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Induced Expression of Pattern Recognition Receptors in Cumulus Oocyte Complexes: Novel Evidence for Innate Immune-Like Functions during Ovulation

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Ovulation is the complex, inflammatory-like process by which the cumulus oocyte complex (COC) is released from a mature, preovulatory follicle through a rupture site at the ovarian surface and requires expression of genes that generate and stabilize the expanded extracellular COC matrix. Gene profiling analyses of COCs at selected time intervals during ovulation revealed that many genes associated with immune related surveillance functions were also induced in cumulus cells. Specifically, cell surface signaling molecules known as pattern recognition receptors that act as sensors of the external environment important for the innate immune system to detect self from nonself or altered self are induced and/or expressed in cumulus cells as well as granulosa cells. These include the complement factor q1, CD14, and the Toll-like receptors (TLRs) 4, 8, and 9 as well as mediators of TLR activation, myeloid differentiation primary response gene 88 and interferon regulatory factor 3. COCs exposed to bacterial lipopolysaccharide exhibit enhanced phosphorylation of p38MAPK, ERK1/2 and nuclear factor-κB and increased expression of *Il6* and *Tnfa* target genes, documenting that the TLR pathway is functional. Cumulus cells and granulosa cells also express the scavenger receptors CD36 and scavenger receptor type B1 and exhibited phagocytic uptake of fluorescently tagged bacterial particles. Collectively, these results provide novel evidence that cumulus cells as well as granulosa cells express innate immune related genes that may play critical roles in surveillance and cell survival during the ovulation process. (*Molecular Endocrinology* 20: 3228–3239, 2006)

OVULATION IS THE LH surge-induced process in which the cumulus oocyte complex (COC) is released from a mature, preovulatory (PO) follicle through a rupture site at the ovarian surface. Because this process involves tissue remodeling similar to wound repair and the production of prostaglandins, it has been likened to an inflammatory-like response (1). Indeed, genes related to inflammatory reactions, such as *Ptgs2* that encodes the rate-limiting prostaglandin synthesizing enzyme, are induced in PO follicles by the LH surge. *Ptgs2* is obligatory for ovulation (2–8), which suggests it has a crucial function. Genes required for the formation of the hyaluronan (HA)-rich extracellular matrix that surrounds the oocyte and is essential for ovulation are also induced (9), providing molecular and physiological evidence that prostaglandins and other inflammation-related matrix factors impact the ovulation process. Although it is easy to envision that the HA-rich matrix serves as a physical barrier to protect the ovulated oocyte, the function of the matrix and the enclosed cumulus cells may involve innate immune-related activities as well.

Gene profiling of COCs collected at specific intervals after LH/hCG induction of ovulation revealed that genes thought to be associated selectively with immune cell function and innate immunity are expressed at high levels in cumulus cells before and after ovulation (10). Factors that are known to be critical for fertility include PTX3 and TNFαIP6, which act to stabilize the matrix (11–13) and CD147/basigin, which is a

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Abbreviations: AREG, Amphiregulin; CG, chorionic gonadotropin; COC, cumulus oocyte complex; Cq1, complement factor q1; EGF, epidermal growth factor; HA, hyaluronan; IF, immunofluorescence; IRF3, interferon regulatory factor 3; LPS, lipopolysaccharide; PRR, pattern recognition receptor; MYD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor-κB; NP-40, Nonidet P-40; PO, preovulatory; PTGS2, prostaglandin synthase 2; SCARBI/ SCARBII, scavenger receptor type B1 and BII; TLR, Toll-like receptor; WB, Western blot; WCE, whole-cell extract.

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transmembrane glycoprotein with IgG-like domains (14, 15). Programmed cell death 1 (*Pdcd1*) and its transcriptional regulator *Runx1* are involved autoimmune diseases (16). Activated leukocyte adhesion molecular (*Alcam*), CD34 (*Cd34*), and CD52 (*Cd52*) are cell surface markers of immune cells (10). Based on the highly uniform expression of these genes in cumulus cells (10) and the dearth of immune cells in ovulating follicles (17), we have proposed that cumulus cells of ovulating follicles acquire specific innate immune-like functions (10).

Further analyses of our COC gene profiling database allowed us to identify additional genes heretofore restricted to immune cells or cells with innate immune-like functions (10). Importantly, these include cell surface signaling molecules known as pattern recognition receptors (PRRs) that discriminate pathogen-coded molecular structures known as pathogen-associated molecular patterns (18, 19). PRRs act as sensors of the external environment to detect self from nonself or altered self. Among these receptors are CD14 and the Toll-like receptors (TLRs) 4, 8, and 9 that directly and selectively bind bacterial lipopolysaccharides (LPS), short-strand RNA, and unmethylated cytosine-phosphate-guanine dinucleotide (CpG) DNA. Also observed was the complement factor C1q that is critical for monitoring a variety of external factors and is expressed abundantly at sites of tissue damage. C1q also plays an initiating role in clearance of apoptotic cells (18, 20). Genes encoding the A- and B-type scavenger receptors including scavenger receptor BI and BII (*Scarb1* and *Scarb2*) as well as *Cd36* were identified. Although these latter receptors regulate cholesterol and lipid trafficking in steroidogenic cells (21, 22), they can also mediate phagocytic activity and the ingestion of bacteria and apoptotic cells (23). Based on these observations, we sought to analyze the expression of these immune cell receptor genes in ovarian cells, to determine whether PRR signaling cascades were operational, and to examine the phagocytic capabilities of cumulus cells and granulosa cells.

RESULTS

PRRs Are Expressed in COCs

TLRs, Complement Factor C1q, and Downstream Signaling Molecules Are Regulated *in Vivo* and in Culture. The TLR system is part of the innate immune surveillance system shown in recent studies to be essential for pathogen recognition (19, 20, 24, 25) (Fig. 1A). TLR4, TLR8, and TLR9 recognize LPS, short-strand RNA, and unmethylated CpG DNA, respectively (18, 19, 25). Small fragments of HA, the backbone of the COC matrix, can also bind and activate TLR4 (26–28). TLR4 interacts with CD14 and possibly C1q to mediate many of its effects via activating the adaptor protein MYD88 (myeloid differentiation primary response gene 88) (18, 29). MYD88, in turn, leads to the activation of downstream signaling molecules such as ERK1/2, p38MAPK, and nuclear factor-κB (NF-κB) (19). In addition, TLR signaling mediated by the transcription factor IRF3 (interferon regulatory factor 3) leads to the expression of target genes such as *Il6*, *Tnfa*, and *Ptgs2* (19, 29). In macrophages, TLR signaling also induces expression of members of the scavenger receptor family *Scarb1* and *Cd36* (20, 24).

To determine whether this PRR system (Fig. 1A) was expressed in ovarian cells during ovulation, mice were primed with eCG (equine chorionic gonadotropin) to stimulate growth of PO follicles and human CG (hCG) to initiate ovulation (10). COCs and granulosa cells were isolated from ovaries at selected time intervals, total RNA was extracted, and semiquantitative RT-PCR analyses were done. *Tlr4*, *Tlr8*, and *Tlr9* mRNA levels were low in COCs isolated from PO follicles (0 h) but increased significantly 8–12 h after hCG, a time that just precedes ovulation (Fig. 1B; *arrow*). Expression of these receptors remained high in ovulated COCs collected from the oviducts at 16 and 24 h. By contrast, *Tlr4*, *Tlr8*, and *Tlr9* mRNAs were at higher levels in granulosa cells than was observed in COCs at 0 h; *Trl4* and, to a lesser extent, *Tlr8* and *Tlr9* mRNAs increased at 4–8 h post-hCG but declined by 12 and 16 h after hCG. Each of the other membrane components, *Cd14*, and *C1q*, as well as downstream molecules (*Myd88* and *Irf3*) and the target gene *Il6*, were induced markedly in COCs before ovulation. In these same samples, *Ptgs2* mRNA was highly induced in COCs and granulosa cells by 4 h. With the exception of *Il6* and *Ptgs2*, none of these genes was markedly induced in granulosa cells (Fig. 1B). *Cd36* and *Scarb1* mRNAs were clearly detected in COCs and granulosa cells at 0 h (Fig. 1C). Although a transient increase in *Cd36* mRNA was observed in COCs at 4 h, no significant changes were observed in granulosa cells, whereas *Scarb1* mRNA increased in both COCs and granulosa cells at 4 h (Fig. 1C).

Western blot (WB) analyses using specific antibodies to TLR4, MYD88, IRF3, and SCARBI show increased expression these genes in COCs collected at 8 and 16 h after hCG compared with those collected at 0 h (Fig. 2A). This pattern contrasts with the lower expression of TLR4 and MYD88 and the relatively constant levels of IRF3 and SCARBI in granulosa cells (Fig. 2A). Although the protein expression patterns do not precisely mimic those of mRNA, the results confirm the presence of these proteins in COCs and granulosa cells. Immunofluorescent analyses using the same antibodies revealed intense staining for TLR4, MYD88, and IRF3 as well as SCARBI in cumulus cells collected from COCs 8 or 16 h after hCG (Fig. 2, B and C). It is important to note that essentially all cumulus cells at the 8 and 16 h time points are immunopositive for these proteins, indicating that these factors are derived from cumulus cells and not from invading immune cells. Although the oocytes also appear to be immunopostive, it is not clear whether or not this is

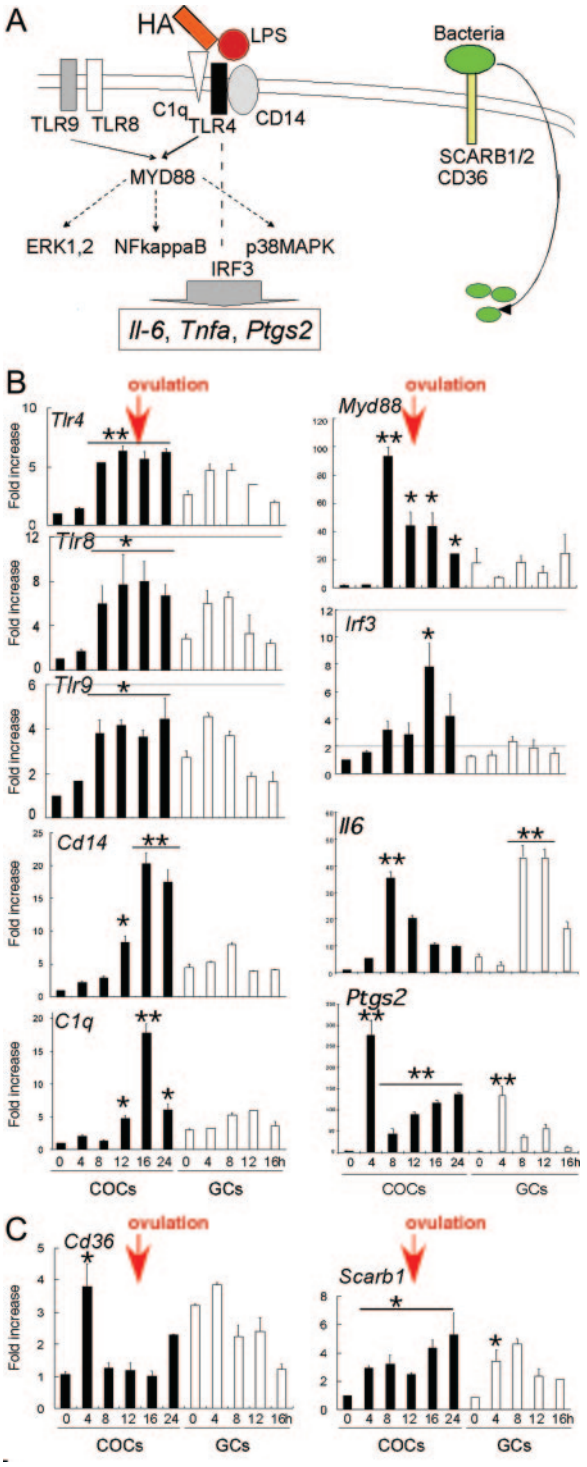

Fig. 1. PRRs, Downstream Mediators, and Target Genes Are Induced in COCs of Ovulating Follicles

Panel A, This schematic depicts selected members of the TLR signaling cascade and the scavenger receptor systems that participate in the innate immune surveillance system characterized in immune cells. TLRs (*rectangles*) interact with coregulatory membrane proteins CD14 and C1q (*oval* and *triangle*, respectively) to stimulate downstream signaling events via the adaptor factor MYD88 leading to the induction of genes such as *Il-6*, *Tnfa*, and *Ptgs2*. TLRs signaling cascades can be activated by the bacterial LPS (*red circle*) and small fragments of HA (*orange rectangle*). The scavenger receptors (SCARB1/2 and CD36; *yellow bar*) have the potential to bind and ingest bacteria depicted as the uptake of fluorescently labeled bacterial particles (*green circles*). B and C, Expression of *Tlr4*, *Tlr8*, *Tlr9*, *Cd14*, *C1qa*, *Myd88*, *Irf3*, *Il6*, *Ptgs2*, *Cd36*, and *Scarb1* mRNAs in COCs and granulosa cells (GCs) isolated from PO follicles of eCG-primed mice at selected time intervals (0–16 h) after an ovulatory dose of hCG, analyzed by semiquantitative RT-PCR, and presented as fold-increase relative to the 0 h COC value for all panels. Representative of two separate experiments that were repeated twice, yielding four separate values that were analyzed to provide mean ± SD. For reference, the 0 h was set as 1 and the data presented as fold-increase. *, $P < 0.05$ compared with 0 h COC; **, $P < 0.01$ compared with 0 h COC.

specific because oocytes frequently exhibit nonspecific staining. Levels of TLR4, MYD88, IRF3, and SCARB1 immunoreactivity were low in nonexpanded COCs isolated from PO follicles at 0 h (*insets* in Fig. 2, B and C), supporting the lower abundance of these proteins at this stage. Cells positive for leukocyte markers (CD4, CD8, CD14, CD45, and CD163) have been reported present in ovulated human COCs (17), leading these investigators to conclude that immune cells were present in the ovulated complexes. However, based on the highly uniform expression of TLR-related genes, including CD14 in mouse cumulus cells (shown herein) and the relative dearth of clear evidence for specific immune cell types in ovulating human follicles (17), the results presented herein indicate that cumulus cells of ovulating follicles in the mouse express a vast array of innate immune-related genes, confirming previous observations (10).

PRRs Were Induced by FSH and Amphiregulin (AREG) in Cultured COCs. LH *in vivo* and FSH in culture stimulate COC expansion, a process mediated in part by the induction of genes encoding the epidermal growth factor (EGF)-like factors amphiregulin (*Areg*), epiregulin (*Ereg*), and betacellulin (*Btc*) (30–32). To determine more directly which signaling cascade(s) might regulate induction of the innate immune-related genes in COCs, COCs were isolated from PO follicles and incubated 16 h with either FSH (100 ng/ml) or AREG (250 ng/ml), used as a representative EGF-like factor. Either factor induced significant expression of receptor (*Tlr4, Cd14, C1qa*), as well as selected downstream effector (*Myd88, Irf3*) mRNAs. FSH and AREG also induced expression of scavenger receptor BI (*Scarb1*) mRNA (Fig. 3A). These results along with the induction of PTX3 by FSH and EGF in isolated COCs (Ref. 11 and our own observations) indicate that genes linked to innate immune functions are induced in isolated cumulus cells devoid of immune cells. Granulosa cells from PO follicles were also cultured 16 h in the presence of FSH, AREG, or FSH+AREG. Whole-cell extracts (WCEs) were prepared, and the levels of TLR4, MYD88, IRF3, and SCARB1 were analyzed by WB. As shown in Fig. 3B, the components of the TLR

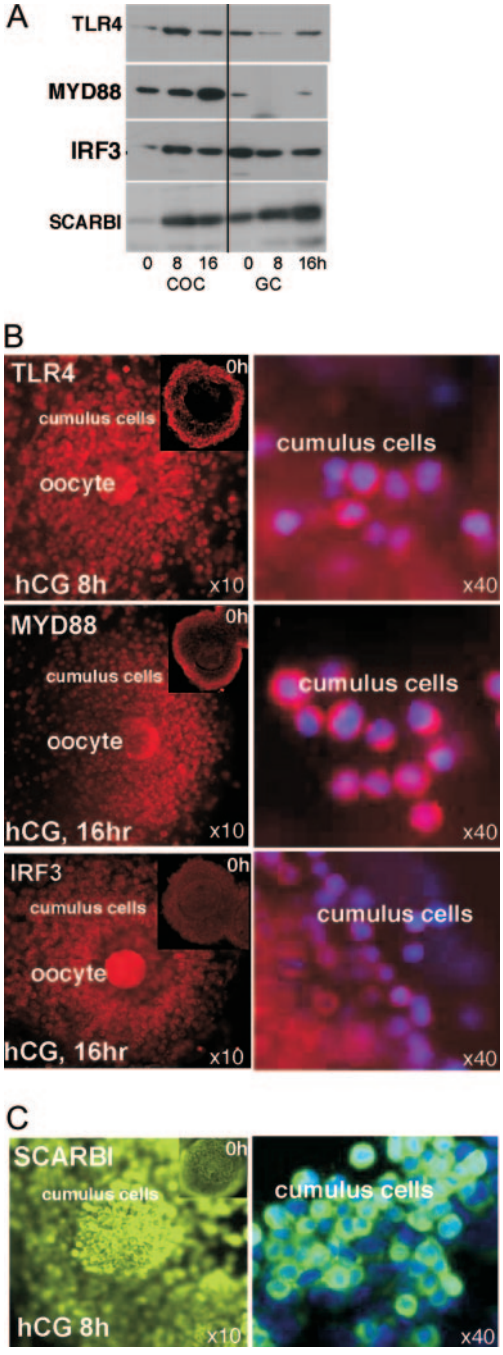

Fig. 2. COCs Express Protein for TLR and Scavenger Receptor Signaling Pathways

A, Western blot analysis of TLR4, MYD88, IRF3, and SCARBI protein in COCs [50 μg/lane and granulose cells (GC) 100 μg/lane] isolated at 0, 8, and 16 h after hCG. B and C, Immunofluorescent localization of TLR4, MYD88, IRF3 (AlexaFluor 594, *red*) and SCARBI (AlexaFluor 488, *green*) in cumulus cells of COCs isolated at either 8 h or 16 h after hCG (as indicated). Nuclei (4′,6-diamidino-2-phenylindole stain) are *blue*. Note the uniform immunostaining for each protein on essentially all cumulus cells. Nonexpanded COCs isolated from PO follicles (0 h) exhibit low immunostaining for TLR4, MYD88, IRF3, and SCARB1. These are shown in the *insets* in the *upper right hand corner* of the panels on the *left*.

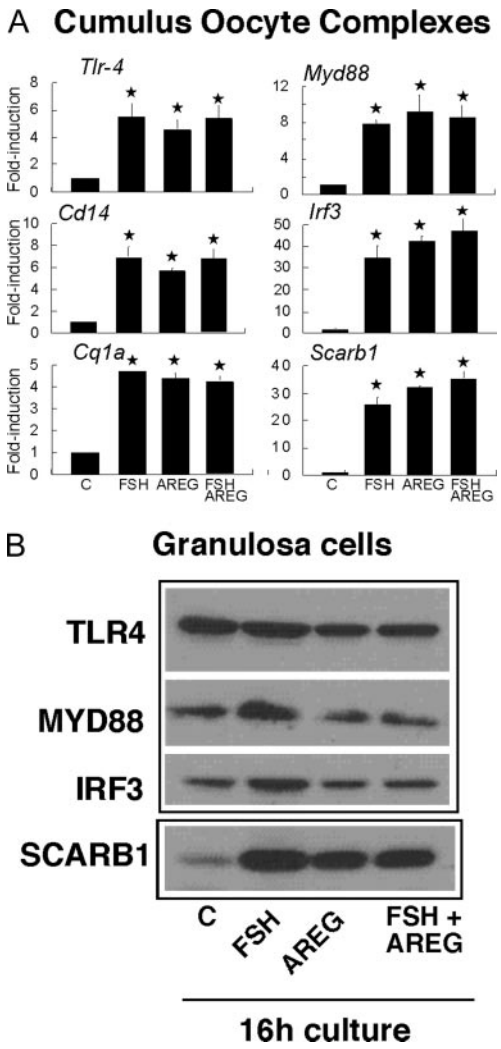

Fig. 3. COCs Were Isolated from PO Follicles and Cultured 16 h with FSH (100 ng/ml), AREG (100 ng/ml), or FSH and AREG

Levels of mRNA encoding TLR signaling components (*Tlr4*, *Cd14*, *Cq1a*, *Myd88*, and *Irf3*) and *Scarb1* were increased significantly ($P < 0.05$) by either FSH or AREG. These experiments have been done on three different sets of samples. Granulosa cells isolated from these same follicles were cultured 16 h in serum-free media containing FSH, AREG, or FSH+AREG as above. Protein extracts were prepared and analyzed by Western blotting for TLR4, MYD88, IRF3, and SCARB1. All data (n = 3) have been analyzed by semiquantitative RT-PCR, represented as fold-induction compared with control samples (mean ± SD); *, $P < 0.05$.

pathway were expressed at relatively constant levels whereas SCARB1 was clearly induced by either FSH or AREG alone. No additive effect was observed when both factors were present, supporting results observed in granulosa cells isolated at specific times *in vivo* (Fig. 2A).

LPS Activates the NF-κB and p38MAPK Pathways Leading to IL-6 Expression in Cumulus Cells and Granulosa Cells. LPS is contained in the cell wall of Gram-negative bacteria. Because LPS serves as a

ligand for several PRRs including TLR4 and TLR8 (19, 20, 25), we determined whether LPS initiated signaling cascades in COCs and/or granulosa cells (Fig. 4, A and B). Because mRNAs encoding the *Tlrs*, *Cd14*, *C1q*, and *Myd88* were all elevated in COCs at 16 h post-hCG, we first analyzed the effects of LPS on COCs isolated from oviducts at this time interval. In COCs, LPS (100 ng/ml) stimulated marked phosphorylation of p38MAPK at 60–120 min and ERK1/2 and NF-κB at 120 min (Fig. 4A). Because expression of *Tlr* mRNAs was high granolosa cells of PO follicles, granulosa cells from eCG-primed mice were isolated. In these granulosa cells, the basal level of phospho-p38MAPK was higher than that in COCs, but LPS also increased the phosphorylation status of each of these factors (Fig. 4B).

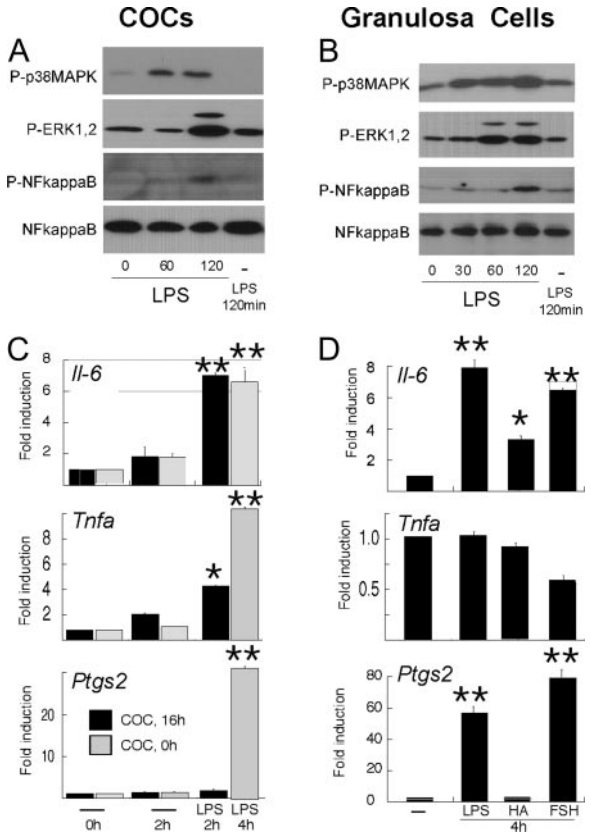

Fig. 4. LPS Activates Cell Signaling and Transcriptional Events in COCs and Granulose

A and B, LPS stimulated phosphorylation of p38MAPK, ERK1/2, and NF-κB in COCs isolated from oviducts of mice at 16 h post-hCG and in granulosa cells isolated from PO follicles of eCG-primed mice. Expression of *Il6* and *Tnfa* but not *Ptgs2* mRNAs was increased in COCs collected from the oviducts of mice 16 h after hCG and stimulated with LPS (100 ng/ml) for 2 h (panel C, *black bars*). *Il6*, *Tnfa*, and *Ptgs2* mRNAs increase in COCs isolated from PO follicles (designated 0 h) and cultured with LPS for 4 h (panel C, *gray bars*). Levels of *Il6* mRNA are increased in granulosa cells isolated from PO follicles cultured with either LPS (100 ng/ml), small HA fragments (~150 kDa; 100 μg/ml), or FSH (100 ng/ml) for 4 h (panel D). Levels of *Ptgs2* mRNA increased in response to LPS and FSH but not HA. All data (n = 3) have been analyzed by semiquantitative RT-PCR, represented as fold-induction to 0 h untreated samples. *, $P < 0.05$; **, $P < 0.01$.

Because down-stream targets of the TLR pathway (19) were induced in COCs 8 h post-hCG *in vivo* (Fig. 1B), additional experiments were done to determine whether LPS activated expression of any of these genes in COCs in culture. Accordingly, COCs were isolated from PO follicles of eCG-primed mice (0 h) and from oviducts of mice16 h post-hCG and cultured with LPS (100 ng/ml). In 16 h COCs, expression levels of *Il6* as well as *Tnfa* mRNA increased 7-fold and 4-fold, respectively, whereas *Ptgs2* mRNA did not (Fig. 4C, *black bars*). In nonexpanded COCs (0 h) where levels of TLRs were lower, LPS nevertheless increased *Il6*, *Tnfa*, and *Ptgs2* mRNAs (Fig. 4C, *gray bars*). Furthermore, FSH enhanced (2-fold) the ability of LPS to increase *Il6* and *Ptgs2* mRNA in PO COCs in culture (data not shown). These results indicate that the TLR receptor system is operative in cumulus cells even before the LH surge but that the effects of LPS are enhanced by the actions of gonadotropins. Likewise, granulosa cells isolated from PO follicles responded to LPS as indicated by the marked increases in *Il6* and *Ptgs2* (but not *Tnfa*) mRNAs within 4 h (Fig. 4D). HA fragments that are known to activate TLR4 also increased expression of *Il6* mRNA in granulosa cells, but the response was not as great as that to LPS. Curiously, FSH mimicked some of the effects of LPS, *i.e.* increased expression of *Il6* and *Ptgs2* mRNAs. Because it is highly unlikely that FSH directly activates TLRs, the effects of FSH may be mediated, in part, by its rapid induction of *Areg* transcripts. AREG is a potent regulator of *Ptgs2* expression in these cells (31) and, as shown in Fig. 1B, the marked induction of *Ptgs2* mRNA 4 h after hCG precedes the increased expression TLR pathway components. Because phosphorylation of p38MAPK and induction of *Il6* and *Tnfa* are downstream events triggered by TLR pathway activation (19), the data presented herein provide strong evidence that CD14, the TLRs 4 and 8, and/or C1q are functional in both cumulus cells and granulosa cells. Furthermore, the regulation and function of the TLR pathway in these cells appears to be closely linked to the actions of FSH and LH.

Cumulus Cells and Granulosa Cells Exhibit Phagocytic Activity

Scavenger receptors regulate cholesterol and lipid transport in endocrine cells. Recent evidence indicates that these receptors can also mediate/facilitate uptake (phagocytosis) of bacterial particles (Figs. 1A and 5A) (21–23). Therefore, we analyzed the phagocytic capabilities of cumulus cells and granulosa cells, by examining their ability to ingest fluorescent-tagged bacterial particles. COCs, isolated 6 h after hCG, were incubated with 5 μg/ml bacterial particles at 37C for 2 h. Intense fluorescence was observed in these intact COCs (Fig. 5A). However, because the resolution was

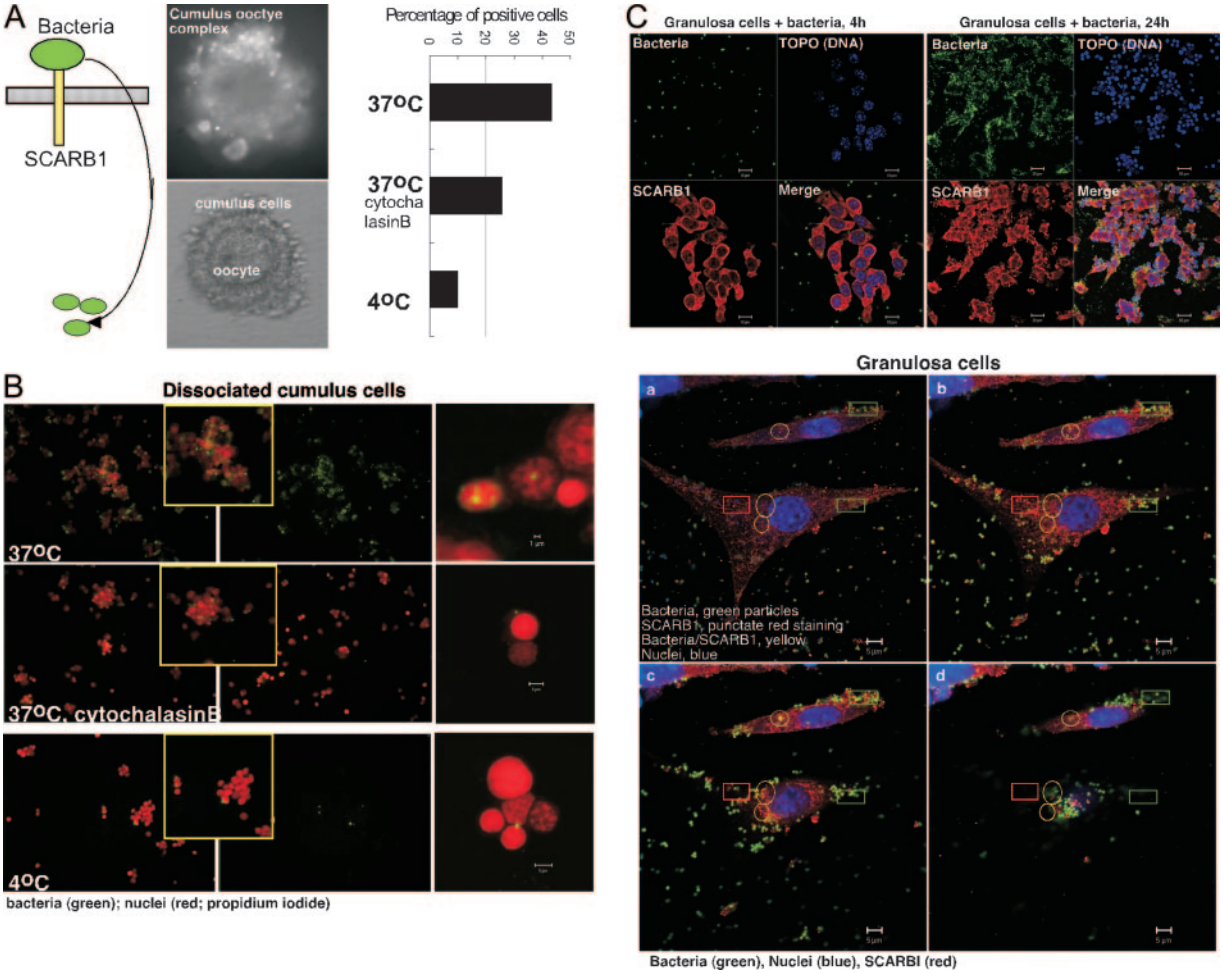

Fig. 5. Cumulus Cells and Granulosa Cells Exhibit Phagocytic Uptake of Bacteria

A, Schematic of bacterial uptake in cells via the scavenger receptors (*left*). Intact COCs were isolated at 6 h after hCG and incubated for 2 h with fluorescent bacterial particles (5 μg/ml) (*middle*); quantitative analyses of bacterial-positive cumulus cells are derived from data presented in panel B (*right*). B, COCs isolated from ovaries of mice 6 h after hCG were incubated with fluorescent bacterial particles for 2 h at 37 C, 37 C in the presence of cytochalasin B, or at 4 C and counterstained with propidium iodide (*red*). *Left panels*, Cumulus cells at $\times$40. The cells in the *yellow inset* are at higher magnification. *Right panels*, Confocal images of selected cells. Note the greater amount of bacteria (*intense green staining*) associated with cumulus cells at 37 C (*upper panels*) than in cells incubated with cytochalasin B (*middle panels*) or at 4 C (*lower panels*). These experiments were done several times but only quantitated once counting 631, 424, and 459 cells, respectively, in each treatment group. *Bright green spots* represent bacteria outside the cells, whereas the *diffuse green* represents bacteria within the cells. C, Granulosa cells isolated from PO follicles were incubated for 4 h or 24 h with bacterial particles, fixed, and costained with SCARBI (Alexa Fluor 594, tagged secondary; *red*) and TOPO-3 (DNA; *blue*). Note the *intense green* staining associated with cells incubated with bacteria for 24 h compared with 4 h. Panel C (images a–d), Four confocal images of the same cells at different planes illustrate bacterial particles associated with SCARB1 in granulosa cells. *Green squares* highlight regions where small bacterial particles appear attached to the cell surface. *Red squares* highlight the punctate staining pattern of SCARB1 to the surface of granulosa cells. *Yellow circles* surround areas where bacterial particles appear colocalized (*yellow dots*) with SCARB1. No costaining of bacterial particles with SCARB1 (*yellow dots*) was observed in cells at 4 h (confocal image not shown).

insufficient to determine whether the particles were ingested, additional COCs were incubated with bacteria for 2 h at 37 C in medium alone or in the presence of 16 μM cytochalasin B (to disrupt microtubule function) or at 4C (control) (Fig. 5B, *upper*, *middle*, and *lower panels*, respectively). After incubation, the cumulus cells were dissociated from the complex, spun onto coverslips, fixed, and stained with propidium iodide (*red*) to reveal intracellular nuclei acids (Fig. 5B). Many cumulus cells were fluorescently tagged when incubated at 37 C (43%; 274 of 631 counted) whereas very few cells exhibited bacterial ingestion at 4 C (10%; 46 of 459 counted). Likewise, cells incubated with cytochalasin B had reduced numbers of fluorescent bacteria (26%; 110 of 424 counted) (Fig. 5A; quantitation of panels in B). When examined by confocal microscopy, apparent binding/uptake (diffuse staining) was observed in cells incubated at 37 C. This

pattern was blocked, and only bright green particles were observed in cells incubated with cytochalasin B or at 4 C (Fig. 5B, *upper*, *middle*, and *lower panels* on the *right side*).

To explore the uptake of bacteria in more detail, we used granulosa cells because they also express the TLRs and SCARB1 receptors, are easier to culture, and require fewer mice to obtain a critical number of cells. Accordingly, granulosa cells were isolated from PO follicles of eCG-primed mice and cultured either 4 h or 24 h on serum-coated coverslips in serum-free defined media with 5 μg/ml bacteria. After fixation and processing as above, confocal imaging localized bacteria (*green*), SCARBI (*red*) and DNA (TOPO3; *blue*) in granulosa cells (Fig. 5C, *upper panels*). At 4 h only a limited number of bacterial particles were attached to the granulosa cell surface, and none were observed within the cells. By contrast, at 24 h, bacteria were attached not only to the surface of all cells but were also present within the cells as noted by the diffuse *green* staining. Additional confocal imaging of single cells (Fig. 5C, *bottom*; panels a–d) localized fluorescent bacterial particles to the surface of the cells (*bright green*) and colocalized many to SCARB1 (*yellow circles*) (see Fig. 5C legend for details). Note that the immunostaining of SCARB1 not associated with bacterial particles is also punctate. These experiments have been repeated three times with similar staining and localization patterns.

To document whether or not any bacterial particles were inside the granulosa cells and associated with lysosomes (23), additional PO granulosa cells were cultured with or without bacteria for 24 h. At that time fresh media containing the lysomosal marker Lyso Tracker DND-99 (100 nM; *red*) was added to all cells for 1 h. The cells exposed to bacteria were then washed, fixed, stained with TOPO3 (DNA), and examined by confocal microscopy. Control cells (no bacteria) were processed in the same manner or were also stained with SCARB1 (*green*). As shown in Fig. 6A, lysosomes (*discrete red dots*) were clearly evident in granulosa cells not exposed to bacteria. Moreover, the lysosomes were not associated with regions of the cell positive for SCARB1 (Fig. 6B). However, in cells exposed to bacteria, many lysosomes appeared as *yellow/orange* organelles indicating that small bacterial particles had been ingested and were present in the lysosomes (Fig. 6C). Bacterial particles not ingested were present on or near the cell surface (*brilliant green*). Lysosomes not containing bacteria were *red* as in panels A and B. These experiments have been repeated three times, and essentially all cells give the same patterns of staining for lysosomes, SCARB1, and bacteria.

DISCUSSION

The observations documented herein provide novel evidence that during ovulation COCs not only generate a physical matrix (protective shield) around the oocyte (9, 10) but cumulus cells within the complex, as well as granulosa cells, express a vast repertoire of immune cell-like functions. Most specifically, we document herein that members of the TLR family are expressed, regulated, and functional in COCs and granulosa cells, and members of the scavenger receptor family contribute to phagocytic activity of these cells. In immune cells, these molecules mediate effects necessary for innate immune responses and tissue repair. These receptors also impact immune cell survival (18–20, 25, 29). Thus, the TLR pathway and the scavenger receptor pathways together may provide cumulus cells and granulosa cells with specific survival mechanisms.

The selective induction of surveillance receptors (C1q, CD14, TLR4, TLR8, and TLR9) in COCs in response to LH *in vivo* as well as to either FSH or AREG in culture indicates that cumulus cells possess mechanisms by which they can respond to a variety of external cues (18, 20). Indeed, we document that LPS not only stimulated phosphorylation of p38MAPK and NF-κB but also induced expression of innate immune-related genes, namely *Il6* and *Tnfa*, in ovulated COCs (19, 29, 33) (Figs. 1–4). These observations combined with the coincident temporal induction of *Tlr4*, *Myd88*, *Il6*, and *Irf3* mRNAs in COCs 8–12 h post-hCG *in vivo* provide additional novel evidence that this pathway is highly regulated and operative during ovulation. Activation of TLRs (most likely TLR4) at this time may be important for regulating ovarian cell survival and the associated inflammatory-like events (Fig. 1) *(*19, 20, 28*).* Our results indicate further that the TLR pathway is present and functional in COCs and granulosa cells isolated from PO follicles (*i.e*. before the onset of ovulation-related events). Specifically, LPS not only induced *Il6* but also *Tnfa* and *Ptgs2* mRNA expression in the PO COCs (before hCG). Moreover, not only LPS but also HA increased *Il6* mRNA in PO granulosa cells, indicating that two distinct activators of TLR4 can induce expression of this cytokine. That FSH itself potently stimulated *Il6* and *Ptgs2*, but not *Tnfa* expression, in PO granulosa cells, suggests that the effects of FSH may be mediated by its rapid induction of *Areg* in these cells (31). AREG is a potent inducer of *Ptgs2* in granulosa cells and cumulus cells (31) and can also increase expression of TLR pathway components (Fig. 3). Thus, FSH may impact TLR signaling by indirect mechanisms that involve the actions of AREG and prostaglandins. Collectively, these pathways may provide granulosa cells with mechanisms that promote cell survival before and during the tissue repair process that occurs during ovulation.

That COCs occupy a unique niche within the follicle and undergo a fate distinct from granulosa cells is reflected by the selective regulation of specific factors in COCs compared with granulosa cells (Fig. 1B) (10). These include components of the TLR pathway, CD14, IRF3, and Cq1. GPI-anchored CD14 is an LPS coreceptor, whereas IRF3 mediates *Tnfa* gene activation

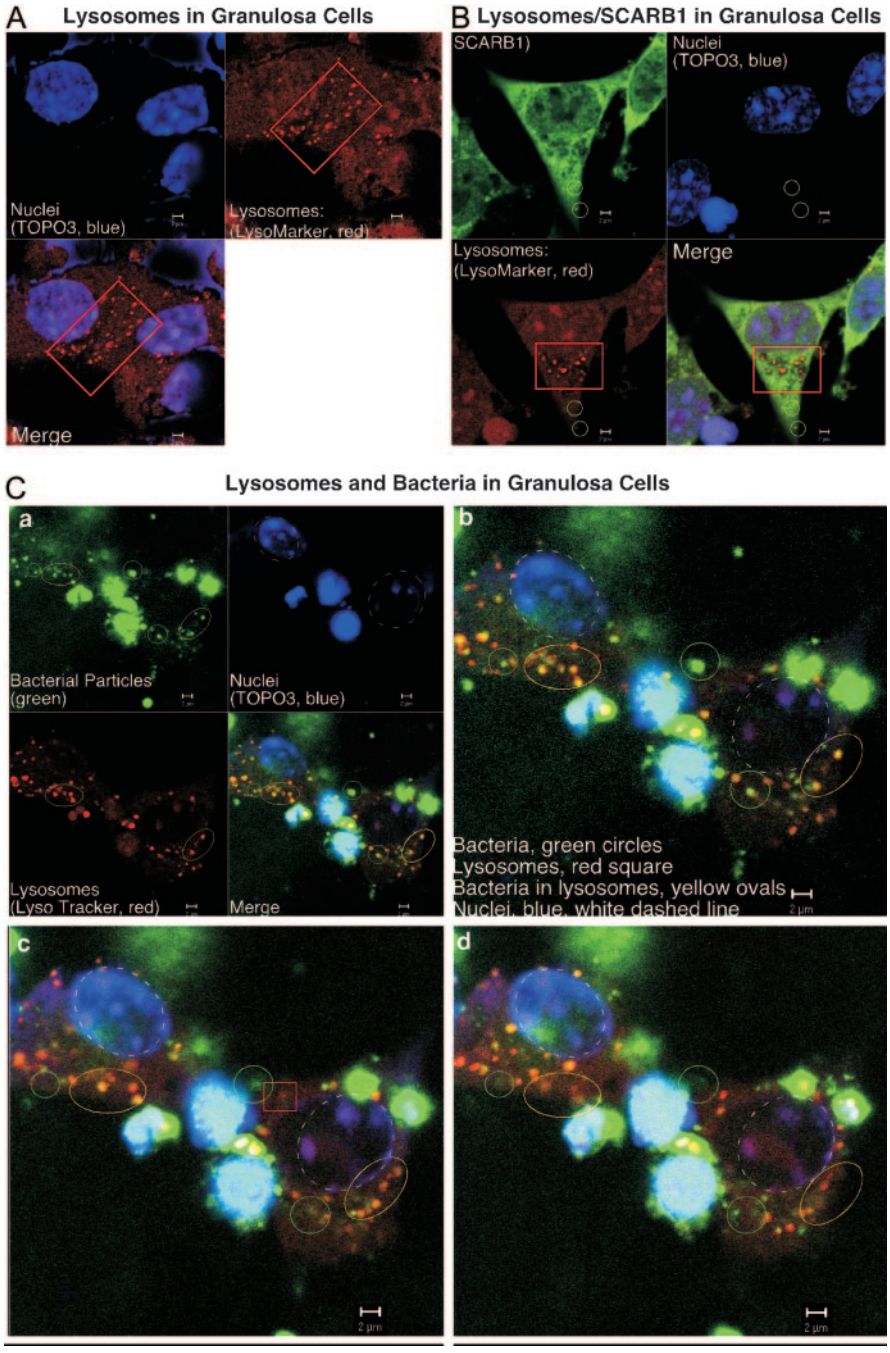

Fig. 6. Bacterial Uptake into Lysosomes

A and B, Granulosa cells were cultured for 24 h in medium alone followed by 1 h in media containing the lysosomal marker (Lyso Tracker, 100 mM; *red*). The cells were fixed and stained with TOPO3 (*blue*) to visualize nuclei or immunostained with SCARB1 antibody (secondary Alexa Fluor 488, *green*) to visualize this cell surface protein. Note the discrete intense staining of lysosomes (several *red spots* highlighted within *red rectangles*) and the punctate staining of SCARB1 (*green circles* delineate two examples). Panel C (a–d), Granulosa cells were cultured for 24 h with fluorescently tagged bacterial particles (5 μg/ml), 1 h with Lyso Tracker (100 nM), fixed, and stained with TOPO3 to visualize nuclei. Panel a represents four separate panels of the same cells (in the same confocal plane) to reveal the individual patterns of bacteria (*green*), nuclei (*blue* and *outlined* with *dotted white line*), lysosomes (*red*), and bacteria with lysosomes (*yellow*). *Green circles* highlight some of the bacterial particles at or close to the cell surface. *Larger green particles* are also present, indicating that although sonicated and vortexed the particles are not of completely homogenous size. *Yellow ovals* surround examples of lysosomes containing bacteria (*red and green* yield *yellow*/*orange* organelles). *Red squares* delineate lysosomes without bacteria. Panel b is an enlargement of that shown in panel a (*lower right*). Panels c and d represent other confocal images taken at different planes within these same cells. The lysosomes present in panel b gradually disappear in panels c and d. Bacterial particles colocalize with 60 $\pm$ 2% of the lysosomes present in 10 cells that were analyzed including those in panels b–d.

(29). The COC-related expression of these genes mimics that of other innate immune-regulatory factors *Pdcd1* and *Cd52*, which are induced in COCs but not granulosa cells of ovulating follicles (10, 11). The complement factor C1q, which is critical for monitoring a variety of external factors, is expressed abundantly at sites of tissue damage and plays an initiating role in clearance of apoptotic cells (18). The coordinate induction of *C1qa* in a pattern similar to that of *Cd14* and the *Tlrs* suggests that this pathway may also play a specific role other than, or in addition to, its ability to recognize IgG complexes. In fact, recent studies indicate that C1q recognizes myelin basic protein and serum amyloid protein [both of which are expressed in cumulus cells (10, 18, 34)] and has been shown to bind DNA, LPS, and some bacterial membranes (18). Curiously, mice null for *C1q*, like mice null for *Pdcd1* and *Runx1*, two other genes induced in COCs (10), develop autoimmune responses with lupus-like disease characteristics (35). However, to date, no overt fertility defects have been reported in these mutant mouse models, perhaps because of redundant and/or overlapping functions of TLR family members.

Although in macrophages and other cells, activation of the TLR pathway is essential for induction of scavenger receptors (20, 24), this does not seem to be the major role of the TLRs in the ovary. SCARBI and CD36 are present and distributed in a punctate manner on the surface of essentially all cumulus cells and granulosa cells before full induction of the TLR pathway and its components (Figs. 2 and 5). However, activation of the TLR pathway may facilitate the functions of the SCARB pathway. Because the process of ovulation releases COCs into the external environment of the oviduct, the presence of CD36 and SCARBI in cumulus cells may provide a mechanism by which the cumulus cells can respond rapidly to any "toxic" or "foreign" material should this type of surveillance response be required. For example, one morphological study showed a cumulus cell within the oviduct phagocytosing a sperm (36), providing a possible role (in addition to regulation of cholesterol transport) for the scavenger receptors on these cells. That cumulus cells (and granulosa cells) have phagocytic activity was documented for the first time herein by their ability to ingest bacterial particles, a process sensitive to temperature (occurring at 37 C but not 4 C) and cytochalasin B. These data support a recent study showing that CD36, SCARBI, and SCARBII have phagocytic capabilities when expressed exogenously in a nonphagocytic cell type and that this process is sensitive to temperature (23, 24). Moreover, testicular Sertoli cells (analogous to granulosa cells) that phagocytose degenerating/apoptotic sperm (37, 38) express functional CD36 and SCARBI providing additional evidence for this kind of activity in gonadal cells (39–41). Mice null for *Scarb1* are infertile and ovulate dysfunctional oocytes, apparently a consequence of abnormal lipoprotein metabolism (42).

In summary, the expression of innate immune cell-like genes in cumulus cells of ovulating COCs, as well as in granulosa cells, provides strong evidence that ovulation not only involves the generation of an extracellular HA-rich matrix but also the acquisition of specific surveillance functions used by immune cells (Fig. 1A). Furthermore, the matrix via HA (and its fragments) may serve to regulate TLR signaling in ovulating COCs and granulosa cells. Toll receptor signaling could therefore mediate cell survival during the wound-healing process that occurs at the site of follicle rupture during ovulation. Additionally, TLRs and scavenger receptors provide mechanisms by which cumulus cells recognize and remove altered self, including apoptotic cells. Thus, cumulus cells and granulosa cells posses and acquire novel, heretofore unexpected, and potentially critical surveillance role(s) during the ovulation process.

MATERIALS AND METHODS

Materials

Gestyl (eCG) was purchased from Professional Compounding Center of America (Houston, TX). Pregnyl (hCG) was purchased from Organon Special Chemicals (West Orange, NJ), and FSH (oFSH-16) was a gift from the National Hormone and Pituitary Program (Rockville, MD). Fetal bovine serum was obtained from Hyclone Laboratories (Logan, UT). Hyaluronic acid (HA) fragments of relatively uniform size (~150 kDa) were obtained from Hyalose (Oklahoma City, OK). Oligonucleotide poly-(dT) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ); AMV reverse transcriptase and Taq polymerase were from Promega Corp. (Madison, WI); [P^{32}]dCTP was from ICN (Los Angeles, CA); oligonucleotide primers for RT-PCR reactions were from Sigma-Genosys (Houston, TX); routine chemicals were from Fisher Scientific (Pittsburgh, PA) or Sigma (St. Louis, MO). Antibodies used for WB, IF, and uptake studies are as indicated: SCARB1 (SR-B1; Novus Biologicals, Littleton, CO): WB, 1:2000; IF, 1:500; Uptake, 1:500. TLR4 (Cell Signaling Technology, Beverly, MA): WB, 1:1000; IF, 1:100; Uptake, 1:100. MYD88 (eBiosciences, San Diego, CA): WB, 1:1000; IF, 1:100; Uptake, 1:100. IRF3 (Cell Signaling Technology): WB, 1:1000; IF, 1:100; Uptake, 1:100. Phospho-p38MAPK: WB, 1:2000. Phospho-ERK1/2: WB, 1:2000. Phospho-NFκB: WB, 1:1000. NF-kB: WB, 1:1000 (Cell Signaling Technology). Secondary antibodies tagged with Alexa Fluor 594 (*red*) and Alexa Fluor 488 (*green*) as well as Lyso Tracker (*red*) DND-99 were purchased from Invitrogen (Carlsbad, CA).

Animals

Immature female C57BL/6 mice were obtained from Harlan, Inc. (Indianapolis, IN). On d 23 of age, female mice were injected ip with 4 IU of eCG (Pregnyl; Organon, West Orange, NJ) to stimulate follicular growth, followed 48 h later with 5 IU hCG (Gestyl; Diosynth, Oss, The Netherlands) to stimulate ovulation (43). Animals were housed under a 16-h light/8-h dark schedule in the Center for Comparative Medicine at Baylor College of Medicine and provided food and water *ad libitum*. Animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, as approved by the Animal Care and Use Committee at Baylor College of Medicine.

Table 1. Specific Primer Pairs Selected for Amplification of cDNA Products

	Primer Pair (5′–3′, Forward and Reverse)	Size (bp)	Cycles
C1q a unit	GAAAGGCAATCCAGGCAATA	402	27
	AAGATGCTGTCGGCTTCAGT		
Cd14	ACATCTTGAACCTCCGCAAC	538	27
	AGTGACAGGTTCCCCACTTG		
Cd34	CCATCTCAGAGACTATGGTCAACTT	326	28
	CTTTAGCCTCCTTGGATATCTGCTA		
Cd36	TCTCTGACACAGAGCTTATGAAATG	340	27
	AGGTTGAATTTAAGGAACGACTTCT		
Il-6	CCGGAGAGGAGACTTCACAG	421	28
	GGAAATTGGGGTAGGAAGGA		
Irf-3	GGTCTTCCAGCAGACACTCTTT	468	27
	CATGTAGGAACAACCTTGACCA		
L19	CTGAAGGTCAAAGGGAATGTG	196	23
	GGACACAGTCTTGATGATCTC		
Myd88	ATGTTCTCCATACCCTTG	365	30
	ACTGCTTTCCACTCTGGC		
Ptgs2	TGTACAAGCAGTGGCAAAGG	433	24
	GCTGTGGATCTTGCACATTG		
Scarb1	CAGGATAAGGAGGCCATTCA	598	27
	GAAAAAGCGCCAGATACAGC		
Tlr4	ACCTGGCTGGTTTACACGTC	455	27
	CAGGCTGTTTGTTCCCAAAT		
Tlr8	CAAACAACAGCACCCAAATG	591	27
	CTGAGGGAAGTGCTGGAAAG		
Tlr9	TGCAGGAGCTGAACATGAAC	297	26
	TAGAAGCAGGGGTGCTCAGT		
Tnfa	AGTCCGGGCAGGTCTACTTT	422	26
	GCACCTCAGGGAAGAGTCTG		

Isolation of COCs and Granulosa Cells

Ovaries of immature mice primed with eCG for 48 h contain multiple PO follicles. COC cells were isolated from these follicles by needle puncture, collected by pipette, pooled, frozen, and stored at −80 C for RNA or protein extraction (10, 13). Granulosa cells that were released by needle puncture of the follicles were also pooled, collected by centrifugation, and frozen at –80 C for protein and RNA analyses. COCs and granulosa cells were also isolated from ovaries of eCG-primed mice after hCG treatment for 4, 8, and 12 h. During this time, COCs expand but have not yet ovulated. Ovulated (fully expanded) COCs were collected by needle puncture of the oviducts of mice 16 h or 24 h after hCG. Collection of each pool of COCs and granulosa cells (15 mice) at each time point was repeated two times (*i.e*. two separate experiments) (10). *For culture*, nonexpanded COCs (~20–30) and granulosa cells were collected from the ovaries of eCG-treated mice, plated in separate wells of a Falcon 24-well plate (Becton Dickinson, Franklin Lakes, NJ) in 0.5 ml of defined medium (13) containing 1% fetal bovine seum without or with either FSH (100 ng/ml), Amphiregulin (AREG; 250 ng/ml) (13), LPS (100 ng/ml), or HA (100 μg/ml). After specific time intervals, as designated in the figure legends, total RNA or protein was extracted from the COCs. In other experiments, ovulated COCs were collected from the oviduct after 16 or 24 h post-hCG injection. Each experiment was repeated two times.

RT-PCR Analyses

Total RNA was obtained from COCs and granulosa cells using the RNAeasy Mini kit (QIAGEN Sciences, Germantown, MD) according to the manufacturer's instructions, and semi-quantitative RT-PCR analyses were performed as described previously (44, 45). Briefly, total RNA was reverse transcribed using 500 ng poly-dT and 0.25 U avian myeloblastosis virus-reverse transcriptase at 42 C for 75 min and 95 C for 5 min. For the amplification of the cDNA products, specific primer pairs were selected as indicated in Table 1. All PCRs were done in the linear range of amplification and contained [^{32}P]dCTP, *Taq* Polymerase, and Thermocycle buffer. cDNA products were resolved on 5% polyacrylamide gels that were dried and exposed to x-ray film. The radioactive PCR product bands were quantified using a Storm 860 PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA). The authenticity of the PCR products was verified by subcloning and sequencing. Total RNA was obtained from COCs and granulosa cells using the RNAeasy Mini kit (QIAGEN Sciences) according to the manufacturer's instructions.

Western Blot Analyses

***In Vivo* Samples.** COCs and granulosa cells were collected from ovaries/oviducts of eCG-primed immature mice at 0, 8, and 16 h after hCG, pooled, and frozen at −80 C. WCEs were prepared by homogenizing each sample in high-salt WCE buffer (46).

***In Vitro Samples*.** Nonexpanded COCs and granulosa cells were isolated from PO follicles of eCG-primed mice and cultured in defined medium plated in separate wells of a Falcon 24-well plate in 0.5 ml of defined medium (13*)* without or with LPS (100 ng/ml) for 0, 60, and 120 min. The COC and granulosa cell samples were collected by centrifugation and extracted in SDS boiling buffer as described previously *(*10). WCE (3 μg protein) and SDS extracts (30 μl) were resolved by SDS-PAGE (10%) and transferred to Immobilon-P nylon membranes (Millipore Corp., Bedford, MA). Membranes were blocked in Tris-buffered saline and Tween 20 [TBST; 10 mM Tris (pH 7.5), 150 mM NaCl and 0.05% Tween 20] containing 5% nonfat Carnation instant milk (Nestle Co., Solon, OH). Blots were incubated with selected primary antibodies overnight at 4 C. After washing in TBST, Enhanced chemiluminescence (ECL) detection was performed using Pierce Super Signal according to the manufacturer's specifications (Pierce) and appropriate exposure of the blots to Kodak x-ray film.

Specific bands were quantified by densitometric analyses using a Molecular Dynamics Personal Densitometer.

IF

COCs were immobilized on polylysine-coated coverslips, fixed with 4% paraformaldehyde, washed with PBS, and used directly or stored in 1% paraformaldehyde. COCs were permeabilized with 0.5% Nonidet P-40 (NP-40), washed, blocked with 5% BSA or Vector M.O.M. immunodetection reagents (Vector Laboratories, Burlingame, CA), and incubated overnight at 4 C with selected primary antibodies as previously (10). Antibody localization was visualized with fluorescein isothiocyanate-labeled antirabbit IgG and Streptavidine AlexaFluor 568 (Molecular Probes). Nuclei were visualized by 4′,6-diamidino-2-phenylindole present in the VECTASHIELD D mounting medium (Vector Laboratories), propidium iodide, or PR-TOPO-3 (Molecular Probes). Digital images were captured using an Axiphot microscope with ×10–40 objectives or a Zeiss Laser Scanning Confocal Microscope (LSM 510; Carl Zeiss, Thornwood, NY).

Phagocytosis Studies

COCs were isolated from ovaries of eCG-primed mice 6 h after hCG, a time selected based on the induction of TLRs, CD14, and MYD88 between 4 and 8 h. COCs were cultured for 4 h in a 12-well culture dish (1 ml defined medium containing 5% serum) with 5 μg/ml of sonicated bacterial (*Escherichia coli* strain K12) particles in the Vybrant Phagocytosis Assay Kit (Invitrogen) (23). The COCs and bacteria were cultured either at 37 C in medium alone, at 37 C in the presence of cytochalasin B (16 μM to inhibit cytoskeletal uptake mechanisms), or at 4 C (control). After 2 h culture, bacterial particles, COCs were washed twice (using a glass pipette) with medium containing 5% serum and then treated with hyaluronidase for 1 min. After a 30-sec vortex (medium speed), the dispersed cells were collected by centrifugation at 10,000 rpm for 1 min. The supernatant was removed, 100 μl of trypan blue quenching solution was added, and the cells were collected again by centrifugation. The cells were washed in serum-free medium twice and then fixed in 2% paraformaldehye for 30 min. Cells were then spun onto (3-aminopropyl) triethoxysilane (Sigma A3648)-coated slides. The immobilized cells were washed in PBS, permeabilized in 0.5% NP-40, and then immunostained as described above using specific antibodies and DNA staining reagents. Granulosa cells obtained from ovaries of eCG-primed mice were plated on serum-coated coverslips in 12-well dishes and cultured in serum-free defined medium at 37 C for 24 h with bacterial particles (5 μg/ml) without or with FSH and additional 1 h in the presence of the lysosomal marker (Lyso Tracker; 100 nM). Granulosa cells were also cultured 24 h on coverslips and then incubated for 4 h with bacterial particles at 37 C. Cells were then washed, fixed in 2% paraformaldehyde 30 min, washed in PBS, permeabilized with 0.5% NP-40, and immunostained as described above and in the figure legends.

Statistics

The semiquantitative RT-PCR data are represented as mean ± SD. Data were analyzed by using GraphPad Prism Programs (ANOVA or *t* test and Neuman-Keuls Multiple Comparison Tests; GraphPad Prism, San Diego, CA) to determine significance. Values were considered significantly different if $^{*}P < 0.05$ or $^{**}P < 0.01$ (Figs. 1, 3, and 4).

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