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TLR4 activates NFκB in human ovarian granulosa tumor cells

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Abstract

Previous studies have demonstrated expression of Toll-like receptors (TLRs) in the surface epithelium of normal ovaries (OSE) and in epithelial ovarian tumors. Most notably, OSE-derived cancers express TLR4, which activates the nuclear factor-kappa B (NF-κB) signaling cascade as a mediator of inflammatory response. Currently, there is considerable interest in elucidating the role of TLR-mediated signaling in cancers. Nevertheless, the expression of TLRs in granulosa cell tumors (GCTs) of the ovary, and the extent to which GCT expression of TLRs may influence cell-signaling pathways and/or modulate the efficacy of chemotherapeutics, has yet to be determined. In the present study, human GCT lines (COV434 and KGN) were utilized to evaluate expression of functional TLR4. TLR4 is expressed in GCT cell lines and ligation of TLR4 with bacterial lipopolysaccharide (LPS) led to IκB degradation and activation of NF-κB. NF-κB activation was confirmed by nuclear localization of NF-κB p65 following treatment with LPS and the naturally occurring ligand, HSP60. Notably, immunoneutralization of TLR4 blocked nuclear localization, and inhibition of NF-κB signaling attenuated LPS-induced *TNFα* plus increased doubling time in both cell lines. Contradictory to reports using human OSE cell lines, inhibition of NF-κB signaling failed to sensitize GCT lines to TRAIL or cisplatin. In summary, findings herein are the first to demonstrate a functional TLR-signaling pathway specifically in GCTs, and indicate that in contrast to OSE-derived cancers, inhibition of NF-κB does not sensitize GCTs to TRAIL or cisplatin.

Keywords

Granulosa cell tumor; Toll-like receptor; TLR; ovary; LPS; lipopolysaccharide

Amongst ovarian carcinomas, granulosa cell tumors (GCTs) are the most common sex-chord neoplasm, yet represent less than 5% of all ovarian cancers. Due to the relative rarity of these malignancies, the etiology, genetics, and pharmacologic management of GCTs have been understudied compared to ovarian surface epithelium (OSE)-derived cancers. As such, the prevailing treatment is largely similar to that of cancers originating from human (h) OSE (the most common form of ovarian carcinoma), despite the fact that granulosa cells and their

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tumor derivatives demonstrate a unique phenotype [1; 2]. Foremost among their defining characteristics is the capacity for steroid production (*i.e.* estradiol), distinctly placing GCTs within the category of an endocrine tumor. The lack of information regarding selective treatment for GCTs is due in large part to the paucity of appropriate *in vitro* model systems, and in particular, transformed granulosa cell lines that maintain expression of functional gonadotropin receptors. Accordingly, the establishment of two GCT lines, KGN [3] and COV434 [4], has proven valuable not only in the study of human granulosa cell function, but has also given rise to new opportunities for developing targeted GCT therapies.

Additionally, there has been recent interest in the role of Toll-like receptors (TLRs) in ovarian biology, including cancer [5; 6; 7; 8]. TLRs represent 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 conserved small molecular sequences found on the surface of invading pathogens, as well as some endogenous molecules. In animal models of ovarian function, TLRs present on granulosa cells have been implicated in ovulation, a naturally occurring inflammatory-like response [5; 9; 10]. In models of OSE-derived cancer, cancerous human OSE cell lines have also been shown to express multiple TLRs [6; 11]. Most notably, OSE-derived cancers express TLR4, which signals through the adaptor protein, MyD88, leading to the phosphorylation and proteasomal degradation of I κ B, and activation and localization to the nucleus of nuclear factor-kappa B (NF- κ B) [6]. This signaling cascade has been shown to increase production of proinflammatory cytokines (*e.g.* tumor necrosis factor- α (TNF α), TRAIL [12]) and promote tumor cell survival and proliferation. Activation of TLR signaling can lead to enhanced tumor cell invasion and metastasis through the regulation of metalloproteinases and integrins [13]. Moreover, in human OSE cancer, NF- κ B has been demonstrated to function as an oncogene and enhance tumorigenesis in chemoresistant cell lines [14]. Due to accruing evidence implicating a role for TLR and NF- κ B signaling in tumor progression, in combination with an emerging role for TLR signaling in granulosa cell function, there is strong impetus to pursue this pathway when developing targeted cancer therapies. Nevertheless, the expression of functional TLRs has yet to be evaluated in human GCTs.

Materials and Methods

Cell culture and reagents

The human GCT lines, COV434 and KGN, were generously provided by Dr. DeGeyter (University of Basel, Switzerland), and Drs. Nishi and Yanase (Graduate School of Medical Sciences, Japan), respectively. COV434 cells were cultured in DMEM + 10% FBS (Invitrogen), while KGN cells were cultured in DMEM:F12 + 10% FBS (Invitrogen). Cells were maintained in a sub-confluent state during all experiments in an atmosphere of 37° C in 5% CO₂/95% humidified air. Recombinant human TRAIL was purchased from Peprotech (Rocky Hill, NJ), while *Escherichia coli* lipopolysaccharide (LPS) and cisplatin (cis-platinum(II)-diammine dichloride) were from Sigma-Aldrich. Heat shock protein 60 (HSP60) was from Enzo Life Sciences (Plymouth Meeting, PA). The effective doses for TRAIL, HSP60, LPS, and cisplatin were empirically determined for each cell line.

Cell viability assay

Cells were seeded in a 96-well cell culture plate at a density of 2×10^4 (COV434) or 1×10^4 (KGN) and treated with TRAIL (100 ng/ml), alone or in combination with LPS (10 μ g/ml) or cisplatin (25 μ M). Following a 24 h culture period, cytotoxic effects were evaluated by monitoring metabolic activity with a methyl tetrazolium salt (MTS) assay (a measure of cell viability) using the CellTiter 96 Aqueous One reagent (Promega, Madison, WI) [15]. Data shown as fold difference as compared to control cells for each cell line.

Western blot and imaging analysis

Western blot analysis was completed as previously described [16]. The rabbit anti-I κ B was from Santa Cruz Biotechnology, Inc. (La Jolla, CA) diluted 1:1000. The mouse monoclonal tubulin was from Abcam (Cambridge, MA) diluted 1:10,000. The horseradish peroxidase-conjugated anti-rabbit and anti-mouse secondary antibodies were from Pierce (Rockford, IL; 1:10,000). Membranes were incubated with ECL Western blotting detection reagent (Pierce) for 1 min, wrapped and exposed to X-ray film for 3–10 min.

For imaging of cell lines, cells were seeded onto cover slips and cultured for 24 h. Cells were rinsed with phosphate-buffered saline (PBS) and subsequently fixed with 2% PFA for 45 min, rinsed again and incubated in blocking buffer, and finally incubated with TLR4 antibody (1:200; Abcam) for 2 h at room temperature. Cells were washed prior to incubation with FITC-conjugated secondary antibodies. For cell surface localization, cover slips were washed and mounted onto slides, and cells were visualized using a Bio-Rad MRC Scanning Confocal System (Hercules, CA). For localization of NF- κ B, cells were seeded onto cover slips, incubated with or without a mouse anti-human TLR4 antibody (1:200; eBioscience, San Diego, CA) for 1 h prior to treatment with or without HSP60 or LPS for 30 min. Cells were then fixed with 2% PFA for 45 min and blocked with 2% BSA, 2% normal goat serum in PBS plus .1% Tween-20 for 1 h. Primary NF- κ B p65 antibody (1:50; Abcam) was added for 1 hour, followed by 3 washes in PBS and FITC-conjugated anti-rabbit secondary antibody for 2 h (1:250). Rhodamine phalloidin was added for 15 minutes and DAPI for 5 minutes following incubation with secondary antibody. Cells were washed 3 times, and imaging was performed on a Nikon Eclipse TE2000-S inverted fluorescent microscope. Cells counts were used to determine the percentage of cells expressing exclusively nuclear NF- κ B following treatment.

PCR amplification of *TLRs* and *MyD88*

In order to determine if TLRs are expressed in the human GCT lines, COV434 and KGN, *TLR2* and *TLR4* along with the adaptor *MyD88* were amplified from reverse transcribed RNA (Promega Reverse Transcription System, Promega, Madison, WI) isolated from freshly collected GCT lines. Primer sequences are described in Table 1. PCR conditions were specific for primer pairs, and included initial denaturing for 3 min at 94 C, followed by 40 cycles of denaturing at 94 C (30 sec), annealing at 51–60 C (30 sec), followed by an extension step at 72 C (60 sec) using *Taq* DNA polymerase (Invitrogen). All PCR products were subsequently sequenced to verify identity.

Real-time quantitative PCR

The transcriptional capability of NF- κ B was monitored through real-time PCR for the known NF- κ B transcriptional target, *TNFA*. Primers for *TNFA* were validated for use with real-time PCR by determining the optimal amplification efficiency and primer concentrations. Template dilutions were performed to verify that amplification efficiency of target genes were the same as that of the 18s rRNA housekeeping gene. Conditions were set to the following parameters: 15 min at 95 C followed by 40 cycles each of 15 s at 95 C, 30 s at 60 C, 30 s at 72 C. The Ct (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 $\Delta\Delta$ Ct method [17]. Values are expressed as fold-difference compared to an appropriate control sample.

Vectors and Retroviral Infection

The pBabe-puro-I κ B-Mut harbors two mutations (S32 to A32, and S36 to A36) that render I κ B resistant to phosphorylation and degradation [18]. This construct was described

previously by Dr. William C. Hahn (Dana Farber Cancer Institute, Boston, MA [19]), and was obtained through the Addgene plasmid repository (Cambridge, MA). The expressed I κ B-Mut results in repression of NF- κ B activation [19]. For retrovirus production, 1 μ g of pBabe-puro vector or pBabe-puro-I κ B-Mut DNA was transfected into the Phoenix-A packaging cell line. Transduction of COV434 and KGN was performed using fresh viral supernatant, followed by puromycin drug selection (2 μ g/ml) for 10 days.

Statistical Analysis

Experiments were independently replicated a minimum of three times unless otherwise stated. Data among replicate experiments were standardized by expressing individual experiments as a fold-difference versus control, or versus mock transformed cells. Transformed data from replicated experiments were analyzed by one-way ANOVA without including values from the control or mock transfected group (arbitrarily set to 1.0), and the Fisher's protected least significant difference multiple range test. Alternatively, in cases where a specific comparison was between two groups, original data from the replicate experiments were analyzed using a Student's t-test.

Results

TLR4 is expressed and functional in COV434 and KGN human GCT lines

TLR4 protein expression was detected in both cell lines, using a rabbit anti-TLR4 antibody in PFA fixed cells (Figure 1A). Negative IgG controls showed little to no background staining, indicative of antibody specificity. Additionally, *TLR4* mRNA, as well as mRNA encoding *TLR2* and the signaling adaptor, *MyD88*, were amplified from both COV434 and KGN (Figure 1B). Stimulation of TLR4 with exogenous ligand, bacterial LPS (10 μ g/ml), led to rapid degradation of I κ B, a requirement for NF- κ B activation, by 10–60 min in both cell lines (Figure 2A; $p < 0.05$). This effect was not due to an increase in cell death, as treatment with LPS had no significant effect on cell viability up to 18 h post treatment (Figure 2B; $p > 0.05$). Furthermore, treatment with the endogenous ligand, HSP60 (10 μ g/ml; Figure 2C, E), or the exogenous ligand, bacterial LPS (10 μ g/ml; Figure 2C, F), for 30 min led to the translocation of NF- κ B from the cytoplasm to the nucleus (HSP60, Figure 2E, 57.3 $\pm$ 4.8% nuclear, $p < 0.05$ vs control; LPS, Figure 2F, 65.3 $\pm$ 6.4% nuclear, $p < 0.05$ vs control). To verify that this effect was indeed mediated by TLR4, a mouse anti-human TLR4-binding antibody (TLR4-NAb) was used to immunoneutralize TLR4. Pretreatment with TLR4 antibody for 1 h effectively blocked the nuclear translocation of NF- κ B (Figure 2C–F; $p < 0.05$).

Inhibition of NF- κ B signaling in GCTs

To evaluate the role of NF- κ B on GCT proliferation and chemosensitivity, both KGN and COV434 cell lines were stably transduced with a mutant I κ B (mutated at S32 and S36), to inhibit NF- κ B activation (I κ B-mut). Expression of I κ B-mut inhibits phosphorylation and degradation of I κ B in a dominant negative fashion, and results in inhibition of NF- κ B signaling [19]. Activity of this dominant negative repressor was verified through the inhibition of the downstream transcriptional target of LPS-induced NF- κ B signaling, *TNFA* (Figure 3A). While LPS significantly upregulated *TNFA* in mock-transduced KGN and COV434 cells, LPS had no effect on *TNFA* transcription in cell lines harboring the I κ B-mut construct. Furthermore, both KGN and COV434 I κ B-mut cell lines exhibited a significant delay in doubling time (KGN 1.806 $\pm$ 0.2 d vs 3.01 $\pm$ 0.25 d; COV434 1.204 $\pm$ 0.1 d vs 2.107 $\pm$ 0.22 d; $p < 0.05$; Figure 3B) as compared to mock transformed cells.

Finally, as reported previously, treatment with the pro-apoptotic TRAIL led to a slight, but statistically significant, reduction in cell viability following an 18 h culture (Figure 3C;

$p < 0.05$ [20]). Additionally, treatment with the conventional chemotherapeutic, cisplatin (25 μM) led to a significant reduction in viability ($p < 0.05$), and pre-treatment with cisplatin followed by treatment with TRAIL led to a further reduction in cell viability in both cell lines. However, inhibition of NF- κB did not sensitize cells to TRAIL as compared to control (Figure 3C). Suppression of NF- κB signaling also did not affect the cytotoxicity of cisplatin, either alone or in combination with TRAIL (Figure 3C).

Discussion

Although TLRs have recently been described in ovarian cancers of epithelial origin [8], they have not previously been described in cancerous human granulosa cells. The results presented herein are the first to describe expression of TLR4 in human ovarian tumor cells specifically of granulosa cell origin. Together, these data demonstrate that TLR4 is expressed and functional in the human GCT lines, COV434 and KGN, as indicated by both gene and protein expression, combined with the degradation of I κB following treatment with LPS, the nuclear localization of NF- κB following treatment with the endogenous ligand HSP60 as well as LPS, and transcriptional activation of *TNFA*. Interestingly, while confocal imaging following TLR4 protein staining clearly indicates that TLR4 is present extra- and intracellularly in KGN cells, immunoneutralization of TLR4 in live KGN cells effectively blocked both HSP60 and LPS activation of NF- κB . These data strongly suggest that the functionality of TLR4 is related to its cell surface expression, and not intracellular expression, as has been reported for some other cell types [21]. It has recently been shown that OSE cancer cells respond to TLR4 activation through the acute activation of NF- κB , and subsequent up-regulation of proinflammatory cytokines. However, OSE cancer cells lacking MyD88 do not activate NF- κB , and do not regulate cytokine production [6]. Because normal granulosa cells express *MyD88*, it was not surprising that GCT-derived cell lines also expressed *MyD88* and that stimulation with TLR4 ligands (either HSP60 or LPS) acutely activated NF- κB in both COV434 and KGN.

It has also been demonstrated in multiple cancer cell types, including human OSE cancer cells, that active NF- κB signaling opposes TRAIL-induced apoptosis. However, in the human GCT lines examined, the activation status of NF- κB had no effect on TRAIL sensitivity as activation of NF- κB via treatment with LPS did not inhibit TRAIL sensitivity, nor did inhibition of NF- κB enhance the effects of TRAIL in COV434 and KGN. These findings indicate that NF- κB does not modulate TRAIL sensitivity in these GCT lines. The data provide further evidence that the role of NF- κB in TRAIL-sensitivity is cell type specific. While an antagonistic role for NF- κB activation on TRAIL-induced cell death has been previously described in multiple cancerous and non-cancerous cell types [22; 23; 24], other reports have demonstrated that NF- κB signaling does not play a role in TRAIL sensitivity [25; 26; 27]. The finding that inhibition of NF- κB does not play a role in TRAIL-sensitivity in these GCT lines is significant, as inhibition of NF- κB has been proposed as a potential molecular target for cancer therapy [28].

In summary, the studies described herein are the first to demonstrate a functional TLR-signaling pathway specifically in human granulosa tumor cells. Additionally, while inhibition of NF- κB has been previously shown to render otherwise TRAIL-resistant cancer cell lines sensitive to TRAIL treatment, this is not the case in KGN and COV434 GCT lines. This finding emphasizes the importance of using specific tumor-derived cell lines in the development of molecular targets to address the pharmacologic management of granulosa cell tumors.

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Abbreviations

GCT	granulosa cell tumor
TLR	Toll-Like Receptor
TRAIL	Tumor necrosis factor related apoptosis inducing ligand
NF-κb	Nuclear factor kappa b
OSE	ovarian surface epithelium

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Figure 1.

TLR4 is expressed in COV434 and KGN GCT lines. **A.** Fluorescent imaging of immunofluorescent TLR4 antigen in COV434 and KGN. Brightfield showing fixed KGN cells is shown on right, with no positive staining fluorescent staining (α -rabbit conjugated FITC shown in inset) **B.** PCR amplification of *TLR4*, *TLR2* and *MyD88* from COV434 and KGN GCT lines.

Figure 2.

TLR4 activates NF-κB in GCT lines. **A.** Western blot analysis of IκB alpha in COV434 and KGN following treatment with LPS (10 μg/ml) for 0–60 min and image quantification (*, $p < 0.05$ by t-test; $n = 3$ replicate experiments). α-tubulin is used for normalization and to indicate protein-loading efficiency. **B.** Cell viability following treatment with LPS (10 μg/ml) for 18 h. Con, control cells, ns not significance ($p > 0.05$). **C.** Immunofluorescent staining of NF-κB p65 in KGN cells. Intracellular localization of the p65 subunit of NF-κB is shown with and without treatment with LPS (10 μg/ml) or HSP60 (10 μg/ml) for 30 min (LPS+/- HSP60+/-). Cells were pretreated with and without a TLR4 neutralizing antibody (TLR4-NA +/-; 1:200 dilution) for one hour prior to LPS or HSP60 challenge. NF-κB is shown in green, DAPI nuclear stain in blue, rhodamine phalloidin actin filament staining in red. Cells were counted and nuclear localization is expressed as a percentage (**D–F**). $n = 3$, * indicates significance, $p < 0.05$.

Figure 3.

Inhibition of NF- κ B does not affect TRAIL sensitivity in GCT COV434 and KGN. **A.** Inhibition of NF- κ B prevented transcriptional activation of *TNF α* . Treatment with LPS (10 μ g/ml) for 18 h increased *TNF α* levels in both COV434 and KGN GCT lines transduced with the p-Babe-puro vector (mock; $p < 0.05$). However, transduction with the dominant negative I κ B (I κ B-Mut) prevented this increase (*, $p < 0.05$ by t-test; $n = 3$ replicate experiments). **B.** Doubling time (shown in days) was increased in COV434 and KGN (*, $p < 0.05$ by t-test; $n = 3$ replicate experiments). **C.** In COV434 (**left**) and KGN (**right**), treatment with TRAIL (100 ng/ml) led to a slight, but statistically significant increase in cell death in cells transduced with the p-Babe-puro vector (mock; A,B: $p < 0.05$). Additionally, in both COV434 (**A**) and KGN (**B**) the efficacy of cisplatin (cis; 25 μ M) on cell death was unchanged following inhibition of NF- κ B, when treated alone or in combination with TRAIL. A,B,C,D: $p < 0.05$).

Table 1

PCR primer pairs.

Gene name	Accession No.	Primers
<i>TLR2</i>	NM_003264.3	GGCCAGCAAATTACCTGTGTG AGCCGGACATCCTGAACCT
<i>TLR4</i>	U88880	CTGCAATGGATCAAGGAC CA AGTTCTCGTTGAAGTCGCTCCATCC
<i>MyD88</i>	U70451	TCTCTGTTCTTGAACGTGCGGACA TTTGGCAATCCTCCTCAATGCTGG
<i>TNFα</i>	M10988	CATGTTGTAGCAAACCCTCAAGC TGATGGCAGAGAGGAGGTTGAC