Published in final edited form as:

Placenta. 2007 ; 28(5-6): 477–481. doi:10.1016/j.placenta.2006.08.004.

LPS Induces Translocation of TLR4 in Amniotic Epithelium

Kristina M. Adams^{1,2}, Joëlle Lucas^{2,3}, Raj P. Kapur^{4,6}, and Anne M. Stevens^{5,6}

¹Department of Obstetrics & Gynecology, University of Washington, Seattle, WA

²Division of Clinical Research, Fred Hutchinson Cancer Research Center, Seattle, WA

³School of Medicine, University of Washington, Seattle, WA

⁴Department of Laboratory Medicine, University of Washington, Seattle, WA

⁵Department of Pediatrics, University of Washington, Seattle, WA

⁶Children's Hospital and Regional Medical Center, Seattle, WA

Abstract

Toll-like receptor 4 (TLR4) mediates lipopolysaccharide (LPS) induced immune responses, which may contribute to preterm labor associated with intraamniotic gram-negative bacterial infections. The study objective was to investigate gestational age and LPS-induced changes in TLR4 subcellular localization within amniotic epithelium, the first line of host defense against intraamniotic bacteria. TLR4 localization in amniotic epithelium was assessed using immunohistochemistry on 24 placentas of different gestational ages: first trimester (n = 6), second trimester (n=6), and third trimester (n=12). Immunofluorescence was used to determine TLR4 localization following *ex vivo* LPS stimulation of amnion from women undergoing cesarean section without labor at term. TLR4 was expressed in the cytoplasm of amniotic epithelium starting at 9 weeks with apical polarization by 25 weeks gestation. TLR4 localization to the basal membrane was significantly associated with chorioamnionitis (p=0.01). After LPS stimulation, TLR4 was expressed sequentially within the apical membrane, cytoplasm, and finally in the basal cellular compartment. This suggests that TLR4 expression in amniotic epithelium is poised to monitor amniotic fluid for pathogens. TLR4 translocation to the basal membrane may decrease LPS signaling early in an infection, but allow the amniotic epithelium to remain competent to invasive or intracellular bacteria.

Keywords

placenta; toll-like receptor 4; amnion; amniotic epithelium; chorioamnionitis lipopolysaccharide; translocation

© 2006 Elsevier Ltd. All rights reserved.

Address correspondence and request for reprints to: Dr. Kristina Adams Fred Hutchinson Cancer Research Center Human Immunogenetics Program 1100 Fairview Ave. N., D2-100 P.O. Box 19024 Seattle, WA 98109-19024..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Immune responses to intrauterine infections during pregnancy are poorly understood and contribute to the majority of early preterm births.[1] Pro-inflammatory cytokines in fetal blood and amniotic fluid are thought to initiate pathways leading to preterm labor, but which placental tissues or cells responsible for initial pathogen recognition are unknown. Mechanisms of placental immune defense are challenging to study, because preterm labor is a late symptom of infection and human placental tissues are inaccessible until birth. Innate immune responses likely predominate, because placental macrophages and NK cells are abundant, while lymphocytes are rare.[2,3] Toll-like receptors (TLR) are a family of innate immune receptors that act as the principal sensors of bacterial pathogens and are highly expressed in murine and human placenta.[4-6] Placental TLR recognition of intrauterine pathogens in the chorioamniotic membranes may be a critical event in the pathogenesis of infection-associated preterm birth. Amniotic epithelium within the membranes represents the first line of host defense to intraamniotic bacteria and changes in TLR expression may determine its functional ability to recognize pathogens within amniotic fluid.

Toll-like receptor 4 (TLR4) is the main mediator of immune responses to lipopolysaccharide (LPS), a component of the plasma membrane of gram-negative bacteria.[7,8] Downstream signaling leads to NF- κ B activation and cytokine gene expression. TLR4 expression in the placenta has been described mainly in trophoblast with only a single report describing increased chorioamniotic expression with intrauterine infection.[9-11] Changes in TLR4 expression or signaling with gestational age and infection may contribute to susceptibility to preterm labor. If TLR4 in amniotic epithelium is concentrated along the apical cell surface similar to that observed in syncytiotrophoblast and undifferentiated intestinal epithelial cells (IEC), TLR4 may be poised to recognize LPS from pathogens in amniotic fluid.[12,13] Interestingly, changes in TLR4 distribution occur with LPS stimulation of undifferentiated IEC resulting in TLR4 trafficking to the basal membrane, which may be important for LPS signaling or to downregulate a TLR4-mediated cytokine response during an infection.[13] We hypothesized that TLR4 is also localized to the apical surface of amniotic epithelium and with LPS stimulation traffics toward the basal membrane to downregulate the immune response.

Materials and Methods

Collection of placental tissues and diagnosis of chorioamnionitis

A total of 24 archival paraffin-embedded, formalin-fixed samples of chorioamniotic membranes between 8 and 41 weeks gestation were collected in accordance with the University of Washington institutional ethics committee guidelines. Six first trimester placental tissues between 8 and 11 weeks were provided by the University of Washington Birth Defects Research Laboratory following elective termination of pregnancy. Additional tissues were collected at the University of Washington from two women undergoing voluntary termination of pregnancy at 16 weeks, from 7 women after preterm delivery between 25 and 37 weeks gestation, and from 9 women between 38 and 41 weeks gestation after spontaneous delivery or cesarean section. Study enrollment was targeted to include women with possible chorioamnionitis. The majority of placentas were obtained from women, who had labored. Only two placentas were obtained from women undergoing cesarean section in the absence of labor. Patients with multiple gestation and fetal congenital anomalies were excluded. A pathologist (RPK) examined hematoxylin and eosin stained membranes for evidence of chorioamnionitis. Chorioamnionitis was diagnosed by the presence of a neutrophilic infiltrate at the chorion-decidua junction (mild) or amniochorion junction (moderate/severe). Chorioamnionitis was diagnosed in 10 cases (mild, n=5; moderate/severe, n=5).

Immunohistochemistry

Immunohistochemistry was performed as previously described with minor changes.[11] Blocking was performed by sequential application of 5% normal blocking serum, avidin, and biotin (Elite Universal, Vector Laboratories, Burlingame, CA), followed by incubation with 2 µg/ml polyclonal goat anti-human TLR4 antibody (sc-8694, Santa Cruz Biotechnology, Santa Cruz, CA) with demonstrated specificity in placenta (1 h, 20°C or 18 h, 4°C).[11] A streptavidin-conjugated secondary antibody-mediated peroxidase development system (Elite Universal Vectastain ABC kit) was used and antibody complexes visualized using DAB (BioFX Labs, Owing Mills, Maryland, USA). Tissues were counterstained with Mayer's hematoxylin and imaged by light microscopy. Images were imported into Adobe Photoshop Elements (Adobe, San Jose, California, USA) and the background white-balanced. The subcellular location of TLR4 was rated as predominantly apical or basal by two observers (KMA, JL) blinded to the diagnosis of chorioamnionitis. Fisher's exact test was performed to compare differences in the proportion of cases with apical or basal TLR4 distribution with and without chorioamnionitis. A p value < 0.05 was considered significant.

Ex vivo LPS stimulation, immunofluorescence, and confocal microscopy

Placentas were obtained within 5 minutes after cesarean section from three women with a normal term pregnancy that had not labored. Eight mm punch biopsies of amniochorion were obtained 3–4 cm from the junction of the membranes and the placental disc. Biopsies were washed in sterile 1X PBS, and placed under mesh inserts (Ted Pella, Inc.) in a 12-well plate containing sterile PBS with 1% bovine serum albumin (BSA). Within 10 minutes of delivery, tissues were stimulated with LPS (10 µg/ml, *Escherichia coli*, O55:B5; Sigma, St. Louis, MO), fixed in ice-cold 4% paraformaldehyde at several time points (0, 30, 60, 120 min, 240 min), and permeabilized (1% Triton X-100, 30 min). Tissues were then blocked (10% donkey and 10% rabbit serum, 30 min) and incubated with the primary antibody for TLR4, (5 µg/ml, sc-8694, Santa Cruz) for 30 min (20°C), washed (1XPBS, x3), and incubated with a secondary antibody (rabbit anti-goat, 5 µg/ml, Alexa Fluor 488, Invitrogen, Carlsbad, CA) for 30 min. Tissues were mounted using Prolong Gold antifade reagent with 4'-6-Diamidino-2-phenylindole (DAPI) to stain the nuclei (Invitrogen, CA). Each experiment had two controls: 1) omission of the primary antibody, and 2) incubation in PBS with 1% BSA without LPS for 240 minutes.

Confocal microscopy (Zeiss Axiovert LSM 510, Germany) was used to acquire images (1 µm per slice, 512 × 512 pixels) in three different areas of each tissue using a 488 nm laser and LSM510 software. DAPI was detected using a two-photon laser at 780 nm (Chameleon, Coherent, Santa Clara, CA). Tissue reconstructions in 3-D were created with Volocity 3.7 (Improvision, Lexington, MA). Image analysis was performed with ImageJ software (Java program) after setting the intensity threshold to eliminate greater than 99% of background fluorescence in control tissues not incubated with primary antibody. Integrated intensity of TLR4 immunofluorescence was calculated by multiplying mean pixel intensity in each focal plane by the area of pixels falling within threshold parameters. Integrated intensities for each focal plane were averaged using 9 images (three placentas, three different areas imaged in each tissue). TLR4 mean integrated intensity was calculated for each subcellular compartment: apical 0–5 µm, intracytoplasmic 6–15 µm, and basal 16–20 µm as measured from the apical membrane.

Results

TLR4 expression was investigated in amniotic epithelium from 8 to 41 weeks gestation. At 8 weeks (n=2), no TLR4 expression was detected in the amniotic epithelium. However, TLR4

expression in the amniotic epithelium was identified in two of four samples at 9 weeks and in all cases older than 10 weeks (Figure 1A, 1B). Between 9 and 16 weeks, TLR4 expression was present diffusely within the cytoplasm with no apical immunoreactivity. After 25 weeks, TLR4 expression was polarized to the apical membrane with varying degrees of apical and intracytoplasmic punctate immunoreactivity (Figure 1C). In 7 of 10 cases of chorioamnionitis, TLR4 expression predominated along the basal membrane often with punctuate intracytoplasmic staining (Figure 1D, 1E). Basal TLR4 immunostaining was significantly associated with chorioamnionitis ($p=0.01$). Although insufficient samples were available to achieve adequate statistical power, no correlation was suggested between severity of inflammation and basal localization of TLR4.

To determine if the basal TLR4 expression observed in infected membranes could have been secondary to gram-negative bacterial infection and LPS signaling, we stimulated biopsies of normal term chorioamnion with LPS *ex vivo* and imaged TLR4 using a fluorescent antibody and confocal microscopy. At baseline, TLR4 fluorescence was concentrated to the apical surface with a diffuse, grainy appearance. After LPS stimulation, TLR4 fluorescence clustered, first along the apical surface and then within the cytoplasm and basal membrane (Figure 2A). Likewise, the integrated intensity of TLR4 fluorescence increased sequentially at the apical membrane, cytoplasm, and finally along the basal membrane (Figure 2B). The fold change in TLR4 integrated intensity (with respect to 0 minutes of LPS stimulation) was calculated for each subcellular compartment and time point and averaged across three experiments: apical membrane (1.0 at 60 minutes, 2.0 at 120 minutes, and 2.1 at 240 minutes), intracytoplasmic (0.9 at 60 minutes, 1.5 at 120 minutes, and 2.7 at 240 minutes), and basal (0.8 at 60 minutes, 1.4 at 120 minutes, and 3.5 at 240 minutes). The mean fold change in TLR4 integrated intensity in amniotic epithelium not exposed to LPS and incubated for 240 minutes in PBS with 1% BSA was similar to baseline (apical 0.8, intracytoplasmic 0.5, basal 0.7). Thus, after LPS stimulation, TLR4 was expressed sequentially within the apical membrane, cytoplasm, and finally in the basal cellular compartment.

Discussion

TLR4 immunoreactivity in paraffin sections from placentas with and without chorioamnionitis and the *ex vivo* studies of LPS-induced TLR4 trafficking were analyzed by different methods, which provided complementary results. Light microscopic analysis of immunoperoxidase stained sections, while not as sensitive or quantitative as immunofluorescence and confocal imaging, clearly resolved a significant apical-to-basal shift in the distribution of TLR4 in association with chorioamnionitis. The static images conveyed from analyses of paraffin sections led to a hypothesis about LPS-induced changes in subcellular receptor distribution that we were able to address with prospective studies of fresh placental tissue and sensitive quantitative immunofluorescence/confocal microscopy analysis.

Our findings provide a dynamic view of TLR4 receptor expression in the amniotic epithelium with advancing gestation and intraamniotic infection. The subcellular distribution of TLR4 immunostaining changes with advancing gestation; TLR4 was not expressed consistently until after 10 weeks gestation and concentrated along the apical membrane at 25 weeks gestation, where it remained until term in non-inflamed placentas. The majority of membranes studied using immunohistochemistry were obtained following labor and vaginal delivery. Although apical localization of TLR4 may represent its normal *in utero* distribution, we cannot exclude the possibility that strong apical expression of TLR4 resulted from LPS stimulation due to vaginal flora during labor, delivery, and post-delivery prior to fixation. However, TLR4 expression in membranes from women with cesarean

delivery in the absence of labor was also associated with apical TLR4 expression, albeit to a lesser degree and with more punctate intracytoplasmic immunoreactivity (Figure 2A; 0 minutes).

Chorioamnionitis was associated with a shift in the intracellular distribution of TLR4 to the basal membrane, which could be modeled *ex vivo* by LPS stimulation. LPS stimulation of chorioamnion *ex vivo* resulted in sequential increases in TLR4 fluorescence along the apical membrane, cytoplasm, and finally along the basal membrane. After 240 minutes of LPS stimulation, there was increased TLR4 expression in all subcellular compartments consistent with TLR4 biosynthesis; the greatest increase in TLR4 expression occurred in the basal compartment (Figure 2B). Membranes with chorioamnionitis studied using immunohistochemistry were likely exposed to LPS for a longer period of time and TLR4 was often expressed exclusively along the basal membrane in these cases (Figure 1D, 1E). Collectively, the findings suggest that LPS induces TLR4 biosynthesis and trafficking within the amniotic epithelium resulting in TLR4 translocation to the basal subcellular compartment. Confocal microscopy allowed quantitation of LPS-induced changes in subcellular receptor distribution in a dynamic *ex vivo* system with greater sensitivity than possible in the initial experiments using immunohistochemistry.

Many features of amniotic epithelial TLR4 expression parallel findings in other epithelia. Concentration of TLR4 to the apical surface has been reported in a human colon cancer cell line (T84), murine colon crypts, inflamed colonic epithelia, rat kidney tubules, and gastric epithelium.[13-16] In the amnion, TLR4 does not localize to the apical membrane until the second trimester. This developmental change is similar to that observed in IEC, in that cellular differentiation is associated with a shift from cytoplasmic to apical TLR4 expression.[13] Polarity in TLR4 expression allows the cell to monitor the apical (luminal) environment for pathogens. Lack of TLR4 expression along apical membranes in early gestation may prevent bacterial signaling at a time when intrauterine bacteria from the lower genital tract are more likely to be present.[17] The shift from cytoplasmic to surface TLR4 expression may coincide with initial production of a protein regulating TLR4 surface expression (Protein Associated with TLR4).[18] The similarity between our findings of amniotic epithelial TLR4 trafficking and those observed in differentiated IEC is also striking.[13,19] In the T84 human IEC line, TLR4 redistribution from apical to basal compartments occurred specifically after LPS stimulation.

The importance of TLR4 redistribution in amniotic or intestinal epithelium is unknown. Trafficking of TLR4 to the basal membrane may reflect a protective mechanism to downregulate immune responses to bacteria. Repeated exposure to LPS leads to a diminishing inflammatory response, which has been correlated with a decrease in surface TLR4 in murine peritoneal macrophages.[20,21] However, intracytoplasmic TLR4 may remain functional as demonstrated in a murine small intestinal epithelial cell line, where colocalization of TLR4 in the Golgi apparatus with internalized LPS was required for LPS-stimulated signaling.[22] Interestingly, toll-like receptor 5 is also activated following bacterial invasion of IEC (T84 cell line), despite the fact that it is expressed only on the basal and lateral cell membranes.[23] In any case, polarized expression of TLR4 in amniotic epithelium and changes in TLR4 distribution with LPS stimulation suggests that this receptor may function in the placental innate immune defense to intraamniotic pathogens.

Acknowledgments

We sincerely thank Kara Kendall for technical assistance and Dr. Julio Vazquez Lopez, David McDonald, and Adrian Quintanilla (Fred Hutchinson Cancer Research Center Scientific Imaging staff) for assistance with confocal microscopy and image analysis. We also thank Jan Hamanishi for assistance in making the figures and the University of Washington Birth Defects Research Laboratory for providing first trimester placental specimens.

This work was supported by National Institutes of Health grants HD01264, AI01759

References

- [1]. Goldenberg RL, Hauth JC, Andrews WW. Intrauterine infection and preterm delivery. *N Engl J Med*. 2000; 342:1500–1507. [PubMed: 10816189]
- [2]. Bulmer JN, Sunderland CA. Immunohistological characterization of lymphoid cell populations in the early human placental bed. *Immunology*. 1984; 52:349–357. [PubMed: 6376338]
- [3]. Bulmer JN, Johnson PM. Macrophage populations in the human placenta and amniochorion. *Clin Exp Immunol*. 1984; 57:393–403. [PubMed: 6380834]
- [4]. Beutler B. Innate immune responses to microbial poisons: discovery and function of the Toll-like receptors. *Annu Rev Pharmacol Toxicol*. 2003; 43:609–628. [PubMed: 12540749]
- [5]. Medzhitov R, Preston-Hurlburt P, Janeway CA Jr. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature*. 1997; 388:394–397. [PubMed: 9237759]
- [6]. Abrahams VM, Mor G. Toll-like receptors and their role in the trophoblast. *Placenta*. 2005; 26:540–547. [PubMed: 15993703]
- [7]. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, Layton B, Beutler B. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science*. 1998; 282:2085–2088. [PubMed: 9851930]
- [8]. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the *Lps* gene product. *J Immunol*. 1999; 162:3749–3752. [PubMed: 10201887]
- [9]. Kumazaki K, Nakayama M, Yanagihara I, Suehara N, Wada Y. Immunohistochemical distribution of Toll-like receptor 4 in term and preterm human placentas from normal and complicated pregnancy including chorioamnionitis. *Hum Pathol*. 2004; 35:47–54. [PubMed: 14745724]
- [10]. Kim YM, Romero R, Chaiworapongsa T, Kim GJ, Kim MR, Kuivaniemi H, Tromp G, Espinoza J, Bujold E, Abrahams VM, Mor G. Toll-like receptor-2 and -4 in the chorioamniotic membranes in spontaneous labor at term and in preterm parturition that are associated with chorioamnionitis. *Am J Obstet Gynecol*. 2004; 191:1346–1355. [PubMed: 15507964]
- [11]. Holmlund U, Cebers G, Dahlfors AR, Sandstedt B, Bremme K, Ekstrom ES, Scheynius A. Expression and regulation of the pattern recognition receptors Toll-like receptor-2 and Toll-like receptor-4 in the human placenta. *Immunology*. 2002; 107:145–151. [PubMed: 12225373]
- [12]. Beijar EC, Mallard C, Powell TL. Expression and subcellular localization of TLR-4 in term and first trimester human placenta. *Placenta*. 2006; 27:322–326. [PubMed: 16338476]
- [13]. Cario E, Brown D, McKee M, Lynch-Devaney K, Gerken G, Podolsky DK. Commensal-associated molecular patterns induce selective toll-like receptor-trafficking from apical membrane to cytoplasmic compartments in polarized intestinal epithelium. *Am J Pathol*. 2002; 160:165–173. [PubMed: 11786410]
- [14]. Ortega-Cava CF, Ishihara S, Rumi MA, Kawashima K, Ishimura N, Kazumori H, Udagawa J, Kadowaki Y, Kinoshita Y. Strategic compartmentalization of Toll-like receptor 4 in the mouse gut. *J Immunol*. 2003; 170:3977–3985. [PubMed: 12682225]
- [15]. El-Achkar TM, Huang X, Plotkin Z, Sandoval RM, Rhodes GJ, Dagher PC. Sepsis induces changes in the expression and distribution of Toll-like receptor 4 in the rat kidney. *Am J Physiol Renal Physiol*. 2006; 290:F1034–1043. [PubMed: 16332927]
- [16]. Schmausser B, Andrusis M, Endrich S, Lee SK, Josenhans C, Muller-Hermelink HK, Eck M. Expression and subcellular distribution of toll-like receptors TLR4, TLR5 and TLR9 on the gastric epithelium in *Helicobacter pylori* infection. *Clin Exp Immunol*. 2004; 136:521–526. [PubMed: 15147355]
- [17]. McDonagh S, Maidji E, Ma W, Chang HT, Fisher S, Pereira L. Viral and bacterial pathogens at the maternal-fetal interface. *J Infect Dis*. 2004; 190:826–834. [PubMed: 15272412]
- [18]. Konno K, Wakabayashi Y, Akashi-Takamura S, Ishii T, Kobayashi M, Takahashi K, Kusumoto Y, Saitoh S, Yoshizawa Y, Miyake K. A molecule that is associated with Toll-like receptor 4 and

- regulates its cell surface expression. *Biochem Biophys Res Commun.* 2006; 339:1076–1082. [PubMed: 16338228]
- [19]. Backhed F, Hornef M. Toll-like receptor 4-mediated signaling by epithelial surfaces: necessity or threat? *Microbes Infect.* 2003; 5:951–959. [PubMed: 12941387]
- [20]. Cross AS. Endotoxin tolerance-current concepts in historical perspective. *J Endotoxin Res.* 2002; 8:83–98. [PubMed: 12028748]
- [21]. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J Immunol.* 2000; 164:3476–3479. [PubMed: 10725699]
- [22]. Hornef MW, Frisan T, Vandewalle A, Normark S, Richter-Dahlfors A. Toll-like receptor 4 resides in the Golgi apparatus and colocalizes with internalized lipopolysaccharide in intestinal epithelial cells. *J Exp Med.* 2002; 195:559–570. [PubMed: 11877479]
- [23]. Gewirtz AT, Navas TA, Lyons S, Godowski PJ, Madara JL. Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression. *J Immunol.* 2001; 167:1882–1885. [PubMed: 11489966]

Figure 1.

Immunohistochemistry and light microscopy was used to detect amniotic epithelial TLR4 expression in early and late placentas (**A-E**). Tissues were developed using DAB (brown) and counterstained with hematoxylin (blue). In the first trimester, TLR4 staining of the amniotic epithelium was only observed consistently after 10 weeks (**A**: absent TLR4 at 9 weeks, **B**: TLR4 expression at 10 weeks). In second and third trimester placentas, patterns of TLR4 expression included variable degrees of polarization to the apical surface (**C**: 38 weeks) and predominant basal membrane staining (**D**: 40 weeks, mild chorioamnionitis; **E**: 41 weeks, moderate chorioamnionitis). Negative controls revealed absent TLR4 staining with omission of the primary antibody (**F**: 41 weeks). In panel A, arrowheads indicate nuclei of amniotic epithelium and the measurement bar represents 20 μm . All photomicrographs were taken at 40 $\times$ magnification.

Figure 2.

LPS stimulation of amniotic epithelium *ex vivo* was performed in a time course (0-240 minutes) and confocal microscopy used to image TLR4 expression by immunofluorescence (**A**). 3-D projections of the tissue were created and rotated on the x-axis to project the 0, 60, 120, and 240 minute time points in cross-section for comparison. The measurement bar represents 20 μm and imaging was done at 40× magnification.. Image analysis of the integrated intensity of TLR4 immunofluorescence was performed at each time point and compared to baseline (**B**). The y-axis is the fold change in mean TLR4 integrated intensity in each 1 micrometer focal plane with respect to 0 minutes. The x-axis represents depth (micrometers) into the amniotic epithelium. Data is representative of three independent experiments.