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TLR signaling pathway orchestrating pro-inflammatory cytokines and differential expression of chemokines in *C.trachomatis* infected primary cervical epithelial cells

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ABSTRACT

Human Primary cervical epithelial cells offer a better opportunity to investigate factors contributing to initial *Chlamydia trachomatis* infection and subsequent pathology. In a time dependent in-vitro study significantly elevated levels of IL-8, IL-6 were detected at 12 hours post infection (hpi) and TNF-α at 18 hpi, ($p<0.001$) in *C. trachomatis* infected primary cervical epithelial cells as compared to uninfected control. IL-8 and IL-6 were found consistently increased from 12 hpi to 72 hpi showing thereby probable involvement of IL-8 in chlamydial differentiation and metabolic activities. In human inflammatory cytokines and receptors pathway array studies at 12 hpi, genes found significantly up-regulated were *ccl1, ccl5, ccr, cxcl10, cxcl12, ifn, il-8 and tnf ($p<0.1$)*, this may be to maintain a long term interaction between host cell and bacteria. In human TLR signaling pathway studies at 6hpi showed significantly up-regulated genes of *Il-1ß, Il-6, Il-8, Irak1, Myd88, Ripk2, Tirap and Tlr2* ($p<0.1$) as compared to uninfected control and there was requirement for TLR-2 in MyD88 in cellular activation during *C. trachomatis* infection.

KEYWORDS

Chlamydia trachomatis;
Primary cervical epithelial cells;
TLR;
Cytokines;
Chemokines.

INTRODUCTION

Diseases caused by chronic or repeated *Chlamydia trachomatis* infection are mainly due to inflammation mediated tissue damage. Inflammatory factors are known to be up-regulated by epithelial cells during chlamydial infection[1]. *C.trachomatis* has a unique developmental cycle that begins with attachment of infectious but metabolically inactive elementary bodies to host cell surface. The host response to primary chlamydial infection at mucosal surfaces occurs within 1–2 day of infection and is characterized by inflammation and mucosal infiltration with neutrophils and small numbers of monocytes[2]. Cervical epithelium is the target cell layer to encounter microbes ascending to the female reproductive tract. Both types of columnar epithelium and squamous epithelium form the physical barrier to invading microbes including *C.trachomatis*, however how they mediate innate immune protection remains unclear. Further the presence of TLR on the epithelial cell is integral between the innate and adaptive immune

Regular Paper

response[3].

The process of development of effective in vitro model against *C. trachomatis* infection would be greatly facilitated by the availability of in vitro model that more accurately predict in vivo efficacy. It was earlier shown that in vitro infection of cervical and colonic epithelial cells with *C.trachomatis* induced the secretion of an array of pro-inflammatory cytokines[4]. There is a critical time in the host response to the infection as the array of chemokines and cytokines produced will determine the nature of the host inflammatory response and the types of cells recruited. Therefore a time frame invitro study in which one is able to associate the gene expression of particular chemokine and cytokine genes is important to extrapolate the mechanism.

There have been number of reports characterizing the chemokines and cytokines response using in vivo mouse infected in the genital tract with *C.trachomatis* or in Hela cells. In general IL-1á, IL-1â, IL-6, IL-8 and TNF-á are elicited and all require active infection of the target cell, strongly indicating that the intracellular growth of chlamydia may be a key factor in stimulating the appropriate intracellular pathways for the production of these molecule[4,5,6,29]. It has been reported that *C.trachomatis* infected epithelial cells also elicit chemokines including CCL-1, CCL-2, CCL-3, CCL5 (RANTES), CCL-11 (eotaxin), CXCL-2, CXCL-9 (monokine induced by IFN-ã) and CXCL-1, 10 (Interferon - inducible protein 10[IP-10]) and 16[7]. IL-8 response is controlled at the level of transcription with multiple promoter elements and the factor that bind to these elements are regulated by cellular signaling pathways of which NF- êâ and extracellular signal related kinase (ERK) independent of p38 and jun N-terminal MAPK (mitogen activated protein kinase) have been shown to be necessary for the endogenous IL-8 response to *C.trachomatis*[8].

Also, TLR signaling elicits a pro-inflammatory response characterized by the secretion of cytokines like tumor necrosis factor (TNF), type I and II interferon (IFNs), and chemokines, which in turn control the recruitment of inflammatory cells to infected tissues[9]. In chlamydia pathogenesis, TLR2 is recruited to intracellular Chlamydia and is required for cellular activation required for IL-8 secretion during infection. Indeed, TLR signals apoptosis through the TLR adapter molecule, myeloid differentiation factor 88 (MyD88), via a pathway involving Fas-associated death domain protein and caspase-8, associated with Fas-mediated apoptosis. It has also been reported that *C.trachomatis* cell wall components interact with TLR-2 and to a lesser extent with TLR-4 on DCs to stimulate IL-12 secretion and therefore a Th1 response[10]. However, there has been no study dedicated to evaluate potential role of secreted and proinflammatory cytokines pathway with chemokine expression pattern and gene expression of TLR in primary cervical epithelial cells that are privilege target for *C.trachomatis* infection. Therefore the study reported herein offers a new perspective in understanding chlamydial pathogenesis.

MATERIALS AND METHODS

Enrollment of subjects

Cervical cells were obtained, after informed consent, from 15 women attending the gynecology OPD of Safdarjung Hospital, New Delhi, India undergoing routine checkup. The Hospital's ethics review committee approved the study. All cytobrush samples had negative results for blood contamination. No cervical specimens were taken from menstruating patients, or if blood was visible in the cervical area or if the epithelium appeared disrupted. Diagnosis of *C. trachomatis* and other STD pathogens as were done as previously reported[11].

Culture of Primary epithelial cells

Cervical samples were centrifuged at 2000 rpm for 10 min at 4° C, and pellet obtained was washed twice with PBS at 1000 rpm. To check the viability of the cells trypan-blue staining was performed on a hemocytometer. Pure populations of primary cervical epithelial cells were isolated using magnetic labeled EpCAM microbeads (Miltenyi Biotech). The final pellet was re-suspended in Keratinocyte –SFM (Gibco) supplemented with epithelial cell growth factor like recombinant epidermal growth factor (0.2 ng / mL) and bovine pituitary extract (25 µg/ ml) into another plate. These cervical epithelial cells

Regular Paper

(2.5 x 10^5 cells) were incubated for 3 days to reach 80-90% confluence in order to avoid contact inhibition of the cells. The epithelial cells were confirmed by staining with a cytokeratin-specific antibody (ICN pharmaceuticals, Costa Mesa, Calif., USA) and the absence of leucocytes was verified by the lack of staining with an antibody specific for the leukocyte common antigen, CD45 (Dako Corp., Carpinteria, Calif., USA).

Infection protocol

For infection, cervical epithelial cells (2 x 10^5 cells/ well) were seeded in tissue culture flask (25 cm^2); after 1 day cells were infected with Chlamydial serovar D as described previously [12]. One multiplicity of infection (MOI) was defined as equivalent to one infectious EB per cell. To infect primary cervical epithelial cells, culture medium (1–2 ml) containing the required number of chlamydial EBs corresponding 1 MOI were added. After infection, cells were centrifuge at 1800 x *g* for 60 min at 37°C. After centrifugation media was replaced to remove nonadherent chlamydial EBs. Infected epithelial cells were suspended in DMEM medium containing 10% (v/v) FCS incubated at 35°C in a 5% CO2 incubator for various time periods. Supernatants were collected at 24 h post-infection, frozen at -80°C and analyzed later for cytokines by ELISA as per manufacture instruction.

Gene expression profile of Inflammatory Cytokines and Receptors and TLR pathway array

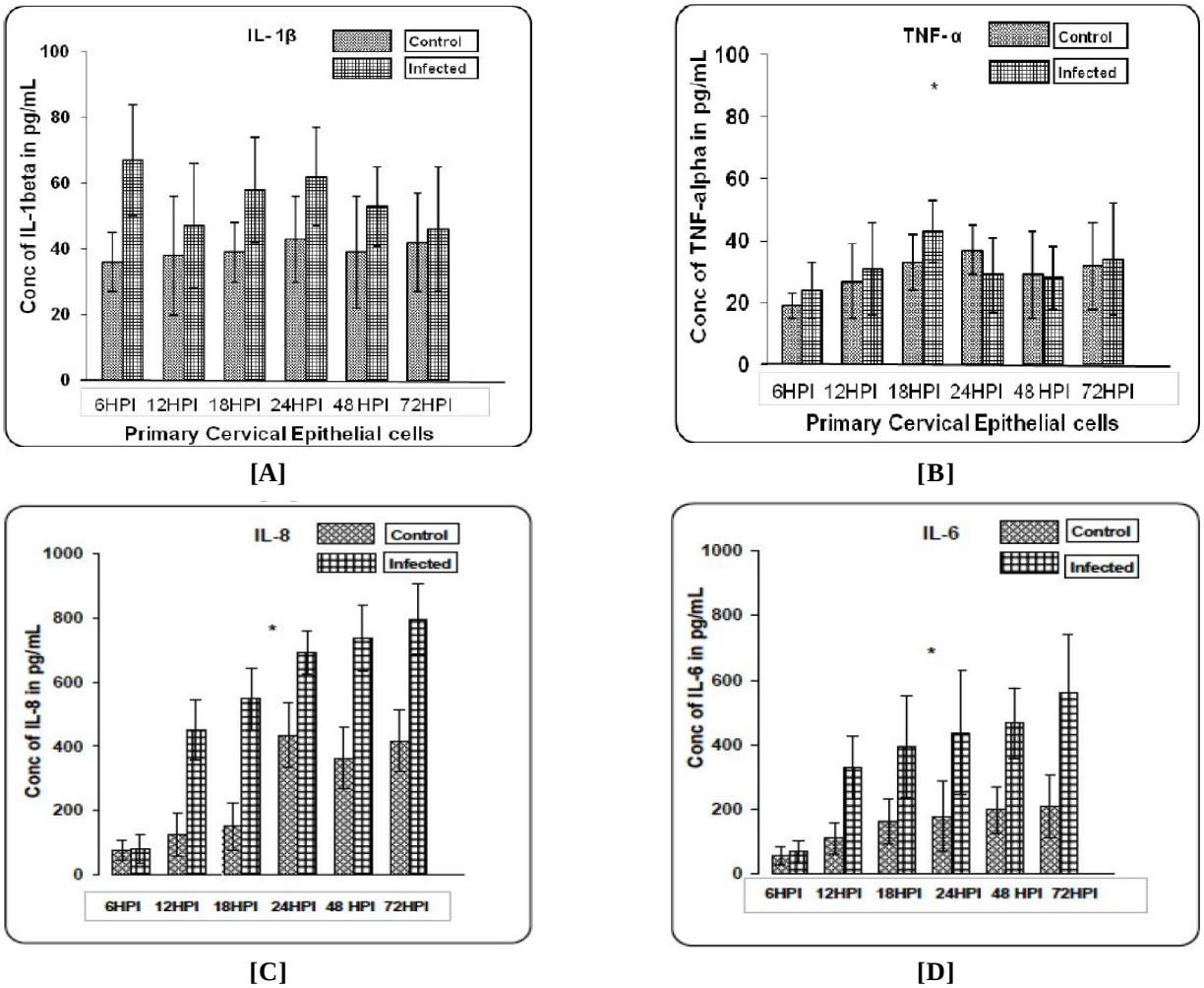

Figure 1 : Concentration of secreted cytokines (pg/ml) in supernatants of *C.trachomatis* infected cervical epithelial cells at various time points. * *P<0.*05, *P<0.*01 as compared to uninfected control epithelial cells. [A] Conc of IL-1β in pg/ml, [B] Conc of TNF-α in pg/ml, [C] Conc of IL-8 in pg/ml, [D] Conc of IL-6 in pg/ml. Control-uninfected cells and C.trachomatis infected epithelial cells.

Regular Paper

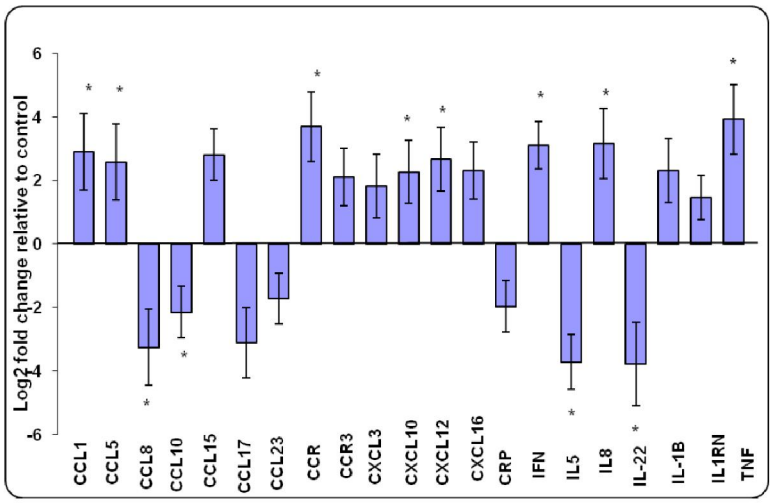

Figure 2 : Inflammatory Cytokines and receptor pathway gene expression profile in *C.trachomatis* infected cervical epithelial cells compared to uninfected cells (control) expressed as log2 fold change.* Significantly different from control ($P < 0.01$).

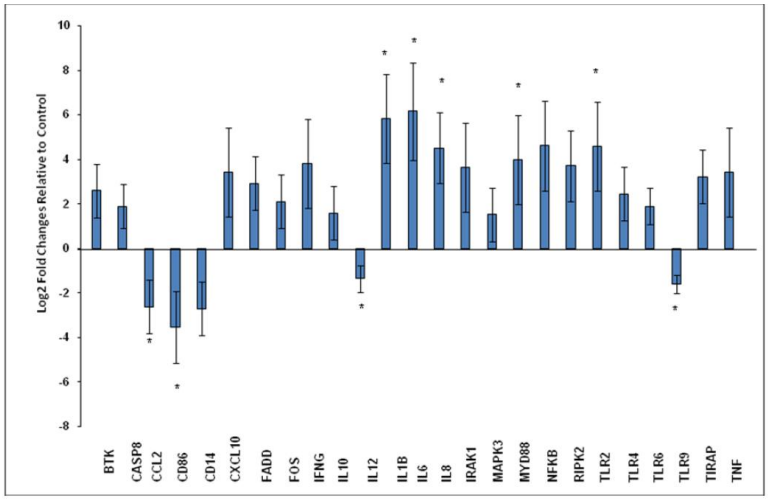

Figure 3 : Toll like receptor signaling pathway gene expression profile in *C.trachomatis* infected cervical epithelial cells compared to uninfected cells (control) expressed as log2Fold change relative to control. Data are presented as means of three individual experiments. * Significantly different from control ($P < 0.01$).

Epithelial Cells were seeded in 6-well plates at a density of 2 x 10^5 cells/well and allowed to attach for 1 day. Epithelial cells were infected with Chlamydial EBs as described before for 6 hr and 12 hr for gene expression profile of TLR pathway and human inflammatory cytokines and receptors pathway array and uninfected cells were taken as control. After exposure, total RNA was extracted from the cells using the RNAeasy Mini Kit according to manufacturer's instructions, as reported earlier[1]. cDNA was generated from 1 mg total RNA using the Reaction Ready First Strand cDNA Synthesis kit (SABioscience, MD, USA) according to manufacturer's instructions. The both human signaling pathway RT2-PCR-Profiler PCR Array (SABioscience, MD, USA) was carried out according to manufacturer's instructions using the Real-Time PCR Detection System (Eppendorf, Hamburg, Germany). The raw data from both the control and the treated groups were obtained and uploaded into GEarray Analyzer software (SABioscience, MD, USA) for analysis and verification of appropriate experimental procedures.

Statistical analysis

The differences between experimental groups were analyzed by using the Student's paired and unpaired *t* test. All data are presented as mean ± SE

Regular Paper

and a difference in mean values was considered significant when the *P* value was <0.05. Gene expression data presented are expressed as mean ± SE. *C. trachomatis* induced changes in gene expression using the RT2-PCR-Profiler PCR Array were statistically analyzed using a Student t test.

SI Units

1 ng/ml: nanogram/milliliter
2 mg: milligram
3 pg/ml: pictogram/millilitre

RESULTS

Secreted cytokines by infected primary cervical epithelial cells

The levels of various cytokines (IL-1ß, IL-6, IL-8 and TNF-á) were quantified in the culture supernatants of infected primary cervical epithelial cells. Significant differences were observed in the levels of TNF-á ($P < 0.001$) and IL-1â ($P < 0.001$) in the supernatants of infected cells as compared to uninfected controls (Figure 1A-B). The median levels of IL-6 and IL-8 were significantly (P<0.05) higher as compared with uninfected controls (Figure 1C-D).

In time dependent study significantly elevated level of IL-8 and IL-6 secretion were detected at 12 hpi, ($P < 0.001$) and TNF at 18hpi (P <0.001) in *C. trachomatis* infected primary cervical epithelial cells compared to control (mock infected epithelial cells) (Figure 1 B-D). Secretion of IL-1â was not significantly elevated at 18 hpi as compared to uninfected control primary cervical epithelial cells, Further highest level of IL-1â seen only at 6hpi ($P < 0.001$). Levels of secreted cytokines IL-8 and IL-6 were found consistently increased from 12hpi to 72hpi as compared to uninfected control primary cervical epithelial cells.

Gene expression profile of Inflammatory cytokine pathway and TLR pathway

In order to assess the gene expression profile of Inflammatory cytokines, primary cervical epithelial cells were infected with *C. trachomatis,* and Human Inflammatory cytokines and TLR pathway real time PCR array (SA biosciences) were used to screen for changes in 84 genes involved in both pathway. The human inflammatory cytokines and receptors pathway array was carried out according to manufacturer's instructions and significant fold changes observed for various genes in the primary cervical epithelial cells infected over uninfected controls were recorded. After 12 h of infection, genes which were found significantly upregulated were *ccl1, ccl5, ccl15, ccr, cxcl10, cxcl12, ifn, il-8 and tnf* (P<0.1) (Figure 2). While *ccl8, ccl10, il-5 and il-22* genes were found significantly down regulated in *C. trachomatis* infected primary cervical epithelial cells as compared to uninfected control (P<0.1) (Figure 2). Data obtained indicate that at 12 h post infection inflammatory cytokines genes were upregulated, in order to maintain a long term interaction between host cell and bacteria.

The human TLR signaling pathway RT2-PCR-Profiler PCR Array was carried out according to manufacturer's instructions and significant fold changes observed for various genes in the infected over uninfected controls were recorded. After 6 h of infection at MOI of 1, genes which were found significantly upregulated were *Il-1ß, Il-6, Il-8, Irak1, Myd88, Ripk2, Tirap and Tlr2* (Figure 3). *Ccl2, Cd 86, Il-12, and Tlr9* genes were found significantly down regulated in Chlamydia infected as compare to uninfected control (P<0.1) (Figure 3). The raw data from both the control and the treated groups were obtained and uploaded into GEarray Analyzer software (SABioscience, MD, USA) for analysis and verification of appropriate experimental procedures.

In this in vitro study, it was observed that in *C.trachomatis* infected primary cervical epithelial cells level of IL-1â was increased significantly after 6 hpi, which is an important agonist for proinflammatory cytokines and that downstream signalling by IL-1â, could be the mechanistic basis of pathology. It has been reported that IL-1á and IL-1â exert a similar biological function by binding to Interleukin -1 receptor IL-1R1, and triggering downstream effects, including induction of proinflammatory cytokines[13]. During chlamydial infection IL-1â is produced in large amounts by infiltrating macrophages and neutrophils, suggesting

Regular Paper

that it is primary cytokine in downstream IL1R activation and IL-1 are the key initiator of tissue destruction and inflammation during *C. trachomatis* infection. There was log twofold change at 12 hpi in Interleukin -1 receptor antagonist IL1RN and IL-10 at the gene expression level in our study. It has been reported that IL1RN acts as blocking agent and in reduced induction of IL-1â and also IL-10 has been reported to diminish the destructive effect of *C.trachomatis* induced by IL-1 on the epithelium.(14) Based on earlier study it is reported that IL-10 inhibit IL- 8, however in the present study constant induction of IL-8 strongly supports that IL-1 is the key initiator of infection and tissue destruction. As is evident from our gene expression data there is significant increase in FADD Ligand that bridges between FAS receptor and caspase 8 death receptor enzymes. Caspase has a strong role in exacerbating the fallopian tube inflammation induced by primary infection and also reported to plays a dominant role in chlamydia induced oviduct inflammation[15, 16, 17, 18]. In our study TNF-á was expressed early at 6 hpi and consistently increased thereafter from 6, 12, 18 and 72 hpi. The markedly late induction of TNF-á at (18 hpi) relative to IL-8 which is also highly significant at 18 hpi onwards and its dependence on bacterial growth and protein synthesis implicate direct intracellular interactions of bacteria and host cell][14,19].

The level of secretory IL-8 increased significantly at 12 hpi ($P < 0.001$) till 72 hour post infection (hpi) in comparison to control cells showing thereby probable involvement of IL-8 in early phase of chlamydial differentiation and metabolic activities at 12 hpi. Elevated level of IL-8 might help epithelial cells at the mucosal surface to communicate with professional immune cells in response to pathogenic insult. Continuous IL-8 production can promote the infiltration of neutrophils that are not only inefficient in resolving chlamydial infections but can also release proteases that damage cells. Also activation of the MAPK/ERK pathway occurs at similar times as IL-8 up-regulation and thus was also likely candidate to be involved in the IL-8 response[14].

In our study the consisting higher trend of IL-6 at different time point implies that IL-6 is a prolonged or sustained response to chlamydia compared to many other cytokines, which is likely important in the diseases setting as is also reported earlier[20,21].

In our in-vitro study expression profile of genes involved in Inflammatory Cytokine and receptors pathway showed the up regulation of genes like *ccl1, ccl15, ccr, cxcl10, cxcl12, IFN, IL-8 and TNF* genes in primary cervical epithelial cells infected with *C. trachomatis* as is also reported earlier[6,22]. Further in this study we observed, after 12 hr infection the host response included a large no of chemokines expression associated with Th1 - CCL1, CCL5, CCL15, CCR, CCR3, CXCL3, CXCL10, CXCL12, CXCL16 were all transcribed at high level, which was noteworthy, although CCR3, CXCL3, CXCL16 were not significantly higher. These findings may have been due to the fact that may be CCR3, CXCL3, and CXCL16 protein level peaked at a time point earlier than the one at which we measured expression or is not expressed in primary cervical region. Also in a previous study it is reported that chlamydia infection in mice suggested that there are regional difference in the immune response between cervical vaginal region and oviduct[23].

Previous studies on chemokines demonstrated that induction of CXCL10 and CCL5 responsible for selective recruitment of the cells during chlamydial infection[23,24]. Therefore these would be important chemokines in controlling the course of infection that participate in up regulating Th1 response IFN-ã which has concomitantly attained high level in our study and also for the initiation of adaptive immune response and the recruitment of memory T cells in case of re-infection.

Our results demonstrated that mRNA expression of TLR 2 was significantly high compared to TLR4 in primary cervical epithelial cells which is essential for infection for maximal expression of inflammatory cytokine indicating a role for TLR2 in inflammation induced by *C.trachomatis*. The decreased expression of TLR-4 reflective of complex microbiota and chemokine receptor molecule associated with this cervical region. It is possible that under conditions of low TLR 4 expression, TLR 2 is activated, either because of redundant or alterna-

tive ligand recognition[25] and expression profile of genes involved in TLR pathway showed the up regulation of genes like *Il-1ß, Il-6, Il-8, Irak1, Myd88, Ripk2, Tirap and Tlr2* genes in *Chlamydia* infected cells. We found a requirement for both TLR2 and MyD88 in cellular activation during chlamydia infection, Furthermore; our data demonstrates that cells are activated not simply by ligands expressed at the surface of the elementary body, but by a ligand or ligands that are expressed during an active and productive intracellular infection.

MyD88 also has an intermediate domain that is crucial in TLR signaling as it interacts with IL-1R associated kinase (IRAKs)[26]. Our data also suggested that *Tirap* gene have important role in TLR2 signaling. Previous study of TIRAP/Mal knockout mice showed normal responses to the TLR3, TLR5, TLR7 and TLR 9 ligands, but were defective in TLR2 ligand- induced inflammatory cytokines production[27,28,29]. Taken together, these studies clearly established that TIRAP/Mal is essential for the MyD88 dependent signaling pathway via TLR2 but not for MyD88 independent pathway. These data also suggested that TLR2 expression is augmented by TNF-á. TLR 4 engagement, which results in the production of TNF-á and PAMPs would precipitate a more vigorous inflammatory response subsequent to increased expression of TLR-2. TLR1, TLR3, TLR 5 and TLR 6 are also present in the human female genital tract, but they do not recognize *C. trachoamtis* PAMPs[30].

ABBREVIATION

C.trcahomatis	:	*Chlamydia trachomatis*
RANTES	:	Regulated on expression, nomal T cell expressed
STD	:	Sexually transmitted disease
TLR	:	Toll like receptor
MOI	:	Multiplicity of infection
EB's	:	Elementary bodies
DMEM	:	Dulbecco's essential medium
FCS	:	Fetal calf serum
ELISA	:	Enzyme linked immunosorbant assay
Hpi	:	hours post infection
RT-PCR	:	Reverse transcript Polymerase chain reaction.
Th1 and Th2	:	T helper cell

CONCLUSION

In our study TLR 2, TLR4, TLR6 are activating MyD88 dependent IL-8 signaling pathway in *C.trachomatis* infected cervical epithelial cells. From our data, we inferred, that TLR9 does not signal via MyD88 dependent, which is unlikely to be involved in sensing chlamydia infection. The data presented here suggests that the co-interaction between epithelia and chemotactic adhesion cells has a greater effect on immunopathological outcomes than monocytic and epithelial cell responses alone. This information will serve as a baseline for future studies of the mechanisms by which production of these mediators are elicited and in development of prognostic biomarker in chlamydial genital infection and will help in finding the mechanism and basis of asymptomatic nature of chlamydia infection.

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Regular Paper

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