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Chlamydia trachomatis antigen induces TLR4-TAB1-mediated inflammation, but not cell death, in maternal decidua cells

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Abstract

Background: During gestation, the decidua is an essential layer of the maternal-fetal interface, providing immune support and maintaining inflammatory homeostasis. Although *Chlamydia (C.) trachomatis* is associated with adverse pregnancy outcomes the pathogenic effects on maternal decidua contributing to adverse events are not understood. This study examined how *C. trachomatis* antigen affects cell signaling, cell death, and inflammation in the decidua.

Methods: Primary decidua cells (pDECs) from term, not-in-labor, fetal membrane-decidua were cultured using the following conditions: 1) control - standard cell culture conditions, 2) 100ng/mL or 3) 200ng/mL of *C. trachomatis* antigen to model decidual cell infection *in vitro.* Differential expression of Toll-like receptor (TLR) 4 (receptor for *C. trachomatis* antigen), signaling pathway markers phosphorylated TGF-Beta Activated Kinase 1 (PTAB1), TAB1, phosphorylated p38 mitogen-activated protein kinases (Pp38 MAPK), and p38 MAPK (western blot), decidual cell apoptosis and necrosis (flow cytometry), and inflammation (ELISA for cytokines) were determined in cells exposed to *C. trachomatis* antigen. T-test was used to assess statistical significance ($p<0.05$).

Results: *C. trachomatis* antigen significantly induced expression of TLR4 ($p=0.03$) and activation of TAB1 ($p=0.02$) compared to controls. However, it did not induce p38 MAPK activation. In addition, pDECs maintained their stromal cell morphology when exposed to *C. trachomatis* antigen showing no signs of apoptosis and/or necrosis but did induce pro-inflammatory cytokine interleukin (IL)-6 (100ng/mL: $p=0.02$ and 200 ng/mL: $p= 0.03$), in pDECs compared to controls.

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Conclusion: Prenatal *C. trachomatis* infection can produce antigens that induce TLR4-TAB1 signaling and IL-6 inflammation independent of Pp38 MAPK and apoptosis and necrosis. This suggests that *C. trachomatis* can imbalance decidual inflammatory homeostasis, potentially contributing to adverse events during pregnancy.

Introduction

Chlamydia (C.) trachomatis is the most common sexually transmitted bacterial infection (STI) in the U.S.[1,2] and is asymptomatic in 60-80% of cases[3]. *C. trachomatis* is an established cause of pelvic inflammatory disease, which can result in subsequent infertility and ectopic pregnancy. In addition, prenatal Chlamydia has been associated with several adverse outcomes, including preterm birth, pregnancy loss[4], and preeclampsia[5]. Despite the prevalence of *C. trachomatis* and potential adverse effects on pregnancy, there continues to be suboptimal Chlamydia screening (<60%)[6-8], delays in treatment (55%), as well as reinfection or persistent infection (9-14%) during pregnancy.[9,10] In addition, it has been shown in non-pregnant individuals that screening and antibiotic treatment does not always prevent long-term sequelae. Thus, it is imperative that we understand the pathogenesis of *C. trachomatis* during pregnancy as this may lead to the development of novel therapeutic approaches. Unfortunately, many questions remain as to the mechanistic effects *C. trachomatis* might have on pregnancy.

Although understudied in pregnancy, *C. trachomatis* may induce excessive inflammation at the maternal-fetal interfaces, such as the decidua-placenta and decidua-fetal membrane junctions, through activation of Toll-like receptors (TLRs). This could result in partial activation of the systemic cytokine networks responsible for the induction of labor, thus triggering preterm birth [11]. Other pathways may also be affected, influencing the risk of other adverse hypertensive pregnancy outcomes. It has been demonstrated that *C. trachomatis* induces chemokine production in both endometrial stromal cells[12] and placenta trophoblasts[13], thus impacting the process of decidualization and altering the maternal-fetal interface. Additionally, *C. trachomatis* infection of placenta trophoblasts has been shown to compromise the biosynthesis of cholesterol, depleting the substrate pool for estrogen and progesterone synthesis and impairing implantation and placentation[14]. Suggesting in humans, *C. trachomatis* is able to induce implantation and placentation dysfunction early in pregnancy. However, *C. trachomatis* murine models do not consistently reproduce this phenotype[15], creating a lack of *in vivo* models to study ascending *C. trachomatis* and other STIs during pregnancy.

Mechanisms by which ascending *C. trachomatis* induces inflammatory changes and upper genital tract tissue damage during pregnancy are yet to be elucidated. In addition, it is unclear if pathogen-released elementary bodies or antigens are responsible for cellular damage. The objective of this study is to investigate the effects of antigen exposure at the maternal decidua during ascending upper reproductive tract *C. trachomatis infection*. To do this, we used an *in vitro* cell culture model, where primary decidual cells were treated with different concentrations of *C. trachomatis* antigen. Changes in cellular morphology, receptor expression, cell signaling, cell death, and inflammation were assessed after 3 or 48 hours. We hypothesized that *C. trachomatis* antigen exposure in primary decidua cells

would induce cellular dysfunction characterized by increased TLR expression, increased stress signaling kinase activation, increased cell death via apoptosis and necrosis, and an increase in pro-inflammatory cytokine production.

Methods

IRB approval

This study protocol is approved by the Institutional Review Board at The University of Texas Medical Branch (UTMB) at Galveston, TX, as an exempt protocol to use of discarded placenta after normal term cesarean deliveries (UTMB 11–251). No subject recruitment or consent was required for this study.

Clinical samples

Discarded placentas from non-laboring caesarian deliveries were collected. Fetal membranes were dissected from the placenta, washed three times in normal saline, and cleansed of blood clots using cotton gauze. We then proceeded with the decidua cell isolation described below. *Inclusion Criteria:* Normal term (39-40 weeks) birth were women with and no pregnancy-related complications. *Exclusion Criteria:* Term labor vaginal deliveries (> 390/7 weeks) were excluded. Subjects with multiple gestations, placenta previa, fetal anomalies, and/or medical interventions or surgeries (intervention for clinical conditions that are not linked to pregnancy) during pregnancy were excluded. Severe cases of preeclampsia or persistent symptoms (headache, vision changes, right upper quadrant pain) or abnormal laboratory findings (thrombocytopenia, repeated abnormal liver function tests, creatinine doubling or > 1.2, or HELLP syndrome) or clinical findings (pulmonary edema or eclampsia) were excluded. Subjects who had any surgical procedures during pregnancy or who were treated for hypertension (pregnancy induced or chronic), preterm labor or for suspected clinical chorioamnionitis (reports on foul-smelling vaginal discharge, high levels of C-reactive protein (CRP), fetal tachycardia), positive group B streptococcus (GBS) screening or diagnosis of bacterial vaginosis, behavioral issues (substance use). If any of these conditions were met, but the sample was still delivered at term, they were excluded.

Isolation and culture of human pDECs

Primary decidual cells (pDECs) were isolated as previously described[16]. Briefly, the chorion layer was mechanically removed, gently rinsed in prewarmed saline, and cut into 5 cm x 5 cm squares. The decidual layer was dissected from the chorion using a scalpel. The tissue was then minced into small pieces and digested in digest buffer-I (0.125% trypsin [Gibco, Cat# 15050-065] and 0.02% DNase-I [Sigma, Cat# D4527] in HBSS] for 30 min at 37°C with intermittent vortexing. Then, the pellet was collected after centrifuging at 3000 rpm for 10 min at room temperature (RT) and resuspended in digest buffer II (0.125% trypsin, 0.02% DNase-I, and 0.2% collagenase type IA [Sigma, Cat#C2674] in HBSS) for 60 min at 37°C. The digestion was neutralized by adding an equal volume of complete DMEM media and filtering the cells through four layers of sterile gauze. The collected cells were centrifuged at 2000 rpm for 10 min at RT, and the pellet was resuspended in 4 mL of the same media that is used in preparing gradient dilutions. The OptiPrep™ Discontinuous Gradient (OptiPrep™) was prepared following the manufacturer's instructions. Briefly, using

40% Optiprep, a working solution (WS) was prepared in the serum-free media by mixing two volumes of OptiPrep with one volume of serum-free media. Starting with the 40% solution, 4 mL of each solution were layered into a 50 mL conical tube using a 5cc syringe fitted with a bore needle (18G) for acquisition, ranging from 4% to 40%. After preparing the gradients, decidual cells were added (1.027-1.038 g/mL, 4-6%) to the top of the gradient, then centrifuged at 3000 rpm for 35 min at RT. The cells were collected, washed with serum-free media (4x volume to collect cells from the column) and centrifuged at 2000 rpm for 10 min at RT. The pellet was resuspended with complete DMEM/F12 and placed in T25 or T75 flasks.

pDEC treatment

Once the cells were 80–90% confluent, they were passed into a variety of culture plates depending on the assay. Cells were cultured under the following conditions for up to 48 hours (minimum of N = 3 each): 1) control - standard cell culture conditions, 2) 100ng/mL of *C. trachomatis* antigen (Native *C. trachomatis* antigen derived from infected McCoy cells, The Native Antigen Company, UK) to model *C. trachomatis* infection *in vitro,* 3) 200ng/mL of *C. trachomatis* antigen within 6 well plates. The concentration of *C. trachomatis* antigen is within the range of endotoxin levels that are seen in the amniotic fluid of women with infection-associated pregnancy compliacation[17,18]. After 3 or 48 hours of treatment, the cells and media were saved for further analysis of protein signaling, cell death, and inflammatory status.

Microscopy

Brightfield microscopy images were captured using a Nikon Eclipse TS100 microscope (10x and 20x) (Nikon, Melville, NY, USA). Three regions of interest per condition were used to determine overall cell morphology.

Western blot analysis

Cells were lysed with RIPA lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, and 1.0 mM EDTA pH 8.0, 0.1% SDS) and supplemented with protease, phosphatase inhibitor cocktail, and phenylmethylsulfonyl fluoride. After centrifugation at 10,000 RPM for 20 min, the supernatant was collected, and protein concentrations were determined using BCA (Pierce, Rockford, IL). The protein samples were separated using precast gels and transferred to the membrane using an iBlot1 gel transfer device (Thermo Fisher Scientific, Waltham, MA, USA). Membranes were blocked in 5% blotting grade blocker made with 1x Tris-buffered saline- Tween 20 (TBS-T) buffer for 1 hour. The primary antibodies were added, and the membrane was left to rock overnight at 4°C. The membrane was incubated with a secondary antibody for 1 hour. For membranes that were stripped, a restore Western blot stripping buffer was used; none of the membranes in this study were stripped more than three times. The following non-human antibodies were used: TLR4 (1:300, Novus, NBP2-24821), PTAB (1:600, Millipore, 06-1334, THR431), TAB (1:1000, R&D Systems, AF3578), Pp38 MAPK (1:300, Cell Signaling, 9211S, T180/Y182), p38 MAPK (1:1000, Cell Signaling, 9212S), Actin (Sigma-Aldrich, A5441).

pDEC apoptosis/necrosis by flow cytometry

To determine the population of cells undergoing apoptosis and/or necrosis, cells were stained using the Dead Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & PI (Life Technologies, Carlsbad, CA) as previously described[19] (Martin et al., 2019). Briefly, cells were harvested by trypsinization and centrifuged for 5 min at 2000 g. Cell pellets were washed with cold 250 μL x PBS and centrifuged at 2000g for 5 min. Pellets were resuspended in 100 μL 1x annexin binding buffer supplemented with 5 μL Alexa Fluor 488 Annexin V and 1 μL 100 μg/mL PI. After a 15-min incubation, 400 μL annexin binding buffer was added, and samples were run immediately on the CytoFlex flow cytometer (Beckman Coulter). Unstained control pDECs were used as negative controls for gating. Data were analyzed using Cytexpert software (Beckman Coulter).

ELISA assays for inflammatory cytokine production

To analyze changes in inflammatory mediators' interleukin (IL)-6 and IL-8 were analyzed from the cell supernatants after treatments (N=4). Supernatants were manually collected from 6 well plates after 48 hours of exposure to one of the three conditions. Standard curves were developed with duplicate samples of known quantities of recombinant proteins that were provided by the manufacturer (BD OptEIA Set Human IL-6 [555220] and BD OptEIA Set Human IL-8 [555244]). Sample concentrations were determined by relating the colorimetric values that were obtained to the standard curve by linear regression analysis.

Statistical analyses

All data were analyzed using Prism 9 software (GraphPad Software, La Jolla, CA, USA). The Shapiro-Wilk test for normality was conducted to check for the normality of the data. A one-tailed student's T-test was used to determine the statistical significance between control vs. treatment groups (i.e., control vs. 100ng/ml or control vs. 200ng/ml antigen). Graphs are shown as Mean ± SEM. A p-value of <0.05 was used to determine significance.

Results

C. trachomatis antigen induces TLR4 expression and TAB1 activation in pDECs

During pregnancy, the decidua surrounds the placenta and fetal membranes forming the maternal-feto interface and providing critical immune support and maintains inflammatory homeostasis. To test if *C. trachomatis* during gestation can induce decidua dysfunction, pDEC were stimulated with *C. trachomatis* antigen (100 ng/mL and 200 ng/mL) for 3 hours and stress signaling pathways were analyzed. Western blot analysis of TLR4, a receptor that can regulate inflammatory events in a cell[20] and its downstream stress-induced cell signaling kinases (i.e., TGF-Beta Activated Kinase 1 [TAB1] and p38 mitogen-activated protein kinases [p38 MAPK]) were examined to assess activated phosphorylation (P) status (Figure 1A). *C. trachomatis* antigen-induced significant upregulation of TLR4 in pDECs within 3 hours compared to the control cells (p=0.03) (Figure 1B). Downstream analysis showed *C. trachomatis* antigen treatment did not induce the activation of Pp38 MAPK; however, it did significantly induce the activation of PTAB1 (p=0.02) compared to controls (Figure 1B). These data suggest that *C. trachomatis* antigen can induce TLR4-TAB1 signaling in the

decidua; however, it is unknown how this stress-induced signaling can affect cell death and inflammation.

C. trachomatis antigen did not induce cell death in pDECs

Although Chlamydia infection is associated with reproductive tract cell death and inflammation[21], the role of *C. trachomatis* in pDECs is not fully understood. To analyze two common cell death pathways, apoptosis and necrosis, pDECs were cultured for 48 hours with or without *C. trachomatis* antigen and flow cytometry for Annexin-V and propidium iodide was conducted. Brightfield microscopy showed pDECs maintained their elongated morphology and did not punctate after *C. trachomatis* antigen treatment (Figure 2A). *C. trachomatis* antigen also did not significantly induce apoptosis (Annexin-V^+), late apoptosis (Annexin-V^+ & propidium $iodide^+$), or necrosis (propidium $iodide^+$) in pDEC cells (Figure 2B). These results suggest that infection during pregnancy does not induce apoptosis or necrosis in pDECs, though whether this infection modulates the *in-utero* environment through other secretory pathways is unknown.

C. trachomatis antigen induces inflammation in pDECs

Maintenance of inflammatory homeostasis is a critical function of the maternal decidua during pregnancy and disruption of the maternal-feto interface environment can contribute to the onset of adverse pregnancy outcomes. To determine if *C. trachomatis* can induce decidua inflammatory dysfunction, pDECs were treated with *C. trachomatis* antigen for 48 hours, and supernatants were analyzed for pro-inflammatory cytokines IL-6 and IL-8. ELISA analysis showed *C. trachomatis* antigen treatment significantly induced pro-inflammatory cytokines IL-6 (100ng/mL: $p=0.02$ and 200 ng/mL: $p= 0.03$), but not IL-8 (100ng/mL: $p=0.37$ and 200 ng/mL: $p= 0.64$), in pDECs after 48 hours compared to controls (Figure 3). This suggests that chlamydia infection can induce pro-inflammatory cytokine production in decidua cells which can disrupt immune homeostasis.

Discussion

C. trachomatis is a prevalent STI that often goes undetected and has been associated with poor pregnancy outcomes such as preterm birth, pregnancy loss, and preeclampsia. While studied more frequently in non-pregnant individuals, the factors that drive upper genital tract ascension and pathology are not well understood. Studies to understand mechanisms during pregnancy are limited to a handful of investigations. This study was performed to further elucidate the mechanisms by which prenatal *C. trachomatis* can induce cell signaling, cell death, and inflammation in decidua cells, thereby contributing to adverse pregnancy outcomes. *C. trachomatis* antigen-exposed decidua cells induced cell signaling through TLR4-PTAB1 upstream and downstream stress signaling pathways but not through p38 MAPK activation. Despite the changes in stress-induced signaling, the *C. trachomatis* antigen did not significantly induce cell death via apoptosis or necrosis in the maternal decidua. However, it did selectively induce the production of pro-inflammatory cytokine IL-6, but not IL-8, in decidua cells after 48 hours. The results of this study suggest that *C. trachomatis antigen* can affect cell signaling and inflammatory homeostasis within the decidua, leading to inflammatory shifts at the feto-maternal interface during pregnancy.

During placental development, localized TGFβ production by resident immune cells and endometrial stromal cell regulate decidua function[22,23] by promoting homeostasis through cell proliferation[24], decidualization[25,26], and inhibiting matrix metalloproteinases activation[27]. Canonical TGFβ signaling occurs through multiple classes of receptors (i.e., TGFβ family proteins to type 1 and type 2 [*Tgfbr*], bone morphogenetic protein, and activin) that leads to downstream canonical (i.e., SMAD2/3 or SMAD1/5/9/4) or non-canonical signaling pathways (i.e., ERK, JNK, p38 MAPK, TAK-TAB1)[28-30]. Physiologically these pathways regulate fundamental cellular and biological functions[31]. However, pathologically, these same pathways can induce cell cycle arrest, cell death, epithelial-to-mesenchymal transitions (EMT), collagen remodeling, and inflammation[32-34], suggesting tight regulation of TGFβ signaling in the decidua to maintain homeostasis. Here we document a non-canonical TGFβ pathway focusing on PTAB1, that could bypass other TGFβ kinases (i.e., SMADs, ERK, JNK, and p38 MAPK) and induce localized inflammation.

TGFβ-TAK1-TAB1 induced p38 MAPK autophosphorylation was recently documented in fetal membrane cells to play an important role in the development of EMT, cellular senescence, and inflammation[35,36]. It has also been shown with other stimulants (i.e., Lipopolysaceride and oxidative stress inducers) that the maternal decidua is resistant to p38MAPK activation leading to cellular senescence compared to fetal membrane cells in culture[37,38]. As p38 MAPK was not activated in this model, we hypothesize that maternal decidua cells respond differently to infectious stress than fetal-derived cells, suggesting TLR4-TAB1 could be an alternate mechanism to induce inflammation. TLR4 signaling has been shown to enhance TGFβ pathways to promote fibrosis and inflammation[39,40], specifically through the TLR3/4-PTAB1-NFkB pathway[41,42] leading to pro-inflammatory cytokine production.

However, it remains unknown if IL-6 production in decidua cells is truly linked to dysfunction during early pregnancy. IL-6 is produced in the reproductive tract and other *in utero* tissues and exerts regulatory function on implantation, placentation, and immune tolerance[43]. Particularly, there is evidence indicating changes in systemic IL-6 production and signaling in women with recurrent pregnancy loss[43]. However, it is widely documented that IL-6 is abundant in the decidua, placenta, fetal membranes, and amniotic fluid during the first trimester[44-46] as these sites are major regulators of IL-6 synthesis. To accurately determine the effect of *C. trachomatis*-induced inflammation in decidua cells, more advanced *in vitro* techniques, such as trans wells or organ-on-chips, or physiologically relevant animal models, are needed.

Interestingly, an activated TAB1 pathway was recently identified among women with *C. trachomatis*-induced infertility using whole-exome sequencing and path analyses[47]. TLR4 is also indicated in upper genital tract ascension of *C. trachomatis* in women with suspected pelvic inflammatory disease[48]. There is a strong scientific premise to suggest that identifying peri-conception contributors to defective decidualization or decidua dysfunction may indicate women who are at risk for various reproductive failures. This insight will provide a wealth of knowledge to understand the effects of *C. trachomatis* on early pregnancy and to derive inferences on pathogenesis through the identification of novel biomarkers and targets for preventative therapeutics that can mitigate tissue damage.

Additionally, advances in understanding pathogenesis will bring us one step closer to vaccine development. Since natural exposure to *C. trachomatis* has been demonstrated to generate partial immunity, it is possible that chlamydia vaccine antigens could be developed[49].

This study is one of the first to investigate the impact of *C. trachomatis* antigen on primary decidua cells. Additionally, this is the first study to document a pathway (i.e., TLR4-PTAB1 signaling leading to IL-6 production) of *C. trachomatis* antigen-induced dysfunction in the decidua. Mechanistic studies should be conducted to confirm this pathway and the presence of other TLR or TGFβ signaling. This model system opens the door for further investigation of how cell stress-induced signaling and cytokine production can alter the maternal decidua after it has transitioned from the endometrium. McCoy cell-derived *C. trachomatis* antigens were used as a proxy for Chlamydia infection in this study; therefore, these findings could be in part induced by components of McCoy cell lysates or inflammatory factors found in the purified antigen. A major limitation of this study is that we did not test the impact of live *C. trachomatis* or elementary body exposure on decidua cells, which could induce a different response than *C. trachomatis* antigen exposure and play a more pivotal role in the adverse effects of Chlamydia infection during pregnancy. An additional limitation of this study was the lack of 30% immune cells within the decidua culture[50-52]. During *C. trachomatis* infection, resident immune cells (i.e., T cells, neutrophils, and macrophages) produce INF-γ and TNFa, which in turn activates cell-autonomous immunity. These cell-cell interactions were not replicated in this study though it is known immune cells play an essential role in the clearance of infection and maintaining feto-maternal interface homeostasis.

C. trachomatis screening is suboptimal in pregnancy, coupled with treatment delays and risk of recurrent/persistent infection; there is a need to improve understanding of chlamydia-induced decidual damage for the development of novel therapies that can be coupled with antibiotic treatment to reduce the burden of disease. We demonstrated that live *C. trachomatis* is not needed to induce dysfunction in the maternal decidua and that *C. trachomatis* antigen exposure is enough to increase cell stress-signaling through TLR4-PTAB1 in the decidua leading to increased inflammation. The ability of this response to affect neighboring cells and developmental processes needs further investigation. Advanced *in vitro* devices such as organ-on-chips or microphysiological systems could address some of these challenges and create novel platforms for studying the effect of ascending STI at the maternal-feto interface during pregnancy.

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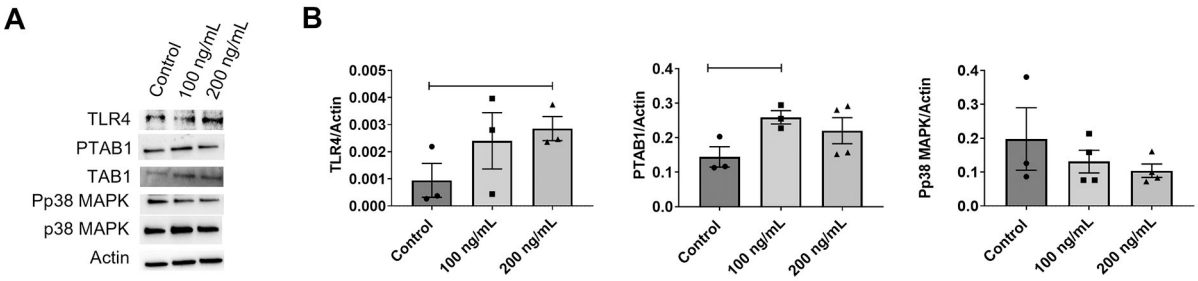

Figure 1: *C. trachomatis* antigen induces TLR4 expression and TAB1 activation in pDECs

A) Western blot analysis was conducted to detect activation of stress-induced TLR4, PTAB1, TAB1, Pp38 MAPK, p28 MAPK, and actin after 3 hours of *C. trachomatis* antigen treatment (100ng/mL and 200 ng/mL) in pDECs.

B) Densitometry of western blot data showed *C. trachomatis* antigen induced a significant increase in TLR4 expression (p=0.03) and significantly activated PTAB1 (p=0.02); However, it did not affect p38 MAPK expression or activation.

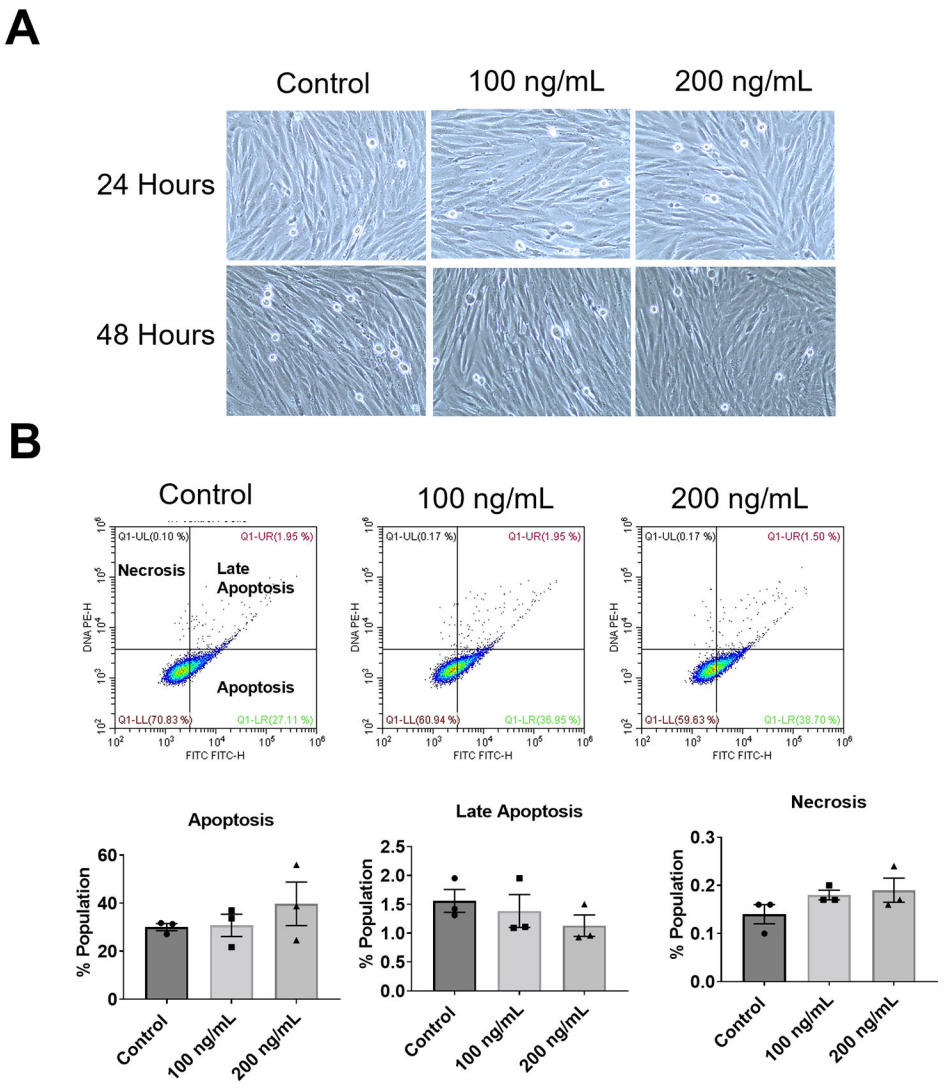

Figure 2: *C. trachomatis* antigen did not induce cell death in pDECs

A) Bright field microscopy images of pDECs maintaining their elongated morphology after 24 hours and 48 hours of *C. trachomatis* antigen treatment (100ng/mL and 200 ng/mL).

B) Flow cytometry did not show a *C. trachomatis* antigen (100ng/mL and 200 ng/mL) induced increase in cellular apoptosis (Annexin-V^+), late apoptosis (Annexin-V^+ & propidium $iodine^+$), or necrosis (propidium $iodine^+$) in pDEC cells after 48 hours.

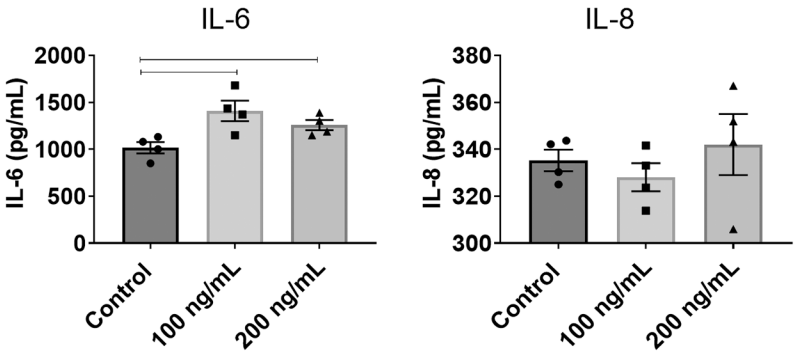

Figure 3: *C. trachomatis* antigen induces inflammation in pDECs

ELISA assay measured media concentrations of pro-inflammatory cytokines IL-6 and IL-8 after 48 hours of treatment. *C. trachomatis* antigen-induced the production of significant levels of IL-6 in 100ng/mL (p=0.02) and 200ng/mL (p=0.03) pDEC cells, but not IL-8.