-domain-containing adaptor protein (TIRAP, or MAL), toll-interacting protein (TOLLIP), IRAK4, transforming growth factor- β activated kinase (MAP3K7), the Tak-1 binding proteins (MAP3K7IP1 and MAP3K7IP2), TNF receptor-associated factor 6 (TRAF6), and NF κ B inducing kinase (MAP3K14; Lynn *et al.* 2003). Functional

Figure 3 LPS inhibits agonist-induced progesterone (P4) from undifferentiated, but not differentiated, granulosa cells via MAPK signaling. (A) RIA targeting progesterone production by undifferentiated granulosa cells. Treatment with LPS (10 µg/ml) attenuates FSH (100 ng/ml)-induced progesterone production. Pre-treatment with the MAP3K2/MAPK inhibitor, U0126 (U0; 10 µM) for 1 h reverses the effect of LPS treatment. $n=4$, $P<0.05$, a, b and c indicate significant differences by ANOVA. (B and C) RIA targeting progesterone production from differentiated granulosa cells from the F2 (B) and F1 (C) follicle. LPS does not effect progesterone production induced by LH (100 ng/ml) or 8-br-cAMP (8-br; 100 nM). $n=3$, $P<0.05$, a and b indicate significance. Note difference in magnitude of P4 for each stage of development.

Figure 4 LPS induces granulosa cell apoptosis. (A) Cell viability assay following 20 h of treatment with LPS in undifferentiated granulosa cells. $n=3$, * indicates significance ($P<0.05$). The presence of cleaved DNA fragments after a 6 h culture confirms activation of apoptosis. (B) Cell viability assay following 20 h of treatment with LPS in differentiated granulosa cells (F1 follicle). $n=3$, * indicates significance ($P<0.05$ by ANOVA).

studies on chicken TLRs demonstrate that the pathways and ligand specificity for each TLR are conserved in the hen (Fukui *et al.* 2001, Leveque *et al.* 2003, Iqbal *et al.* 2005, Philbin *et al.* 2005).

Results reported herein demonstrate the expression of mRNA encoding TLRs and key signaling components in both undifferentiated and differentiated granulosa cells from hen ovarian follicles. Multiple TLRs have been recently described in hen follicles (including the granulosa layer (Subdei *et al.* 2007)), and it has been postulated that TLR-mediated immune responses may participate in the protection of ovarian tissues from invasive pathogens (Shimada *et al.* 2006, Herath *et al.* 2007). Unlike a previous report that indicates the presence of only *TLR4* and *TLR5* in hen granulosa cells (Subdei *et al.* 2007), *TLR4*, *TLR2*, and *TLR15* mRNAs were detected in both undifferentiated and differentiated granulosa cells from hen follicles. This finding is significant, because *TLR4* and *TLR2* (type 2) have been shown to recognize bacterial LPS (Fukui *et al.* 2001, Spiller *et al.* 2007). Additionally, while genes encoding key chicken TLR-signaling components have been identified *in silico*, this is the first study to verify their presence in granulosa cells. These data, in combination with the ability of LPS to modulate granulosa cell

function, establish a functional TLR signaling pathway in hen granulosa cells.

Not surprising was the ability of LPS to modulate *TLR2* and *TLR4* mRNA expression levels *in vitro*. Similar to the results obtained from F3 (third largest preovulatory follicle), granulosa layers extracted from hens treated intravenously with LPS (Subdei *et al.* 2007), both *TLR2* and *TLR4* mRNA expressions were increased following treatment with LPS for 20 h, but not at the earlier time point of 4 h. The timing of up-regulation, however, differed for the largest (F1) preovulatory follicle, in which treatment with LPS elicited a significant increase in *TLR2* and *TLR4* mRNA levels after only 4 h. These data suggest a differential sensitivity to LPS-induced TLR mRNA based on stage of maturation, whereby an accelerated response is elicited in the follicle next to be ovulated.

This differential sensitivity also occurred following treatment with LH. Treatment with LH led to a rapid (within 4 h) increase in *TLR2* mRNA in granulosa cells from the largest preovulatory follicle, in contrast to granulosa cells from the second largest preovulatory follicle in which the increase in *TLR2* mRNA occurred later (within 20 h). This finding is not entirely unexpected, due to the significant increase in LH responsiveness in the F1 follicle as compared with all other stages of

Figure 5 Inhibition of caspase-8 cleavage inhibits LPS-induced apoptosis. Cell viability assay in undifferentiated (A) or differentiated (B; from F1 follicle) granulosa cells. Cells were treated with or without Z-IETD-FMK (IETD; 20 µM) for 1 h followed by treatment with or without LPS (10 µg/ml) for an additional 20 h. $n=3$, A, B and C indicate significance ($P<0.05$ by ANOVA).

Figure 6 *IL1B* mRNA expression in granulosa cells following treatment with LPS. (A) Differentiated granulosa cells (from F1 follicles) were treated with or without LPS (10 µg/ml) for 4 h. $n=3$ * indicates significance ($P<0.05$) by *t*-test. (B) Undifferentiated granulosa cells (from follicles 3–8 mm in diameter) were treated with or without LPS (10 µg/ml) for 20 h. $n=3$, not significant (ns) $P>0.05$ by *t*-test.

maturation (Calvo *et al.* 1981, Johnson *et al.* 1996). It is also consistent with a recent report demonstrating gonadotropin stimulated TLRs in murine granulosa cells (Shimada *et al.* 2006). Significantly, this differential sensitivity suggests that TLR-signaling may modulate ovarian function specifically at the preovulatory stage, while providing protective mechanism against infection in follicles prior to selection. Notably, in undifferentiated granulosa cells from 3 to 8 mm follicles, treatment with LPS did not up-regulate *TLR2* mRNA and resulted in the down-regulation of *TLR4* mRNA.

The ability of LPS to modulate steroid production in hen granulosa cells is also stage dependent. In undifferentiated granulosa cells, treatment with LPS for 20 h abrogated FSH-induced progesterone production. Furthermore, pre-treatment with U0126 prevented this decrease in progesterone, indicating that the actions of LPS on progesterone production are dependent upon downstream MAPK signaling. However, under similar conditions, LPS did not modulate the level of progesterone produced in response to LH or 8-br-cAMP in differentiated granulosa cells from F2 or F1 follicles. Together, these findings are congruous with previously reported data revealing that the effects of MAPK on progesterone production in the hen are stage dependent (Woods *et al.* 2007). Moreover, accruing evidence from both mammalian and avian ovarian models indicate that the actions of LH are dependent upon MAPK signaling (Su *et al.* 2002, Park *et al.* 2004, Jammongjit *et al.* 2005, Woods *et al.* 2007). Thus, while TLR-induced MAPK signaling may be inhibitory in undifferentiated granulosa cells, there is precedence that it may transition to facilitory at the preovulatory stage. Concomitantly, the abrogation of gonadotropin-induced progesterone production in hen granulosa cells may represent an inherent mechanism to potentially block a follicle from being selected into the preovulatory hierarchy. Such a mechanism could contribute to the prevention of transovarian transmission of *S. serovar* spp. This mechanism may be of importance, as *S. enteritidis* has been

demonstrated to preferentially invade prehierarchical follicles (Howard *et al.* 2005).

In addition to the differentiation-dependent effects of LPS-induced MAPK activity, LPS-induced cell death is also varied based upon stage of maturation. Whereas a significant decrease in cell viability was measured following treatment with 10 µg/ml LPS in differentiated granulosa cells, undifferentiated granulosa cells are more sensitive, undergoing apoptosis following 5 µg/ml LPS. Additionally, pre-treatment with the caspase-8 inhibitor, Z-IETD-FMK, only partially reversed LPS-induced cell death in undifferentiated granulosa cells while fully reversing the effects of LPS on cell death in differentiated granulosa cells. These data are consistent with numerous studies demonstrating that undifferentiated granulosa cells are inherently more sensitive to death than their differentiated counterparts (reviewed in Johnson 2000). This sensitivity has been attributed to elevated levels of pro-apoptotic proteins and reduced levels of anti-apoptotic proteins (reviewed in Johnson 2000), reduced levels of the anti-apoptotic AKT (Johnson *et al.* 2001), and relatively rapid rate of proliferation (Woods *et al.* 2005). Moreover, these data further support a role for a TLR-mediated immune response in attenuation of transovarian transmission of *S. enteritidis* at the pre-selection stage of follicle maturation, whereas latter stages of development appear much less affected.

The ability of the caspase-8 inhibitor to prevent the effects of LPS on viability in differentiated follicles indicates a requirement for the extrinsic cell death pathway, likely involving the up-regulation of one or more cytokines, or alterations in intracellular adaptor and executioner genes. Many of the chicken intracellular adaptor and executioner genes required for extrinsic cell death have recently been identified (Johnson *et al.* 2007), along with orthologous chicken death-inducing cytokine genes, including *TNFSF10*, *TNFSF15*, and *IL1B*. To date, the chicken ortholog to mammalian TNF has not been identified, and it has been proposed that *TNFSF15* fulfills this role in the chicken (Takimoto *et al.* 2005). Notably, *IL1B* mRNA was increased following treatment with LPS in undifferentiated granulosa, while levels of *TNFSF10* and *TNFSF15* mRNAs remained unchanged (data not shown). This is consistent with a recent report in which i.v. LPS increased *IL1B* mRNA in granulosa layers from hen preovulatory follicles (Subdei *et al.* 2007). While *IL1B* was the only cytokine found to be transcriptionally regulated following stimulation with LPS in either study, these data do not preclude additional mediators of LPS-induced cell death. Rather, these data are indicative of a complete and fully functional TLR-signaling pathway in hen granulosa cells, with *IL1B* as an indicator of downstream transcriptional activation.

The reported decrease in *TLR4* mRNA expression together with the impediment of gonadotropin-induced progesterone production and viability in undifferentiated granulosa cells is consistent with immune cell function.

Down-regulation of TLR4 expression following LPS stimulation represents a critical component of LPS tolerance. Functionally, this process serves to prevent excessive inflammation (Ziegler-Heitbrock 1995). This prevention of inflammation may mechanistically lead to the destruction of only infected follicles without excessive cytokine production that could potentially target neighboring healthy follicles. While not entirely effective at preventing transovarian transmission of *S. enteritidis*, this mechanism may assist in the prevention of debilitating infertility in the face of infection.

Alternatively, given the up-regulation of TLRs in preovulatory follicle granulosa cells in response to LPS or gonadotropin, coupled with a lack of attenuation of steroidogenesis, it is possible that TLR signaling may mediate physiological processes at or around the time of ovulation. In fact, multiple endogenous ligands for TLRs have been reported (Zhang & Schluesener 2006), many of which are present in hen ovarian follicles. These include hyaluronan fragments, fibronectin, and heparan sulfate. Of these, hyaluronan fragments and fibronectin are most highly expressed in the F1 follicle (Jackson *et al.* 1991, Asem & Novero 1994), thus have the potential to activate TLRs at or around the time of ovulation. Notably, fibronectin is produced by hen granulosa cells in response to LH and epidermal growth factor (EGF; Asem & Novero 1994, Asem & Conkright 1995), which are requisite for ovulation and steroid production in preovulatory follicles (Bahr & Johnson 1984, Woods *et al.* 2007). In murine granulosa cells, TLRs have been shown to bind hyaluronan fragments (Shimada *et al.* 2006). Hyaluronan is endogenously synthesized, forming part of the matrix required for cumulus expansion in preovulatory murine follicles (Eppig 1979). Interestingly, hyaluronan production has been shown to significantly increase in cultured rat ovarian cells following treatment with IL1B (Kokia *et al.* 1993), which shown herein is significantly up-regulated in preovulatory follicles in response to TLR-signaling.

In summary, these data demonstrate a functional TLR signaling cascade within hen ovarian granulosa cells. Activation of TLR signaling results in stage of maturation-dependent effects on steroidogenesis, granulosa cell apoptosis, and cytokine production. In particular, these findings support a putative mechanism by which TLR signaling could modulate ovarian function, independently from pathogenic stimuli, in the latter stages of follicle maturation. The inflammatory-like response associated with ovulation includes extensive tissue remodeling occurring at and around the time of follicle rupture. TLRs may play a role in tissue remodeling (Zhang & Schluesener 2006), thus contributing to structural changes occurring in the ovary due to ovulation (Girling & Hedger 2007). While additional studies are required to determine what, if any, effects impacting follicle development are mediated through TLR signaling in granulosa cells *in vivo*, effects of both

microorganisms as well as endogenously produced ligands have the potential to mediate ovarian function through TLRs expressed on granulosa cells.

Materials and Methods

Animals and reagents

Single-comb white leghorn hens (Creighton Bros., Warsaw, IN, USA), 25–40 weeks of age and laying regular sequences of, at minimum, six eggs, were used in all studies described. Birds were housed in individual laying batteries, with free access to feed (Purina Layena Mash, Purina Mills, St Louis, MO, USA) and water, and were exposed to a photoperiod of 15 h light:9 h darkness. Individual laying cycles were monitored by the daily timing of oviposition. Hens were euthanized ~16–18 h prior to a mid-sequence ovulation by cervical dislocation. All procedures described herein were reviewed and approved by the University of Notre Dame Institutional Animal Care and Use Committee, and were performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals.

Tissues collected for cell culture and isolation of total RNA included bone marrow and ovarian granulosa cells from the largest (F1) preovulatory follicle, second largest (F2) preovulatory follicle, and granulosa cells from prehierarchal (prior to selection into the preovulatory hierarchy) follicles 3–8 mm in diameter. For cell culture experiments, granulosa cells were collected and dispersed as described previously (Tilly *et al.* 1991, Johnson & Bridgham 2001). Cells were incubated for up to 6 h at 40 °C in 12 × 75 mm polypropylene tubes at a density of 5×10^5 , or for 20 h in 6- or 96-well polystyrene culture dishes (Thermo Fisher Scientific, Rockford, IL, USA) at a density of 1×10^6 and 1.5×10^4 respectively in DMEM (Invitrogen), containing 2.5% FBS (Invitrogen), 0.1 mM non-essential amino acids, and 1% antibiotic–antimycotic mixture (Invitrogen). All time points were evaluated based on previously established endpoints for granulosa cell function and viability (Tilly & Johnson 1988, Johnson & Bridgham 2001). Recombinant human FSH and ovine LH were provided by the National Hormone and Pituitary Program (HUMC, Torrance, CA, USA). The cAMP agonist, 8-br-cAMP, and the purified *S. enteritidis* LPS were from Sigma–Aldrich. U0126 (a selective MAPK extracellular signaling-regulated kinase (MAPK1) kinase (MAP3K2) inhibitor) was purchased from BioMol (Plymouth Meeting, PA, USA), while the caspase-8 inhibitor, Z-IETD-FMK, was from R&D Systems (Minneapolis, MN, USA).

PCR amplification of TLR adaptor and signaling components

In an initial effort to verify a functional TLR signaling pathway in granulosa cells, *Gallus* orthologs of mammalian TLR2 (type 2), TLR4, and TLR15 were amplified from RT RNA (RT system, Promega) isolated from freshly collected granulosa cells (3–8 mm diameter or F1 follicles), as well as bone marrow. Additionally, intracellular signaling components and cytokines were amplified to verify the presence or absence in ovarian granulosa cells. Accession numbers and PCR primer pairs

Table 1 PCR primer pairs for TLRs and related signaling components and downstream transcriptional targets.

Gene name	Accession no.	Primers	Size	Annealing temperature (°C)
<i>TRAF6</i>	XP_421089	GCAAAGATGGAGACGCAAAACACTC ATTAGCACACCGCTGAGCACTTGG	264	56.5
<i>TOLLIP</i>	NP_001006471	AACCCACACAGCACATAATGGAGC TTCCTTCTTTGTCATCACCTGCC	233	56
<i>MAP3K7</i>	XP_001233504	GGTGTTCACAGTGTCCCAAGGAG TTCAGGTGCCATCCAAGCAG	203	54.2
<i>MAP3K7IP1</i>	XP_416787	TTGAACACCGCAAAGACCTGG TGGCTGAGATACCATTACCCGAGG	248	56.7
<i>MAP3K7IP2</i>	XP_419660	CAGTTTCTCCAATAAGCCCTGC AGTTCTCGTTGAAGTCGCTCCATCC	253	57
<i>MAP3K14</i>	NP_001026098	GGAAGAATACTTGGTGGACGCTTG CGCACTGAAATCCTGTCTGCTTGTC	281	59.3
<i>LY96</i>	XP_001232093	TCCTGGAGTCAGTGGATTTTCTG GCAATGCTCTGGCACCTTCATAC	215	53.6
<i>CD14</i>	XP_001232016	GACTTCCCACCTCCTCATCCG CACACCTTGCTTTTACAATGTTT	152	57.2
<i>MAL/TIRAP</i>	NP_001020000	TTTCCTCCTCCTCCTGCTTG ATCCTTCCTGTCAATGTCTTCAG	422	60.2
<i>IRAK4</i>	NP_001025909	GGCATTGTGTTCAAAGGCTACATC CAAGCAAGTCTGTCAAGCAGCG	236	54.2
<i>IRAK2</i>	NP_001025776	TTCATTCTCTCTCCACCACCTC TGTTGTGCCTCTGACCTTTGTAGC	251	55.7
<i>TLR4</i>	NM_001030693	CTACTTTGATGATTGCCTTCTCC AATAGTTCTGGACCTTCACTCTCCC	287	56.7
<i>TLR2</i>	NM_204278	GATTGTGGACAACATCATTGACTC AACGCTGCTTCAAGTTTCCC	294	55.4
<i>TLR15</i>	NM_001037835	GTTCTCTCTCCAGTTTGTAAATAGC GTGGTTTATTGGTTGTTTTAGGAC	262	55.1
<i>TNFSF15</i>	NM_001024578	CCTGAGTTATCCAGCAACGCA ATCCACGAGCTTGATGTCATAAC	292	55.9
<i>TNFSF10</i>	AB114678	CAAAGACAGAGTGGCTGACCCC ATTCGTTCCAACAAACCCGAG	417	58.1
<i>IL1B</i>	NM_204524	CACAGAGATGGCGTTCGTTC AGGTGACGGGCTCAAAAACC	293	56.8
<i>MYD88</i>	NM_0001030962	GAAAGACCTTCAGTTTGTCCAGGAG AGATGCTGTAGGAACACCGTGG	458	56.4

specific for *Gallus* TLRs and signaling components are listed in Table 1. While PCR conditions were specific for primer pairs, amplification conditions included an initial denaturing for 3 min at 94 °C, followed by 40 cycles of denaturing at 94 °C (45 s), annealing at 51–62 °C (45 s), followed by an extension step at 72 °C (60 s) using *Taq* DNA polymerase (Invitrogen). All PCR products were subsequently sequenced to verify nucleic acid identity.

Progesterone analysis

Progesterone in cell culture medium samples was measured by RIA as previously described (Tilly & Johnson 1988). The replicate experiments were combined, and the mean ($\pm$ S.E.M.) values are expressed as fold difference versus control (untreated) cells.

Quantitative (real-time) PCR

Forward and reverse primers encoding type 2 TLR2, TLR4, IL1B and 18s rRNA (used for standardization) were generated using MacVector software (Table 1), and were subsequently validated for use with real-time PCR by determining the optimal amplification efficiency and primer conditions as described by

the system manufacturer (Applied Biosystems, Foster city, CA, USA). Random-primed, RT cDNA synthesis reactions were performed using the Promega Reverse Transcription System (Promega), according to conditions described by the manufacturer. Template dilutions were performed to verify that amplification efficiency of TLR2, TLR4, IL1B was the same as that of the 18s rRNA housekeeping gene. For real-time PCR, primers were added to 25 μ l total reaction volume using reagents provided in the ABgene Absolute QPCR Sybr Green Mix (ABgene, Epsom, UK). Reactions were completed on the ABI 7700 Thermocycler (Applied Biosystems). Amplification conditions included an initial denaturing for 15 min at 95 °C followed by 15-s denaturing at 95 °C, 1 min annealing at 60 °C and 1 min extension at 72 °C for 40 cycles. The C_t (defined as the cycle number at which the fluorescence exceeds a threshold level) was determined for each reaction (run in triplicate) using the Sequence Detection software (v.1.6.3), while quantification was accomplished using the $\Delta\Delta C_t$ method (Livak & Schmittgen 2001).

Cell density assays

Immediately following collection, 1.5×10^4 cells/well were seeded into a 96-well plate. Cells were treated in the presence or absence of LPS (1–10 μ g) for 24 h. All treatments in each replicate experiment were performed in triplicate. Cell density

was monitored using the colorimetric CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), as a measure of metabolic activity and viability. The indicator solution was added directly to the plate after 24 h culture, incubated for an additional 1–4 h, and the absorbance values were read at 490 nm. Where applicable, the caspase-8 inhibitor, Z-IETD-FMK, was added 1 h prior to the addition of LPS. Apoptosis was verified through the qualitative analysis of oligonucleosome formation as described previously (Johnson *et al.* 1998).

Data analysis

Experiments were independently replicated a minimum of three times unless otherwise specified. Standardized values for the combined replicate experiments were expressed as a fold difference (mean $\pm$ S.E.M.) versus control cultured cells. Data were analyzed by one-way ANOVA without including data from the control group (arbitrarily set to 1.0), followed by Fisher's protected least significant multiple range test for *post hoc* analysis. In instances where a *t*-test was used to compare two related treatments, individual comparisons were made using original, non-transformed, data.

Declaration of interest

The authors D C W, J S S, and A L J have nothing to declare related to the material being published.

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