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Mechanisms of Interleukin-1Ã¢ Production in Chlamydia trachomatis Infected Placental Trophoblasts

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Mechanisms of Interleukin-1β Production in *Chlamydia trachomatis* infected placental trophoblasts

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Abstract

In a normal pregnancy the immune system plays the critical task of protecting the fetus from the constant threat posed by pathogens while limiting the effects of the mother's immune response on the fetus itself. Viral and bacterial infections can cause preterm labor and intrauterine growth restriction, and the placental trophoblasts express pattern recognition receptors (PRRs) to help detect invading pathogens and initiate an effective immune response. *Chlamydia trachomatis (Ct)* is the most common sexually transmitted bacterial infection in the United States and can infect the decidua and placenta. *Chlamydia* infection of first trimester placental trophoblasts induces a change in the cytokines and chemokines present at the maternal-fetal interface and results in production of the pro-inflammatory cytokine interlukin-1β (IL-1β). We hypothesize that the IL-1β response is mediated through toll-like receptor 2 (TLR-2) for transcription, and inflammasome forming Nod-like receptors (NLRP-3 or IPAF) for its processing and secretion. Our work in the trophoblast cell lines H8 and Sw.71 demonstrated that *Ct* induces the transcription of IL-1β mRNA and translation of intracellular pro-IL-1β as early as 12h, and the secretion of active-IL-1β starting at 24h post infection. The induction of IL-1β is independent of MyD88, as there was no change in IL-1β production when we blocked MyD88 by using dominant-negative (DN) MyD88 cells. The production of IL-8 and IL-6 by trophoblasts after *Ct* infection is mediated through MyD88 and TLRs as described in other cell types, which confirms that our DN-MyD88 system is functional. Further we demonstrated that preventing NALP-3 inflammasome activation by blocking

potassium (K^+) efflux from cells partially inhibited IL-1β secretion in both H8 and Sw.71 cells. This suggests a role for NLRP-3 inflammasome in mediating the processing and secretion of IL-1β. In summary we have shown that *Chlamydia* is able to act through different pathways to affect its host. It can induce IL-1β transcription through PRRs in a MyD88 independent manner, it can potentially induce the processing of pro-IL-1β through the NALP-3 inflammasome, and it can stimulate the secretion of active-IL-1β from the cells. The presence of *Chlamydia trachomatis* in the placenta, and the many mechanisms it uses to modulate the immune system, like IL-1β secretion, can result in negative pregnancy outcomes, miscarriages and preterm labor.

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Introduction

Chlamydia trachomatis (Ct) is a gram negative obligate intracellular bacterium that affects an estimated 90 million people per year worldwide. In the United States *Chlamydia* is the most common sexually transmitted bacterial infection and the Centers for Disease Control and Prevention (CDC) estimate up to 3.5 million asymptomatic infections per year. Despite the fact that almost half of these infections are thought to spontaneously clear, they still have a large public health impact [1]. The best estimate of *Chlamydia* prevalence is 7.4%, with the highest rates among young sexually active women between the ages of 15 and 24. The majority of women that have *Chlamydia* infections, up to 60-70%, are asymptomatic and thus do not seek treatment [1]. Both symptomatic and asymptomatic disease however, can have a negative impact on a woman's reproductive potential, with up to 40% resulting in pelvic inflammatory disease (PID), which can lead to infertility and ectopic pregnancies [2]. Intrauterine bacterial or viral infections have also been associated with pregnancy complications such as preterm labor, intrauterine growth restriction (IUGR) and pre-eclampsia [3]. Moreover there is evidence indicating that *Chlamydia* infection can be associated with pregnancy complications like still birth, spontaneous abortion and prematurity [4].

Chlamydia trachomatis exists as multiple serologically distinct serovars [5]. The ocular serovars A–C cause ocular trachoma which is the most common cause of preventable blindness wordwide; serovars D–K infect the urogenital tract; and serovars L1–L3 cause Lymphogranuloma Venerium [6]. *Ct* primarily

infects epithelial cells of the eye and genital tract, but it can also invade a wide range of other cell types including monocytes, dendritic cells, fibroblasts and endothelial cells. In addition, some clinical studies have demonstrated that *Ct* is present in the placenta and decidua [5, 7-9]. Chlamydia invades cells as a metabolically inert elementary body (EB) and once inside the cytoplasm starts transforming its endocytic vesicle into an inclusion (a special vesicle containing host lipids and *Ct* proteins). The EBs differentiate into the non-infectious but metabolically active reticuloid bodies (RBs) and start replicating within the inclusion. After replication, the RBs re-differentiate into EBs and get released from the host cell by cell lysis or exocytosis at approximately 2-3 days after infection and proceed to infecting the neighboring cells [10].

Chlamydia trachomatis has many mechanisms to evade or modulate the immune system. The bacterium has antigenically diverse surface proteins, but because it replicates within a membrane bound inclusion it helps it avoid detection. During an infection *Chlamydia* can also modify the host cell and the immune response by secreting virulence factors through a type III secretion system. Some factors prevent the fusion of the inclusion with lysosomes, they block or down-regulate the caspases involved in apoptosis and secrete proteases (CPAF) that degrade transcription factors needed for the up-regulation of MHC class I and II. Moreover, another protease encoded by the CT441 gene cleaves the NFkB p65 and interferes with the NFkB signaling pathway that is downstream of some of the immune receptors involved in pathogen recognition [1]. *Chlamydia trachomatis* infection of human immune cells can lead to the generation of an

inflammatory immune response through the production of cytokines and chemokines like IL-6, IL-8, IL-1 and IL-18 [24, 30-32].

The maternal-fetal interface at the level of the placenta serves as an active barrier against microorganisms and is thought to involve primarily the innate immune system. The majority of the immune cells present in the placenta in the first trimester are NK cells (70%), non-inflammatory macrophages (20-25%) and dendritic cells (~2%), which promote trophoblast invasion and spiral artery remodeling needed for a successful placentation [11]. Placental trophoblasts also have the ability to sense pathogens and have been shown to express pattern recognition receptors (PRRs), like Toll-like receptors (TLRs) and NOD-like receptors (NLRs), which recognize highly conserved molecules on pathogens known as pathogen-associated molecular patterns (PAMPs) [11-12]. In humans 10 TLRs have been identified so far, and they are expressed on mononuclear, endothelial, epithelial and trophoblast cells. TLRs are transmembrane proteins which recognize PAMPs like bacterial lipopolysaccharide (LPS), petidoglycan, flagellin, lipoproteins or viral dsRNA. Upon binding of their ligands, they signal through the adapter protein MyD88 to activate the NFkB or MAPK pathways and thus induce the transcription of genes encoding for cytokines and chemokines [13-14]. Through these receptors, the trophoblasts can initiate a pro-inflammatory innate immune response. This will recruit immune cells at the site of infection, trigger the adaptive immune system and protect the fetus [7,15].

The trophoblast cells are unique in that they constitutively secrete an array of cytokines and chemokines that ensure the proper immune cell balance in the

uterus to maintain a healthy pregnancy. The activation of TLR-4 in trophoblasts by binding of LPS induces the up-regulation of their constitutive secretion of cytokines and chemokines like IL-8, MCP-1, GROα, RANTES. This response is involved in the migration of NK cells, monocytes and PMNs to the placental interface [11-12]. The increased presence of these inflammatory cytokines and immune cells was seen in placentas from preterm deliveries and are thought to have triggered the parturition [16]. *Chlamydia* has been shown to activate TLR-4 and TLR-2 in macrophages and dendritic cells [17]. The *chlamydial* LPS is weakly endotoxic and while it is able to stimulate TLR-4, in animals it does not lead to a change in infection clearing. However, TLR-2 plays a larger role in initiating an inflammatory response [18]. TLR-2 has been shown to recognize *chalmydial* LPS [19] and in genital tract epithelial cells infected with *Chlamydia*, TLR-2 and its adapter protein MyD88 were shown to be recruited to the *chlamydial* inclusion membrane and be required for the production of IL-8, a potent neutrophil attractant [20]. Studies in TLR-2 knockout mice showed greatly reduced pathology after *Chlamydial* infections compared to the wild type animals suggesting a role for TLR-2 in mediating the immune response to this pathogen [17].

The pro-inflammatory immune responses to *Chlamydia* infections in cervical epithelial cells that leads to the production of IL-1β, IL-8, IL-6 and GROα does not always occur until a day after the initial infection when the organisms is already inside the cell [21]. This suggests that intracellular PRRs like the NLRs could also sense the presence of the pathogen and play a role in the

immune response. One family of NLRs is the NOD (nucleotide-binding oligomerization domain) proteins which are a general cytosolic sensor of gram positive and gram negative bacteria. NOD proteins recognize peptides derived from peptidoglycan and activate the NFkB and MAP-kinase pathways which result in an inflammatory response [11]. NOD1 generally detects meso-diaminopimelic acid (meso-DAP), which is present in gram negative bacteria while NOD2 detects muramyl-dipeptide (MDP), a large motif common to both gram positive and gram negative bacteria [22].

First trimester trophoblast cells have been shown to express both NOD1 and NOD2 and after stimulation with MDP, the common NOD-2 ligand, the trophoblasts up-regulate their production of the pro-inflammatory cytokines IL-6, IL-8, MCP-1, GROα and RANTES [12]. A similar response is seen when trophoblasts are stimulated with the NOD1 agonist iC-DAP, which proves that the NOD receptors are functional in these cells [12]. The trophoblasts also up-regulate their expression of NOD1 and NOD2 via the NFkB pathway when TLR-4 is stimulated with bacterial LPS [23]. NOD1 and NOD2 have also been implicated in the recognition of *Chlamydia* [21], and as described above at least two of the TLRs (TLR-2 and TLR-4) are involved in mediating an immune response to *Chlamydia* infections [19-20]. These findings come to suggest that there is a tight connection between the roles of TLRs and NODs in recognizing *Chlamydia trachomatis*, and that they might work together in mounting an effective immune response.

Another class of NLRs potentially involved in detecting invading bacteria is the NLR proteins. The NALPs like the NALP1, NALP3 and NLRC4 (IPAF) have leucine rich repeats (LRR) able to recognize bacterial toxins, pathogen PAMPs and danger signals like free ATP resulting from cellular damage. Upon activation, NALPs assemble into "inflammasomes". The inflammasomes are multi-protein complexes composed of ASC (apoptosis-associated speck-like protein containing a caspase recruitment domain) a sensing NLR protein and pro-caspase-1, which lead to the activation of the inflammatory caspases-1. NALPs oligomerize through their NACHT domains and associate with the adapter protein ASC via the PYD region [22]. ASC in turn contains a CARD domain, which recruits pro-caspase-1 and leads to its dimerization and activation [22]. While the exact signals for the assembly of NALP inflammasomes are still being worked out, it appears that K^+ efflux from the cells and formation of ROS is a common trigger for NALP1 and NALP3 inflammasomes [24]. Unlike the NALP1 and NALP3, the NLRC4 (IPAF) inflammasome is known to sense virulence factors from gram negative bacteria, and can activate caspase-1 even in the absence of ASC [22].

Caspase-1 (also known as interleukin-1 converting enzyme or ICE) is a cysteine protease composed of 2 subunits (10kDa and 20kDa) present in cells as an inactive zymogen. After activation it has been shown to process the cytokines pro-IL-1β, pro-IL-18 and pro-IL-33 into their active forms [25]. Caspase-1 activation occurs in HeLa epithelial cells after *Chlamydia* infection, but it was not until recently that it was shown that this activation was dependent on the NALP3

inflammasome [26]. Caspase-1 activation and IL-1β production in response to *Chlamydia* infection was also shown in macrophages, monocytes and dendritic cells [24].

IL-1β is one of the most important mediators of inflammation in response to infections, and it has also been found to be implicated in autoimmune disorders and even Alzheimer's disease [27]. IL-1β has multiple beneficial roles including mediating host responses to microbial invasion, like fever and transmigration of leukocytes into the tissues, but also promotes tissue damage and tumor invasiveness. In pregnancy IL-1β is associated with initiation of labor and its early up-regulation presence at the maternal-fetal interface due to infections could result in preterm birth [28]. Most of the cells that usually express IL-1β do not do so in a constitutive manner. The mRNA and protein production results from stimulation of either TLRs or NODs and is tightly controlled [27]. Thus the activation of pro-IL-1β into its biologically active form by caspase-1 and its rapid secretion is thought to involve a second stimulus [27]. This requirement suggests that more than one receptor such as TLRs and/or NLRs are involved in the production and secretion of IL-1β. The messages from these 2 signaling pathways would have to be integrated before the release of this highly potent inflammatory cytokine.

Previous work performed in our laboratory de la Torre *et al*. (2009)[1] showed that *Chlamydia trachomatis* serovar D infects first trimester placental trophoblast cells (HTR-8 and Sw.71 cell lines) and alters the milieu of cytokines and chemokines secreted by the trophoblasts [1]. It was observed that both the

HTR8 and the Sw.71 trophoblast cell lines up-regulated the secretion of IL-1β, IL-8, IL-6 and down-regulated the secretion of MCP-1 (CCl2), GROα, and RANTES in response to *Chlamydia* infection [1]. Moreover there was minimal cell death noticed initially in the infected trophoblasts that is consistent with previous work reporting that *Chlamydia* inhibited apoptosis [1]. These changes in the cytokine levels could have an effect on the types of immune cells recruited to the maternal-fetal interface, which could play a role in causing pregnancy complications. PRRs like TLRs (TLR-2, TLR-4) and NLRs (NOD1, NOD2, NALP1 and NALP3) are expressed in the trophoblast cells, and responses to *Chlamydia*l infections in other cell types seem to be mediated through both TLRs and NLRs. We would like to investigate the possible roles of TLR2, TLR4 and the NALP3 inflammasome in mediating the IL-1β production seen after trophoblast infection with *Chlamydia trachomatis*. Our goal of the project for the thesis was to test the following hypothesis.

Hypothesis

Chlamydia trachomatis infection of first trimester placental trophoblasts induces a change in the immune cytokines and chemokines present at the maternal-fetal interface and results in production of the pro-inflammatory cytokine IL-1β. We hypothesize that the IL-1β response is mediated through the pattern recognition receptors TLR-2 or TLR-4 for transcription, and the NLRP-3 inflammasome for processing and secretion.

Specific Aims

1. Investigate which PRRs are activated and play a critical role in the secretion of the inflammatory cytokine IL-1β upon *Chlamydia trachomatis* infection of placental trophoblasts. Explore the roles of TLRs (TLR-2 and TLR-4) through inhibition of their adapter protein MyD88.

2. Study the role of inflammasomes in the pro-inflammatory immune response (IL-1β secretion) to *Chlamydia trachomatis* infection. Investigate if *Chlamydia* infection leads to an increase in caspase-1 activation, and examine if NLAP-3 and the adapter ASC play a role in the IL-1β production.

Materials and Methods

Cell lines

We used two human first trimester trophoblast cell lines. The SV40-transformed HTR8 cells (referred to as H8) were a gift from Dr. C. Graham (Queens University, Kingston, Ontario, Canada), and the Sw.71 cells, were immortalized by telomerase-mediated transformation. The H8 cells were maintained in RPMI 1640 (GIBCO-Invitrogen) and the Sw.71 cells were grown in high-glucose DMEM (GIBCO-Invitrogen). Both media were supplemented with 10% FBS (HyClone), 10 mM HEPES, 0.1 mM MEM nonessential amino acids, 1 mM sodium pyruvate, and 100 U/ml penicillin/streptomycin (GIBCO-Invitrogen). The human-derived cervical carcinoma cell line HeLa was obtained from American Type Culture Collection and was grown in Iscove's modified high-glucose DMEM supplemented with 10% FBS, 1% nonessential amino acids

(GIBCO-Invitrogen). The human 293T human embryonic kidney cell line, a gift from Dr. Jim Riley (Univ. of Pennsylvania, Philadelphia, PA) was maintained in DMEM supplemented with 10%FBS, 2mM glutamine and 100U/ml penicillin/streptomycin (GIBCO-Invitrogen). All cell lines were maintained at 37°C/5% CO_2.

Generation of stably transfected cell lines

H8 cells were stably transfected with the pDeNy expression plasmid containing human DN-MyD88 (dominant negative) (InvivoGen #pdn-hmyd88). The DN-MyD88 (aa 161-296) contains a truncated form of MyD88 that contains the C-terminal TIR domain but lacks the death domain and is unable to homodimerize and activate the signaling cascade. $2x10^5$ H8 trophoblast cells were seeded in a T25 flask in 4 mL RPMI +10% FBS and allowed to attach overnight, then washed with serum free OptiMEM (Gibco-Invitrogen). Cells were transfected for 18h with 2μg of DNA using the Fugene 6 transfection reagent (Roche Diagnostics) in a 1to 3 ratio in 3 mL OptiMEM. Following transfection the cells were allowed to recover for 24h in RPMI growth media, then selection for stable expression was initiated. The cells expressing DN-MyD88 or DN-ASC were selected for using Zeocin (InvivoGen #ant-zn) at 1000μg/mL for 10-14 days. Untransfected cells served as wild-type controls. Further, the antibiotic selected cells were seeded in 48-well plates at 1 cell/well concentration. The plates were screened for single cell colonies under a microscope and further cultured in RPMI with Zeocin at 500μg/mL for maintenance. The DN-Myd88

individual clones were further tested for IL-8 activity, which served as a functional selection marker (IL-8 is MyD88 dependent).

Real time PCR

Total RNA was isolated from cells using the RNeasy Mini Prep kit from Qiagen following the manufacturer's protocol. Reverse transcription of the RNA into cDNA was done on 5μg of total RNA using the Sprint RT Complete Oligo (dT)18 kit from Clontech following the manufacturer's protocol (incubate at 70°C for 10 min then at 42°C for 1h). Real-time RT-PCR was performed using commercially available Applied Biosystems probes for IL-1β (Hs00174097_m1) and the control GAPDH (Hs99999905_m1), Taqman Universal Mastermix, and a Thermal Cycler (iQ5; Bio-Rad Laboratories, Hercules, CA). Samples were analyzed in duplicate and the target gene expression was normalized to that of GAPDH.

Cell Viability Assays

The effects of increased extra-cellular potassium ($K^{+)}$ on wild-type H8 and Sw.71 trophoblast cell viability were determined using the CellTiter 96 viability assay (Promega) following the manufacturer's protocol. Cell lines were plated in 96-well plates at 1 x 10^4 cells/well in growth medium and cultured overnight. The medium was then replaced with serum-free Opti-MEM (Invitrogen) or Opti-MEM+ 50, 70 or 90mM KCl and the cells were cultured for 36h. The CellTiter 96 substrate was added to all wells and following 1-2h incubation at 37°C, ODs

were read at 490 nm. All samples were assayed in triplicate and cell viability was presented as a percentage relative to the untreated control.

Chlamydia trachomatis culture and stock preparation

Chlamydia trachomatis (Ct) serovar D rif-r was a gift from Dr. Robert DeMars (University of Wisconsin, Madison, WI). Ct was propagated in HeLa cells grown in antibiotic-free DMEM (GIBCO-Invitrogen) as previously described. Briefly, HeLa cells were seeded in T75 cm^2 flasks (Falcon; BD Biosciences) (80% confluency) and allowed to attach overnight. The cells were treated with DEAE-Dextran (Sigma #D9885-10G) to increase infectivity. The flasks were washed with Hank's Balanced Salt Solution (HBSS) (Invitrogen) and then incubated for 30min at room temperature with 3mL HBSS + 30μg/mL DEAE-Dextran. The cells were washed with HBSS and then infected at a multiplicity of infection (MOI) of 5-10 IFU (infection forming unit) in 3ml of DMEM. The flasks were rocked (two excursions per minute on a Bellco rocker platform) for 1 h at room temperature followed by resting for 1 h. Following this, the medium was aspirated and replaced with fresh DMEM containing 10% FBS and 1 μg/ml cycloheximide (Calbiochem). The infected cells were then transferred into a humidified incubator at 37°C/5% CO_2 for 48 h. Next, the HeLa cells were washed with 10mL PBS, collected by scrapping in 3mL PBS, and then transferred into 14-ml round-bottom tubes (Falcon). The tubes (2 ml cell solution/tube) were then placed in ice water in a disruptor cup horn of a sonicator (Branson Digital Sonicator/S250D). The cells were sonicated at 200 W and 78% amplitude during three rounds of 20 s and one last round of 10 s. The cells were then centrifuged for

10 min at 200 x *g* at 4°C. The supernatant was collected and further centrifuged for 1 h at 30,000 x *g* at 4°C in a Sorvall centrifuge. The supernatant from this step was discarded and the pellet slowly pipetted up and down to resuspende with sucrose-phosphate-glutamate (SPG) buffer (200 mM sucrose, 20 mM NaH_2PO_4, 20 mM Na_2HPO_4, 5 mM L-glutamate, and dH_2O (pH 7.2), filtered), aliquoted, and stored at –80°C

Inclusion-forming unit (IFU) recovery

HeLa cells were seeded in a 6-well plate and grown overnight. The cells were infected with 1:10 dilutions of Ct EBs in DMEM. After rocking/resting the plates for 2 h at room temperature, the medium was replaced with DMEM/10% FBS supplemented with 1 µg/ml cycloheximide and the cells were grown for 48h. The cells were collected and stained for Ct-LPS and the number of infected cells was detected using Flow cytometry (see staining protocol below). The number of IFUs per HeLa cell was calculated as follows: using the Poisson distribution, the mean number of IFUs infecting the HeLa cells was calculated using the percent uninfected cells ($Po = e^{-m}$). We then multiplied (IFU per cell) by the number of cells plated per well to give us the number of IFUs per well. Based on the dilution of the stock and the volume used for infection, we determined the total number of IFUs in the stock. The following calculation was then performed: total IFUs recovered divided by percent infected HeLa cells x total number of HeLa = number of IFU recovered per infected cell.

Infection of trophoblast cells with Ct

Trophoblast cells (1 x 10^5) were seeded into wells of a 6-well plate and allowed to attach overnight. The next day, the cells were washed with 10mL PBS and infected at a multiplicity of infection (MOI) of 0.5 - 2 in 2 ml of SPG by spinning at 350 x *g* at 8–10°C for 40 min in a Eppendorf centrifuge with a plate holder. This MOI was calculated based on an assay performed using HeLa cells. The trophoblast cells were then washed with PBS to remove any unattached bacteria. Fresh serum-free OptiMEM (GIBCO-Invitrogen) was then added to the plates, and the cells were cultured at 37°C for 12–48 h.

Intracellular staining and flow cytometry

Infection rates in both the trophoblast cells and the HeLa cells were analyzed by flow cytometry. Postinfection (24-48h), the cells were washed with PBS, detached with 0.05% trypsin-EDTA (Invitrogen), and collected with Staining Buffer (SB) (PBS, 1% FBS, and 0.1% sodium azide (NaN_3); Sigma-Aldrich). The suspensions were transferred to a 5-ml polystyrene round-bottom tube, snap cap (Falcon; BD Biosciences), and centrifuged at 200 x *g* for 5 min at 4°C. The cell pellet was then suspended with SB and centrifuged two more times. After this, the cells were fixed in 1 ml of 3.7% formaldehyde (Calbiochem) for 15–20 min at room temperature. The cells were then centrifuged and washed twice in 1 ml of cold Perm/Wash buffer (BD Biosciences #554723) and then incubated for 40 min on ice. After this, cells were centrifuged and then resuspended in 100 ml of Perm/Wash and add 2-3µg mouse IgG Ab (Jackson ImmunoResearch #015-000-002) to block non-specific binding, and incubate for

10 min on ice, then add 2 µl of a FITC-conjugated anti-Ct-LPS mAb (ViroStat #1649) and incubate for 25 min on ice in the dark. After adding 1 ml of cold Perm/Wash buffer and centrifugation, the cells were washed with SB buffer and then resuspended in SB. For resuspending pellets, the tubes were gently flicked, medium was added, and a quick touch with a vortexer on low speed was performed. Cells were analyzed using the FACSCalibur (BD Biosciences). Data were acquired using CellQuest (BD Biosciences) and analyzed using FlowJo software (Tree Star), respectively.

Cytokine studies

The changes of cytokine/chemokine production in H8-wt vs.DN-MyD88 trophoblasts infected with *Ct* were determined by multiplex technology. Following infection of trophoblast cells in a 6-well plate with Ct for 12-48 h, the culture supernatants were collected and passed through a 13-mm, 0.2-µm pore size filter (Pall) to remove cell debris and any EBs. The supernatants were then stored at –80°C until analysis was performed. Supernatants were then analyzed for a full panel of cytokines and chemokines using the custom Bio-Plex Human Cytokine 19-Plex from Bio-Rad with detection and analysis using the Luminex 100 IS system (Upstate Biotechnology) as recently described [12]. The 19 cytokines/chemokines analyzed were IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p70, IL-13, IL-17, G-CSF, GM-CSF, GRO-α, IFN-γ, MCP-1, MIP-1β, RANTES, and TNF-α. The H8 or Sw.71 trophoblasts cytokine production was measured by ELISA for human IL-1β (R&D Systems #DLB50), human IL-8 (Assay Designs #900-156) and human IL-6 (Assay Designs #900-033) following

the manufacturer's protocols on filtered supernatants as described above. The samples were read on a BioRad 550 microplate reader and analyzed in Excel. The IL-1β secretion by H8 cells was measured using 5x concentrated supernatants because the concentration was too low to be aqurately detected by ELISA. The supernatants were concentrated using Amicon ultra centrifugal filters with a 10k membrane (Millipore #UFC501024).

Western blot analysis

For analysis of intracellular proteins, cells were lysed using 1% Nonidet P-40 and 0.1% SDS in the presence of protease inhibitors (Roche #11697498001). Protein concentrations were calculated by bicinchoninic acid assay (Pierce Biotechnology). Proteins were then diluted with gel loading buffer to 20 μg and boiled for 5 min. Proteins were resolved under reducing conditions on 12% SDS-PAGE gels and then transferred onto polyvinylidene difluoride paper (PerkinElmer). Membranes were blocked at room temperature for 1 h with 5% fat-free powdered milk in PBS/0.05% Tween 20 (PBS-T) that was filtered. Following three washes for 10 min each with PBS-T, membranes were incubated overnight at 4°C with the rabbit anti-human IL-1β Ab (Cell Signaling #2022) that detects both the pro-form (31kDa) and the cleaved form (17kDa) of the protein. Following this incubation, membranes were washed three times as before and then incubated at room temperature for 1 h with the goat anti-rabbit secondary Ab conjugated to peroxidase (Vector Laboratories) in PBS-T/1% fat-free powdered milk. Following three washes for 10 min each with PBS-T and three washes for 10 min each with distilled water, the peroxidase-conjugated Ab was detected by

ECL (enhanced chemiluminescent substrate) (PerkinElmer). Membranes were then stripped and subsequently probed for rabbit anti-human Caspase-1 Ab (Cell Signaling #06-503) that detects both the pro-form (45kDa) and the active form (20kDa) of the protein and exposed as above. Further the membranes were stripped and probed for rabbit-anti-human β-actin Ab (Sigma #A2066) (45kDa). For confirming gene knockdown in the shRNA expressing cells rabbit anti-human NALP3 Ab (Sigma #HPA012878) (118kDa) and rabbit anti-human ASC Ab (Calbiochem #ST1121) (22kDa) were used and the protocols described above were used. Images were recorded using the Gel Logic 100 (Kodak) and Kodak MI software.

Statistical analysis

Data are expressed as mean ± SD. Statistical significance ($p < 0.05$) was determined using either Student's *t* tests or, for multiple comparisons, one-way ANOVA followed by Bonferroni's post-test or multiple regression analyses. For all statistical analysis GraphPad Prism 4 was used. All experiments were performed at least in triplicate.

Note: all the above experiments and reagent preparations were performed by Crina Boeras with the exceptions below. The Abrahams Lab in the OB-Gyn Department at Yale helped run the multiplex cytokine studies and one of the IL-1β ELISAs. However, the sample acquisition and data analysis was performed by Crina Boeras.

Results

Culture of Rifampin-resistant Chlamydia trachomatis serovar D

Chlamydia trachomatis serovars D and L1 were successfully grown in and purified from the HeLa epithelial cell line as described by de la Torre *et al*. [1] and more details are presented in the methods section. The *Ct* serovar D more commonly infects the female genital tract [29] and we wanted to use this serovar for all our studies. However, the *Ct* serovar D was no longer available in our laboratory and we received a gift from Dr. Robert DeMars of the Rifampin-resistant (rif-r) *Chlamydia trachomatis* serovar D. We have successfully grown the Ct rif-r serovar D in HeLa cells and titrated the EB stock as described in detail in the methods.

Studies done in HeLa cells showed an increased efficiency of infection with *Ct* when the cells were pre-treated with DEAE-Dextran 30μg/ml for 30 min at 25ºC prior to the infection. We have either treated the HeLa cells with HBSS (Hank's balanced salt solution) or HBSS+DEAE Dextran prior to the *Ct rif-r* infection and 24h post-infection we have intracellularly stained the cells with a *chlamydial* anti-LPS Ab conjugated to FITC as described in the methods. The cells were analyzed by flow cytometry (measured 10.000 events for each sample) at 24h post-infection and the rate of infection was determined. The cells treated with DEAE-Dextran showed an increase in infection rate by ~20-30% (35% vs. 48%) compared to the untreated cells (data not shown). These results were observed in two individual experiments. When the DEAE-Detran treatment was

used to prepare Chlamydia stock, the titers obtained were higher than prior 1-1.4x10^8 vs. ~2-5x10^7 IFU/mL.

Rifampin-resistant Chlamydia trachomatis infects first trimester trophoblast cells

Chlamydia trachomatis, which preferentially infects epithelial cells, has been shown to also infect a variety of immune cells like monocytes and dendritic cells. Recently, de la Torre *et al*. [1] showed that *Chlamydia trachomatis* serovars D and L1 can infect the first trimester trophoblast cell lines HTR8 (H8) and Sw.71. Moreover this infection led to the production of viable infectious EBs that could infect trophoblast or HeLa epithelial cells. The infection protocol was optimized and was found that centrifuging the *Ct* serovar D onto the cells leads to a higher infection rate in H8 cells (14.4% vs. 67.8%) and Sw.71 cells (7.35% vs. 27.4%) at 36h post-infection [1]. We used the *Ct rif-r* serovar D (referred to as *Ct* for the remainder of the manuscript) and followed the infection, anti-LPS staining and flow cytometry protocols described by de la Torre *et al*. [1] for all of the experiments.

H8 and Sw.71 trophoblast cells (1x10^5) were seeded into wells of a 6-well plate and allowed to attach overnight. The next day, the cells were infected with *Ct* at a multiplicity of infection (MOI) of ~1 in 2mL of SPG (Sucrose Phosphate Glutamate solution) by spinning at 350xg at 8–10°C for 40 minutes. The trophoblast cells were then grown in serum-free OptiMEM at 37°C for 24 –48h. Initially the rates of infection in H8 cells were determined by anti-LPS staining and flow cytometry at 36h post-infection and ranged between 27-36%, with some cell death being observed. While looking at the cells under a microscope at 24h

post-infection we observed the majority (over 50%) of cells had *Ct* containing inclusions, which did not correlate with the rates of infections seen at 36h post-infection (data not shown). We hypothesized that the Rifampin resistant strain of *Ct* was more aggressive than the regular serovar D*,* and by 36h post-infection a significant number of the infected cells would lyse and could no longer be detected by flow cytometry. Another posibility was that the amount of *Ct* EBs used was greater than calculated (MOI ~2) which resulted in an increased infection rate and was killing the cells sooner than expected.

We therefore decided to compare the rates of infection at 24h and 36h post-infection with 2 different MOIs (1 and 2) in the H8 cells. Indeed, we found that both the increased MOI and the longer time had an effect on infection rates. At MOI=1 there was a significant drop in the measured infection rates between 24h and 36h at 60.6% vs. 45.5%, and at MOI=2 the infection rates were 81% at 24h vs. 50% at 36h (data not shown). Moreover, the cell death observed with at MOI=2 was significantly reduced when an MOI=1 was used. This supported our hypothesis that by 36h the infection rate drops due to cell lysis and release of *Ct* inclusions, and that assaying infection rates at 24h post-infection would give a more accurate measurement. Also, the decrease in MOI from 2 to 1, leads to a greater cell survival in this more aggressive *Ct* strain. Similar experiments were performed in Sw.71 cells and the same trends were observed.

In Figure **1** we show a representative experiment for both H8 and Sw.71 cells. In panel **A**. we show the scatter plots for the uninfected (left) and infected (right) cells which indicate their size and internal composition. We can observe

that in many but not all cells the infection changes their scatter profiles (increased side scatter SSC-H on the y-axis) due to the presence of the *Ct* inclusions. The flow cytometry histogram plots (Figure **1.B**) show the uninfected cells in red and infected cells in blue. The blue graph shows two distinct populations of cells: the *left-hand peak* represents the uninfected cells and the *right-hand peak* with the marker represents the infected trophoblast cell population. The infection rates at 24h post-infection were 62.1% for H8 cells and 40.1% for Sw.71 cells. Similar infection rates were seen in all subsequent experiments. In conclusion, we have successfully infected H8 and Sw.71 cells with *Ct rif-r* serovar D and were able to optimize the infection parameters. An adequate MOI to balance infections rate and cell death is an effective MOI=1, and the best time to assay the infection efficiency by anti-LPS staining and flow cytometry is at 24h post-infection. We used these parameters for all our subsequent experiments.

Ct infection of H8 and Sw.71 trophoblasts induces the production of IL-1β both at the mRNA and protein levels

Recently, de la Torre *et al.* [1] showed that placental trophoblasts secrete pro-inflammatory cytokines like IL-1β and IL-8 after *Ct* infection. While these cells constitutively secrete some IL-8, they do not secrete IL-1β unless stimulated. We evaluated the effect of *Ct* infection on IL-1β production at the transcriptional, translational and secreted level in trophoblasts.

H8 cells were either non-infected (NI) or infected with *Ct* at an MOI=1. At 24h post-infection the cells were lysed and total RNA was extracted. The RNA was transcribed into cDNA and the IL-1β mRNA expression was assayed using quantitative PCR (QPCR) as described in detail in the methods.

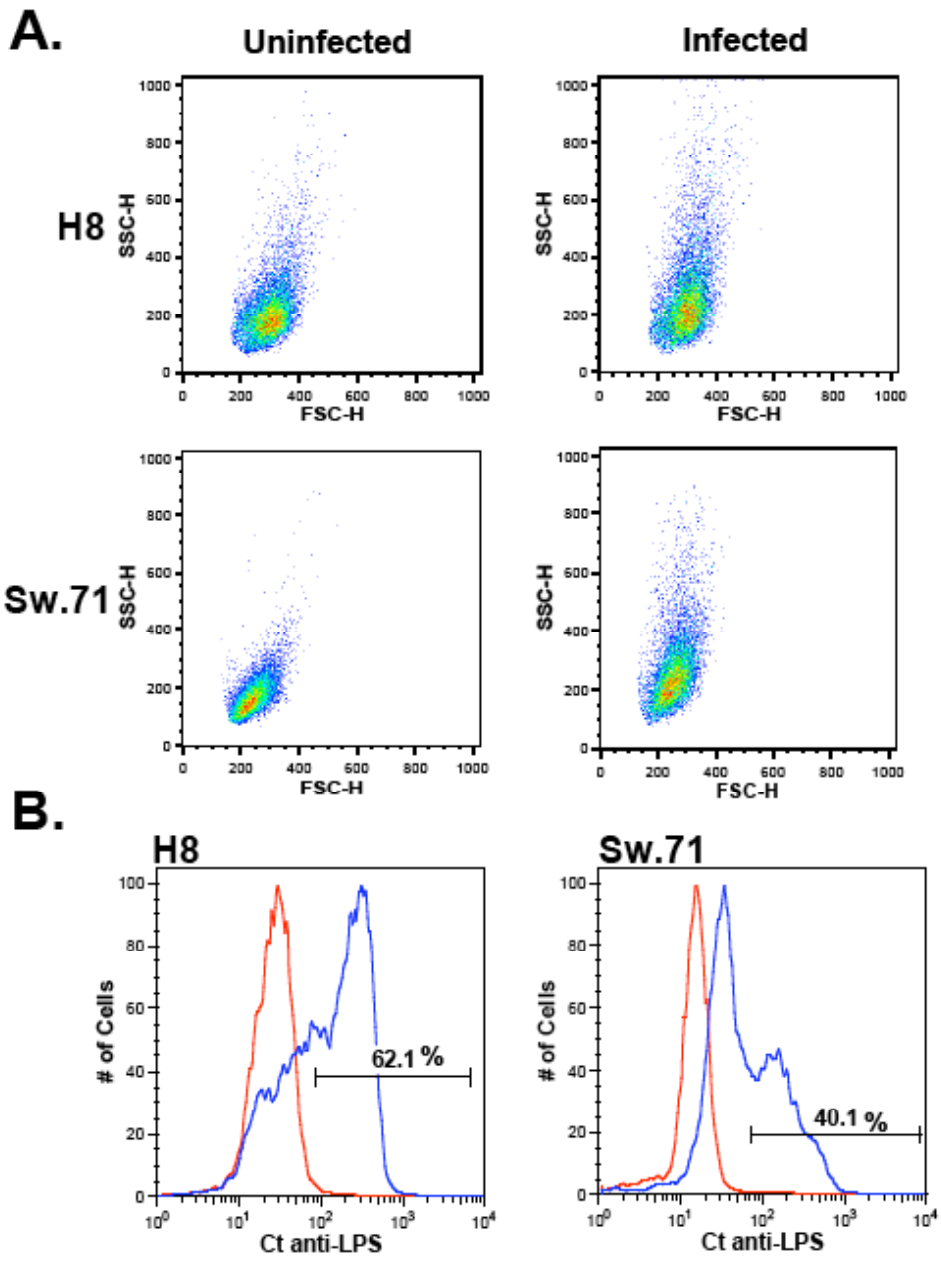

Figure 1. Infection rates of trophoblast cells infected with Ct by centrifugation. H8 and Sw.71 trohpoblasts were infected with Ct at a MOI ~1 by centrifugation. At 24h post-infection the cells were stained intracellularly with a FITC-conjugated mouse anti-Ct LPS mAb. The infection rates were then analyzed by flow cytometry. **(A)** The flow cytometry dot plots show the side and forward scatter of the cells, which indicate their size and internal composition. **(B)** The flow cytometry histogram plots show the uninfected cells in red and infected cells in blue. The *right-hand peak* in the blue graph with the marker represents the infected trophoblast cell population.

The IL-1β mRNA levels were normalized to the levels of GAPDH mRNA (a housekeeping gene) and the results are seen in Figure 2.A. There was a substantial (8-10 fold) increase in the IL-1β mRNA levels in the *Ct* infected compared to the non-infected cells with a $p<0.01$. The experiment was performed in triplicate (n=3).

Next, we looked at the levels of intracellular IL-1β by western blot. H8 and Sw.71 cells were either non-infected (NI) or infected with *Ct* at an MOI=1, and at 12h, 24h, 48h post-infection the cells were lysed and protein was extracted. The IL-1β levels were analyzed by Western blot using an antibody that recognizes both the full-length pro-IL-1β (31kDa) and the processed active IL-1β (17kDa). A representative picture of the western blot is shown in Figure 2.B for H8 cells and Figure 2.D for Sw.71 cells. The protein bands were analyzed by densitometry and the levels were normalized to the corresponding β-actin levels which served as a loading control. The relative densitometry data is shown in Figures 2.B and D in bar graph format (n=7 for H8 cells, n=3 for Sw.71 cells). The H8 and Sw.71 have none or minimal expression of pro-IL-1β, but there is a significant induction after *Ct* infection as early as 12h post infection with *= $p<0.05$ or **= $p<0.01$. While the Sw.71 pro-IL-1β induction did not reach significance at 24h and 48h, the image shows a definite increase in the levels in the *Ct* infected cells. Both the H8 and Sw.71 cells have a small amount of intracellular IL-1β regardless of infection status. In the H8 cells by 48h post-infection the levels significantly decrease with $p<0.05$, presumably because the active IL-1β is being secreted and is lost in the media.

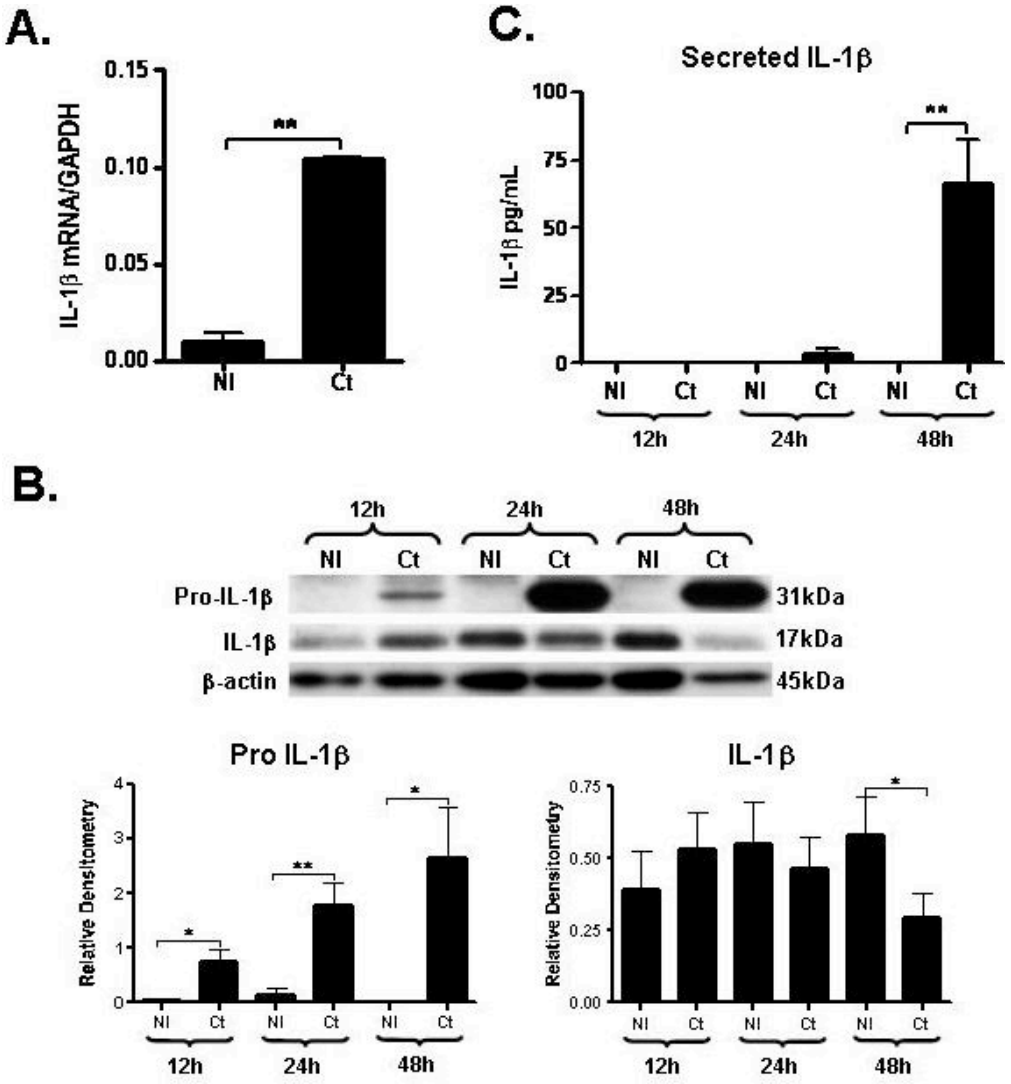

Figure 2. In trophoblasts *Ct* infection (MOI~1) induces the production of IL-1β mRNA and protein. **(A)** H8 cells were either uninfected (NI) or infected (*Ct*) and grown for 24h, then IL-1β mRNA levels were assayed by real-time PCR and levels were normalized to GAPDH levels. **(B)** H8 cells were either NI or *Ct* infected and at 12h, 24h, and 48h post infection (PI) protein lysates were made and analyzed by Western blot using an IL-1β antibody that detects both the pro and active form of the protein. Densitometry of the Pro-IL-1β and IL-1β bands was calculated and normalized to the corresponding β-actin levels. The relative densitometry graphs are shown. (n=7). **(C)** The cell-free/EB free supernatants of the NI and *Ct* infected cells from the 12h, 24h, 48h PI time points were analyzed for secreted IL-1b by ELISA. Bar graphs show the IL-1β levels (n=7). (* $p<0.05$, ** $p<0.01$)

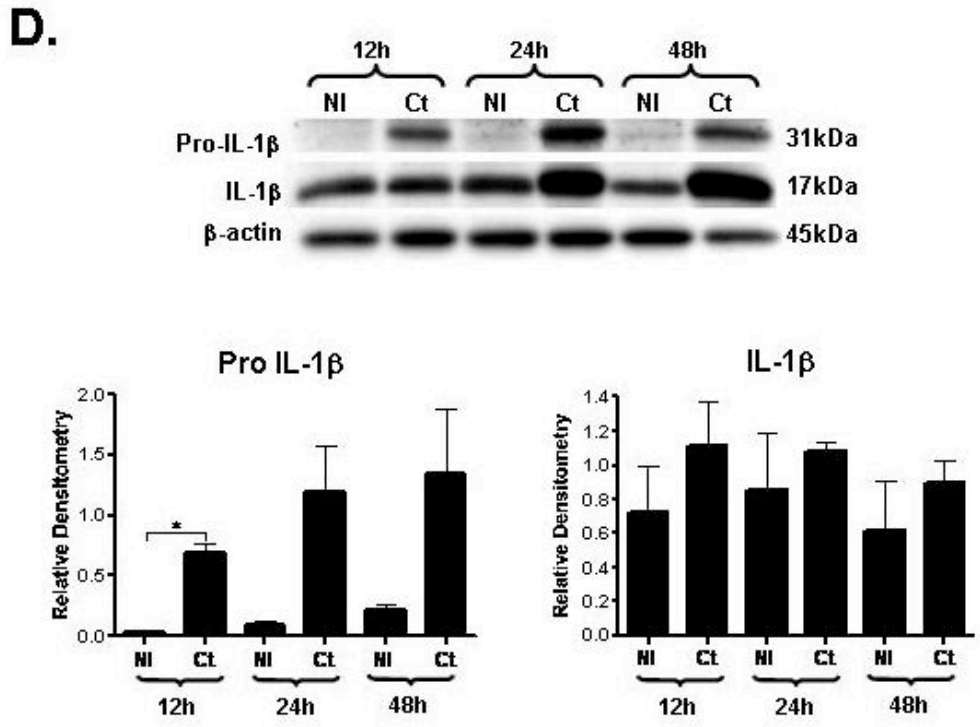

Figure 2 (cont.). (**D)** Sw.71 cells were either uninfected (NI) or infected (*Ct*) and at 12h, 24h, and 48h post infection (PI) protein lysates were made and analyzed by Western blot using an IL-1β antibody that detects both the pro and active form of the protein. Densitometry of the bands was calculated and normalized to β-actin levels and the relative densitometry graphs are shown (n=3). (* $p<0.05$)

To verify this presumption we further analyzed the cell supernatants for IL-1β by ELSIA. The cell free/EB free supernatants from the NI or *Ct* infected H8 cells from all time points (12h, 24h and 48h post-infection) were collected and analyzed. Figure 2.C shows the levels of secreted IL-1β. The uninfected cells do not secrete any of their intracellular IL-1β while the *Ct* infected cells start processing and secreting IL-1β at 24h and by 48h post-infection there is a significant increase in IL-1β secretion $p<0.01$ (n=7). This correlates with the western blot data.

Generation of a stably transfected DN-MyD88 cell line

TLR-2 and TLR-4 have been shown to recognize *chlamydial* LPS in immune cells like macrophages and dendritic cells [17-18]. Also in genital tract epithelial cells infected with *Chlamydia*, TLR-2 and its adapter protein MyD88 have been shown to be recruited to the *chlamydial* inclusion membrane and be required for the production of IL-8 [20]. Studies have shown that both TLR-2 and TLR-4 signal through the adapter protein MyD88. Thus, we wanted to investigate the potential role for these TLRs in inducing an inflammatory response in *Ct* infected trophoblasts. We wanted to block the action of MyD88 and assay the secretion of cytokines after *Ct* infection.

We stably transfected H8 cells with the pDeNy expression plasmid containing the human DN-MyD88 (dominant negative) construct as described in detail in the methods section. The DN-MyD88 (aa 161-296) seen in Figure 3**.A** contains a truncated form of MyD88 that has the C-terminal TIR domain but lacks the death domain (DD) and is unable to homodimerize and activate the signaling cascade. The cells expressing DN-MyD88 were selected using the antibiotic Zeocin at 1000μg/mL for 10-14 days. Untransfected cells served as H8 wild-type controls. The antibiotic selected cells were then cloned by limiting dilution and cultured further.

As seen in Figure 3**.B**, supernatants from H8 cells and five of the DN-MyD88 individual clones referred to as C1→C5 were tested for IL-8 levels by ELISA after stimulation for 72h with the IIC5 antibody (20μg/mL). The IIC5 Ab. has been shown to induce a MyD88 dependent IL-8 response in trophoblasts

[59g]. Since the IL-8 production was shown to be MyD88 dependent, a decreased production of IL-8 from a given clone would suggest a functional DN-MyD88 construct. The IL-8 levels in the unstimulated clones were normalized to match the baseline of the unstimulated H8 cells and the treated cells were adjusted by the same factor. The clones with the least IL-8 induction, C3 (374μg/mL) and C5 (358μg/mL) (black bars), compared to H8 cells (624μg/mL) were selected for further analysis.

H8 cells and the DN-MyD88 cells (C3 and C5) were either non-infected (NI) or *Ct* infected with an MOI=1. At 48h post infection the cell-free/EB free supernatants were collected and analyzed for IL-8 by ELISA (n=3) and the results are shown in Figure 3.**C**. The baseline IL-8 level in the non-infected DN-MyD88 cells (C3 and C5) was normalized to match that of the non-infected H8 cells. At 48h post infection the IL-8 response in the C3 and C5 cells was significantly lower than H8 cells, with $p<0.05$ and $p<0.01$ respectively, suggesting an efficient knockdown of the MyD88 function. We used the C5 clone for our next experiments. To further prove that these cells are truly DN-MyD88 in the furutre we can also stimulate them with LPS and look for NFkB pathway activation which is MyD88 dependent.

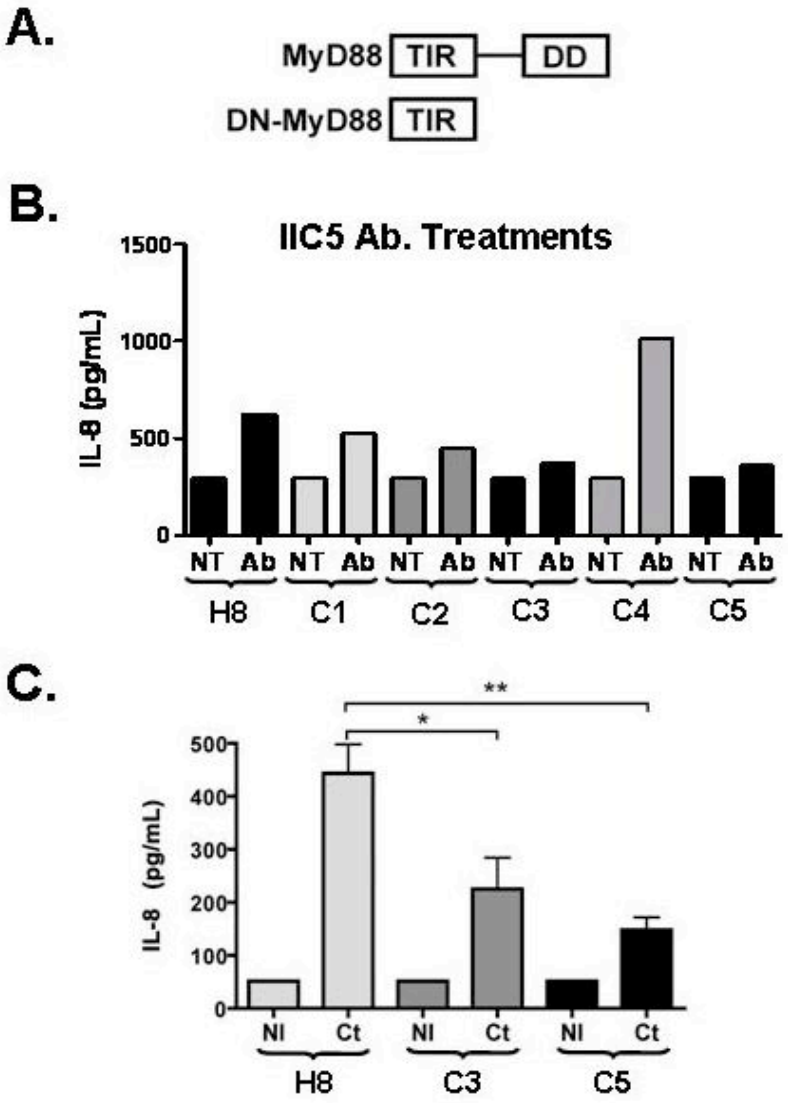

Figure 3. Selection of DN-MyD88 cell lines using a functional approach. **(A)** The dominant negative (DN) MyD88 has the TIR domain that interacts with the TLRs, but lacks the death domain (DD) that leads to downstream signaling. **(B)** H8 cells and 5 different clones (C1 thru C5) of DN-MyD88 cells were treated with 20μM IIC5 Ab (which induces a MyD88 dependent IL-8 response) for 72h and the supernatants were analyzed for IL-8 by ELSIA. The clones with the least IL-8 induction (C3 and C5) compared to *wt* H8 cells were analyzed further. **(C)** H8 cells and DN-MyD88 cells (C3 and C5) were either NI or *Ct* infected at MOI~1. At 48h post infection cell-free/EB free supernatants were collected and analyzed for IL-8 by ELISA (n=3). The baseline IL-8 secretion in the NI cells was normalized to match that of the NI *wt* H8 cells. The IL-8 response in the DN-MyD88 (C3, C5) cells was significantly lower than the *wt* H8 suggesting an efficient knockdown of MyD88. (* =$p<0.05$, ** = $p<0.01$)

Chlamydia trachomatis rif-r infects DN-MyD88 trophoblasts

After we have generated and selected functional clones for DN-MyD88 cells (as described above) we wanted to see if suppressing the MyD88 function might interfere with the ability of *Chlamydia* to infect these trophoblast cells.

H8 and DN-MyD88 trohpoblasts were infected with *Ct* at a MOI =1 by centrifugation as described in the methods section. At 24h post-infection the cells were stained intracellularly with an anti-chlamydial LPS mAb that is FITC-conjugated. The infection rates were then analyzed by flow cytometry.

In Figure 4 we show a representative experiment for both H8 and DN-MyD88 cells. In panel **A**. we show the scatter plots for the uninfected (left) and infected (right) cells which indicate their size and internal composition. We can observe that the infection increases the side scatter (on the y-axis) due to the presence of the *Ct* inclusions within both H8 and DN-MyD88. The flow cytometry histogram plots (Figure 4.**B**) show two distinct populations of cells, the uninfected cells in red and infected cells in blue. In the blue graphs the *right-hand peak* with the marker represents the infected trophoblast cell population. The infection rates at 24h post-infection were 62.1% for H8 cells and 64.3% for DN-MyD88 cells. Similar infection rates were seen in all subsequent experiments. We have successfully infected the H8 and the DN-MyD88 cells with *Ct rif-r* serovar D. The knockdown of MyD88 does not seem to affect the infectibility of the cells. The infection rates were similar and there was no increased cell death seen in the transfected cells compared to the wild-type H8 cells.

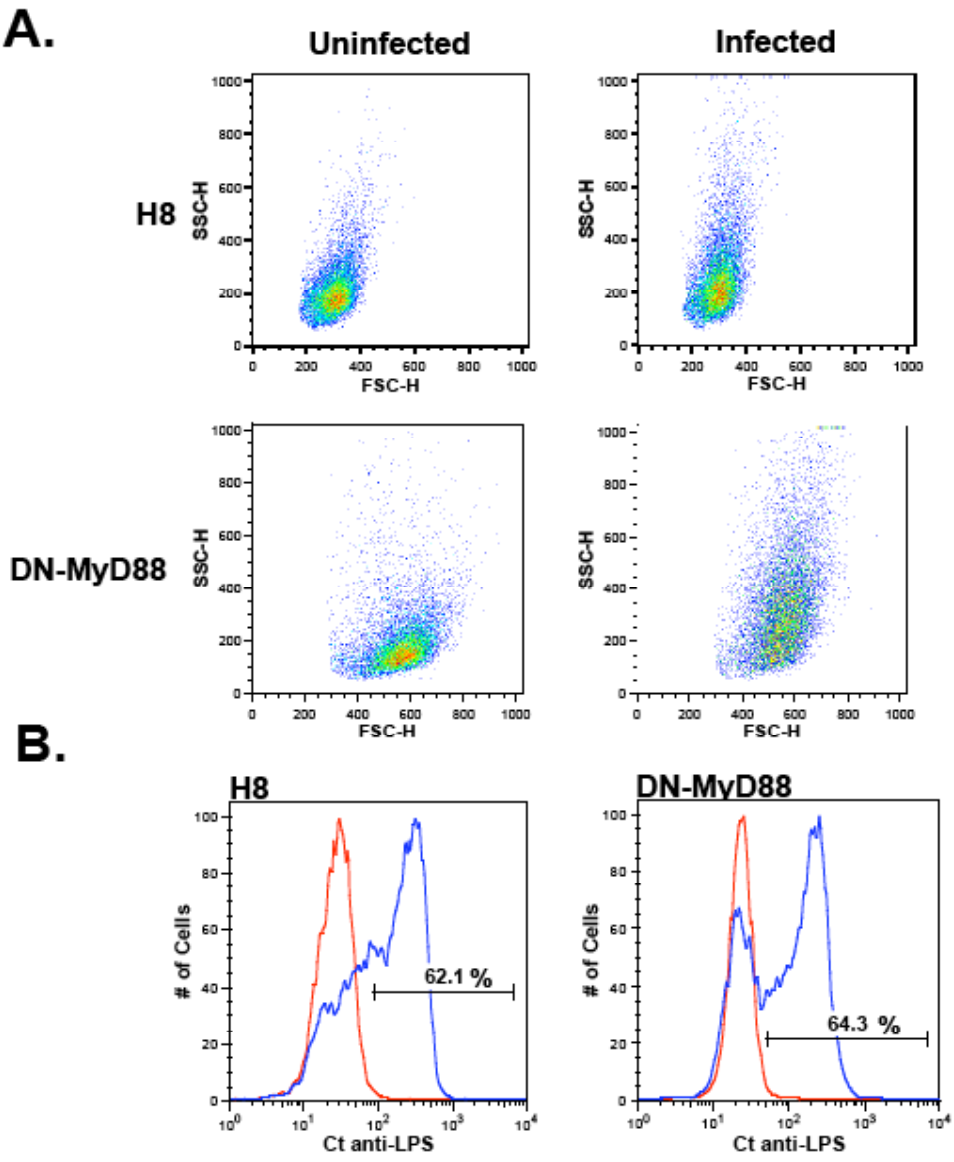

Figure 4. Infection rates of H8 and DN-MyD88 trophoblast cells infected with Ct by centrifugation. H8 and DN-MyD88 trohpoblasts were infected with Ct at a MOI~1 by centrifugation. At 24h post-infection the cells were stained intracellularly with a FITC-conjugated mouse anti-Ct LPS mAb. The infection rates were then analyzed by flow cytometry. **(A)** The flow cytometry dot plots show the side and forward scatter of the cells which indicate their size and internal composition. **(B)** The flow cytometry histogram plots show the uninfected cells in red and infected cells in blue. In the blue graph the *right-hand peak* with the marker represents the infected trophoblast cell population.

IL-1β production in H8 trophoblast cells after Chlamydia infection is independent of MyD88

We generated a functional DN-MyD88 cell line and wanted to test if the production of IL-1β resulting from the *Ct* infection was mediated through a MyD88 dependent TLR such as TLR-2 or TLR-4. We decided to explore this at the mRNA level, the intracellular protein level and the secreted cytokine level.

H8 and DN-MyD88 cells were either non-infected (NI) or infected with *Ct* at an MOI=1. At 24h post-infection the cells were lysed and total RNA was extracted. The RNA was transcribed into cDNA and the IL-1β mRNA expression was assayed using quantitative PCR (QPCR) as described in detail in the methods. The IL-1β mRNA levels were normalized to the levels of GAPDH mRNA and the results are seen in Figure 5.**A**. In both the H8 cells (as shown in figure 2) and DN-MyD88 cells there was a substantial (8-10 fold) increase in the IL-1β mRNA levels in the *Ct* infected cells compared to the non-infected cells with a $p<0.01$ for H8 and $p<0.05$ for DN-MyD88. The experiment was performed in triplicate (n=3). This showed that the inhibition of MyD88 did not affect the induction of IL-1β mRNA after *Ct* infection.

We then looked at the secreted cytokine levels in response to *Ct* infection in H8 vs. DN-MyD88 cells. The cells were non-infected or infected with MOI=1 and cell/EB free supernatants were collected at 48h post infection. The samples were analyzed by multiplex technology for 19 cytokines/chemokines. Of these factors, the levels of the pro-inflammatory cytokines IL-1β, IL-8 and IL-6 were of interest to us. In Figure 5.**B** we see the levels of secreted IL-1β at 48h post-infection of both H8 and DN-MyD88 cells. As seen at the mRNA level, there is a

significant induction in IL-1β production after *Ct* infection in both cell types (H8 $p<0.01$, DN-MyD88 $p<0.05$) (n=3). However there is no difference in the ability to produce IL-1β between the H8 cells and the DN-MyD88 cells suggesting that this response is independent of MyD88.

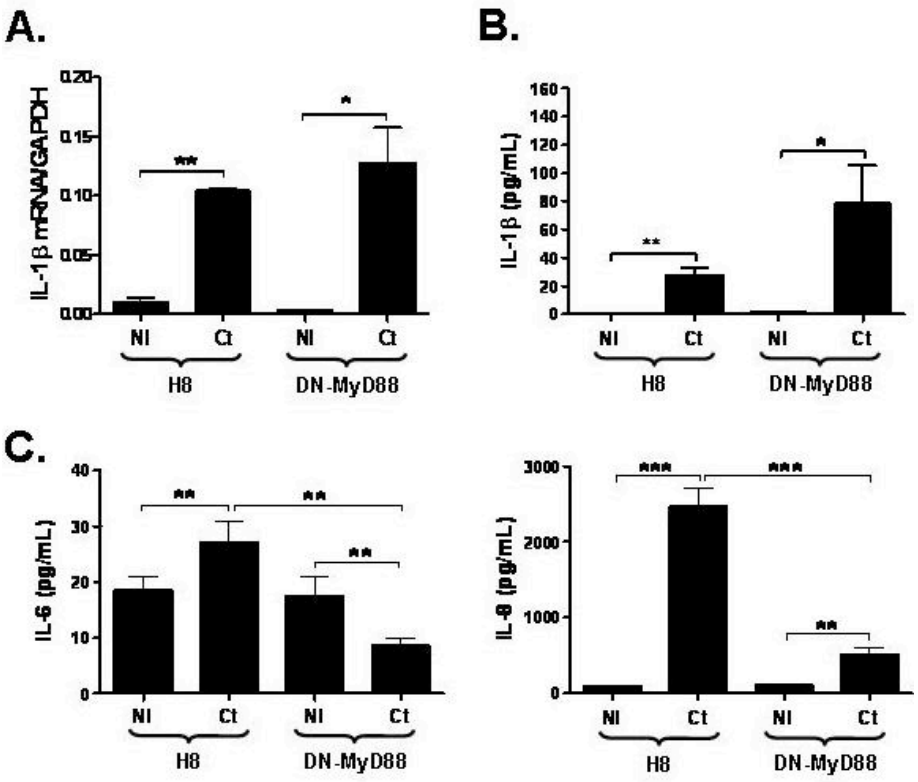

Figure 5. Cytokine profiles of H8 and DN-MyD88 cells with Ct infection (MOI~1). (**A)** H8 and DN-MyD88 cells were either uninfected (NI) or infected (*Ct*) and grown for 24h, then IL-1β mRNA levels were assayed by real-time PCR and levels were normalized to GAPDH levels (n=3). H8 and DN-MyD88 cells were either uninfected (NI) or infected (*Ct*) and grown for 48h, then the cell-free/EB-free supernatants were analyzed for cytokines by Luminex/multiplex analysis. Bar graphs show the levels of **(B)** secreted IL-1β or **(C)** IL-6 and IL-8 after NI or *Ct* infection (n=3). (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

The DN-MyD88 cells have already been selected for reduced IL-8 production (as described in the prior section) and the results seen on Figure 5.**C** in the right panel confirm this. We see a significant induction of IL-8 production

after *Ct* infection in both H8 and DN-MyD88 cells as compared to non-infected cells (H8 $p<0.001$, DN-MyD88 $p<0.01$). However, the DN-MyD88 infected cells have a 5 fold reduced response compared to the H8 infected cells ($p<0.001$) (n=3). In the left panel we see the effect of *Ct* infection and inhibition of MyD88 on IL-6 production which is also thought to be MyD88 dependent. There is a moderate induction of IL-6 with *Ct* infection in H8 cells ($p<0.01$), but a significant decrease in IL-6 production in DN-MyD88 cells ($p<0.01$). This suggests that the IL-6 production in trophoblasts is MyD88 dependent.

Next we looked at the levels of intracellular IL-1β in the cells we collected the supernatants from. H8 and DN-MyD88 cells were either non-infected (NI) or infected with *Ct* at an MOI=1, and at 12h, 24h, 48h post-infection protein was extracted. The IL-1β levels were analyzed by Western blot. A representative picture of the western blot is shown in Figure 5.**D**. The protein levels were normalized to the corresponding β-actin levels and the relative densitometry data is shown in bar graph format (n=7 for H8 cells, n=3 for DN-MyD88 cells). The H8 and DN-MyD88 have none or minimal expression of pro-IL-1β, but there is a significant induction after *Ct* infection as early as 12h post infection, with *= $p<0.05$ or **= $p<0.01$. This induction increases further by 24h and 48h post infection. While the DN-MyD88 pro-IL-1β induction did not reach significance at 24h and 48h, the image shows a definite increase in the levels in the *Ct* infected cells. Both the H8 and DN-MyD88 cells have a small amount of intracellular IL-1β regardless of infection status. These results support the fact that the production of IL-1β after Ct infection is independent of MyD88 and possibly TLR-2 and

TLR-4. Another possibility is that a very small of MyD88 activity is sufficient for IL-1 β production, while a larger amount is needed for IL-8 and IL-6.

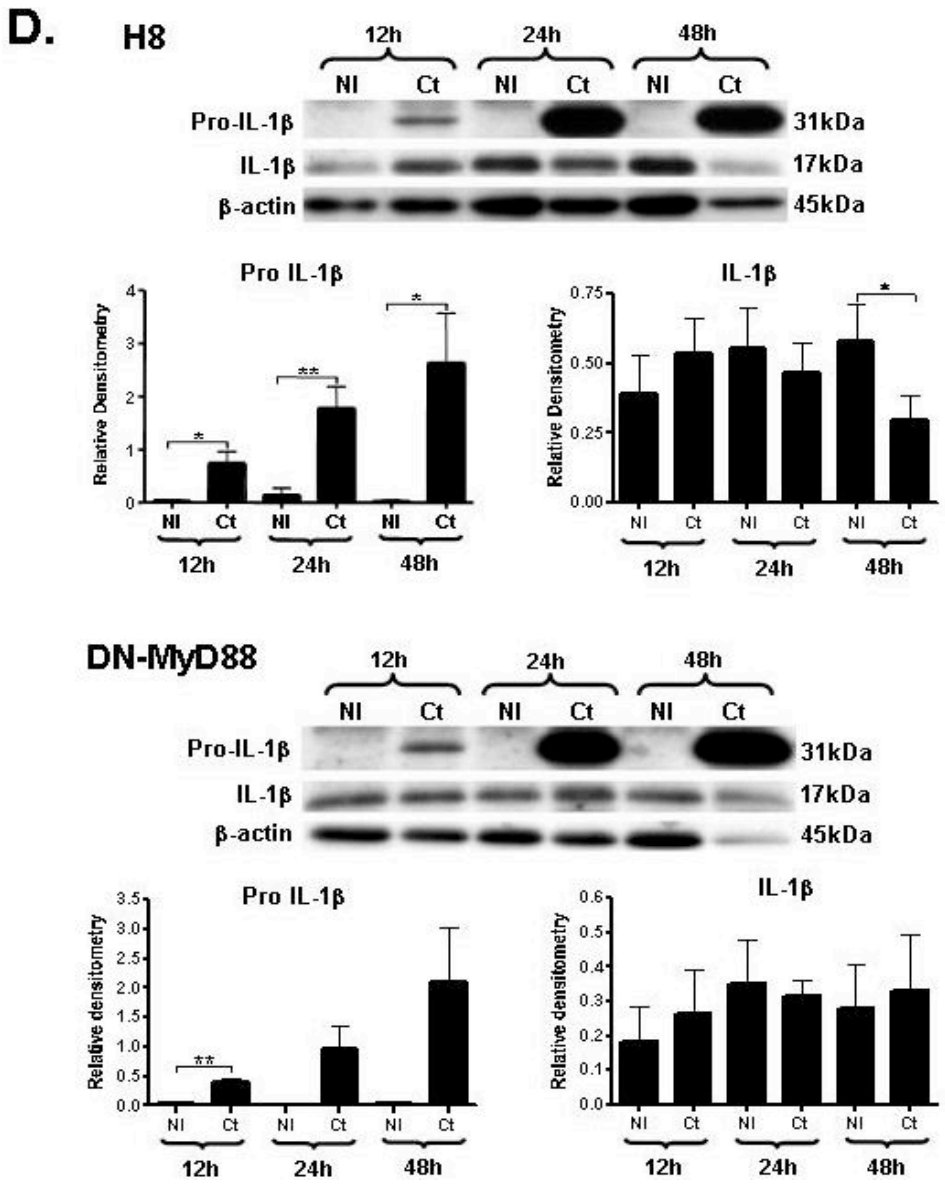

Figure 5 (Cont). Cytokine profiles of H8 and DN-MyD88 cells with Ct infection (MOI~1). **(D)** H8 (*top*) and DN-MyD88 (*bottom*) cells were either NI or *Ct* infected and at 12h, 24h, and 48h post infection (PI) protein lysates were made and analyzed by Western blot using an IL-1β antibody that detects both the pro and active form of the protein. Densitometry of the Pro-IL-1β and IL-1β bands was calculated and normalized to the corresponding β-actin levels. The relative densitometry graphs are shown (n=3). (*$p<0.05$, **$p<0.01$)

The trohpoblasts' response to increased extracellular KCl suggests a role for NLRs (NALP3) in their IL-1β induction upon Ct infection

Activation of the inflammasome leads to the activation of Caspase-1 and the subsequent processing of pro-inflammatory cytokines like IL-1β, IL-18 and IL-33. One of the best studied inflammasomes, the NALP3 inflammasome, is activated in response to microbial stimuli including LPS, MDP, bacterial RNA as well as endogenous molecules like uric acid crystals, silica and ATP [33]. Exposure to extracellular ATP leads to the opening of a cation channel (P2X7R) and release of intracellular potassium and NALP3 inflammasome activation. Conversely, increased levels of extracellular potassium block the ion efflux and the NALP-3-mediated caspase-1 activation [33-34].

We wanted to investigate if the NALP-3 inflammasome is involved in the processing of the IL-1β produced by trophoblasts after *Ct* infection. Therefore we decided to expose the trophoblasts to increased extracellular KCl (potassium) and look at IL-1β secretion. First we determined the effect of the extra KCl on cell survival. As seen in Figure 6.**A** we incubated both H8 and Sw.71 cells with media containing increasing amounts of KCl (50mM, 70mM and 90mM) for 36h and then measured cell viability/survival. The untreated cells were considered to have 100% survival and the treated cells' survival was expressed as a percentage of this number. The cells had a linear increase in death with increasing amounts of KCl, but at 50mM the survival was unaffected. We used 50mM KCl in our subsequent experiments.

H8 and Sw.71 cells were either non-infected or infected with *Ct* at MOI~1 and 50mM KCl was added to the media after infection. The cells were incubated

for 36h and then the cell free/EB free supernatants were the assayed for the levels of IL-1β by ELISA. (n=3). As seen in Figure 6.**B** the increased extracellular KCl significantly inhibited the secretion of IL-1β in both H8 and Sw.71 cells compared with the untreated cells (* $p<0.05$, ** $p<0.01$). This suggests that the reduced ionic gradient resulted from the higher KCl outside the cells precluded the activation of the NALP-3 inflammasome and reduced the caspase-1 activity that is needed for IL-1β secretion.

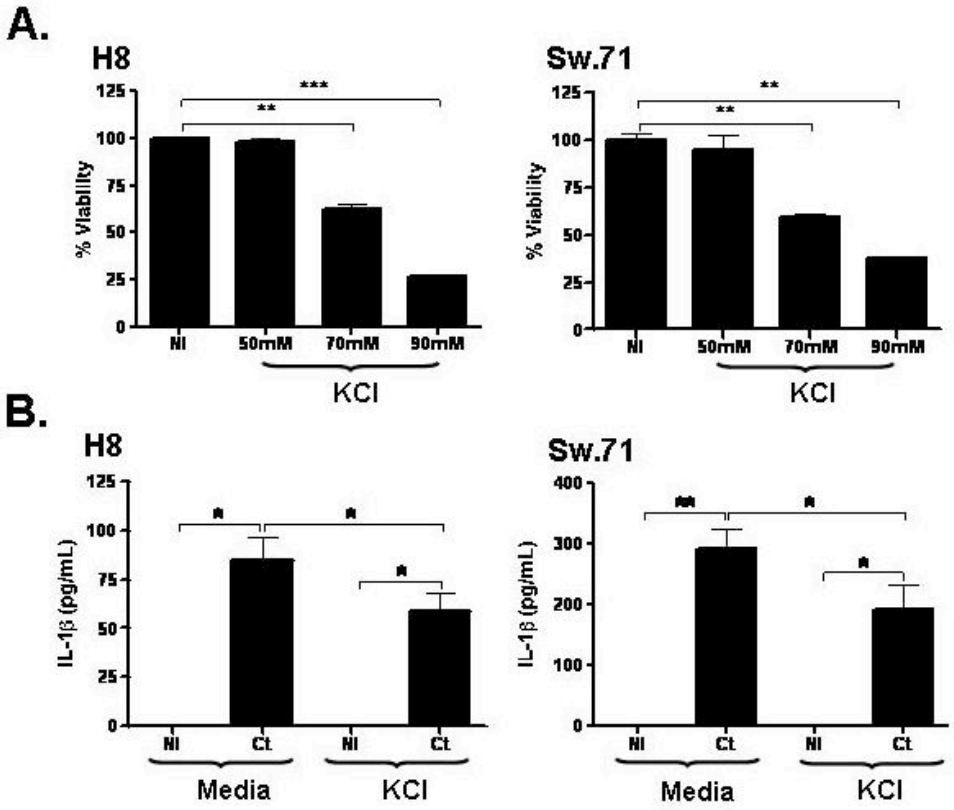

Figure 6. In trohpoblasts the response to increased extracellular KCl suggests a role for NLRs in the IL-1β induction upon Ct infection. (**A)** H8 and Sw.71 cells were incubated with increasing amounts of KCl (50mM→ 90mM) in the media for 36h. Cell viability was assayed and plotted with non- treated cells representing 100%. **(B)** H8 and Sw.71 cells were infected with Ct at MOI~1 and 50mM KCl was added to the media after infection. The cells were incubated for 36h and then the levels of IL-1β from the supernatants were measured by ELISA. (n=3). The KCl treatment inhibited the secretion of IL-1β in both H8 and Sw.71 cells compared with the untreated cells. (* $p<0.05$, ** $p<0.01$, *** $p<0.001$)

Chlamydia infection of placental trophoblasts causes some changes in active caspase-1 levels

Caspase-1 is the only protease known to process the pro-IL-1β into its active form that gets secreted and exerts its biological functions. As mentioned above, caspase-1 is present as an inactive zymogen and it requires the assembly of an inflammasome to be able to oligomerize and become activated [22,33]. Trophoblast cells have been shown to express small amounts of active caspase-1 without stimulation, however we wanted to investigate if infection with *Chlamydia* would lead to an increase in the active caspase-1 levels, which would in turn result in the increased levels of secreted IL-1β we observed.

We looked at the levels of intracellular active caspase-1 by western blot. H8 and Sw.71 cells were either non-infected (NI) or infected with *Ct* at an MOI=1, and at 12h, 24h, 48h post-infection protein was extracted. The caspase-1 levels were analyzed with an antibody that detects the cleaved form of the protein. A representative picture of the western blot is shown in Figure 7.**A** for both the H8 cells (top) and Sw.71 cells (bottom). The protein bands were normalized to the corresponding β-actin levels and the relative densitometry data is shown in Figures 7.**B** in bar graph format. (n=7 for H8 cells, n=3 for Sw.71 cells). As described before, we observe that both H8 and Sw.71 cells express active caspase-1 at baseline. The infection with Ct resulted in an increase in active caspse-1 in H8 cells at 12h post-infection (*$p<0.05$), equal levels at 24h and a significant decrease in the levels by 48h post infection (*$p<0.05$). This response was not seen

in the Sw.71 cells where the levels of active caspase-1 remained largely unchanged.

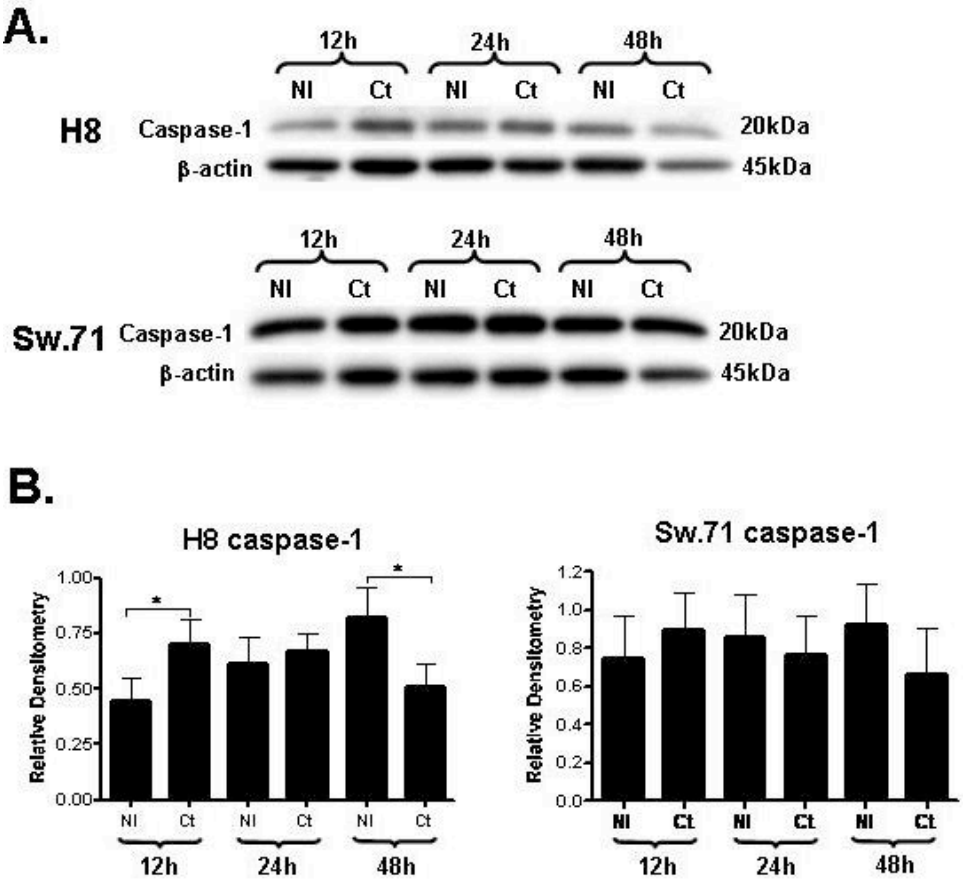

Figure 7. In trophoblasts *Ct* infection (MOI~1) induces slight changes in active Caspase-1 levels. **(A)** H8 and Sw.71 cells were either uninfected (NI) or infected (*Ct*) and at 12h, 24h, and 48h post infection protein lysates were made and analyzed by Western blot using a Caspase-1 antibody that detects the active form of the protein. **(B)** Densitometry of the Caspase-1 bands was calculated and normalized to the corresponding β-actin levels. The relative densitometry graphs are shown. (H8, n=7; Sw.71, n=3). (*$p<0.05$)

Discussion

The placental trophoblast cells are the first line of defense at the maternal-fetal interface and they express TLRs and NLRs in order to detect invading pathogens and mount a rapid and effective immune response. They are distinctive

cells in that they constitutively secrete chemokines like IL-8, MCP-1 and GRO-α which recruit monocytes and macrophages in the absence of a threat [11, 35]. They are responsible for modulating the immune response to ensure that the "foreign" fetus is not attacked by the mother's immune system. These cells are also thought to play a role in trophoblast invasion and remodeling of the spiral arteries to ensure adequate placental development [11,35]. However, in the presence of pathogens like *Chlamydia* these resident immune cells could become infected and activated and further contribute to the inflammatory response.

Previously published studies from our laboratory showed that *Chlamydia trachomatis* serovar D successfully infected two first trimester trophoblast cell lines, H8 which was transformed with SV40 large T Antigen, and Sw.71 which was immortalized by retroviral transduction of the telomerase gene [1]. The infection resulted in a change in the cytokines and chemokines produced by the trophoblasts. Most notably was the induction of the pro-inflammatory cytokine IL-1β, and the increase in the levels of the constitutively secreted IL-8 and IL-6 [1]. Our results confirm the IL-1β, IL-8 and IL-6 cytokine secretion after *Ct* infection, and further demonstrate that *Chlamydia* induces the transcription of IL-1β mRNA, the translation of intracellular pro-IL-1β as early as 12h post infection and the processing and secretion of active IL-1β starting at 24h. While the production of IL-8 and IL-6 after *Chlamydia* infection is mediated through MyD88, the induction of the pro-inflammatory cytokine IL-1β is independent of MyD88. Further we demonstrated that preventing activation of the NALP3 inflammasome by blocking K^+ efflux from cells inhibited IL-1β secretion in both

H8 and Sw.71 cells suggesting a role for NLRs and inflammasomes in mediating this immune response.

While de la Torre *et al*. (2009) [1] saw minimal cell death until 72h post-infection; in our studies we observed an increase in cell death, presumably due to lysis and EB release as early as 48h post-infection. Chlamydia has been shown to block apoptosis in order to complete its life cycle. The differences seen are likely due to the use of a rifampin resistant strain of *Chlamydia* serovar D, which is more aggressive in invading both trophoblasts and HeLa cells, and was observed to complete its life cycle faster. As a result we focused most of our studies in the first 48h after infection.

Chlamydia trachomatis had antigenically diverse surface proteins and can be recognized by multiple TLRs. Some studies show that both chlamydial LPS and the *Ct*-HSP60 protein stimulate TLR-4 [36] and through the activation of caspases it leads to trophoblast apoptosis [37]. However, chlamydial LPS can also signal through TLR-2 [19], and infection with the whole organism also leads to TLR-2 activation [36]. While in animal macrophages TLR-2 stimulation by *Ct* leads to IL-6 and TNF-α production [17], in human epithelial cells we see an up-regulation of IL-8 [20]. This IL-8 response had been shown to be mediated through TLR-2 and its associated adapter protein MyD88, which are recruited to the *Chlamydia* inclusion membrane. The presence of a dominant negative (DN) form of MyD88 results in a reduction in the IL-8 levels [20]. This is consistent with our data in trophoblasts that showed a reduction in IL-8 secretion in all the DN-MyD88 clones in response to both the anti-phospholipid antibody (IIC5)

stimulation and *Chlamydia* infection. This suggests that the DN-MyD88 construct is functional in our cells. To further substantiate that these cells are truly MyD88 dominant negative we can also stimulate them with LPS and look at the NFkB activation, or look for the DN-MyD88 plasmid expression in these cells. We observed a decrease in the IL-6 production in the DN-MyD88 cells which points to a MyD88 dependent TLR, like TLR-2, for mediating the IL-8 and IL-6 secretion by trophoblasts after a *Chlamydia* infection.

IL-1β is a potent pro-inflammatory cytokine that is released by trophoblasts after *Chlamydia* infection. In this study we demonstrated that *Ct* induces the transcription of IL-1β mRNA and the subsequent translation of pro-IL-1β. These processes are thought to be mediated through the NFkB or MAPK pathways in response to a microbial stimulus propagated through PRRs [42]. In addition to TLRs, other PRRs like the NODs can also activate the NFkB pathway and lead to transcription of pro-inflammatory genes. In human macrophages, stimulation of TLR-4 with LPS resulted in induction of IL-1β mRNA and pro-IL-1β [38] which are mediated through the adapter protein MyD88 and the activation of the NFkB pathway. Moreover, MyD88 is believed to be essential for inducing an immune response to Chlamydia infections [36], and while we confirmed this for the secretion of IL-8 and IL-6, MyD88 did not play a role in the production of IL-1β by trophoblasts. Knocking down MyD88 in H8 cells had no effect on the induction of IL-1β mRNA, nor on the levels of intracellular pro-IL-1β or the secreted active-IL-1β. This suggests that MyD88 dependent TLRs are either not stimulated by *Chlamydia*, or the stimulation does not result in the initiation of IL-

1β production. Another possibility is that the induction of IL-1β requires minimal MyD88 activity and a complete inhibition of MyD88 was not achieved. The IL-8 secretion is MyD88 dependent which could be a result of direct stimulation of TLR-2 by *Ct*, or a secondary response mediated by the action of the secreted IL-1β on the IL-1 receptor, which can also associate with and signal through MyD88.

The other potential players in the induction of IL-1β are the NLRs, like the intracellular NOD receptors. The NOD proteins have a C-terminal LRR domain able to recognize PAMPs, a central NOD domain that allow their self-oligomerization and an N-terminal CARD domain that allows them to interact with caspases [21]. NOD1 and NOD2 are present in the cytosol and recognize bacterial products that result from the degradation of peptidoglycan [12]. NOD1 is an ubiquitous receptor for peptidoglycan from gram negative bacteria, and *Chlamydia* was shown to stimulate the NOD1 receptor and activate the NFkB pathway in epithelial cells [21]. The H8 trophoblasts express NOD1 and upon its activation they secrete IL-8 and IL-6 suggesting that NOD1 is functional in these cells [23]. The expression of NOD1 in turn is up-regulated through TLR-4 stimulation by LPS and depicts a close relationship between the different PRRs in mediating an inflammatory response [23]. Therefore, the IL-1β transcription and translation in H8 cells infected with *Chlamydia* could be mediated through NOD1 and further studies could shed more light on the exact mechanisms involved.

IL-1β depends for its processing and secretion on the activity of caspase-1. Caspase-1 activation can occur upon the assembly and activation of an inflammasome, which is a complex of proteins that contains a sensor NLR (like

NALP1, NALP3 or IPAF), the adapter ASC and pro-caspase-1. Inflamamsomes are thought to first undergo ”priming” (transcriptional up-regulation of their components) mediated through NFkB or sometimes TNF in response to stimuli such as bacterial infections, and then assembly into the active forms [22]. Monocytes and macrophages infected with *Chlamydia* have been shown to secrete caspase-1 dependent IL-1β, and to assemble inflammasomes for its processing [26]. Recent studies in HeLa cervical epithelial cells showed that infection with *Chlamydia trachomatis* leads to caspase-1 activation and this process is dependent on the activation of the NALP-3 inflammasome and the presence of the adapter ASC [26]. The NALP-3 inflammasome can be activated in response to a variety of molecules, but K^+ efflux from cells seems to be one of the main up-stream events. The inhibition of K^+ efflux by increased extracellular potassium (KCl) resulted in reduced caspase-1 levels and presumably reduced NALP-3 inflammasome activation in epithelial cells [26]. This study however could not study the role of the NLAP-3 inflammasome on IL-1β secretion since the epithelial cells do not produce this cytokine.

As we observed an up-regulation of pro-IL-1β and the accumulation of intracellular IL-1β in our *Ct* infected trophoblast cells, we wanted to investigate whether the NALP-3 inflammasome and caspase-1 play a role in this process. Our results show a statistically significant decrease in IL-1β production in both H8 and Sw.71 trophoblast cells infected with *Chlamydia* when the cells are incubated with high extracellular KCl and the NALP-3 inflammasome activation is inhibited. This suggests a role for the NALP-3 inflammasome in the IL-1β

processsing in trophoblast cells after *Ct* infection, which is consistent with studies done in other cell types. To further test if the KCl treatments truly inhibited the NALP3 activation we can also look at the activation of caspase-1 or the production of IL-1β in response to known NALP3 activators like ATP in the presence of high KCL levels.

To further substantiate the role of NALP-3 and ASC in inducing IL-1β in trophoblasts we pursued studies to knock down the function of these proteins by using shRNA technology. The shRNA constructs transfected into the cells would bind to the NALP-3 or ASC mRNA and target it for degradation thus lowering the levels of the expressed proteins. We used shRNA constructs obtained from Dr. Jenny Ting, which showed a NALP-3 and ASC dependent IL-1β immune response to the influenza-A virus in monocytes [39]. In Sw.71 trophoblast cells there was more than 50% reduction of mRNA for both ASC and NALP-3; however there was no reduction in the levels of NALP-3 protein. ASC protein levels were reduced by 40-50%, however this was not sufficient to observe an effect on the production of IL-1β (data not shown). We are now pursuing the use of commercially available shRNA constructs from Sigma as described in [26] to obtain more efficient protein knockdown. If NALP-3 and ASC prove not to be the major mediators of IL-1β processing in the trophoblast cells, another possibility is the NLRC4/IPAF inflammasome which can assemble and lead to caspase-1 activation in the absence of ASC [22]. The IPAF inflammasome is activated by flagelin and other components of gram negative bacteria that have type III secretion systems such as *Chlamydia,* [43] which make IPAF it a likely candidate.

While it was shown that *Ct* induces the activation of caspase-1 in epithelial cells [26], the levels of active caspase-1 obtained in our western blot analysis showed only a modest increase in the H8 infected cells, and no change in the Sw.71 cells. The discrepancy can be due to the fact that the trophoblasts constitutively express caspase-1 and these low levels of constitutive caspase-1 present in the cells could be sufficient for the processing of the pro-IL-1β induced by *Chlamydia* infection. The constitutive caspase-1 activation could also be responsible for the presence of active IL-1β seen in these cells at baseline. Further studies looking at active caspase-1 levels by other methods or inhibiting the activity of caspase-1 would help determine the requirement for caspase-1 activation in the IL-1β secretion by trophoblasts.

The processing and secretion of IL-1β is thought to be tightly coupled and occur is fast succession in the presence of a stimulus that activates an inflamamsome [22]. However, we observed that both the uninfected and infected H8 and Sw.71 trophoblast cells have intracellular IL-1β, but only the infected cells secrete it. This suggests yet another signal requirement/checkpoint for the secretion of this highly potent inflammatory cytokine. The trophoblasts appear to make and store small amounts of IL-1β to have readily available for secretion in the case of a pathogen invasion. The secretion mechanisms for IL-1β are still poorly understood, and IL-1β does not have the hydrophobic signal sequence required for entering the classical secretory pathway involving the endoplasmic reticulum and the Golgi apparatus [27]. A non-classical pathway hypothesized to result in IL-1β secretion involves exocytosis from secretory lysosomes, shedding

of plasma membrane microvesicles and export through the plasma membrane using specialized transporters [27]. This wide variety of mechanisms involved offers the opportunity for pathogens like *Chlamydia* to act and modulate the secretion of IL-1β or other cytokines.

In summary we have shown that *Chlamydia* is able to induce IL-1β mRNA transcription in a MyD88 independent manner in trophoblasts, which is a novel observation and is not consistent with mechanisms described in other cell types. Secondly we have some evidence that processing of pro-IL-1β occurs through the NALP-3 inflammasome. Thirdly, *Chlamydia* infection can stimulate the secretion of active-IL-1β from trophoblast cells. This last observation uncovers yet another step at which *Chlamydia* can act and modulate the immune response in trophoblast cells. The requirement of at least two separate signals to produce and secrete inflammatory cytokines like IL-1β highlights the close relationships between the different receptors and pathways in ensuring a tight regulation of pro-inflammatory immune responses.

In a normal pregnancy the immune system plays the critical task of protecting the fetus from the constant threat posed by pathogens while limiting the effects of the mother's immune response on the fetus itself. Both viral and bacterial infections have been linked to preterm labor, spontaneous abortions [3] and intrauterine growth restriction [40,41]. Moreover, evidence of intrauterine inflammation and chorioamnionitis was noted in up to 85% of placentas from preterm deliveries even when the amniotic fluid cultures were negative [16]. *Chlamydia trachomatis* is one of the organisms detected in the deciduas and

placentas from complicated pregnancies [5, 7, 8]. The placental trophoblasts attract a variety of immune cells to the maternal-fetal interface in response to pathogens like *Chlamydia*, and an amplified inflammatory response, as seen by the secretion of IL-1β, can lead to initiation of early parturition. The inflammation and scarring of the upper genital tract after *Chlamydia* infections has also been well documented and it can prevent implantation and further successful pregnancies. Through the many mechanisms *Chlamydia* possesses to modulate the immune system, as described above for IL-1β, it can have a negative impact on pregnancy outcomes and a woman's reproductive potential.

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