Cervical Mucosal Immune Responses and Role of Antigen Presenting Cells During *Chlamydia trachomatis* Infection

THESIS

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by

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Under the Supervision of

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CERTIFICATE

This is to certify that the thesis entitled **'Cervical Mucosal Immune Responses and Role of Antigen Presenting cells During *Chlamydia trachomatis* Infection'** which is submitted for award of Ph.D. Degree of the Institute, embodies original work done by her under my supervision.

Signature in full of the Supervisor Aruna Singh

Name in capital block letters **Dr ARUNA SINGH**

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Date: 20-5-2010

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I actually feel that happiness lies in the pursuit as much as in reaching the goal, therefore, at this juncture I bow my head with great reverence to Him who is omnipresent and the cause behind every effect.

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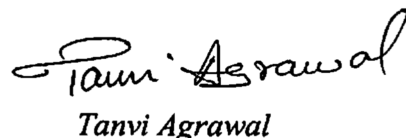

May 20th, 2010 *Tanvi Agrawal*

BSA	Bovine Serum Albumin
CBA	Cytokine Bead Array
cDNA	Complementary DNA
cHSP	Chlamydial Heat Shock Protein
CT	*Chlamydia trachomatis*
Cm	Centimeter
CRP	C-reactive protein
CPM	Count Per Minutes
CL	Cervical lymphocyte
DC	Dendritic cells
DFA	Direct Fluorescent Assay
ddH_2O	Double Distilled water
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
dTTP	Deoxythymidine tri phosphate
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbant assay
FCS/FBS	Fetal Calf/Bovine Serum
FD	Fertility Disorders
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
Hr/hrs	Hour/hours
HRP	Horseradish peroxidase
IFU	Infection forming unit
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
Kb	Kilo base
LPS	Lipopolysaccharide
M	Molarity
MPC	Mucopurulent Cervicites
mDC	Myeloid Dendritic Cells
Mg	Milligram

MHC	Major histocompatibility complex
Min/mins	Minute/Minutes
mL	Millilitre
mm	Milimeter
mM	Milimolar
N	Normailty
$NaHCO_3$	Sodium bicarbonate
ng	Nanogram
NO	Nitric oxide
^0C	Degree Celsius
OD	Optical Density
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate buffered Saline with Tween-20
PCR	Polymerase chain reaction
pDC	Plasmacytoide Dendritic cells
PHA	Phytohaemaglutinin
pg	Picogram
RNase	Ribonuclease
rpm	Revolution per minute
RT-PCR	Reverse transcription- PCR
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SE	Standard error
TAE	Tris acetate EDTA
TLR	Toll Like Receptor
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) amino acid
WHO	World Health Organization

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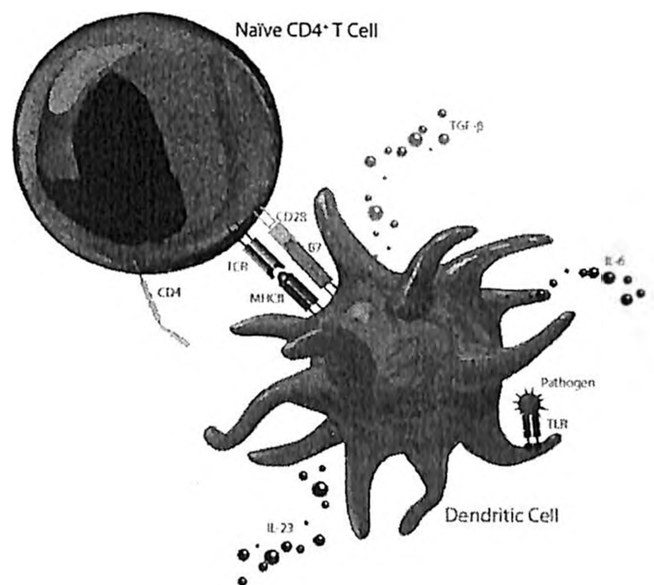
ABSTRACT

Everything is everywhere, the environment selects

M.W. Beijerinck



Abstract

Infection with *Chlamydia trachomatis* can lead to a variety of diseases including ectopic pregnancy, infertility and blindness. Exposure of host to *C. trachomatis* stimulates multiple innate and adaptive immune effectors that contribute towards controlling bacterial replication. However, these effectors are often insufficient and continued presence of *C. trachomatis* within the host chronically induces inflammatory cytokines leading to pathology. A vaccine that can stimulate immunity while avoiding pathology is still lacking as till date no data on mucosal immune response in human females infected with *Chlamydia* is available. Further, role of antigen presenting cells like dendritic cells in modulating immune response to *Chlamydia* is also not well understood.

Thus, in the present study mucosal host-pathogen interactions during *Chlamydia trachomatis* infection were assessed. The study revealed that IFN-γ levels were significantly higher in cervical washes of women with recurrent chlamydial infections and can act as single best predictor of women at risk of developing sequelae to *C. trachomatis*. Further, in women with fertility disorders high levels of both pro (IFN-γ) and anti-inflammatory cytokines (IL-10) were found to be present leading to incomplete clearance of bacteria and thus persistence. *Chlamydia* stimulated cervical cells from women with recurrent infection responded significantly to chlamydial heat shock proteins compared to chlamydial surface antigens. This confirmed that persistent *Chlamydia* was responsible for developing pathology rather than active infection. Differences in cytokine secretion pattern of cervical cells, stimulated with chlamydial Elementary Bodies (EBs)) from women with (secretion of IL-6, IL-8 and IL-10) or without (IFN-γ and IL-12) pathological conditions suggests that changes in secreted cytokine pattern by host can be responsible for development or non-development of

pathology. Both myeloid (mDCs) and plasmacytoid dendritic cells (pDCs) were recruited to the cervical mucosa during chlamydial infection. pDCs were found to be higher in women with fertility disorders with significant correlation with inflammatory factors like C-reactive protein. In contrast, mDCs correlated significantly with protective immune responses revealing that while mDCs provide protection, pDCs are involved in pathogenesis.

Both TLR2 and TLR4 were detected on cervical monocytes and were upregulated when pulsed with chlamydial EBs showing their role in recognition of *Chlamydia.* Late induction of iNOS expression with nitric oxide production showed its involvement in direct killing of bacteria. Production of IL-12 by cervical monocytes was dependent on TLR4 suggesting role of TLR4 in initiation of Th1 immune response. Inhibition of MyD88 reduced IL-12 production by monocytes but not completely, suggesting the presence of both MyD88 dependent and independent downstream signalling in cervical monocytes.

Chlamydia pulsed dendritic cells efficiently presented chlamydial antigens to $CD4^+$ T cells. The activated T cells secreted IL-2 and IFN-γ. However, pretreatment of cells with estradiol significantly reduced TLR4 expression and up-regulated IL-10 secretion modulating the Th1 type immune response to Th2 type enhanced infection in case of women with high estradiol levels (hormonal imbalance, pregnancy or contraceptives).

Thus, extrapolation of these mucosal and *in vitro* results will help in designing of an effective strategy to develop vaccine against *Chlamydia trachomatis.*

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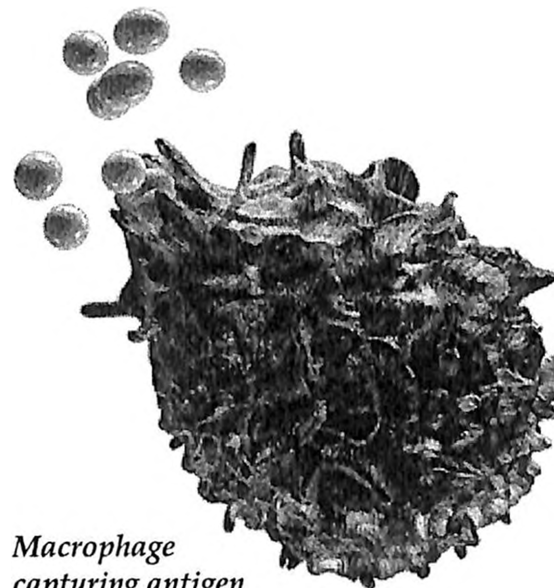
Chapter 1
INTRODUCTION

There is nothing more difficult to take in hand, more perilous to conduct or more uncertain in its success than to take the lead in the introduction of a new order of things

Niccolo Machiavelli



Macrophage capturing antigen

Introduction

Chlamydiae are obligate intracellular bacterium causing an array of diseases in both humans and animals [Schachter & Caldwell, 1980]. *C. trachomatis* and *C. pneumoniae* are human pathogens while *C. psittaci* and *C. pecorum* are primarily animal pathogens. *C. trachomatis* is a leading cause of sexually transmitted bacterial infections and trachoma, the leading cause of preventable blindness [Jones, 1995; Pannekoek, 2000]. *C. trachomatis* infection causes urethritis, epididymitis and prostatitis in males [Stamm *et al.*, 1984] while in females it causes cervicitis, endometritis, salpingitis and pelvic inflammatory diseases (PID). It also has long term consequences include infertility, ectopic pregnancy and chronic pelvic pain that are secondary to scarring of fallopian tubes and ovaries [Hare & Thin, 1983]. Infection with *Chlamydia* can also cause premature birth or spontaneous abortions [Nyari *et al.*, 2001]. In addition, infection with *C. trachomatis* felicitates the transmission of Human Immunodeficiency Virus (HIV) [Antilla, 2001] and might be a co-factor in human papilloma virus (HPV)-induced cervical neoplasia [Gopalkrishna *et al.*, 2001; Miller *et al.*, 1992]. *C. pneumoniae* has been recognized as a major cause of respiratory infections and has been associated with new onset of asthma, exacerbation of chronic asthma, atherosclerotic disease [Grayston, 1992; Saikku *et al.*, 1988] myocarditis and recently Alzheimer's dementia [Balin *et al.*, 1998]. *C. psittaci* causes endemic avian chlamydiosis resulting in ocular, respiratory and gastrointestinal infections and life threatening pneumonia in humans [Storz, 1988].

Latest international estimates show that around 92 million new cases of chlamydial infections are occurring every year (Figure 1.1). The highest prevalence of *Chlamydia* in adults has been reported in South-East Asia (43 million) followed by Sub-Saharan Africa (15 million)

[WHO, 2001]. *C. trachomatis* infection remains sub clinical in a high proportion of infected individuals (70-90% of women and 30-50% of men).

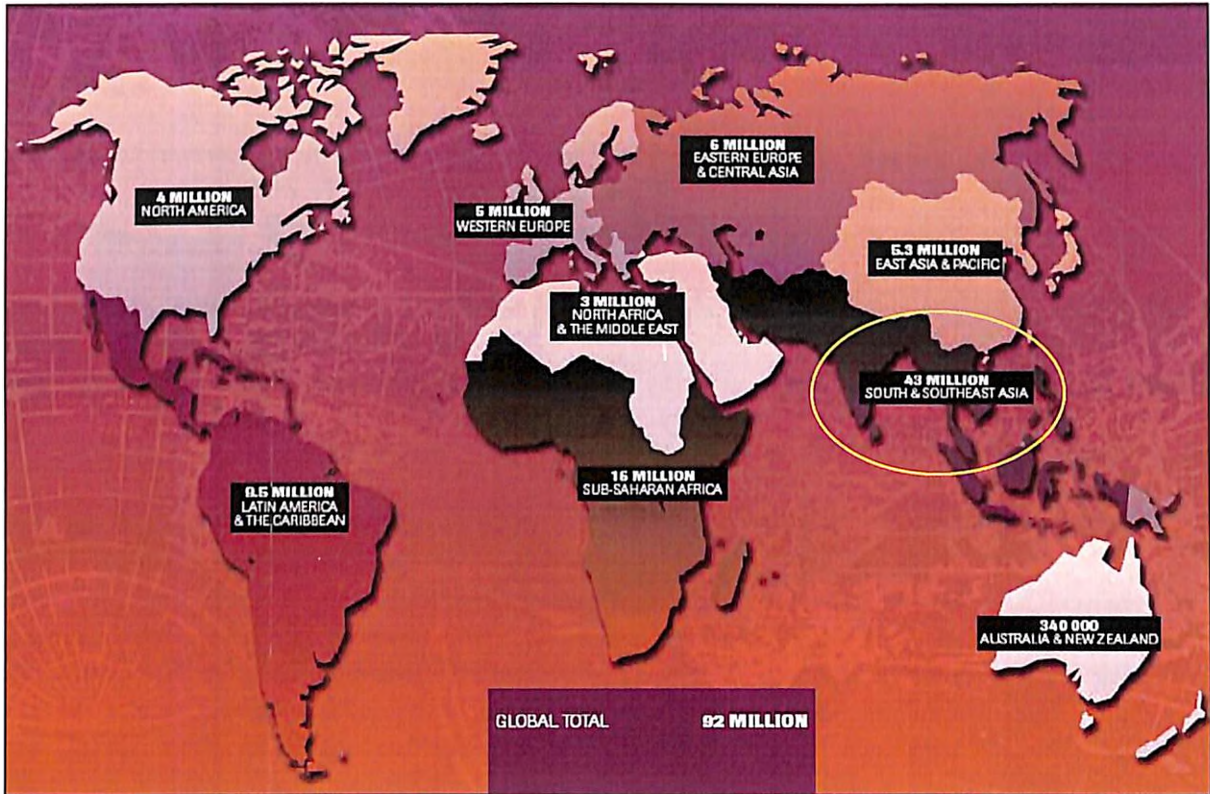


Figure 1.1 Global prevalence and incidence of *Chlamydia trachomatis*

In India also, a high prevalence of chlamydial infection in symptomatic women (upto 40% in cervicitis and 36% in infertility) has been reported [Mittal *et al.*, 1993] with a high percent positivity among urban slum dwellers due to poor living and unhygienic conditions [Singh *et al.*, 2002]. There is growing concern over the association of cervical *C. trachomatis* infection with poor obstetric outcome, including still birth, preterm delivery and spontaneous abortion. We found that 21% of pregnant women were infected with *C. trachomatis* of which 16% gave birth to still born while 26% have premature births [Rastogi *et al.*, 1999]. Spontaneous abortions were also reported in 15% of pregnant women with chlamydial infection having highest percentage in multigravidae women [Rastogi *et al.*, 2000].

The chlamydial developmental cycle involves a metabolically inactive non replicating infectious form called elementary body (EB) which, after entry into the host cells, differentiates into metabolically active Reticulate Body (RB). The organism infects the epithelial cells, often inducing an acute inflammatory response which is exacerbated during persistent chlamydial infections, or by repeated infections with the bacterium.

Both humoral and cellular responses can be readily detected in patients suffering from *C. trachomatis* infection. Since infection is intracellular, neutralizing antibodies have little relevance in resolving infection [Morrison & Caldwell, 2002]. Antibodies specific for *C. trachomatis* can neutralize infection in tissue culture *in vitro* [Byrne, 1993], however, in humans high titers of *C. trachomatis* specific antibodies do not correlate with resolution of infection and in fact, more strongly correlated with increased severity of sequelae of infection, such as tubal infertility [Punnonen *et al.*, 1999]. In contrast, there is good evidence that T-cell mediated immune responses play a major role in clearance and resolution of chlamydial infections and T-cell responses are critical in host resistance to *C. trachomatis*. Transfer of T lymphocytes into naïve mice have also been shown to protect the mice against *C. trachomatis* infection [Ramsey & Rank, 1991].

After infection of host with *C. trachomatis,* dendritic cells (DCs) and macrophages/monocytes are the main antigen presenting cells (APC's) encountering the bacteria. DC's are key players in immunity that dictate the type of immune response generated to a particular antigen. They are professional antigen presenting cells that have extraordinary capacity to stimulate naive T-cell and initiate primary immune response. Immature DC present in the epithelium of cervix and vagina capture microbial antigens,

process them and present them to T lymphocytes. Infection with *Chlamydia muridarum* has been shown to stimulate DC's to produce IL-12 (T_H1 type response) [Su & Caldwell, 1998]. Toll like Receptors (TLR's) comprise a family of cell surface receptors that recognize pathogen associated molecular patterns (PAMP's), including lipopolysaccharide (LPS) and hypomethylated CpG- rich DNA as well as double stranded and single stranded RNA. Toll like receptors detect microbial infection and have an essential role in the induction innate and adaptive immune responses [Iwasaki & Medzhitov, 2004]. A recent hypothesis states that differential expression and engagement of TLR family members at the surface of dendritic cells and macrophages influences the type of immune response that is induced by a microbial pathogen. It is not confirmed which particular TLR's expressed by dendritic cells are engaged by *Chlamydia* spp., however, TLR2 has been shown to have an important role in the activation of DC's by *C. pneumoniae* [Prebeck, 2001]. Furthermore, signaling through TLR2, but not TLR4, was associated with increase fallopian tube pathology in *C. muridarum* infected mice [Darville, 2003], indicating that engagement of TLR2 is a potential common pathway in both the immunity and immunopathology induced by *Chlamydia* spp. Given the high level of expression of TLR's by DC's and there ability to polarize immune responses, the identification of the role of DC's in *Chlamydia* specific immune responses is crucial for understanding the type of immune response that is elicited and therefore also for designing a vaccine against infection with *Chlamydia trachomatis* [Gervassi *et al.*, 2004].

Studies in the animal models have clearly established that T cells have a crucial role in the resolution of infection with *Chlamydia* spp [Lu & Zhong, 1996]. Specifically protection seems to be mediated by $CD4^+$ T cells that produce IFN-γ [Su & Caldwell, 1995]. The role and effector mechanisms of *Chlamydia* positive $CD8^+$ T cells are less clear. MHC class I

peptide presentation to $CD8^+$ T cells is not essential for clearance of infection with *Chlamydia* spp. In some situation $CD8^+$ T cells might be important in elimination of cells infected with *Chlamydia* spp.[Reddy *et al.*, 2004]. Also adoptive transfer of $CD8^+$ T cell lines specific for serovar L2 of *C. trachomatis* protected mice against infection with *C. trachomatis* through a mechanism involving the production of IFN-γ [Starnbach, 2002]. Stimulated T cells produce a variety of cytokines for clearance of chlamydial infection including IFN-γ and IL-12. Most of the cytokines secreted by T cells and macrophages are T helper1 (Th1) cytokines, which have a role in polarizing the immune response to *Chlamydia* spp towards a protective Th1 type response. By contrast, cytokines such as TNF, IL-1α and IL-10 might be involved in the pathology associated with infection with *Chlamydia* spp. IFN-γ, the main T_H1 type cytokine is essential for the clearance of chlamydial infections from genital tract. It controls the *in vitro* growth of *C. trachomatis* through inducing production of enzyme indoleamine-2,3-dioxygenase (IDO) [Beatty *et al.*, 1994]. Activation of IDO by IFN-γ leads to degradation of tryptophan and lack of this essential amino acid causes the death of *C. trachomatis* through tryptophan starvation [Beatty *et al.*, 1994]. Additional immune effector mechanisms induced by IFN-γ include induction of nitric oxide production, which inhibits growth of *C. muridarum* [Ramsey, 2001] and the promotion of Th1 type protective immune response, which downregulate non protective Th2 type responses, thereby, promoting persistent infection [Wang *et al.*, 1999]. Persistent infection induces the secretion of proinflammatory cytokines, leading to chronic inflammatory cellular response and tissue damage [Cappuccio *et al.*, 1994; Hogan *et al.*, 2004].

Our understanding of the exact pathological mechanism involved during chlamydial infection is largely unknown. It is very difficult to understand how the infection is

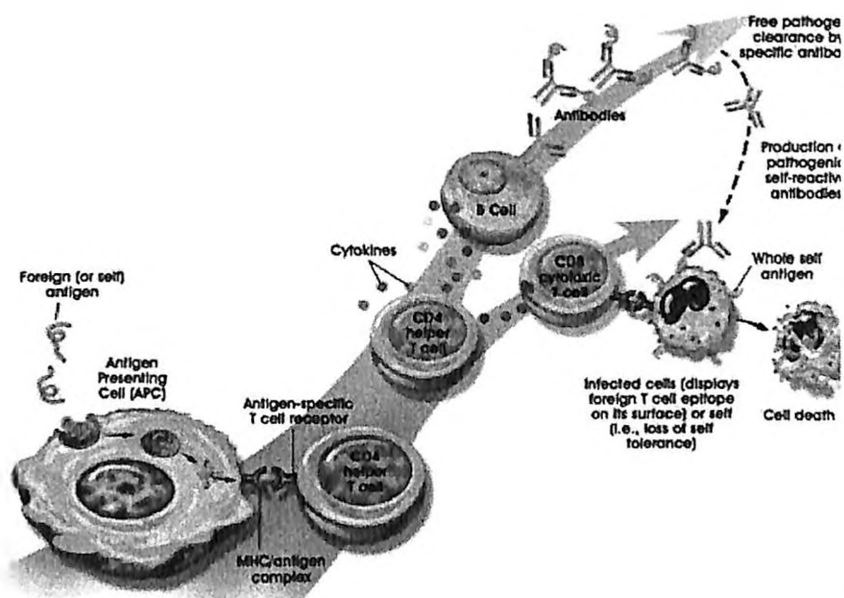
established and then cleared from the genital tract. Further, it is necessary to elucidate the dynamics of major cytokine responses in cervix and the role of dendritic cells (DCs) and Toll like receptors (TLRs) in the development of protective immunity towards genital *Chlamydia trachomatis* infection. Further, role of sex hormones in modulation of immune responses to *Chlamydia* is also largely unknown. Therefore, this study was planned with the following objectives:

1. Evaluation of mucosal immune responses to *Chlamydia trachomatis* in human females
2. Enumeration of cervical dendritic cell subsets and their role in modulation of immune response to *Chlamydia trachomatis* infection.
3. Innate immune response (TLRs and nitric oxide) to *Chlamydia trachomatis* in protection against infection
4. *In vitro* presentation of chlamydial elementary bodies by monocyte derived dendritic cells to autologous $CD4^+$ T cells.

Chapter 2
REVIEW OF LITERATURE

Each problem that I solved became a rule, which served afterwards to solve other problems

Rene Descartes



Review of Literature

Chlamydiae

Phylum *Chlamydiae* comprises exclusively of gram negative obligate intracellular bacterial pathogens that shows a characteristic biphasic developmental cycle, including metabolically inert elementary bodies (EBs) and actively dividing reticulate bodies (RBs), which thrive within a host-derived vacuole termed inclusion [Hammerschlag, 2002]. This phylogenetically well-isolated group of closely related bacteria constituted the single family *Chlamydiaceae* of the order *Chlamydiales*, which form a separate phylum in the domain *Bacteria*. *Chlamydiae* are ancient bacteria; the last common ancestor of symbiotic and parasitic *chlamydiae* was already adapted to intracellular survival in early eukaryotes about 700 million years ago [Horn *et al.*, 2004].

History and taxonomy

The *Chlamydiae* are non-motile, gram-negative bacterial pathogens that were once mistakenly thought to be viruses because of their obligate intracellular developmental cycle. Historic evidence of infection caused by *Chlamydia* exists as long as 4000 years ago and since the description of the characteristic features of a chlamydial inclusion by Halbersteadter and Provazek in 1907, *Chlamydia* has been studied extensively. In 1909, Lindner found same typical intracytoplasmatic inclusions in conjunctival scrapings from newborn affected by *opthalmia neonatorum*. Lindner and others described the occurrence of these inclusions in cervical cells from mothers of babies with inclusion conjunctivitis, as well as in urethral cells from fathers of such babies and in men with nongonococcal urethritis. Thus trachoma, inclusion conjunctivitis of the newborn and infection of the adult genital tract were caused by similar infective agents (now known as *Chlamydia trachomatis*) all of which were capable of

passing filters that otherwise generally retained bacteria. This property, coupled with inability of these agents to grow in artificial media led to the erroneous belief that these agents were viruses.

The isolation of *Chlamydia psittaci* was reported in 1930, and Levinthal Coles and Lillie independently described minute basophilic particles in Giemsa stained, blood and tissue from the infected birds and human patients. Bedson *et al.*, 1930 soon proved their aetiological relationship with psittacosis and went on to define the characteristics of chlamydial developmental cycle during which large basophilic 'initial bodies' undergo a series of fissions to form smaller elementary bodies [Bedson & Bland, 1932]. Ten years later, the agent causing lymphogranuloma venereum (LGV) [Rake, 1940] was isolated; today this is classified as a subtype of *Chlamydia trachomatis.* As early as 1934, Thygeson, an ophthalmologist, had drawn attention to the resemblances between the development and morphology of the inclusions seen in trachoma and inclusion conjunctivitis and of those found in psittacosis. The finding of a common complement-fixing antigen strengthened the idea that these agents and those of LGV and of mouse pneumonitis were related within the same unique group.

In 1944, Macchiavello reported the successful culture of the trachoma agent in the yolk sac of fertile hens' eggs, but the first isolation is usually credited to T'ang and his coworkers (1957); their finding were confirmed and extended by Collier & Sowa (1958) who isolated similar agents in The Gambia, demonstrated their morphological similarity of these of psittacosis and LGV, and showed them to contain the genus specific compliment-fixing antigen. The aetiological relationship of this organism with trachoma was proved in 1958 by the inoculation of human volunteers [Collier *et al.*, 1958].

Using the same procedure, i.e. the inoculation of the yolk sac of embryonated hens' eggs, the isolation of the so-called TRIC (Trachoma Inclusion Conjunctivitis) virus from ocular and genital material was reported mainly by workers in Great Britain and the United States. With the development of a cell culture method for clinical samples in 1965 by Gordon and Quan, the modern age of clinical chlamydial research had started. However, it was not until the 1970s that the important role of *C .trachomatis* in the etiology of nongonococcal urithitis (NGU) in males and cervicitis in female [Hilton *et al.,*1974; Oriel & Ridgway 1982] was firmly established and accepted as a clinical reality.

***Chlamydia* taxonomy**

Although *chlamydiae* were originally thought to be protozoa and later virus, it becomes clear that *chlamydiae* had all requisite properties of bacteria. *Chlamydia spp.* has been placed in their own bacterial division because they have an evolutionary pathway deeply separated from other bacteria [Pace, 1997]. Significantly, most of the different species within *Chlamydiaceae* similarly separated from each other in their evolution [Stephens, 2002] resulting in a very broad spectrum of pathoadaptive species [Pallen & Wren, 2007]. Chlamydiae are classified in the order *Chlamydiales,* which has only one family *Chlamydiaceae*, one genus *Chlamydia* and four recognized species *C. trachomatis, C. pneumoniae, C. psittaci, C. pecorum* [Ward, 1983; Graston *et al.*, 1986; Fukushi & Hirai 1992; Black 1997]. In 1999, it was proposed to assign chlamydial strains in the single genus, *Chlamydia,* to two genera, *Chlamydia* and *Chlamydophila,* based on the apparent differential clustering of 16S rRNA gene [Everett *et al.,* 1999]. The differences between the old and the new models of classification are shown in Figure 2.1. However, I6s rRNA gene phylogenetics analyses demonstrated a split between *C. trachomatis* group and all other

Chlamydiae as distribution of proteins was not consistent with this early separation [Gupta & Griffith, 2006]. Chlamydial plasmid present in both the proposed genera was also very similar [Griffith *et al.,* 2006]. Thus, this split of genus is still not been accepted by the scientific community [Schachter *et al.*, 2001; Stephens *et al.*, 2009]. It is confirmed now that *Chlamydiae* have one genus, *Chlamydia* and nine species [Draghi et al., 2007], thereby showing spectrum of divergence among *Chlamydiae* without generic difference.

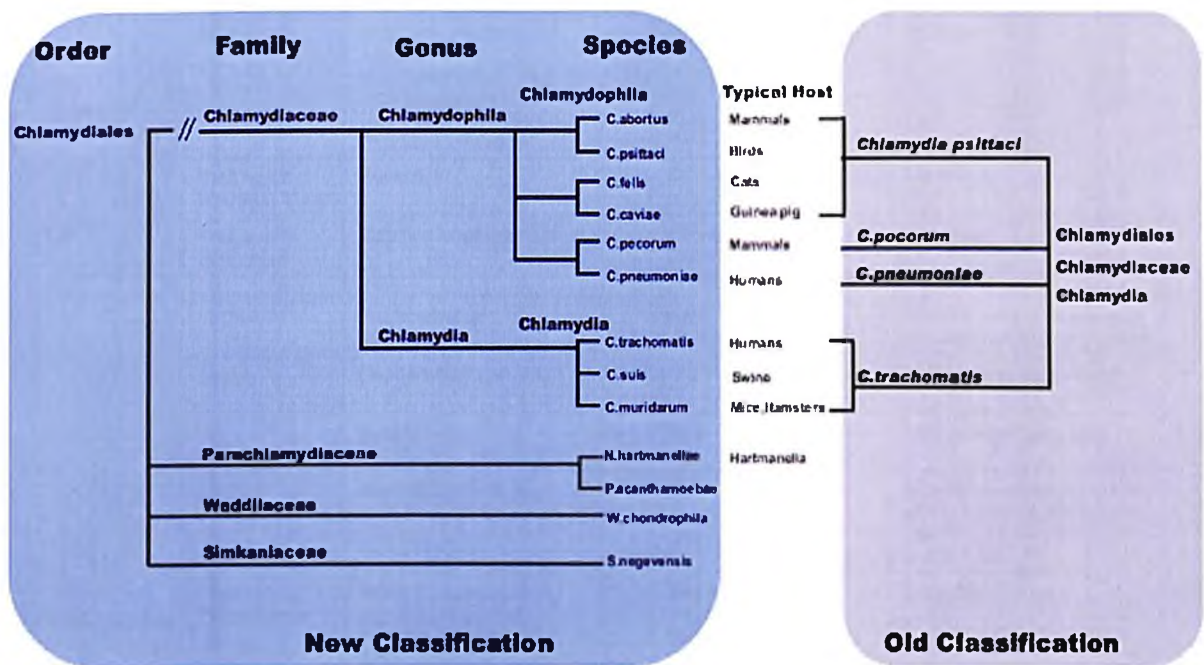


Figure 2.1 Old versus new classification of *Chlamydiales*. Adapted from Bush & Everett, 2001.

Chlamydia: host and disease

Chlamydiae have a broad host spectrum. They are clinically and epidemiologically important throughout the world, both in human and in veterinary medicine. Human pathogenic chlamydiae typically cause infections of the eye, or of the urogenital or respiratory tracts. These infections are often initially not recognized or misinterpreted, as they are difficult to diagnose and the symptoms are mild. Chronic and repeated infections may lead to

irreversible damage, including blindness (trachoma) after infection of the eye and tubal infertility after infection of the female genital tract. Table 2.1 gives an overview of the significance of the different *Chlamydia* species and their spectrum of disease.

TABLE

Diseases caused by Chlamydiae in man and in animals

Genus	Species	Diseases in man	Frequency	Diseases in animals
Chlamydia	muridarum	Non-pathogenic in man		
	suis	Non-pathogenic in man		Pneumonia, enteritis, conjunctivitis (pig)
	trachomatis	Urogenital infections Adnexitis Reactive arthritis Swimming pool conjunctivitis Neonatal conjunctivitis Neonatal pneumonia	Frequent in adolescents Occasional Occasional Rare Rare Rare	Unknown
	trachomatis, trachoma biovar	Trachoma	Frequent in tropical countries	Unknown
	trachomatis, LGV biovar	Venereal lymphogranuloma	Occasional in tropical countries, also in HIV-positive men in Europe	Unknown
Chlamydophila	abortus	Septic infection Abortion	Rare	Enzootic ovine abortion, also occasionally abortion in cattle
	caviae	Non-pathogenic in man		Conjunctivitis (guinea pig)
	felis	Conjunctivitis	Rare	Keratoconjunctivitis (cat)
	pecorum	Non-pathogenic in man		Enteritis, abortion, conjunctivitis, pneumonia, encephalomyelitis, polyarthritis (ruminants)
	pneumoniae, TWAR biovar	Respiratory infections Atypical pneumonia Reactive arthritis Infestation of the arteriosclerotic plaque	Very frequent Occasional Occasional Probably frequent	Uncertain
	pneumoniae, koala biovar	Non-pathogenic in man		Uncertain
	pneumoniae, equine biovar	Non-pathogenic in man		Uncertain
	psittaci	Respiratory infections Pneumonia Myocarditis Hepatitis Encephalitis	Occasional Occasional Rare Very rare Very rare	Psittacosis/ornithosis (true parrots, domestic poultry)
Simkania	negevensis	Respiratory infections	Probably frequent	Unknown
Parachlamydia	acantamoebae	Non-pathogenic in man		Under study
Waddlia	chondrophila	Non-pathogenic in man		Under study

Table 2.1 Diseases caused by *Chlamydiae* in man and animals.

Complications after infection—most importantly reactive arthritis—are presumably linked to the immunological response. Moreover, chlamydiae have been linked etiologically to a series of chronic inflammatory processes, particularly artherosclerosis.

Developmental cycle

Chlamydiae undergo a distinct developmental cycle, converting between two morphologically and functionally discrete forms, the elementary body (EB) and the reticulate body (RB). The basic cycle follows this sequence: I) attachment and internalization, II) EB to RB differentiation, III) remodeling of the parasitophorous vacuole ("inclusion") and bacterial replication, IV) inclusion expansion and transition of RB into EB, and V) release of bacteria from the host cell and infection of new target cells by EBs (Figure 2.2).

Attachment and entry

The EB, the infectious bacterial form, attaches to and is internalized by the host cell. Multiple bacterial adhesins and ligands have been proposed, and host receptor utilization depends on both the host cell type and chlamydial species [Dautry-Varsat *et al.*, 2005]. Bacterial factors such as glycosaminoglycan (GAG) [Menozzi *et al.*, 2002], major outer membrane protein (MOMP) [Su *et al.*, 1996], OmcB [Fadel *et al.*, 2007], and PmpD [Wehrl *et al.*, 2004] have been proposed as adhesins or ligands for receptor interactions. Previously proposed host receptors include heparan sulfate, mannose receptor, mannose 6-phosphate receptor, and estrogen receptor [Campbell *et al.*, 2006]. Recently, two distinct roles were uncovered for cell surface exposed protein disulfide isomerase (PDI) in internalization: a structural one involved in EB attachment, and an enzymatic one required for bacterial entry [Conant *et al.*, 2007, Abromaitis *et al.*, 2009]. The exact mechanism of endocytosis remains unclear due to conflicting studies using pharmacological inhibitors and dominant-negative constructs

[Dautry-Varsat *et al.*, 2005]. These results reflect that multiple redundant strategies likely exist to ensure chlamydial entry, and the route is dependent on the *Chlamydia* species or features of the host cell type being invaded.

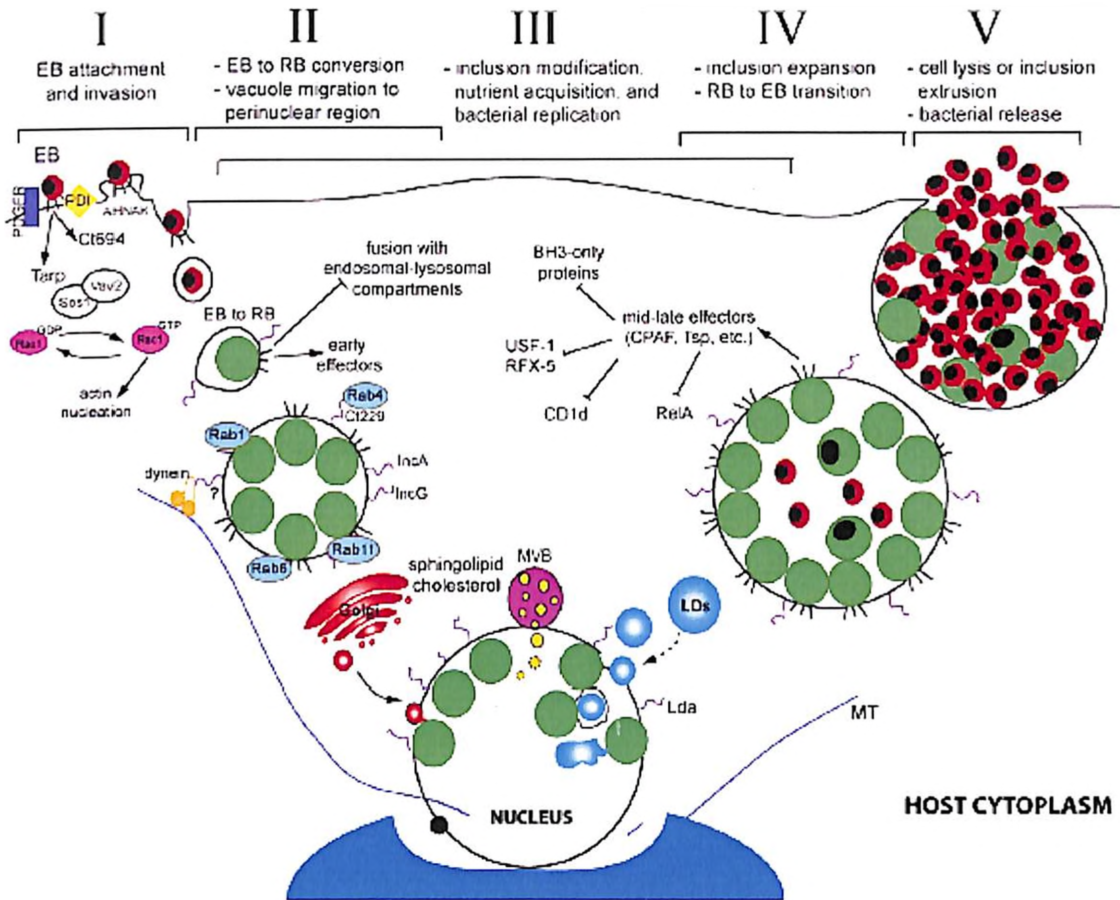


Figure 2.2 Developmental cycle of *Chlamydia trachomatis*

Transition from EB to RB

EBs are stable in the extracellular environment by virtue of extensively cross-linked outermembrane proteins [Newhall *et al.*, 1983]. These disulfide bonds are reduced during internalization [Hackstadt *et al.*, 1985] followed by nucleoid decondensation and initiation of bacterial transcription. Within 15 minutes, new bacterial proteins are already being produced

and RNA expression can be detected as early as one hour [Plaunt *et al.*, 1988, Belland *et al.*, 2003]. RBs replicate by binary fission inside inclusion boundaries. Roughly midway through infection, replication becomes asynchronous as RBs begin differentiating back into EBs.

Inclusion expansion

The developing inclusion must expand to accommodate increasing bacterial numbers. Inclusion growth is likely fueled by attainment of nutrients and lipid precursors from the host. Unlike other intracellular pathogens that remain individually surrounded by tight membrane compartments, the inclusion is a large, relatively spacious organelle. The structural integrity of the inclusion is preserved by a meshwork of host cytoskeletal structures primarily composed of F-actin and intermediate filaments (IFs). The Head domains of IFs surrounding the inclusion are progressively cleaved by chlamydial protease activating factor (CPAF) generating structural changes in the filaments as the inclusion ages. The purpose of inclusion stabilization is unclear but may limit cytoplasmic exposure of lumenal contents. This would benefit the pathogen by reducing activation of innate immune responses via microbial pattern recognition receptors. [Kumar & Valdivia, 2008]

EBs exit and dissemination

At developmental cycle completion, EBs must exit the cell to initiate subsequent rounds of infection. Exit can occur via two discrete mechanisms. First, cell lysis involves the sequential disruption of inclusion and cellular membranes by cysteine proteases, and the host cell is destroyed. Alternatively, the inclusion can remain membrane-bound and be pushed out, or "extruded", from the host cell. One chlamydial effector protein, *Chlamydia* protein associating with death domain, or CADD, can interact with Tumor necrosis factor (TNF) receptor death domains and induce Fas-related apoptosis upon ectopic expression [Stenner-

Liewen *et al.*, 2002]. Yet, the biological relevance of this is unknown since host cell-death induction at the late developmental stages appears non-apoptotic [Ying *et al.*, 2006].

Clinical manifestation of *C. trachomatis* infection

C. trachomatis has been divided into three biovars: trachoma, lymphogranuloma venereum (LGV), and murine (mouse pneumonitis [MoPn] agent). DNA homology studies of genomic DNA and comparison of DNA sequences of specific genes have shown that the trachoma and LGV biovars appear to be essentially identical and the murine biovar is more distantly related. The trachoma and LGV biovars are distinguished by significantly different clinical features. Serovars of *C. trachomatis* are determined by serological assays, and not by DNA sequencing and are currently divided into 19 serovars, according to the specificity of major outer membrane protein (MOMP) epitopes [Schachter, 1999]. Serovars A, B, Ba and C are the agents of trachoma, a major cause of blindness in Africa, the Middle East, Asia and South America. Serovars D–K, including D, Da, E, F, G, Ga, H, I, Ia, J and K, are the most common sexually transmitted bacteria, and serovars L1, L2, L2a and L3 are the agents of transmission of lymphogranuloma venereum (LGV). However, most of these infections produce few or no symptoms in approximately 70% of women and 50% of men [van de Laar & Morre, 2007] and thus remain undetected. Clinical manifestations of *C. trachomatis* infection are given in Table 2.2. Representative symptoms of a few manifestations are shown in Figure 2.3.

Serovars	Clinical Manifestations	Complications	Distribution
A, B, Ba, C	Trachoma	Blindness	Asia and Africa
D to K	Disease of eye and genitals Urethritis, proctitis mucopurulent cervicitis Respiratory System: Infant pneumonia	Epidymitis Endometriosis, salpingitis Pelvic pain, ectopic pregnancy Infertility Reactive arthritis	Worldwide
LGV1 LGV2 LGV3	Lymphogranuloma venerum (LGV)	Fibrosis, rectal stricture	Worldwide

Table 2.2 Clinical manifestations of *C. trachomatis* infection

Trachoma

Trachoma is a characteristic ocular disease caused by infection with *C. trachomatis* serovars A, B and C [Grayston & Wang, 1975; Jones 1975]. Trachoma is the common infectious cause of blindness in the world and an estimated 7 million people blind as a result of trachoma. Most of these individuals are middle-aged and early adult. Active trachoma often has its onset within the first 1-2 years of life. Recurrence is common during childhood, but cease spontaneously by 10-15 years of the age, presumably because of acquired immunity [Mabey & Bailey, 1996]. The *C. trachomatis* serovars that produce trachoma are spread by direct contact with contaminated secretion carried by fomites such as facecloth or eye-seeking flies. Perinatal exposure to *C. trachomatis* from maternal genital tract infection is not an important route for trachoma transmission

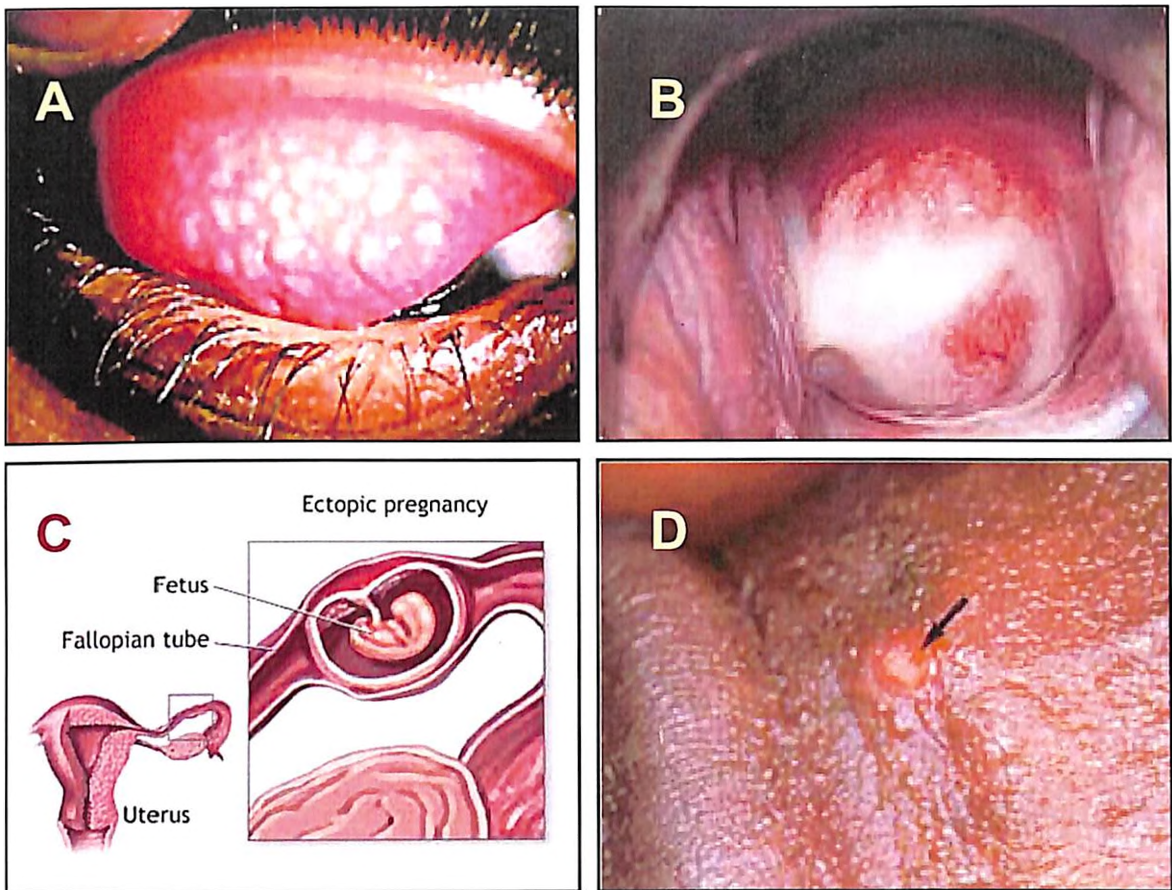


Figure 2.3 Representation of *C. trachomatis* clinical manifestations (A) Trachoma (B) cervicitis (C) Ectopic pregnancy and (D) LGV

Infections in women

Women with cervicitis can be asymptomatic or may complain of mucopurulent vaginal discharge or postcoital bleeding. Oedema, congestion and bleeding of the cervix have been observed. Urethral infection can be associated with cervicitis. A culture-negative leucocyturia finding is suggestive of *C. trachomatis* infection. Ascending infections can result from cervicitis. Endometritis is frequently associated with this and may produce irregular uterine bleeding. Salpingitis or pelvic inflammatory disease (PID) is often subclinical. It seems possible that, *C. trachomatis* is the cause of at least 60% of cases of acute PID [Rogstad, 2008]. Salpingitis may lead to tubal scarring and severe reproductive

complications. Two-thirds of all cases of tubal factor infertility and one-third of all cases of ectopic pregnancy could be due to chlamydial infection [Peipert, 2003; Paavonen & Eggert-Kruse, 1999]. Chronic pelvic pain linked to the presence of peritoneal adhesions may occur in more than 15% of women with previous episodes of PID [Rogstad, 2008].

Fitz-Hugh–Curtis syndrome, a perihepatitis observed after or in conjunction with salpingitis, is more commonly associated with chlamydial than with gonococcal infections. There is little evidence, and this is conflicting, to implicate *C. trachomatis* in chorioamnionitis and adverse pregnancy outcome [Rogstad, 2008]. Postpartum endometritis occurs in 30% of women with antenatal chlamydial infection. In both men and women, *C. trachomatis* may be involved in conjunctivitis by auto-inoculation from the genital tract.

Infections in men

C. trachomatis is the major cause of non-gonococcal urethritis and post-gonococcal urethritis. Urethritis can be complicated by acute epididymitis in young men. After 7–21 days of incubation, the symptoms include dysuria, and a moderate clear or whitish urethral discharge [Peipert, 2003]. Acute proctitis can be associated with oculo-genital serovars, but is usually milder than that associated with LGV serovars. There is no evidence of the role of *C. trachomatis* in prostatitis [Hicks, 2008], and chlamydial infection does not significantly contribute to male infertility [Barbeyrac de *et al.,* 2006]. Reiter's syndrome (urethritis, conjunctivitis, arthritis and mucocutaneous lesions) or reactive arthritis have also been associated with genital *C. trachomatis* infections, with a high male/female ratio [Hicks, 2008].

Neonatal infections

Infants of mothers with chlamydial infections can be infected at delivery. The transmission rate via infected vaginal secretions is high (50–70%). Approximately 30–50% of infants of infected mothers will have conjunctivitis 5–10 days after delivery. At least 50% of infants with conjunctivitis will have nasopharyngeal infection [Peipert, 2003]. Chlamydial pneumonia develops in approx. 30% of these cases, after 2–3 weeks of incubation. The untreated infection acquired at birth can persist for months or years [Hammerschlag, 1994; Stenberg & Mardh, 1986].

Lymphogranuloma venereum (LGV)

LGV is associated with L serovars, which are more invasive than D–K serovars, affecting submucosal connective tissue layers, and being able to disseminate to locoregional lymph nodes. LGV proctitis can be misdiagnosed as inflammatory bowel disease [Manavi, 2006], and lead to rectal stricture. The persistence of LGV cases that may contribute to the transmission of human immunodeficiency virus infection highlights the importance of the need to control this infection.

Diagnosis of *Chlamydia trachomatis*

There have been major developments during the past 30 years. As *C. trachomatis* is an obligate intracellular bacterium, cell culture remains a reference method, but many commercial non-culture-based assays are now available for diagnosis.

Specimens

The type of specimen depends on the clinical picture, the diagnosis conditions, and the laboratory technique used for detection, with the conditions of transport and storage being adapted to the particular technique. Invasive specimens include urethral swabs in men, and

endocervical or urethral swabs, and specimens taken from the upper genital tract, in women (liquid from Douglas's pouch, endometrium and tubal specimens). Other sites include the conjunctiva, nasopharynx or deeper respiratory tract. Non-invasive self-collected specimens include first-void urine (FVU), vulvovaginal swabs, anal swabs and penile swabs. The bacterial load of these specimens is a major aspect of their suitability for the diagnosis, which can be made only by using nucleic acid amplification tests (NAATs) [Michel *et al.,* 2007]. Self-collected vaginal swabs have a lower bacterial load than endocervical swabs, but a higher load than FVU, and are very well adapted to screening programmes [Schachter, 2005]. FVU is a suitable sample type for men [Gaydos *et al.,* 2008].

Cell culture

Cell culture has near 100% specificity. However, it is not recommended for routine use, because of its lack of sensitivity, its technical complexity, the long turn-around time, the requirements concerning transport and storage of specimens, and the limited number of appropriate specimens [Essig, 2007; Black , 1997]. Owing to the detection of only viable organisms, it remains the method of choice in medico-legal situations and for antibiotic susceptibility testing [Warford *et al.,* 1999].

Antigen-based detection methods (direct fluorescent staining with monoclonal antibodies (DFA) and enzyme immunoassay (EIA)

DFA is rapid to perform and specific, but is subjective, and not suitable for a large number of specimens [Black, 1997]. EIA tests can be automated. They are more reproducible than DFA, and the sensitivity of the best EIA is comparable to that of culture and lower than that of NAATs. They can give false-positive results due to cross-reactions with the lipopolysaccharide (LPS) of other microorganisms, and all positive EIA results must be

confirmed. Rapid or 'point-of-care' tests are proposed for patients who are unlikely to return for test results. They are not suitable for non-invasive specimens, have moderate sensitivity, and are not recommended for laboratory settings. A rapid test for diagnosis of chlamydial infection, recently developed by the Wellcome Trust [Michel *et al.*, 2006; Mahilum-Tapay *et al.*, 2007], is based on a second-generation signal amplification EIA for chlamydial LPS in a dipstick type format.

Nucleic acid hybridization tests

DNA probing (with Pace 2, Gen Probe) was the first molecular DNA test for *C. trachomatis,* and was largely used before the advent of NAATs. The performance of these tests is comparable to that of the better antigen detection and cell culture methods. Pace 2 can be used with endocervical or urethral swabs, but is not recommended for use with noninvasive specimens [Schachter, 2005]. The Digene Hybrid Capture II test is a nucleic acid hybridization test that is signal amplification-based. Its sensitivity is substantially higher than that of the Pace 2 test and is comparable to that of PCR [Leber, 2006].

Nucleic Acid Amplification Tests (NAATs)

Because of their high sensitivity and specificity, and their possible use for a large range of sample types, including vulvovaginal swabs and First void urine (FVU), NAATs are the tests of choice for the diagnosis of *C. trachomatis* genital infections. Several commercial NAATs are available [Leber, 2006], and make use of different technologies: PCR and real-time PCR (Roche Diagnostics, Abbott, USA); strand displacement amplification (Becton Dickinson, USA); transcription-mediated amplification (Gen Probe); and nucleic acid sequence-based amplification (bioMerieux, Nancy L'Etoile, France). The major targets for amplification-based tests are generally multiplecopy genes, e.g. those carried by the cryptic plasmid of *C.*

trachomatis, or gene products such as rRNAs. These assays are automated and can be used for screening programmes. Their primary disadvantage is the cost, which could be reduced by pooling specimens. Another drawback is the presence of inhibitors in specimens, which can be overcome by different procedures. Their specificity is very high. The necessity of confirmatory testing of positive specimens, previously recommended in low-prevalence populations, is controversial [Schachter, 2006]. In 2006, a new *C. trachomatis* variant belonging to serovar E, with a 377-bp deletion in the cryptic plasmid, was described in Sweden [Ripa & Nilsson, 2006], where it was reported in high proportions (10–65%) of the infected patients. There is currently no evidence that the variant has spread widely across Europe [Lynagh, 2007; Moghaddam & Reinton, 2007]. This new variant can obviously not be detected by amplification tests targeting the deleted area, but can be detected by amplification targeting a chromosomal gene, e.g. ompA or a rRNA gene. The goal for the future is to improve the diagnosis of sexually transmitted infections by using multiplex tests, in particular DNA microarray technology.

Treatment of chlamydial infection

The treatment of *C. trachomatis* infection depends on the site of the infection, the age of the patient, and whether the infection is complicated or uncomplicated. Treatment also differs during pregnancy. For uncomplicated genitourinary chlamydial infection, the Centre for Disease Control (CDC) recommends 1 gm azithromycin orally in a single dose, or 100 mg doxycycline orally twice per day for seven days. For the treatment of *Chlamydia* infected pregnant women doxycycline and ofloxacin are not recommended during pregnancy; therefore, the CDC recommends erythromycin base or amoxicillin for the treatment of chlamydial infection in pregnant women.

If symptoms suggest recurrent or persistent cervicitis or urethritis, the CDC recommends treatment with 2 gm metronidazole orally in a single dose plus 500 mg erythromycin base orally four times per day for seven days, or 800 mg erythromycin ethylsuccinate orally four times per day for seven days. The CDC does not recommend repeat testing for *Chlamydia* after completion of the antibiotic course unless the patient has persistent symptoms or is pregnant. Because reinfection is a common problem during chlamydial infection, the CDC recommends that women with chlamydial infection should be rescreened three to four months after antibiotic completion. Complication with an effective antibiotic regimen has nevertheless reportedly been associated with an increase frequency of recurrent infection [Dean, *et al.,* 2000; Stamm, 2001], tubal infertility [Patton, et *al.,* 1994]. An *in vitro* study of latent genital chlamydial infections using polarized endometrial epithelial cells reported that a persistent form of *C .trachomatis* did not have the same susceptibility to antibiotics as compared with actively growing *Chlamydiae*, with persistent *Chlamydiae* phenotypically resistant to azithromycin [Wyrick, *et al.,* 2004]. It has been hypothesized that women with high chlamydial load may be increased the risk of antibiotic treatment failure [Horner, *et al.,* 2006].

Immunobiology of *C. trachomatis*

Host responses to C. trachomatis

Although *C. trachomatis* has evolved to survive intracellularly within the host cell, the host has in turn evolved an elaborate system to detect as well as to control infection. The immune response to *C. trachomatis* is a co-ordinated event where innate immune cells, B cells, and T cells act in concert and where each of these immune effectors have roles in recognizing different stages of the infection.

Innate immunity to C. trachomatis

During genital infection with *C. trachomatis*, the mucosal barrier of the genital tract provides the first line of host defense. The ability of the *Chlamydia* organisms to enter this physical barrier is influenced by the stage of the estrus cycle. Shedding of the endometrial epithelium during certain stages of this cycle can limit the ability of *C. trachomatis* to establish a robust infection [Tuffrey *et al.*, 1986; Ramsey *et al.*, 1999]. Urethral samples of infected individuals also contain defensins that have been shown to inhibit *C. trachomatis* infection *in vitro* [Porter *et al.*, 2005]. When *Chlamydia* organisms are able to enter the mucosal lining and establish productive infection, innate immune effectors provide the next line of defense against the bacteria. Although epithelial cells are not classically considered critical players within the innate immune system, they are capable of initiating and propagating innate immune responses [Quayle, 2002].

Toll-like receptors and dendritic cells.

Toll-like receptors (TLRs) detect microbial infection and have an essential role in the induction of innate and adaptive immune responses [Iwasaki & Medzhitov, 2004]. A recent hypothesis states that differential expression and engagement of TLR-family members at the surface of dendritic cells (DCs) influences the type of immune response that is induced by a microbial pathogen [Iwasaki & Medzhitov, 2004]. Infection with *C. muridarum* has been shown to stimulate DCs to produce IL-12 (a cytokine that polarizes immune responses to Th1-type responses) [Su *et al.*, 1998; Lu & Zhong, 1999] and CXCL10 (a chemokine that recruits T cells) and to express CC-chemokine receptor 7 (CCR7; a chemokine receptor that is required for the migration of DCs to local lymph nodes) [Shaw *et al.*, 2001]. Although it is not confirmed which particular TLRs expressed by DCs are engaged by *Chlamydia* spp.,

TLR2 might have an important role in the activation of DCs by *Chlamydophila pneumoniae* [Prebeck *et al.*, 2001]. Furthermore, signaling through TLR2, but not TLR4, is associated with increased fallopian-tube pathology in *C. muridarum* infected mice [Darville *et al.*, 2003], indicating that engagement of TLR2 is a potential common pathway in both the immunity and immunopathology induced by *Chlamydia* spp. TLR2 appears to be more essential for signalling pro-inflammatory cytokine production following *Chlamydia* infection [O'Connell *et al.*, 2006]. *Chlamydia* appear to co localize with TLR2, but the *Chlamydia* pathogen associated molecular patterns (PAMP) that signals through TLR2 remains to be identified [O'Connell *et al.*, 2006]. Given the high level of expression of TLRs by DCs and the ability of DCs to polarize immune responses [Steele *et al.*, 2004], the identification of the role of DCs in *Chlamydia* specific immune responses is crucial for understanding the type of immune response as DCs that is elicited and therefore also for designing a vaccine against infection with *C. trachomatis*. Since DCs recognize pathogen by TLRs and then activate T cells and B cells, therefore, they provide an essential link between innate and adaptive immunity (Figure 2.4).

DCs have been found in mouse vaginal and cervical mucosae [Parr *et al.*, 1991] and are recruited to the site of inflammation in response to infection with *Chlamydia* spp. [Zhang *et al.*, 1999]. Evidence indicates that sampling of microbial antigen across the epithelia of the vagina is accomplished by migratory DCs that carry antigens to peripheral lymph nodes, where antigen is presented to naïve T cells [Neutra *et al.*, 1996]. Mature DCs are highly effective at presenting antigen and priming protective adaptive immune responses. Accordingly, adoptive transfer of DCs pulsed with *C. muridarum* elementary bodies protects mice against subsequent infection [Su *et al.*, 1998].

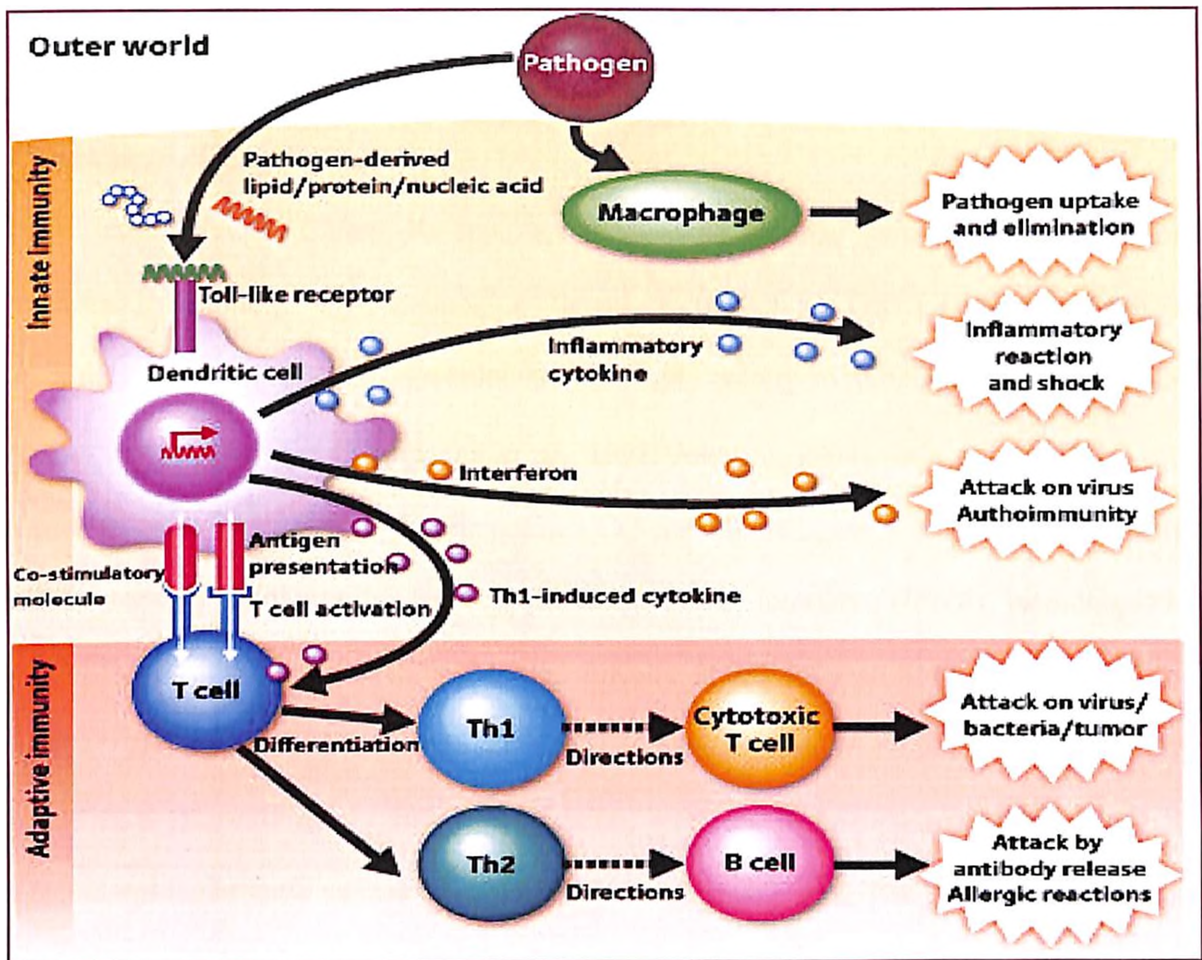


Figure 2.4 Linking of innate and adaptive immunity by dendritic cells

Live and inactivated *C. muridarum* induce different levels of DC maturation, and adoptive transfer of DCs pulsed with live *C. muridarum* has been shown to be even more effective at providing protective immunity than DCs pulsed with inactivated bacteria [Rey-Ladino *et al.*, 2005]. These observations might help to explain why vaccination with whole inactivated *C. trachomatis* was only partially protective in human trials [Grayston & Wang, 1978]. Immature DCs and regulatory DCs have also been described to be associated with immune tolerance [Steinman *et al.*, 2000] and therefore might have a role in promoting disease pathogenesis, although this has not yet been studied for *Chlamydia* spp. Studies of DCs that

reside in the genital tract will be essential to enable the design of vaccines against infection with *C. trachomatis*.

Cytokines in chlamydial infection

After infection with *Chlamydia* spp., epithelial cells produce various pro-inflammatory mediators, including CXC-chemokine ligand 1 (CXCL1), CXCL8 (also known as interleukin-8, IL-8), CXCL16,granulocyte/ monocyte colony-stimulating factor (GM-CSF), IL-1α, IL-6 and TNF-α [Rasmussen *et al.,* 1997; Johnson, 2004]. Infected epithelial cells also upregulate expression of the chemokines CC-chemokine ligand 5 (CCL5) and CXCL10, and they secrete cytokines that promote the production of Interferon (IFN)-γ, including IFN-α, IFN-β and IL-12 [Johnson, 2004; Maxion & Kelly, 2002]. Infected fibroblasts secrete IFN-α, IFN-β and nitric oxide [Devitt *et al.,* 1996], whereas infected macrophages produce TNF-α and IL-6 [Darville *et al.,* 2003]. In addition, secretion of chemokines such as IL-8 by infected epithelial cells can recruit classical innate immune cells [Buchholz and Stephens, 2006]. These include natural killer (NK) cells and phagocytes such as neutrophils, macrophages and DCs, which are abundant in the genital mucosa [Parr & Parr, 1990; 1991]. The recruited cells can in turn produce more inflammatory cytokines such as TNF-α, leading to the restriction of *C. trachomatis* growth within the infected epithelial cells [Dessus-Babus *et al.*, 2002]. Most of these are T helper 1 (Th1)-cell cytokines, which have a role in polarizing the immune response to *Chlamydia* spp. towards a protective Th1-type response [Johnson, 2004]. By contrast, cytokines such as TNF-α, IL-1α and IL-6 might be involved in the pathology associated with infection with *Chlamydia* spp. [Darville, 2003] Together, these cytokines trigger inflammation and promote the recruitment of immune cells, thereby actively contributing to the development of innate and adaptive immune responses.

Another major inflammatory cytokine produced by innate cells is IFN-γ, which can control *Chlamydia* replication through multiple distinct mechanisms. IFN-γ can upregulate the phagocytic potential of macrophages, thereby promoting the engulfment and destruction of extracellular EBs [Zhong & de la Maza, 1988]. IFN-γ can also directly inhibit *Chlamydia* growth within infected cells. Upregulation of inducible nitric oxide synthase in response to IFN-γ treatment can kill intracellular *Chlamydia* organisms in infected cell lines [Chen *et al.*, 1996; Igietseme *et al.*, 1997]. In addition, when human fibroblasts are treated with IFN-γ, indoleamine 2,3-dioxygenase production is induced [Gupta *et al.*, 1994]. Indoleamine 2,3-dioxygenase can inhibit *Chlamydia* growth by catalysing the catabolism of tryptophan, one of the amino acids that *Chlamydia* scavenges from the host cell. Lastly, IFN-γ-mediated downregulation of the transferrin receptor may also limit *Chlamydia* growth by limiting the intracellular stores of iron available to the organism [Byrd & Horwitz, 1993; Freidank *et al.*, 2001]. Interferon-g has also been shown to be required for *Chlamydia* clearance during experimental animal infections. Both IFN-γ and IFN-γ receptor knockout mice are more susceptible to infection with *C. trachomatis* [Cotter *et al.*, 1997a; Johansson *et al.*, 1997; Perry *et al.*, 1997; Lampe *et al.*, 1998; Ito & Lyons, 1999].

Adaptive immune response to *C. trachomatis*

B cell responses

Although innate immunity provides an early line of defence against *C. trachomatis* replication, cells of the adaptive immune system are necessary to limit the infection and provide protection during a future encounter with *Chlamydia*. Indeed, severe combined immunodeficiency mice, which lack an adaptive immune system, are extremely susceptible to *Chlamydia* infection [Magee *et al.*, 1993]. One key element of adaptive immunity is B

cells, which recognize soluble antigen via the B cell receptor. The concept that B cells and the antibodies they produce can mediate immunity to *C. trachomatis* originated early, when it was observed that the presence of *Chlamydia*-specific antibodies correlated with protective immunity against *C. trachomatis* infection in humans [Jawetz *et al.*, 1965; Barenfanger & MacDonald, 1974]. Subsequently, it was demonstrated that monoclonal antibodies against *Chlamydia* Major Outer Membrane Protein (MOMP) could neutralize *Chlamydia* infection *in vitro* [Peeling *et al.*, 1984; Peterson *et al.*, 1991] and provide a modest level of protection against infection when passively administered to mice [Cotter *et al.*, 1995]. In addition to neutralization, there is also evidence that Fc receptor mediated activities of antibodies also play an important role in combating infection [Moore *et al.*, 2002; 2003]. However, during primary infection, B cells may not play a critical role in controlling *Chlamydia* infection. Su *et al.* [1997] showed that B cell-deficient mice control primary *Chlamydia* genital infection as efficiently as wild-type mice. However, *Chlamydia* clearance following secondary infection is slightly delayed in the absence of B cells [Su *et al.*, 1997; Williams *et al.*, 1997], suggesting that B cells may play a somewhat larger role in the memory response to *C. trachomatis*, although this protective effect may not hold true for all *Chlamydia* serovars [Johansson & Lycke, 2001].

T-cell responses to C. trachomatis

Although antibodies can neutralize *Chlamydia* infectivity and enhance phagocytosis of EBs, they are unable to access the *Chlamydia* organisms that have established intracellular infection. At this stage, T cells become crucial for recognizing infected cells and orchestrating *Chlamydia* clearance.

The importance of T cells for controlling *Chlamydia* infection was documented over 20 years ago when it was observed that nude mice, which lack T cells, establish chronic infection with *Chlamydia* whereas wild-type mice clear infection within 20 days [Rank *et al.*, 1985]. Conversely, transfer of polyclonal *Chlamydia*-specific T cells into *Chlamydia*-infected T cell-deficient mice facilitates bacterial clearance [Ramsey & Rank, 1991; Thoma-Uszynski *et al.*, 1998]. Studies using gene knockout animals or antibody-mediated depletion of $CD4^+$ or $CD8^+$ T cells demonstrated that both $CD4^+$ and $CD8^+$ T cells can contribute to controlling *Chlamydia* infection, with $CD4^+$ T cells appearing to play a bigger role during natural infection [Landers *et al.*, 1991; Magee *et al.*, 1995; Morrison *et al.*, 1995; Williams *et al.*, 1997]. Furthermore, it has been demonstrated that $CD4^+$ and $CD8^+$ T cell clones can confer protection against *Chlamydia* infection when transferred into nude mice [Igietseme *et al.*, 1993; 1994]. Protection has also been observed following transfer of *Chlamydia*-specific $CD4^+$ and $CD8^+$ T cells into immunocompetent mice [Starnbach *et al.*, 1994; Su & Caldwell, 1995; Roan & Starnbach, 2006; Roan *et al.*, 2006]. Collectively, data from multiple groups suggest that both $CD4^+$ and $CD8^+$ T cells can provide protective effects during *Chlamydia* infection *in vivo*.

$CD4^+$ and $CD8^+$ T cells recognize antigens from different compartments

$CD4^+$ and $CD8^+$ T cells recognize antigens that are processed through different pathways. $CD4^+$ T cells typically recognize antigens that are engulfed by professional antigen presenting cells (APCs). These antigens are processed by proteases within the lysosomal compartments of APCs, and the resulting peptides are presented by major histocompatibility complex (MHC) class II molecules to $CD4^+$ T cells [Trombetta & Mellman, 2005]. *Chlamydia* EBs in the extracellular space within tissues can be phagocytosed by professional

APCs and thereby serve as a source of $CD4^+$ T-cell antigen. RB antigens can also be presented to $CD4^+$ T cells by professional APCs that have engulfed infected cells harboring RB organisms. $CD4^+$ T cells may therefore recognize antigens from multiple stages of *Chlamydia* infection. In contrast, $CD8^+$ T cells typically recognize antigens that have access to the cytosol of infected cells. These cytosolic antigens are processed by the proteasome into peptide fragments, which are eventually shuttled to the cell surface in complex with MHC class I to activate $CD8^+$ T cells [Cresswell *et al.*, 2005]. Although *Chlamydia* are confined to the inclusion during the intracellular stages of the developmental cycle, a number of *Chlamydia* proteins have access to the host cytosol. These include proteins that are secreted into the cytoplasm [Zhong *et al.*, 2001; Clifton *et al.*, 2004] as well as inclusion membrane proteins that have domains which extend into the cytosol of the host cell [Bannantine *et al.*, 2000; Fling *et al.*, 2001]. *Chlamydia* proteins that have access to the cytosol have been demonstrated to serve as $CD8^+$ T-cell antigens [Fling *et al.*, 2001; Starnbach *et al.*, 2003].

Effector functions of $CD4^+$ T cells

Activated $CD4^+$ T cells are important producers of effector cytokines. In general, the types of effector cytokines produced by $CD4^+$ T cells are largely dependent upon the nature of the infection and the types of cytokines present in the environment during T-cell differentiation. Classically, the two major lineages of $CD4^+$ effector T cells are Th1 and Th2 cells. Th1 cells produce IFN-γ, a cytokine also produced by innate effectors, as discussed earlier. In addition to its role in directly limiting *Chlamydia* replication, IFN-γ also plays an important role in adaptive immunity as it can enhance the presentation of antigens to both $CD4^+$ and $CD8^+$ T cells [Gaczynska *et al.*, 1993; Steimle *et al.*, 1994]. $CD4^+$ T cell-derived IFN-γ appears to protect against infection as an IFN-γ-producing $CD4^+$ T-cell clone, but not an IL-4-producing

$CD4^+$ T-cell clone, protected mice against *Chlamydia* genital infection [Hawkins *et al.*, 2002]. In contrast to Th1 cells, Th2 cells produce IL-4, IL-5 and IL-13. They do not appear to protect against *Chlamydia* infection and may even indirectly enhance *Chlamydia* load by inhibiting the development of protective Th1 responses [Brunham & Rey-Ladino, 2005]. The inability to completely eliminate *Chlamydia* organisms from hosts with inadequate Th1 responses can result in continuous production of inflammatory cytokines which can lead to tissue destruction. The factors that determine whether a Th1 or a Th2 response develops following *Chlamydia* infection is largely unknown, but will be important to understand in order to develop a vaccine that will stimulate protective and not pathological outcomes. Recent experiments comparing immune responses against different species of *Chlamydia* have suggested that NKT cells may be an important determinant of the Th1/Th2 bias during *Chlamydia* infections [Joyee *et al.*, 2007]. In addition to cytokine production, $CD4^+$ T cells are also crucial activators of other immune effectors, such as B cells and $CD8^+$ T cells. Mice deficient in $CD4^+$ T cells therefore have defects in multiple arms of adaptive immunity. This may in part explain why mice deficient in $CD4^+$ T cells are more susceptible to *Chlamydia* infection than mice deficient in only B cells or $CD8^+$ T cells [Morrison *et al.*, 1995; Williams *et al.*, 1997].

Effector functions of $CD8^+$ T cells

Like $CD4^+$ T cells, $CD8^+$ T cells are also potent producers of effector cytokines such as IFN-γ. $CD8^+$ T cell-derived IFN-γ can contribute significantly towards controlling *C. trachomatis* infection. Whereas adoptive transfer of IFN-γ-producing *Chlamydia*-specific $CD8^+$ T cells into naïve mice confers protection against *C. trachomatis* challenge, *Chlamydia*-specific $CD8^+$ T cells unable to produce IFN-γ do not confer protection [Lampe *et al.*, 1998]. In

contrast to CD4$^+$ T cells, which recognize peptides presented by MHC class II expressed mainly on professional APCs, CD8$^+$ T cells recognize peptides presented by MHC class I, which is expressed on virtually all nucleated cells. Therefore, CD8$^+$ T cells may provide an essential source of IFN-γ in response to *Chlamydia* infection of the genital mucosa, where bacterial replication occurs predominantly in epithelial cells. In addition to cytokine production, CD8$^+$ T cells also have the ability to directly kill target cells, hence the name cytotoxic T lymphocytes. CD8$^+$ T cell-dependent cytolysis is very specific for cells expressing the appropriate peptide-MHC class I complex, thereby ensuring the elimination of infected cells while sparing neighboring healthy ones. T cell-mediated lysis of *Chlamydia*-infected cells has been observed *ex vivo* [Starnbach *et al.*, 1994; Roan & Starnbach, 2006]. It has always been tempting to predict that lysis of infected cells should contribute to controlling infection as it could deprive *C. trachomatis* of its intracellular niche and lead to the release of non-infectious RBs. However, a role for lysis in protection against *C. trachomatis* infection *in vivo* has yet to be demonstrated.

Antigen-specific T-cell responses to C. trachomatis

Although it has long been appreciated that both CD4$^+$ and CD8$^+$ T cells respond to *C. trachomatis* infection, it is only recently that investigators have begun to examine how T cells specific for defined *Chlamydia* antigens respond to the infection. The ability to now identify and characterize T cells specific for defined *Chlamydia* antigens will allow to identify homing molecules which direct *Chlamydia*-specific T cells to the genital mucosa. In order to determine how the properties of individual antigens can influence T-cell responses, it will be valuable to determine when during the developmental cycle these *Chlamydia* antigens are expressed, and to correlate this information with the kinetics, magnitude, effector

functions and protective capacities of T cells specific for these antigens. T cells recognizing antigens expressed early during the developmental cycle may play a bigger role in controlling infection because they may recognize infected cells before noninfectious *Chlamydia* RBs have differentiated back into infectious EBs. In contrast, T cells recognizing antigens expressed late during the developmental cycle may be ineffective at containing infection because recognition and destruction of *Chlamydia*-infected cells late in the developmental cycle may simply result in the release of infectious EBs which can go on to infect new sites. It will be important to compare T-cell responses against a range of antigens expressed at different stages of the developmental cycle in order to identify the window of time when recognition of infected cells has the largest impact in terms of limiting *Chlamydia* replication and dissemination.

T-cell responses may also be influenced by the subcellular localization of *Chlamydia* protein antigens. In particular, $CD8^+$ T cells typically recognize proteins that have access to the cytosol of non-professional APCs. However, $CD8^+$ T cells can be initially primed against not only cytosolic antigens but also non-cytosolic antigens. Priming of non-cytosolic antigens can occur via cross-presentation, a process whereby professional APCs phagocytose and present antigens from infected epithelial cells to T cells. Although $CD8^+$ T cells specific for non-cytosolic antigens can be primed, during the subsequent effector phase these T cells may never recognize epithelial cells infected with *C. trachomatis* as epithelial cells are unable to crosspresent non-cytosolic antigens. As a consequence, these $CD8^+$ T cells specific for non-cytosolic antigens may not recognize the typical cell type infected with *C. trachomatis*, and may therefore not contribute significantly towards containing the infection.

***C. trachomatis* persistence and immunopathology**

Despite the ability of the immune system to detect and respond to *Chlamydia* infection, *Chlamydia* appears to persist within the host. There is evidence of *Chlamydia* persistence both *in vitro* and *in vivo*. *In vitro*, low levels of IFN-γ can induce *Chlamydia* to develop into an aberrant, dormant state that may represent a persistent form of the organism [Beatty *et al.*, 1993]. Incubation of infected cells under stressful conditions such as nutrient deprivation or in the presence of some antibiotics can also induce *Chlamydia* to differentiate into a phenotypically similar form [Coles *et al.*, 1993; Raulston, 1997; Hogan *et al.*, 2004]. These dormant forms can revert to metabolically active forms following IFN-γ removal or the elimination of stressful conditions, showing that persistence *in vitro* is reversible.

Evidence for *Chlamydia* persistence *in vivo* is indirect. *Chlamydia* antigen, DNA and RNA have been detected in human patients that were culture-negative [Rahman *et al.*, 1992; Patton *et al.*, 1994; Gerard *et al.*, 1998], suggesting the presence of unculturable *Chlamydia* organisms that remain within hosts. *Chlamydia* persistence may lead to the pathological outcome associated with infection. If the organisms are not completely cleared by the immune system, they may persist within the host. As the immune response to the organisms wanes, persistent *Chlamydia* may reactivate and replicate, and in turn restimulate inflammation and recruitment of immune effectors to the site of infection. The inflammatory effectors deployed by the immune system may then drive the organisms back into a persistent form. Repeated cycles of *Chlamydia* persistence alternating with reactivation may stimulate the chronic inflammation associated with *Chlamydia*-induced disease.

Immune evasion by C. trachomatis

Chlamydia persistence may be mediated in part by mechanisms *Chlamydia* has evolved to avoid recognition by the host. *C. trachomatis* can directly downregulate the expression of MHC molecules within infected cells through the degradation of RFX-5 and USF-1, transcription factors that induce expression of MHC class I and MHC class II respectively [Zhong *et al.*, 1999; 2000]. Degradation is mediated by CPAF, a chlamydial protease that is secreted into the host cytosol [Zhong *et al.*, 2001]. Downregulation of MHC expression on infected cells can potentially limit their recognition by *Chlamydia*-specific $CD4^+$ and $CD8^+$ T cells, but may also render the infected cells susceptible to lysis by NK cells. Indeed, human NK cells are able to lyse *Chlamydia*-infected epithelial cells [Hook *et al.*, 2004]. The role of CPAF in immune evasion *in vivo* remains to be determined. Another class of factors that may play a role in evading T cells are the deubiquitinases (DUBs) encoded by *C. trachomatis* [Misaghi *et al.* 2006]. In addition to downregulating MHC expression, CPAF has more recently been demonstrated to also degrade pro-apoptotic BH3-only proteins [Pirbhai *et al.*, 2006]. The ability of *Chlamydia* to degrade BH3-only proteins, as well as sequester pro-apoptotic proteins and induce the expression of antiapoptotic factors, may explain the longstanding observation that *Chlamydia*-infected cells are largely resistant to immune-mediated induction of apoptosis *in vitro* [Byrne & Ojcius, 2004; Miyairi & Byrne, 2006]. Evading apoptosis may be important for *Chlamydia* in order to allow the bacteria to complete their replication cycle and prevent the release of non-infectious RBs. However, the influence of *Chlamydia* on host cell apoptosis is complex as *Chlamydia* has also been documented to induce apoptosis [Perfettini *et al.*, 2000]. Apoptosis can be mediated through the expression of *Chlamydia* protein associated with death domain, which may promote apoptosis through

association with mammalian death receptors [Byrne & Ojcius, 2004; Miyairi & Byrne, 2006]. In addition, *Chlamydia* induction of TNF-α may promote apoptosis of both infected as well as bystander cells [Perfettini *et al.*, 2000]. Indeed, TNF-α produced by *Chlamydia*-infected macrophages has been shown to induce T-cell apoptosis in a co-culture system [Jendro *et al.*, 2004]. Although it appears contradictory that *Chlamydia* can both inhibit and induce apoptosis, both these processes may help protect the bacterium against immune effectors *in vivo. Chlamydia* may restrict apoptosis long enough to complete the developmental cycle, thereby allowing time for differentiation of RBs back into infectious EBs. Towards the end of the developmental cycle, it may be beneficial for the organism to induce apoptosis in order to avoid necrosis, which can stimulate inflammation and enhance *Chlamydia*-specific immune responses. Apoptosis of *Chlamydia*-infected cells towards the end of the developmental cycle may also aid in the release of infectious *Chlamydia* EBs. *In vivo*, the evidence for immune evasion by *C. trachomatis* is indirect. Previous exposure of humans to *C. trachomatis* does not provide robust immunity against re-infection, perhaps because *Chlamydia* infection induces the development of inadequate immune effectors [Brunham & Rey-Ladino, 2005].

Vaccines against C. trachomatis

The development of a *Chlamydia* vaccine could significantly reduce the prevalence of infection, leading to a decrease in *Chlamydia*-associated diseases. This would also lead to a relief in the socio-economic burden associated with diagnosis and treatment of the infection, and a relief in the significant financial burden associated with treatment of *Chlamydia*-induced sequelae such as infertility, pelvic inflammatory disease and ectopic pregnancy. The major challenge in developing an effective *Chlamydia* vaccine is to generate long-lasting and

sterilizing immunity while avoiding immunopathology. In the 1960s, a human vaccine trial for trachoma was carried out using non-viable whole organisms. The results of the trial suggested that immunity is serovar-specific and short-lived. In addition, post-vaccination exposure to *Chlamydia* in some individuals resulted in more severe disease than that seen in unvaccinated individuals [Grayston & Wang, 1978]. The disappointing results of this trial have directed attention away from whole organisms towards a focus on a subunit vaccine approach. Most subunit vaccine efforts have focused on MOMP because this protein is abundant (comprising 60% of the total protein mass in the outer membrane), surface-exposed, and elicits T-cell responses and neutralizing antibodies [Caldwell *et al.*, 1981]. Although immune responses against MOMP can be elicited, protection against *Chlamydia* infection following MOMP immunization is rather poor [Stagg, 1998; de la Maza & Peterson, 2002]. The disappointing results of MOMP as a vaccine candidate may in part be due to the failure to obtain the native conformation of MOMP in these immunization protocols [Pal *et al.*, 2001]. Regardless, efforts are under way to identify other *Chlamydia* antigens as a subunit vaccine consisting of multiple antigens is more likely to be effective at inducing protective immunity. An ideal subunit vaccine should elicit immunity superior to that observed following natural infection, as it has long been observed that previous exposure to *C. trachomatis* does not provide significant immunity against re-infection [Brunham and Rey-Ladino, 2005]. It would seem that stimulating multiple arms of the adaptive immune system would be the ideal approach to induce robust immunity. A subunit vaccine that stimulates B cells to produce neutralizing antibodies against cell-surface proteins, such as MOMP [Peeling *et al.*, 1984; Peterson *et al.*, 1991], may decrease the number of *Chlamydia* organisms that are able to establish intracellular infection. But in order to be effective, a

subunit vaccine also should also stimulate immune effectors that can recognize the *Chlamydia* organisms once they establish intracellular infection. Identifying appropriate targets for subunit vaccine incorporation will be crucial in eliciting sterilizing immunity against *C. trachomatis*.

Gaps in existing research

More than two-third of the chlamydial infections cases occur in the developing countries, where diagnostic and treatment services are almost absent. An estimated 15 million new cases are occurring in Africa and 45 million new cases in Southern Asia every year [Brunham & Rey- Ladino, 2005]. Because of its effect on reproduction, programmes to control *C. trachomatis* have been implemented in many developed countries but many regions are now showing an increase in the number of infected individuals [Gotz, 2002]. Since current programmes for the control of *C. trachomatis* are not affordable for much of the developing countries, vaccine development have been identified as an essential to controlling infection with *C. trachomatis*.

Innate immune cells, B cells and T cells all respond to infection with *C. trachomatis*. Some of these responses are effective at controlling infection, whereas others are not. Ineffective responses which fail to eliminate the *Chlamydia* organisms from the host may induce chronic inflammation which can lead to tissue pathology. The balance between protective versus pathological responses determines the final outcome of *Chlamydia* infection. Unfortunately, in many cases the outcome is lack of *Chlamydia* clearance and tissue damage. Factors that may influence whether the immune response to *C. trachomatis* is protective or pathological include the nature of the antigens presented during infection, the effector functions deployed

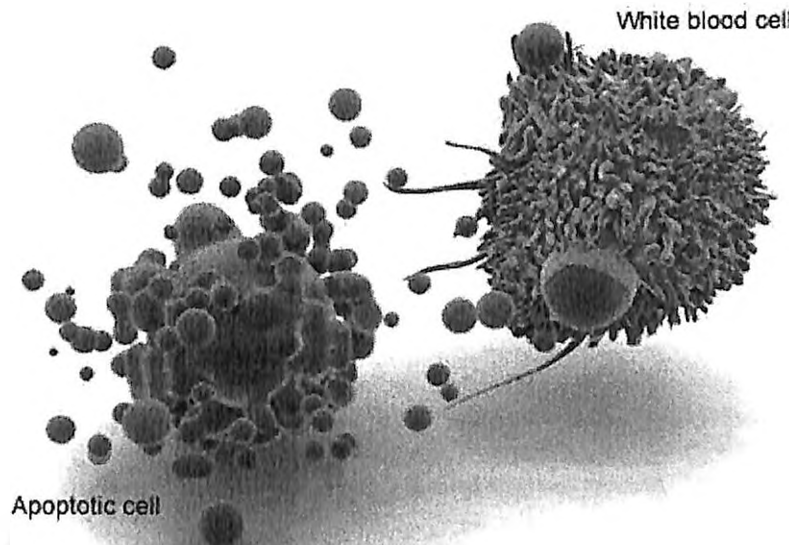
by immune effectors, as well as immune evasion strategies implemented by the *Chlamydia* organisms are largely unknown.

Mouse models of chlamydial infections with *C. muridarum* have provided information on the immune mechanisms of clearance of infection and resistance to reinfection, but there are several important differences between *C. muridarum* and *C. trachomatis* that might effect the immunobiology of infection. Firstly, *C. trachomatis* infection in humans is much more prolonged than *C. muridarum* infection in mice [Parks *et al.*, 1997]. Secondly, immune-evasion mechanisms also differ. These differences limit the direct extrapolation of findings from *C. muridarum* infection to *C. trachomatis* infection. Thus, a better definition of human immune response correlates with *C. trachomatis* protective immunity and disease pathogenesis needs to remain an important research priority if we are to develop a vaccine against *C. trachomatis* infection that has protective and not deleterious effects.

A better understanding of characteristics which define protective versus pathological responses will be instrumental for developing an effective vaccine that can generate sterilizing immunity without inducing tissue pathology.

Chapter 3
AIMS AND OBJECTIVES

He who seeks for methods without having a definite problem in mind seeks in the most part in vain
David Hilbert



Aims and Objectives

The aim of the study is to understand host-pathogen interactions during *Chlamydia trachomatis* infection of human females in terms of both the innate and the adaptive immune response. The study will provide a better definition of human immune response correlates with *C. trachomatis* protective immunity and disease pathogenesis. The specific objectives of the present study have been defined as under:

1. Evaluation of mucosal immune responses to *Chlamydia trachomatis* in human females.

Patients attending the Gynecology out patient department of Safdarjung Hospital, New Delhi and controls will be enrolled after obtaining informed consent. Cervical samples and sera will be collected for studying immune responses. Quantification of cervical immune cell population will be done by flow cytometry and cytokines and sex hormone levels will be evaluated by ELISA. Cervical cells will be pulsed with chlamydial antigens (MOMP and cHSPs) cytokine secretion pattern will be studied. Local markers (Biomarkers-cervical cytokines and antibodies) will be identified which would help in better prediction and screening of women at higher risk of developing sequelae to chlamydial infection. Further, association between chlamydial infectious load and immune correlates will be done to gain better understanding of infectious load regulation.

2. Enumeration of cervical dendritic cell subsets and their role in modulation of immune response to *Chlamydia trachomatis* infection.

Cervical dendritic cell subsets (myeloid and plasmacytoid) will be enumerated in cervical samples by flow cytometry in both pre and post-treatment stage and in acute and chronic

conditions in women infected with *Chlamydia trachomatis* to understand their role in modulation of immune response to *Chlamydia trachomatis*.

3. Innate immune response (TLRs and nitric oxide) to *Chlamydia trachomatis* in protection against infection.

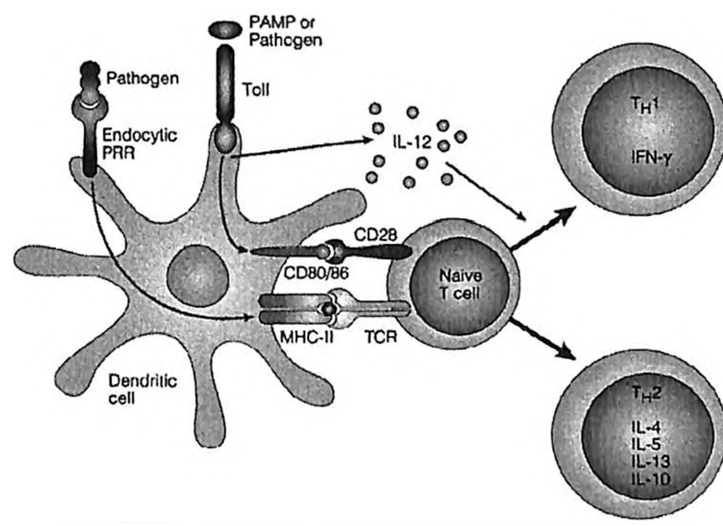
Expression of TLR and inducible nitric oxide synthetase (iNOS) genes will be studied by Real time Polymerase Chain Reaction (PCR). Signalling pathways will be studied by Real time based PCR arrays and western blotting. This study will help in understanding the recognition of *Chlamydia* by antigen presenting cells and the role of innate immune responses in clearance/persistence of infection.

4. *In vitro* presentation of chlamydial elementary bodies by monocyte derived dendritic cells to autologous $CD4^+$ T cells.

Monocyte derived dendritic cells will be pulsed with live chlamydial EBs and cytokine responses will be measured. Pulsed DCs will be co-cultured with autologous $CD4^+$ T cells and proliferation of T cells will be studied. Secreted cytokine will be measured by ELISA to assess protective/pathogenic immune response. Effect of β-estradiol on modulation of dendritic cell function will also be studied.

Chapter 4
Evaluation of mucosal immune responses to Chlamydia trachomatis in human females

If you're not part of the solution, you're part of the precipitate
Henry J. Tillman



Evaluation of mucosal immune response to Chlamydia trachomatis in human females

Introduction

Chlamydiae are intracellular bacterial pathogens that cause a spectrum of clinically important diseases in humans and are the most prevalent sexually transmitted bacterial infections worldwide [West *et al.*, 1991]. *C. trachomatis* undergo a common intracellular biphasic growth cycle [Fields & Hackstadt, 2002] which starts with the entry of elementary bodies (EBs), the infectious form and reticulate bodies, the metabolically active but noninfectious form of chlamydial organisms. All *Chlamydia* species can accomplish their entire biosynthesis, replication, and differentiation within the cytoplasmic vacuole known as inclusion.

Little is known, however, about the pathogenesis of acute human chlamydial infections, and our knowledge of these had been derived largely from animal models [Rank *et al.*, 1985]. Both innate and acquired immunity appear to contribute to this outcome, but, in most cases, the host responses to chlamydial infection are associated with long-term tissue damage [Buchholz & Stephens, 2006]. The complications of chlamydial infection are seen disproportionately in women in whom it may develop pelvic inflammatory disease (PID), ectopic pregnancy, tubal infertility or chronic pelvic pain [McNeely, 1992]. While, chlamydial infection persists in some individuals, spontaneous clearance of infection has been observed in others without therapy [Parks *et al.*, 1997], suggesting that immune responses are often effective in controlling chlamydial infection.

Immunity to chlamydial infection has been shown to involve both humoral and cell-mediated immune responses [Cain & Rank, 1995; Cotter, 1995]. Studies in both animal and human

models have established that both cytokine producing T cells and antibody producing B cells [Bailey *et al.*, 1995; Cohen *et al.*, 2000; Morrison & Caldwell, 2002; Yang, 1999] are critically involved in the clearance of chlamydial infection and resistance to reinfection. Recruitment of inflammatory and antigen presenting cells such as macrophages and dendritic cells, to the site of infection with subsequent release of proinflammatory cytokines appears to be crucial for innate resistance to *Chlamydia.* During reinfection or reactivation of a persistent infection, T cells infiltrate more rapidly and in larger numbers as compared with the host response to a primary infection [Grayston *et al.*, 1985].

Epithelial cells at the mucosal surfaces secrete chemoattractants and proinflammatory cytokines in response to infection with *Chlamydia.* These cytokines are unique to chlamydial interaction with the host cells of the female genital tract [Stephens, 2003; Yang, 2003]. Numerous studies have indicated that rapid and early recruitment of certain immune effectors especially Th1 cells to the local genital mucosa are crucial for antichlamydial immunity [Igietseme *et al.*, 1998; Patton & Rank, 1992; Stagg *et al.*, 1998]. Upon infection, mononuclear cells are triggered to release a number of proinflammatory cytokines including TNF-α, IL-1, IL-6 and IL-8 [Heinemann *et al.*, 1996; Redecke *et al.*, 1998]. CD4 and CD8 T cells when induced, secrete various cytokines especially IFN-γ which has the major role in antichlamydial immune response [Johansson *et al.*, 1997; Perry *et al.*, 1997]. In contrast to these results which depicted the protective role of IFN-γ, other studies have shown that higher levels of cervical IFN-γ secretion in response to *Chlamydia* induced stress proteins like chlamydial heat shock proteins which may lead to tissue damage.

Heat shock proteins (HSPs) are members of a family of stress response proteins, which protects the cells from a variety of insults [Lindquist & Craig, 1988]. A number of infectious

diseases have been associated with activated humoral and cellular responses to microbial HSPs [Zugel & Kaufmann, 1999]. The chlamydial 60 and 10-kDa heat shock proteins (cHSP60 and 10) are thought to be major target antigens, which stimulate a strong pathogenic inflammatory response [Cerrone *et al.*, 1991] in both animal models and in humans with chlamydial genital tract infections. Strong association of serum cHSP60 antibodies with tubal factor infertility has been demonstrated [Hartog *et al.*, 2005]. A specific role for cHSP60 in the pathogenesis of salpingitis has also been suggested by an experimental monkey model of infection [Patton *et al.*, 1994]. However, cHSP expression by *Chlamydia* in human females, its modulation by IFN-γ and its role in persistence of *Chlamydia* leading to development of sequalae in human females still remains to be elucidated.

Further, studies in mice as well as other animal models show that hormones play an important role in controlling immune responses to chlamydial infection and in determining the outcome of infection [Patton & Rank, 1992]. In mice, establishment of infection is dependent either on stage of the estrus cycle or requires high infectious doses [Ito *et al.*, 1984]. In human studies, an association has been observed between the onset of chlamydial infection and the stage of menstrual cycle [Sweet *et al.*, 1986]. Enhanced susceptibility was observed during the proliferative part of the menstrual cycle when β-estradiol levels were high. Oral contraceptives have also been shown to increase susceptibility to chlamydial infections and other sexually transmitted diseases [Washington *et al.*, 1985]. However, till date no study on human sex hormone levels and its association with chlamydial infection has been done.

Mucosal surfaces represent the largest area of contact for microbial agents with the body's immune system [Podoisky, 1999]. Given that chlamydial organisms infect epithelial cells of the cervix, the local mucosal responses should reflect more directly, the host response to chlamydial infection. Chlamydial infection has been known to modulate local immune responses by secretion of cytokines from immune cell aggregates recruited to the mucosal tissues. Our understanding of the exact pathological mechanism involved during chlamydial infection is largely unknown. It is very difficult to understand how the infection is established and then cleared from the genital tract. Further, it is necessary to elucidate the dynamics of major cytokine responses in cervix and their role in the pathogenesis or the development of protective immunity towards genital *Chlamydia trachomatis* infection. Therefore, this study was planned with the following objectives:

1. To study cytokine levels in cervix of *Chlamydia* infected women and their association with sex hormone (β-estradiol and progesterone) levels.
2. To study cytokine secretion pattern of cervical cells obtained from *Chlamydia* positive women with or without pathological disorder.
3. To characterize and compare mucosal and peripheral immune response to chlamydial heat shock proteins in women with primary and recurrent chlamydial infections.
4. Finding local markers which could help in better prediction and screening of women at higher risk of developing sequelae to chlamydial infection.
5. To determine association between chlamydial infectious load and immune correlates to gain better understanding of infectious load regulation.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plasticware and glassware used in cell culture and immune assays was purchased from Greiner, Germany.

Experimental Methods

Study population and patient classification

After obtaining informed written consent, 853 patients attending the gynecology outpatient department, Safdarjung Hospital, New Delhi, India, were enrolled for the study. Healthy age matched controls attending the family planning department for birth control measures were also enrolled. The study received approval from the hospital's ethics review committee. The procedures followed for sample collection were in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki of 1975 (revised in 1983). At recruitment, a detailed clinical questionnaire was administered to each patient to collect information on the reasons for referral, gynecology history, including menstruation, symptoms of genital and urinary tract infection, obstetric and medical histories.

Inclusion criteria

The *Chlamydia*-positive patients included in the study comprised of (a) *Chlamydia* positive fertile asymptomatic women attending a family planning clinic, (b) *Chlamydia* positive fertile women with mucopurulent cervicitis (MPC) [thick discharge and inflammation with number of polymorphonuclear leukocytes (PMNLs) > 30], (c) *Chlamydia* positive women with Fertility Disorders (FD) and (d) *Chlamydia* positive women having recurrent chlamydial infections and being tested positive for infection on ≥2 consecutive visits separated by three months.

Fertile women (asymptomatic women and women with MPC) were described as those having last childbirth within the last 4 months to 1 year and testing positive for *Chlamydia* at the time of conception. Women with fertility-related disorders included those with infertility and Multiple Spontaneous Abortions (MSA). Infertile women were identified as those who lacked recognized conception after 1.5–2 years of regular intercourse without the use of contraception. The infertile group included women with referred diagnostic laparoscopy [Reddy *et al.*, 2004] and women with unknown reasons. Women with MSA (more than 2) have been described as those having delivery of a previable fetus before the 20th week of gestation.

Exclusion criteria

Patients taking oral contraceptives, having a positive urine pregnancy test, recent antibiotic therapy, a history of recently treated sexually transmitted infection other than *Chlamydia* and genital tuberculosis were excluded from the study. Further, women with male factor-related infertility, endometriosis or any other factors such as hormonal imbalances, which could be considered as a cause for infertility, were also excluded. Patients with overlapping group characteristics were also excluded to keep the groups well segregated.

Collection of samples

Cervical samples were collected during midcycle (median 13 days, range 9th–15th day of the menstrual cycle) to avoid variations in sex hormone levels. None of the patients had sexual intercourse 3 days or more before collection of the sample.

The vulva was examined for lesions and the cervix for warts, ulcers, ectopy, erythema and discharge if any. After cleaning the endocervix with a sterile cotton swab, 3 endocervical swabs (HiMedia, Mumbai, India) were collected from patients and controls for diagnosis of

C. trachomatis and other sexually transmitted pathogens. For cell culture, one swab was collected in 1mL of SPG (sucrose, 75g; KH_2PO_4, 0.52g; Na_2HPO_4, 1.22g; glutamic acid, 0.72g; water, 1L; and pH 7.4-7.6) transport media. An additional vaginal cotton swab was collected for screening of pathogens like *Candida* spp, bacterial vaginosis and *Trichomonas vaginalis*.

For collection of cervical cells, a cytobrush was placed within the endocervical canal so that the cells from the endocervical region and the zone between the endocervical and ectocervical region (transformation zone) could be obtained. The cytobrush was then transferred to a sterile centrifuge tube containing sterile Phosphate Buffer Saline (PBS) (pH 7.2) supplemented with 100 U penicillin/mL, 100 µg streptomycin/mL, and 100 µg glutamine/mL. All cytobrush samples had negative results for blood contamination. Cervical washes were collected in 5 mL of sterile saline administered through a sterile Pasteur pipette and recovered after thoroughly washing the cervix and were, then stored at 4^0C until, transported to the laboratory within one hour. 2 mL non heparinised blood for separating serum and 10 ml of venous blood was collected into heparinized vials for isolation and culture of lymphocytes. At the laboratory serum and cervical wash samples were aliqouted and stored at -80^0C until assay and the cervical cells were processed within one hour.

Microbiology

5mm spots were made on clean glass slides using endocervical swabs. These were stained with flourescein isothiocyanate (FITC) conjugated monoclonal antibodies to *C. trachomatis* major outer membrane protein using *Direct Specimen Test kit* (Microtrak, Palo Alto, USA) according to the manufacturer's instructions. A sample was considered to be positive when at least 10 elementary bodies (EB's) were detected. Samples with greater than 1 and less than

10 EBs were further confirmed for positivity by PCR analysis using primer specific for 517 bp plasmid of *C. trachomatis* [Singh *et al.*, 2002].

Gram stained cervical and vaginal smears were examined for the presence of yeast cells (Candidiasis) and clue cells for diagnosis of bacterial vaginosis. Gram stain showing predominance of *Lactobacillus* morphotype was interpreted as normal. When showing *Gardnerella* morphotype or mixed flora they were interpreted as consistent with bacterial vaginosis. Wet mount microscopy was done for diagnosis of *Trichomonas vaginalis*. For detection of *Neisseria gonorrhoeae* cervical specimens were incubated at 35^0C in humidified CO_2 incubator for 48 hrs on Thayer Martin medium. Colony growth was noted and *N. gonorrhoeae* was identified on basis of gram stained smears. Pleuropneumonia-Like Organisms (PPLO) broth was used for the identification of *Mycobacterium hominis* and *Ureaplasma urealyticum* by diluting the cervical samples in arginine-containing and urea-containing liquid media, respectively, and thereafter, incubating the media at 37^0C until there is change in colour after 5-7 days for *M. hominis* and 2 days for *U. urealyticum*.

Isolation of endocervical cells

Cervical cells were isolated from the cytobrush by vigorously rotating it against the sides of the transport tube after incubating the sample with 5mM DL-dithiothreitol (Sigma, St. Louis, USA) at 37^0C for 15 min (to reduce the mucus component of the sample). The cell suspension obtained was then filtered through a sterile 70-μm nylon cell strainer (Becton Dickinson, San Diego, USA) to make a homogenous preparation of cells. These were then centrifuged at 200 *g* for 10 min; the resulting pellet yielding endocervical cells. Viability of cells was determined by Trypan Blue Exclusion assay. The population of epithelial cells and

lymphocytes in the cytobrush sample was counted with a haemocytometer and samples containing less than 1 million lymphocytes/mL, were excluded.

Flow Cytometry

Quantification of different cervical immune cells was performed by standard flow cytometry. Cervical cells were stained with Fluorescein isothiocyanate (FITC) conjugated anti-CD4, -CD8, -CD14 and -CD19 antibodies, and PE conjugated anti-CD3 (Becton Dickinson, San Jose, USA) for 25 min on ice. Appropriate isotype-matched control antibodies were also used to rule out nonspecific fluorescence. Preparations were then washed with stain buffer (PBS supplemented with 0.1% NaN_3 and 2% Fetal Bovine Serum) and acquired using Fluorescence-activated cell sorter (FACS) Caliber (BD Biosciences, San Jose, USA). A total of 10,000 events were acquired and analysis was done using CELLQUEST software (Becton Dickinson).

Cell culture for determination of infectious load

Quantitative cultures were performed as described previously [Brunham *et al.,* 1983], with modifications [Mittal *et al.,* 1993]. Cervical specimens in SPG transport media were vortexed and a 0.1-mL portion was inoculated to a McCoy cell monolayer (grown on coverslips) in each of two wells of a 24-well cell culture plate. In addition, two culture wells were inoculated with 0.1mL each of specimen for passage to three new culture wells each. After incubation for 48 h at 35^0C and 5% CO_2, chlamydial inclusions were detected with a genus-specific antichlamydial lipopolysaccharide monoclonal antibody conjugated with fluorescence. The number of inclusions was determined either by counting all inclusions in each well (if <100 IFUs) or by averaging the number of inclusions in each of the three fields and multiplying that value by the number of fields per well. The average number of

inclusions per well was multiplied by 10 to yield the number of $IFUsmL^{-1}$ of transport medium. If no inclusions were seen in any field, the entire coverslip was scanned to assess the titer. If no inclusions were seen on scanning the entire coverslip, but inclusions did appear when the specimen was blindly passed, the specimen was defined as having <10 $IFUmL^{-1}$.

Lymphoproliferative assay

Peripheral blood mononuclear cells (PBMCs) were prepared by ficoll-Hypaque density gradient centrifugation. The endocervical cells and PBMCs were then washed three times with Hank's balanced salt solution (Sigma, St Louis, MO, USA) and suspended in RPMI 1640 medium (Sigma) supplemented with 10% heat inactivated human AB^+ serum for lymphoproliferation assay. Briefly, the cells were cultured in triplicates in round bottom 96 well plates (5×10^4cells/well) with or without antigen in a total volume of 200 µl. Cultures were incubated in humidified 5% CO_2 at 37^0C for 6 days. Synthetic biotinylated peptides corresponding to epitopes of Major Outer Membrane Protein (MOMP) antigen (VLGTSMAEFISTNVIS) and *C. trachomatis* heat shock protein 60 and 10 [Microsynth, India] were used as antigens at a protein concentration of 2.5µg/ml. The peptides corresponded to amino acids 151-162 (SANNDAEIGNLI) for cHSP60 and 79-87 (SGQELTVEG) for cHSP10 [Yi *et al.,* 1993; La Verda & Bryne, 1997]. Phytohaematogluttinin (Sigma, 1µg/ml) was used as a positive control mitogen in each experiment. Optimum concentrations of antigens and mitogen were determined in preliminary experiments as minimum concentrations giving maximum proliferations. Tritiated thymidine [^3H] (Bhabha Atomic Research Centre, Mumbai, India) was added to the cultures before last 18 hrs of incubation. Proliferative responses were measured as counts per

minutes (cpm) of incorporated radioactivity using a liquid scintillation counter (Packard Biosciences, Downers Grove, USA). Results were expressed as stimulation indices (SI=mean cpm in presence of antigen divided by mean cpm in its absence). A SI value >2 was considered as a positive response. After 6 days cells were pelleted down by centrifugation and supernatants were collected and stored for detection of antibodies against cHSP60 and 10.

Cell cultures

PBMCs and endocervical cells were isolated and counted as described above. Endocervical cells were washed three times with Hank's balanced salt solution (Sigma, St Louis, MO, USA) and suspended in RPMI-1640 medium (Sigma) supplemented with 10% heat-inactivated human AB^+ serum. The cells were then cultured in triplicate ($2x10^5$ mononuclear cells/well) in round-bottomed 96-well plates (for mRNA expression and estimation of secreted cytokines) and in 96-well PVDF-bottomed plates (Millipore, Danvers, USA) for ELISPOT assays, with or without antigen in a total volume of 200μl. Subsequently, cultures were incubated in humidified 5% CO_2 at 37^0C for 12 hours (for RT-PCR), 18 hours (for ELISPOT) and 3 days (for ELISA). Cells were stimulated with/without *C. trachomatis* serovar D (a human clinical genital isolate) whole EBs at multiplicity of infection 2. Phytohaemaglutinin (PHA 2μg/ml) (Sigma Aldrich, St.Louis, USA) was used as positive control mitogen in each experiment. *C. trachomatis* serovar D was grown on confluent McCoy cell monolayers as described previously [Mittal *et al.*, 1993] and EBs were harvested and purified on Renograffin gradients. Optimum concentrations of antigen and mitogen were determined in preliminary experiments as minimum concentrations giving maximal proliferation at different time intervals post stimulation.

RNA extraction from cervical cells and RT-PCR analysis for cytokines.

Total RNA from stimulated cervical cells was isolated using RNeasy Mini Kit (Qiagen, California,USA), in accordance with the manufacturer's instructions and stored at -70^{0}C. Complementary DNA (cDNA) was prepared using a SuperScript^{TM} First-Strand Reverse Transcriptase kit (Invitrogen, California, USA), in accordance with the manufacturer's instructions. Sequences for housekeeping Hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene and cytokine genes (IL-1β, IL-4, IL-6, IL-10, IL-12 and IFN-γ) and cycling parameters used in this study were same as mentioned earlier [Habibi *et al.*, 2001]. The cDNAs were normalized on the basis of HPRT expression. The reaction mixture (20 µl) for PCR contained cDNA, 200 µmol/L each dNTP, 1.5 µmol/L, $MgCl_2$, 25 pmol of each primer, and 0.5 U of Taq DNA polymerase and PCR was performed in a thermal cycler (Eppendorf AG, Hamburg, Germany). The amplified products were electrophoresed on 1.2% agarose gel stained with ethidium bromide and visualized on Alpha Imager gel documentation system (AlphaInnotech, San Leandro, USA). Cytokines expression levels were calculated as expression index (signal intensity of cytokine/ signal intensity of HPRT as determined by densitometry).

Antibody assays

Sera of patients and controls were assayed for Immunoglobulin (IgG) antibodies to *C. trachomatis* surface components using a commercially available ELISA kit (Ridascreen, AG, Germany) according to manufacturer's instructions. Results were obtained as mean absorbance of duplicated samples at 450 nm. An optical density (O.D.) >1.1 was considered as positive.

Cervical washes of patients and controls were assayed for presence of IgG and IgA antibodies to synthetic biotinylated peptides for cHSP60, 10 and MOMP antigen (Microsynth, New Delhi, India). The peptide sequence for the antigens is as described under 'lymphoproliferative assay'. Briefly, the peptides were bound to the wells of a microtiter plate (2.5μg/well) in carbonate buffer (14.2mM Na_2CO_3, 34.9mM $NaHCO_3$, 3.1mM NaN_3, pH 9.5) and were incubated overnight at 4^0C. After washing the unbound peptides the non specific binding sites were blocked with phosphate-buffered saline (PBS)-0.5% bovine serum albumin at 37^0C for 60 min. 100μl of cervical wash was then added and after incubation at 37°C for 120 min. 100μl of 1:10,000 dilution of peroxidase-conjugated goat antibody to human IgA and IgG (Jackson Immunoresearch, Baltimore, USA) was added to each well. After further incubation of 60-min at 37°C, the peroxidase substrate, tetramethylene benzidine (TMB) was added. The reaction was stopped with 0.5 M H_2SO_4 and the plates were read at 450 nm. Known positive and negative controls (cervical washes of known *C. trachomatis* positive and negative women) were always assayed in parallel to test samples. A positive sample was defined as one yielding an optical density value that was at least 2SD above the mean value of known negative samples for all three antigens.

Quantification of cytokines

Quantification of cytokines (IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, and IFN-γ) in cervical washes, sera and culture supernatants was done by using commercially available ELISA kits (eBiosciennces, San Diego, USA), in accordance with manufacturer's instructions. Briefly, 96 well ELISA plates were coated overnight with anti-human capture antibodies for above mentioned cytokines. Unbound coating antibody was removed and nonspecific protein binding sites were blocked with assay diluent as per the manufacturer's

instructions. Duplicate serial dilutions of test samples or controls were incubated for 2 hours at room temperature. Detector antibodies were added and incubated for 1 hour followed by incubation with peroxidase conjugated anti mouse IgG for 30 min. To each well 50μl of TMB was added and color development was stopped after 15 min by addition of 1N sulfuric acid. Absorbance was read at 450 nm with a reference absorbance at 650nm. A log-log standard curve was generated and unknowns were interpolated. The sensitivities of cytokine kits were 2 pg/mL. Positive IFN-γ level in cervical washes was defined as one that was at least 2SD above the mean value of IFN-γ levels in known *Chlamydia trachomatis* negative samples.

Cytokine Analysis using ELISPOT assay

The numbers of IL-4, IL-10, IFN-γ and IL-12 producing cells in cervical cells were measured by ELISPOT assay (Diaclone, Cedex, France) as per the manufacturer's instructions and the number of cytokines secreting cells were counted on a Bioreader® 4000 ELISPOT Reader (Biosys, Leeds, UK).

Protein estimation:

Total protein levels in cervical secretions were estimated by Bradford method. Briefly, 5μl of Bovine serum albumin protein standards and cervical wash samples in triplicate were added to separate wells in a 96 well plate. To these wells, 250μl of Bradford reagent (Sigma, Saint Louis, USA) was added and incubated at room temperature for 30 minutes. Absorbance was read at 595 nm. A standard curve was generated and unknowns were interpolated.

Hormonal assay

Levels of β-estradiol and progesterone in sera of patients and controls were measured using commercially available ELISA kits (DRG, International Inc, USA) as per the manufacturer's instructions.

Determination of C-reactive protein (CRP) levels

CRP was determined using high sensitivity hs-CRP ELISA (Calboitech, USA) according to the manufacturer's instructions and CRP levels above 2 microgram per milliliter were considered to show higher risk of chronic inflammation.

Statistical analysis

The Kruskal-Wallis non parametric test was used to compare continuous variables among multiple groups. The Mann-Whitney U test was used for comparing two groups. Categorical variables were compared using $\chi 2$ test. Correlation was tested with Spearman's correlation coefficient. The prognostic values of single tests as well as test combination for fertility disorders was determined by calculating sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and odds ratio (OR). A stepwise forward logistic regression analysis was used to select the best combination of tests. The results were presented with 95% confidence interval (CI) and P value <0.05 was considered significant.

Results

Characteristics of study population

Cervical *C. trachomatis* infection was diagnosed by Direct Fluorescence Assay/PCR in 309 patients. 28 of these patients were found to be infected either with *Candida* spp, bacterial vaginosis, *T. vaginalis*, *M. hominis*, *U. urealyticum*, or *N. gonorrhoeae* in the cervix and were excluded from the study. Six *Chlamydia*-positive patients and two control were

excluded, as the lymphocyte count in the cervical samples was less than 2 million. Based on clinical history the *Chlamydia* positive women were divided into four groups: (a) *Chlamydia* positive fertile asymptomatic women attending a family planning clinic (n=43), (b) *Chlamydia* positive fertile women with MPC (thick discharge and inflammation with number of PMNs>30) (n=96), and (c) *Chlamydia* positive women with fertility disorders (FD) (n=78) and (d) *Chlamydia* positive women having recurrent chlamydial infections and tested positive for infection on ≥2 consecutive visits separated by three months (n=58). All women in group (d) received antibiotic treatment after their first visit. All women with multiple spontaneous abortions were having atleast one child and are thus not infertile. All healthy controls tested negative for a current *C. trachomatis* infection (absence of *C. trachomatis* IgM and IgG antibodies). Three healthy controls, which were positive for *C. trachomatis* IgG antibodies with no current chlamydial infection, were also excluded from the study. None of the patients enrolled as asymptomatic or with MPC had any previous history of chlamydial infection as confirmed by their medical history and low titer of IgG antibodies against *C. trachomatis* in sera of these women and were collectively termed as women with primary chlamydial infection in some results. However, some women with fertility disorders had recurrent chlamydial infections as shown by their medical history and high titers of IgG antibodies against *C. trachomatis*. The clinical profiles of these patients have been summarized in Table 4.1. There were no significant differences in the median age (27, 26, 27 and 28 years respectively) or in the parity status of the patients.

Table 4.1. Clinical Characteristics of patients

Clinical Findings	Control (n=59)	Asymptomatic CT positive women (n=43)	*Chlamydia* positive with MPC (n=96)	Recurrent *Chlamydia* Infection (n=58)	*Chlamydia* positive with fertility disorders (n=78)
Age, median (range) yrs	28 (20-35)	27 (20-45)	26 (19-34)	27 (22-43)	28 (19-48)
Parity Status					
Nulliparous	14 (24)	9 (21)	37 (39)	6 (10)	10 (13)
Multiparous	46 (78)	38 (89)	60 (62)	52 (90)	0 (0)

NOTE: Data are no. (%) of women positive for the clinical condition, unless otherwise noted.

Immune cell population in cervical mucosa

Flow-cytometric analysis demonstrated an increase in the number of $CD14^+$ monocytes, $CD3^+CD4^+$ T cells and $CD3^+CD8^+$ T cells in all the *Chlamydia* positive groups compared to controls. Higher number of $CD4^+$ T-lymphocytes and $CD14^+$ monocytes per 10,000 events were observed in cervical mucosa of women with MPC as compared to controls. Within Chlamydia positive groups women with MPC have higher number of both $CD4^+$ and $CD14^+$ cells although the difference was non significant (CD4 – 573 versus 375 and 468: CD14 – 474 versus 259 and 287 for women with MPC versus asymptomatic women and women with FD respectively). $CD8^+$ T cell population in the cervix was higher during chlamydial infection compared to controls, however, the difference was again non-significant (348, 385 and 404 for asymptomatic women, women with MPC and women with FD respectively (Figure 4.1).

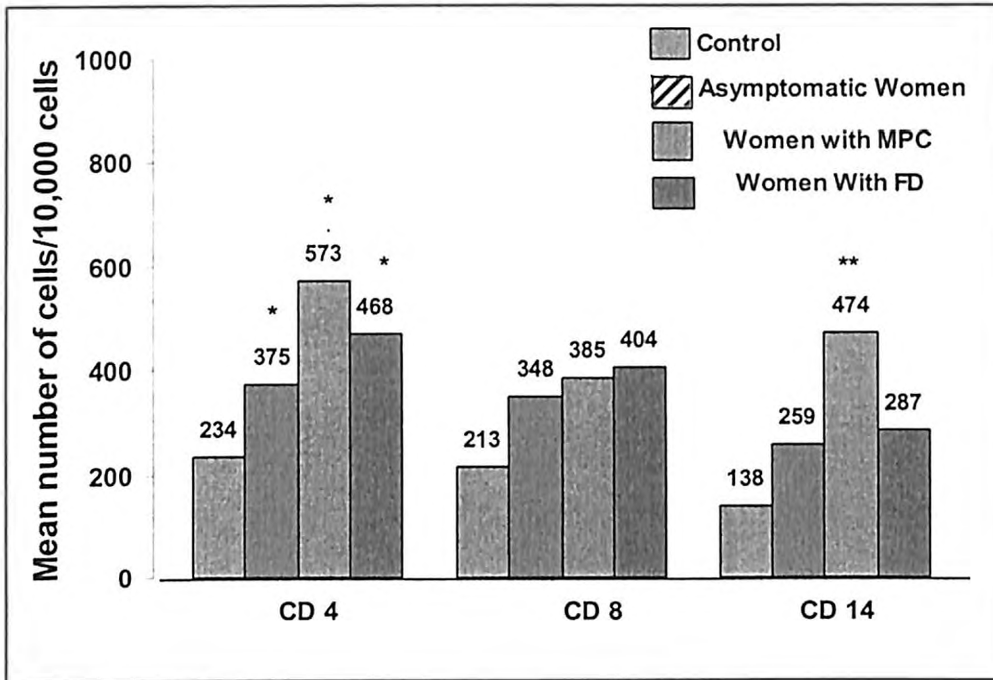


Figure 4.1. Lymphocyte subsets ($CD3^+CD4^+$ and $CD3^+CD8^+$ T-cells) and monocytic cells ($CD14^+$) in cervical mucosa of controls, *C. trachomatis* positive asymptomatic women, women with MPC and women with fertility disorders (FD) as detected by flow cytometry and expressed as mean number of cells per 10,000 events on *y*-axis.

Cervical cytokine concentration

Total protein levels in cervical washes ranged from 0.2-0.5 mg/mL. The median levels of IL-1β, IL-6, IL-8, IL-10, and IFN-γ in cervical washes of *Chlamydia* positive women along with controls are given in Table 4.2. IL-1β, IL-6 and IL-8 levels were significantly higher ($P<0.05$) in both women with MPC and FD compared to asymptomatic women and controls. Significantly higher levels of IL-10 and IFN-γ ($P<0.05$) were detected in women with FD compared to other groups. Median concentration of TNF-α and IL-4 was below detection limit in all the patient groups and was thus not considered for any further analysis.

Table 4.2: Cytokine levels in cervical washes of *Chlamydia* positive women and controls

Cytokines	Control	Asymptomatic CT positive	*Chlamydia* positive with MPC	*Chlamydia* positive with fertility disorders
IL-1β	35 (UD-360)	37.62 (UD-99.86)	96.90 (8.70-398.0)	387.6 (111.7-615.0)[b]
IL-6	13 (UD-201)	09.36 (UD-39.63)	70.96 (UD-190.60)	784.0 (223.7-1677.8)[b]
IL-8	67 (4-356)	137.4 (13.74-375.9)	551.58 (32-1074.0)	971.9 (322.41-1992.6)[b]
IL-10	7 (UD-20.5)	06.64 (UD-22.96)	11.68 (UD-30.91)	107.6 (26.3-414.8)[a]
IFN-γ	114 (6-622)	103.24 (7.33-566.6)	126.96 (UD-330.6)	245.3 (144.1-460.3)[a]

Note: Data represents median values. Figure in parentheses denote range. UD; Under detection limit, IL; interleukin, IFN; interferon, CRP; C-reactive protein; [a]$P < 0.05$ compared to other groups, [b]$P<0.05$ compared to *Chlamydia* positive asymptomatic women and control.

Correlation among cytokines and with estrogen and progesterone levels

The correlation of local cervical cytokines with serum β-estradiol and progesterone levels of patients and controls was investigated to determine the role of sex hormones in providing a protective/pathogenic immune response during chlamydial infection. The median β-estradiol and progesterone levels are given in Table 4.3. Women with primary chlamydial infections (asymptomatic women and women with MPC) had lower levels of estrogen as compared to controls and women with recurrent infections or FD who had lower levels of progesterone compared to controls or women with primary infections although the differences were non significant. Further, women with FD have higher levels of estradiol although not significant.

Table 4.3. Levels of β-estradiol and progesterone in women with primary or recurrent chlamydial infections and controls

	Control	Primary Infection	Recurrent Infection and FD	P
β-estradiol	173 (142-257)	153 (73-154)	275 (156-243)	NS
Progesterone	3.7 (0.1-5.1)	4.4 (0.1-6)	2.3 (0.5-1.9)	NS

NOTE.: Data are median levels of β-estradiol and progesterone in picograms per milliliter and nanogram per milliliter respectively. Figure in parentheses denote range.

In cervical washes of healthy controls IL-1β and IL-6 were found to be significantly correlated (r=0.786; P<0.01). In patients with primary infection cytokines showing significantly high correlations were IL-6 and IL-10 (r=0.702; P<0.001) (Table 4.4). No significant correlation was found among cytokines in cervical washes of women with recurrent infection.

In patients with recurrent infections or FD, significant negative correlation was found between IL-1β and progesterone (r = -0.78; P<0.01), however, no significant correlation was found between any cytokine and progesterone levels of healthy controls or patients with primary infections. β-estradiol levels in women with primary infections showed significant negative correlations with cervical IL-10 (r = -0.8; P<.0001), IL-1β (r = -0.61; P<.01) and IL-6 (r = - 0.619; P<0.01) (Table 4.4). No significant correlation was found between β-estradiol levels and cervical cytokines in controls and women with recurrent infections.

Table 4.4. Correlation among cytokines in cervical washes of women with primary or recurrent chlamydial infections and controls

Correlation	r[a]	P
Controls		
IL – Iβ and IL – 6	0.786	<.01
IL – 10 and IFN–γ	0.580	<.05
Primary infection (asymptomatic & MPC)		
IL–6 and IL–10	0.702	<.001
IL-1β and β-estradiol	-0.61	<.01
IL-6 and β-estradiol	- 0.619	<0.01
IL-10 and β-estradiol	-0.8	<.0001
Recurrent infection & FD		
IL-1β and progesterone	-0.780	<.01

Note. [a] - Spearman Correlation Coefficient. IL-interleukin; IFN-interferon

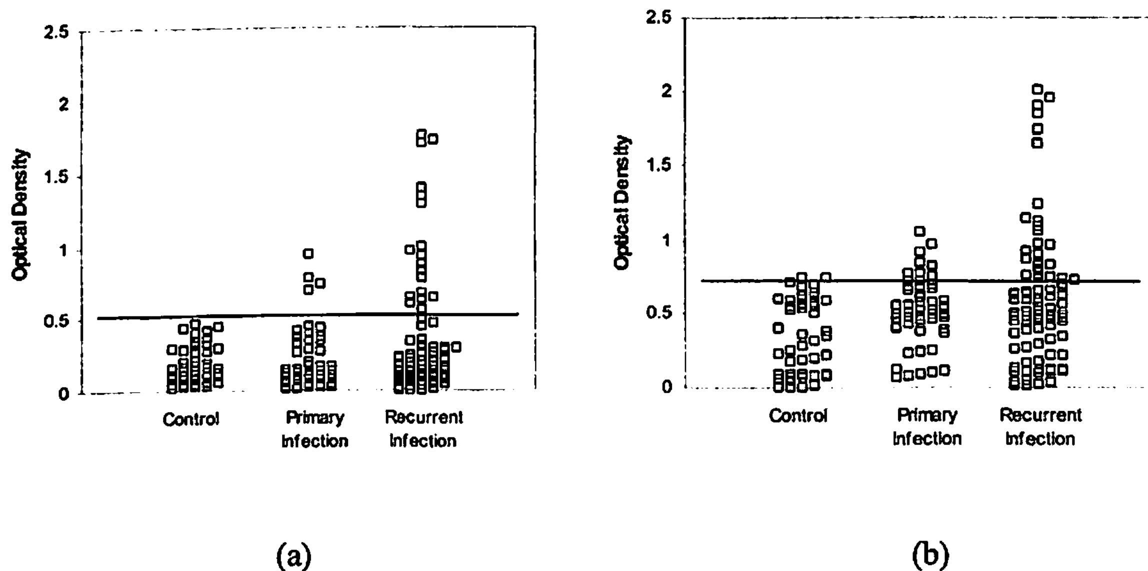
Cervical antibodies to *C. trachomatis* and heat shock proteins

The prevalence of antibodies in cervical washes is shown in Table 4.5. The prevalence of IgG and IgA antibodies to *C. trachomatis* MOMP antigen was significantly higher in patients with primary infections than patients with recurrent infections or FD ($P < 0.0001$ and $P < 0.05$). In contrast, the prevalence of IgG and IgA antibodies against peptides of cHSP10 and IgG antibodies to cHSP60 was significantly higher ($P < 0.01$) in patients with recurrent infections and FD compared to other groups. Individual results for testing for local IgA antibodies to peptides of cHSP60 and 10 are shown in Figure 4.2a and 4.2b.

Table 4.5. Prevalence of antibodies in cervical secretions of *Chlamydia* positive women

	Control (n=59), no (%)	Primary Infection (n=139), no (%)	Recurrent Infection & FD (n=136), no (%)
Ctr-IgG+	4 (7)	108 (78)[a]	31 (23)
Ctr-IgA+	4 (7)	72 (52)[b]	50 (37)
cHSP10 IgG+	7 (12)	28 (20)	58 (43)[c]
cHSP10 IgA+	0 (0)	13 (9)	34 (25)[d]
cHSP60 IgG+	5 (8)	35 (25)	60 (44)[e]
cHSP60 IgA+	5 (8)	38 (27)	45 (33)

Ctr = *C. trachomatis*; [a]$P < 0.0001$; [b]$P = 0.04$; [c]$P = 0.0008$; [d]$P = 0.007$; [e]$P = 0.004$
P values are in comparison with both controls and patient groups



(a) (b)

Figure 4.2. Immunoglobulin (Ig)A antibodies to synthetic peptides corresponding to epitopes of (a) cHSP10 and (b) cHSP60 in cervical washes of women with primary or recurrent infection and controls. The horizontal line indicates the lower boundary of a positive antibody response.

Prevalence of positive tests for cHSP60 and 10, CRP and cervical IFN-γ levels

The prevalence of positive tests has been shown in Table 4.6. The prevalence of IgA antibodies for synthetic peptides of cHSP60 and 10, positive CRP test and cervical IFN-γ

levels were significantly higher in patients with fertility disorders as compared to women without such complications.

Table 4.6 Prevalence of markers for persistent chlamydial infection and chronic inflammation in women with or without fertility disorders.

	***Chlamydia* positive fertile women (n=139)**	***Chlamydia* positive women with fertility disorders (n=78)**	**P**
IFN-γ level[a]	78 (56)	6 (8)	<0.05
hs-CRP[b]	64 (46)	18 (23)	<0.05
cHSP60 IgA[c]	67 (48)	19 (24)	<0.05
cHSP10 IgA[d]	44 (32)	18 (23)	NS

Note: Values depict number of women positive for various markers. Values in parentheses are percentages. [a] Cut off limit : 200 picograms per milliliter; [b]Threshold concentration for positivity: >2 microgram per milliliter; [c] cut off O.D. value: 0.7; [d] cut off O.D. value: 0.5;

Prediction of fertility disorder

As shown in Table 4.7 of all the single tests, positive IFN-γ level in cervical wash was the single best predictor for fertility disorders in women (OR 15.42). Thus, further all the test combinations were made such that they included IFN-γ levels. A forward stepwise logistic regression analysis revealed that adding the hs-CRP test improved the predictive value of the IFN-γ levels (OR of test combination 37.9). Adding the presence of cHSP 60 IgA antibodies to the combination of hs-CRP and IFN-γ lead to an OR of 47.7, but this increase was not significant in comparison to combination of hs-CRP and IFN-γ. When three or four tests were combined the sensitivity decreased to 15% but the specificity of the test was 100%.

levels were significantly higher in patients with fertility disorders as compared to women without such complications.

Table 4.6 Prevalence of markers for persistent chlamydial infection and chronic inflammation in women with or without fertility disorders.

	***Chlamydia* positive fertile women (n=139)**	***Chlamydia* positive women with fertility disorders (n=78)**	**P**
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hs-CRP[b]	64 (46)	18 (23)	<0.05
cHSP60 IgA[c]	67 (48)	19 (24)	<0.05
cHSP10 IgA[d]	44 (32)	18 (23)	NS

Note: Values depict number of women positive for various markers. Values in parentheses are percentages. [a] Cut off limit : 200 picograms per milliliter; [b]Threshold concentration for positivity: >2 microgram per milliliter; [c] cut off O.D. value: 0.7; [d] cut off O.D. value: 0.5;

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Table 4.7. Predictive value of various markers as single test and test combinations (including positive IFN-γ levels) for fertility disorders as multiple spontaneous abortion or infertility.

No. of test done	IFN-γ	hs-CRP	cHSP60 IgA	cHSP10 IgA	No. of patients with positive test	No. of patients with positive test and fertility disorder	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	OR	95% CI
One test	+				79	63	56	92	78	80	15.4[a]	8.2–29.0
		+			99	52	46	77	53	73	2.9[b]	1.8–4.8
			+		107	53	47	75	50	73	2.7[b]	1.6–4.3
				+	95	49	44	78	52	72	2.7[b]	1.6–4.5
Two tests	+	+			43	3	38	99	93	75	37.9[b]	11.4–126.5
	+		+		60	41	37	91	60	73	5.74	3.1–10.6
	+			+	54	39	35	93	72	73	6.8	3.5–13.2
Three tests	+	+	+		22	21	19	100	95	69	47.7[b]	6.3–360.3
	+	+		+	21	19	17	99	90	69	21.1	4.8–92.6
	+		+	+	20	17	15	99	85	69	12.2	3.5–42.7
Four tests	+	+	+	+	17	17	15	100	100	69	–	–

[a]Versus.
[b]$P < 0.05$.
cHSP, chlamydia heat-shock protein; CI, confidence interval; hs-CRP, high sensitivity C-reactive protein; IFN-γ, interferon-gamma; NPV, negative predictive value; OR, odds ratio; PPV, positive predictive value.

Relationship of *C. trachomatis* infection forming unit (IFU) with clinical manifestations

The number of *C. trachomatis* IFU per mL^{-1} was transformed to the base 10 logarithm, and results were expressed as $<10^1$, 10^2, 10^3, 10^4, or $>10^4$, respectively, if <10, 10 to 99, 100 to 999, 1,000 to 9,999, or -10,000 inclusions ml^{-1} of transport medium were detected. Table 4.8 shows the distribution of chlamydial inclusion counts in women enrolled in the three groups. *Chlamydia* positive fertile women showed significantly higher inclusion counts compared to women with FD showing lower recovery of *Chlamydia* from the cervix of these women.

Table 4.8: Distribution of chlamydial inclusion counts in *C. trachomatis* positive women

IFUs	*Chlamydia* positive asymptomatic women	*Chlamydia* positive with MPC	*Chlamydia* positive with FD
$<10^1$	4	1	20
10^2	25	7	49
10^3	30	29	13
10^4	38	47	12
$>10^4$	3	16	6

Note: Data expressed as percentage of women having IFU in particular range, IFUs; Infection forming units mL^{-1} of transport media, MPC; Mucopurulent Cervicitis

Immune cell population: Significant positive correlation was observed between CD4 cell number and chlamydial recovery in all the groups (Table 4.9). CD8 cells on the other hand showed significant negative correlation with chlamydial burden in asymptomatic women and women with FD, however showed positive correlation with chlamydial recovery in women with MPC (Table 4.9). Number of CD14 cells in the cervix showed significant positive correlation with chlamydial burden only in asymptomatic women and none of the other group (Table 4.9).

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Cervical cytokine correlation: In asymptomatic women IL-2, IL-12 and IFN-γ showed significant positive correlation while IL-8 showed significant negative correlation with chlamydial load (Table 4.9). In women with MPC, IL-1β, IL-8, IL-10 and IFN-γ showed significant correlation with chlamydial burden (Table 4.9), however, chlamydial load in women with FD showed significant positive correlation with IL-10 levels and negative correlations with IL-8 and IFN-γ levels (Table 4.9).

Cervical antibodies to C. trachomatis and heat shock proteins: Chlamydial MOMP IgA antibodies showed significant positive correlation with chlamydial load in all the groups. In contrast, cervical IgA antibodies to cHSP60 and cHSP10 showed significant negative correlation to chlamydial burden in women with FD (Table 4.9).

Correlation of infectious load with estrogen and C-reactive protein levels: Significant positive correlation was observed between chlamydial load and estradiol levels in both women with MPC and FD (Table 4.9). CRP on the other hand showed significant positive correlation with infectious load in women with MPC and negative correlation in women with FD.

Table 4.9: Correlation of Infection Forming Units (IFUs) with various immune factors

Immune parameter	Asymptomatic *Chlamydia* positive (n=43)	*Chlamydia* positive with MPC (n=96)	*Chlamydia* positive with fertility disorders (n=78)
CD4	0.57#	0.63#	0.70#
CD8	-0.78#	0.72#	-0.80#
CD14	0.49#	0.38	0.32
IL-1β	0.11	0.77#	0.10
IL-2	0.75#	0.02	0.29
IL-6	0.27	-0.01	0.42
IL-8	-0.84#	0.79#	-0.57#
IL-10	0.42	0.74#	0.83#
IL-12	0.62#	0.23	-0.16
IFN-γ	0.58#	0.82#	-0.48#
β-estradiol	0.37	0.57#	0.65#
CRP	0.15	0.59#	-0.71#
CTR MOMP IgA	0.66#	0.49#	0.62#
cHSP60 IgA	0.04	0.18	- 0.79#
cHSP10 IgA	0.12	0.41	- 0.81#

Note: Data are spearman's correlation coefficients obtained between various immune parameters and chlamydial IFUs. #P<0.05 by spearman correlation coefficient

IL; interleukin, IFN; interferon, CTR; *Chlamydia trachomatis*, cHSP; chlamydial heat shock protein

Lymphocyte Proliferative responses to synthetic peptides for MOMP antigen.

Cell mediated immune response was assessed by stimulating local endocervical lymphocytes and PBMCs with synthetic peptides for MOMP antigen. Positive responses (SI>2) to MOMP were more common in both cervical lymphocytes (CL) and PBMCs of patients with primary infections (Table 4.10) but was statistically not significant from other groups. No significant difference was observed in median SI of CL or PBMCs to MOMP (CL - controls SI 1.14; range 0.5-1.9; primary infection SI 1.71; range 0.72-6.22; recurrent infection SI 1.4; range

0.7-4.83) and (PBMCs – controls SI 1.21; range 0.71-2.14; primary infection SI 1.89; range 0.73-5.46; recurrent infections SI 1.25; range 0.62-3.96) but the difference was not statistically significant.

Lymphocyte Proliferative responses to synthetic peptides of cHSP10

Positive responses (SI>2) to cHSP10 were more common in patients with recurrent infections (Table 4.10) and was statistically significant (P<0.05) from other groups in case of CLs. No significant difference was observed in median SI of CLs or PBMCs to cHSP10 (CLs - controls SI 1.2; range 0.2-3.9; primary infections SI 1.1; range 0.6-4.4; recurrent infection SI 1.78; range 0.7-8.2) and (PBMCs – controls SI 1.52; range 0.62-2.71; primary infections SI 2.05 range 0.7-6.4; recurrent infections SI 1.9; range 0.79-5.42) (Fig. 4.3a). No correlation was found between local IgA or IgG antibodies to cHSP10 and proliferative responses of CLs or PBMCs to cHSP10 in any group.

Lymphocyte Proliferative responses to synthetic peptides of CHSP60

Cell mediated immune response to synthetic peptides of cHSP60 did not differ significantly between groups in either CLs or PBMCs (CLs - controls median SI 1.4; range 0.8-1.9; primary infections SI 1.4; range 0.6-5.4; recurrent infections SI 1.85; range 0.8-8.43) and (PBMCs – 1.65; range 0.52-2.91; primary infection SI 1.94; range 0.73-5.77; recurrent infections SI 1.98; range 0.51-6.71) (Fig 4.3b). Positive responses (SI>2) to cHSP60 were more in patients with recurrent infections in both CLs and PBMCs (Table 4.10) but was statistically not different from women with primary infections. A significant positive correlation (r=0.54; P<0.01) was found between cervical IgA antibodies to cHSP60 peptide and proliferative responses of CLs of patients with recurrent infections to peptides of cHSP60.

Table 4.10. Lymphoproliferative response to chlamydial antigens

	Positive LP response to no (%)					
	Cervical Lymphocytes			PBMCs		
	MOMP	cHSP10	cHSP60	MOMP	cHSP10	cHSP60
Control (n=45)	4 (9)	3 (7)	3 (7)	5 (11)	4 (9)	4 (9)
Primary Infection (n=44)	13 (30)	4 (9)	6 (14)	17 (38)[c]	7 (16)	6 (14)
Recurrent Infection (n=81)	9 (11)[d]	21 (26)[b]	18 (22)	20 (25)	11 (14)	17 (21)

Note: Figure in parentheses denote percentage; LP = Lymphoproliferative; [a]P< 0.05 compared with all others: [b]P = .01 compared with all others: [c]P<0.05 compared to controls: [d]P<0.05 compared to primary infection group.

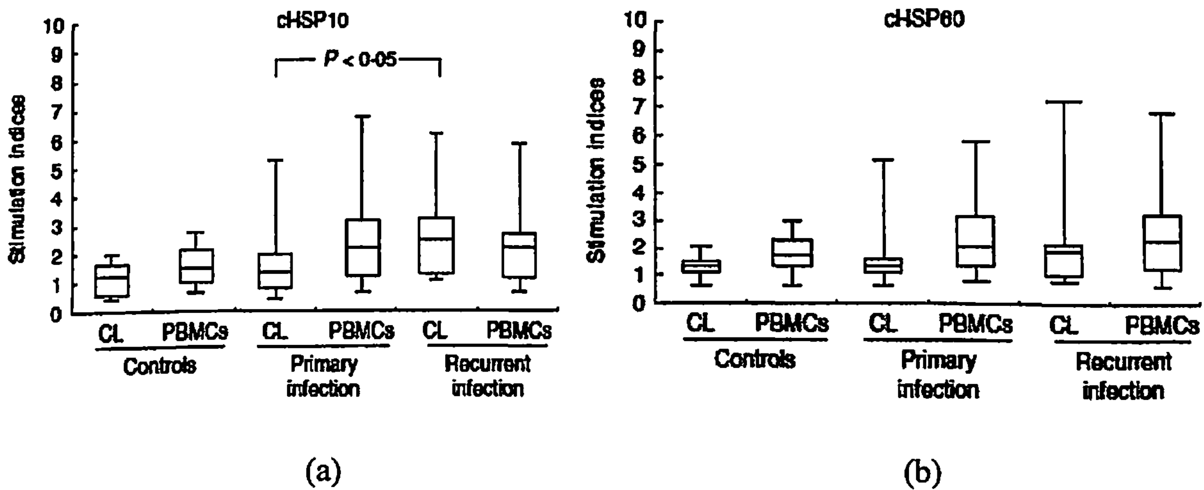


Figure 4.3. Proliferative responses (stimulation index) of cervical lymphocytes and PBMCs to (a) cHSP10 and (b) cHSP60 in women with primary or recurrent *C. trachomatis* infection

along with controls. The horizontal line in the middle of the box is the median value of the responses and the lower (upper) 25th (75th) percentile.

Cytokine mRNA expression levels in stimulated cervical cells

mRNA for IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-gamma and TNF-α was detected in all the stimulated cervical cells from both the patient groups and controls. IL-4 was detected in 62-73% of stimulated cells for patients and controls. On stimulation of cervical cells with chlamydial EBs, significant increase in mRNA expression levels of IFN-γ (P=0.001) and IL-12 (P<0.05) was observed in cervical cells obtained from *Chlamydia* positive fertile women compared to other groups (Figure 4.4).

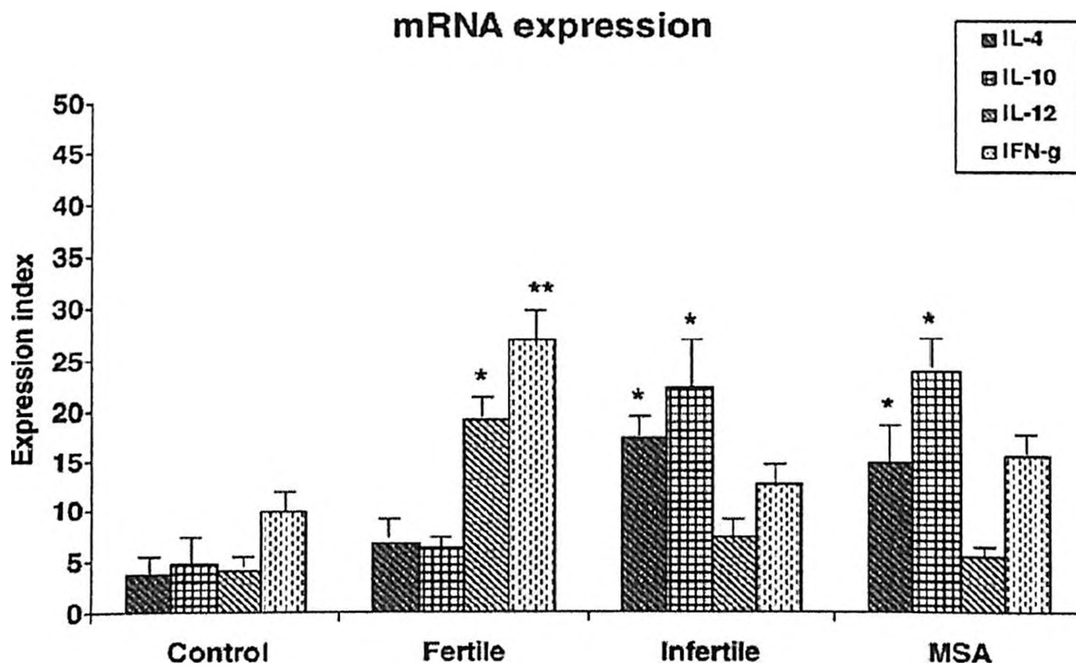


Fig. 4.4 Estimation of mRNA expression of IL-4, IL-10, IL-12 and IFN-γ in stimulated cervical cells. The graphs show results as an expression index represented by bars, defined as the ratio of the intensity of cytokine with respect to the HPRT gene. Bars represent mean ± s.e.m. for all experiments.

In contrast, IL-10 and IL-4 mRNA expression levels were significantly higher ($P<0.05$) in cells obtained from *Chlamydia* positive women with FD compared to other groups. No significant difference was observed in mRNA expression levels of IL-1β, IL-6, IL-8 and TNF-α in cervical cells from *Chlamydia* positive women with FD compared to *Chlamydia* positive fertile women.

ELISA for cytokines in supernatants of stimulated cervical cells

Cytokine levels were quantified in culture supernatants of induced cervical cells obtained from *C. trachomatis* positive women and controls. Significantly higher levels of IL-1β, IL-6, IL-8 and IL-10 were observed upon stimulation of cervical lymphocytes obtained from *Chlamydia* positive women with fertility disorders (FD) as compared to the other two groups (Table 4.11). In contrast, cervical cells obtained from *Chlamydia* positive fertile women secreted significantly higher levels of IL-6 and TNF-α compared to controls and significantly higher levels of IL-12 and IFN-γ compared to both women with FD and controls. IL-4 was below detection limit in all the culture supernatants.

ELISPOT analysis of IL-4, IL-10, IL-12and IFN γ producing cells in stimulated cervical cells.

On the basis of ELISA and RT-PCR results, IL-4, IL-10, IL-12 and IFN-γ spot forming cells (SFCs) were enumerated in stimulated cervical cells obtained from *Chlamydia* positive groups and healthy controls. In controls and *Chlamydia* positive fertile women 72-80% of the samples showed no IL-4 secreting cells. In rest of the cases median IL-4 SFCs were highest in *Chlamydia* positive women with FD (Table 4.12). Median number of IL-10 SFCs was comparable in controls and *Chlamydia* positive fertile women but were significantly higher ($P=0.01$) among cervical cells obtained from *Chlamydia* positive women.

Table 4.11. Cytokine concentrations in culture supernatants of stimulated cervical cells

	Control	CT positive fertile women	CT positive women with FD	
			Infertility	MSA
	(n=59)	(n=139)	(n=34)	(n=44)
IL-1β	125.2 (54.2- 450.14)	178.8 (65.8-490.6)	388.8 (80.5-6003.4)[#]	411.9 (102.8- 5923.6)[#]
IL-6	153.6 (40.5-418.0)	325.8 (36.4-598.3)[‡]	848.2 (212.3-1598.9)[#]	794.0 (194.4-1538.6)[#]
IL-8	489.0 (75.1 -677.6)	497.9 (112.9-967.0)	924.7 (298.35-1896.8)[#]	874.3 (264.4-1938.7)
IL-10	263.7 (97.2-635.9)	463.7 (164.8-867.3)	1002.8 (378.4-1593.2)[#]	993.8 (376.8-1529.9)[#]
IL-12	154.7 (135.7-279.8)	397.4 (136.0-275.2)[ǂ]	139.8 (76.9-694.7)	128.9 (94.9-708.2)
IFN-γ	277.0 (95.1-489.5)	468.6 (193.4-573.7)[ǂ]	236.4 (136.8- 450.8)	2298.8 (132.7- 425.8)
TNF-α	122.6 (34.8-356.1)	294.9 (59.8-418.4)[‡]	198.5(95.2-498.9)	225.4 (109.7-546.7)

NOTE. Data represent median values. Figures in parentheses depict range. CT, *Chlamydia trachomatis*; FD; fertility disorders, MSA; Multiple spontaneous abortions

[a]P<0.05 CT positive women with FD compared to other groups by Mann Whitney U test.

[‡]P<0.01 CT positive fertile women compared to controls by Mann Whitney U test.

ǂP<0.05 CT positive fertile women compared to other groups by Mann Whitney U test

with FD. For IL-12, median SFC count was significantly higher in *Chlamydia* positive groups compared to controls but was much higher among cells from *Chlamydia* positive fertile positive women (Table 4.12). Frequency of IFN-γ secreting cells was highest among cells obtained from controls followed by cells from *Chlamydia* positive fertile women. In *Chlamydia* positive women with FD, significantly ($P<0.05$) lower number of IFN-γ secreting cells were present (Table 4.12).

Table 4.12. Frequency of Spot Forming Cells (SFCs) among stimulated cervical cells by ELISPOT assay.

Patients	IL-4	IL-10	IL-12	IFN-γ
Controls	6 (0-23)	66 (37-103)	68 (44-86)	134 (77-221)[ǂ]
CT positive Fertile women	9 (0-37)	64 (59-87)	344 (277-521)[#]	133 (119-184)[ǂ]
CT positive with infertility	16 (4-89)[#]	96 (79-235)	241 (115-450)	59 (39-159)
CT positive with MSA	13 (3-75)[#]	102 (79-206)[#]	235 (125-464)[‡]	65 (39-172)

Note: Median number of cytokine spot forming cells per 2×10^5 cervical mononuclear cells at 18 hours post stimulation using ELISPOT. Figure in Parenthesis depict range of SFCs for each group. CT; *Chlamydia trachomatis,* MSA; Multiple spontaneous abortions

[#]$P=0.01$ compared to the other groups; [‡]$P<0.05$ compared to controls; [ǂ]$P<0.05$ compared to women with fertility disorders.

Discussion

The immune response to genital chlamydial infection is very complex; it clears infection and confers short-term protection but at the same time sensitizes the host for development of immunopathological changes [Rietmeijer *et al.*, 2002]. In women, chlamydial infections are often asymptomatic and subsequent reinfections leads to inflammatory responses with pathological sequelae [Lehtinen & Paavonen, 1994]. Chlamydial infection generates and produces a variety of cytokines responses, both by direct infection of the epithelial cells lining the mucosal surfaces of the body and by interaction with cells of the immune system. T helper1 (T_H1) cytokines have a role in polarizing the immune response to *Chlamydia* spp towards a protective T_H1 type response [Johnson, 2004]. By contrast, cytokines such as TNF-α, IL-1α, IL-6 and IL-10 have been reported be involved in the pathology associated with infection with *Chlamydia* spp, [Darville *et al.*, 2003]. Therefore, the present study was designed to explore certain aspects of local and systemic immunity in terms of immune modulation leading to clinical outcome of chlamydial infection.

Our study showed higher IL-1β levels in women with recurrent infections, which suggest its possible role in persistence of chlamydial infection as it may hamper the growth and differentiation of chlamydial EB's causing them to persist in a subdued state. Our findings show significantly high levels of IFN-γ in cervical washes of women with recurrent chlamydial infections compared to women with primary chlamydial infections. IFN-γ is considered as the main cytokine essential for the clearance of chlamydial infections from genital tract and considerable *in vitro* and *in vivo* evidence have show that production of IFN-γ by *C. muridarum* specific T cells is essential for clearance of *C. trachomatis* from genital tract [Rottenburg *et al.*, 2003]. Gerard *et al.*, 1998, have proposed increased levels of IFN-γ

transcripts in RNA preparation of synovial tissues of *Chlamydia* infected patients with reactive arthritis. IFN-γ affects human host cells by inducing indoleamine 2, 3-dioxygenase, a non constitutive enzyme that catalyzes the initial step in the degradation of tryptophan. This depletion correlates with growth inhibition of chlamydiae in wide spectrum of human host cells [Byrne & Krueger, 1983; Byrne *et al.*, 1986]. The lower levels of IFN-γ in patients with primary infections than those of controls and slightly higher levels of IL-10 and IL-6 are in concordance with previous studies suggesting downregulation of T_H1 cytokines and upregulation of T_H2 cytokines as IL-10 during primary infection [Reddy *et al.*, 2004]. The lower levels of IFN-γ could also lead to increased susceptibility to other serious infections as HIV [Cohen *et al.*, 1999].

Significantly higher levels of IL-10 and IFN-γ were observed in women with FD compared to other groups. IL-10 is not always an inflammatory/inhibitory cytokine; instead higher levels of IL-10 probably prevent the pathological effect of the inflammatory cytokines like IL-1β, IFN-γ and TNF-α. During chlamydial infection IL-10 has been reported to be associated with typical pathological changes like fibrosis and granuloma formation [Conti *et al.*, 2003]. These results show that higher secretion of IFN-γ for clearance of infection is counteracted by higher secretion of IL-10 resulting in incomplete removal of bacteria thus causing persistence and disease outcome. On the other hand, IL-1β, IL-6 and IL-8 levels were significantly higher in both symptomatic women (with MPC and FD) compared to asymptomatic women. It is been shown previously that synovial tissues from chronic arthritis patients with synovial *C. pneumoniae* infection have significant levels of mRNA for IL-1β and IL-8 [Gerard *et al.*, 2002]. Further, pathogenic role of IL-6, has also been shown by many studies and IL-6 levels were also found to be increased in silent tubal infections of *C.*

trachomatis in a previous study [Li & Liang, 2000]. IL-12 levels were significantly lower in women with MPC as compared to other groups suggesting that during acute inflammation lower levels of IL-12 are secreted.

Correlation among various cytokines in cervical washes within the patient groups was investigated to find out if the cytokines regulate the production of each other or their contribution towards chlamydial pathogenesis. We found significant correlation between IL-6 and IL-1β in cervical washes of controls and patients with primary infection. Significant correlation between IL-1β and IL-6 in controls and during primary infection suggest that *Chlamydia* induced cytokines released from immune cells may contribute to development of inflammation and clearance of infection which also happens in case of controls where no infection is present. The results also show significant correlation between IL-6 and IL-10 in women with primary infection suggesting the regulation of both pro and anti-inflammatory mechanisms during primary chlamydial infections, which lead to clearance of infection without having any serious pathological damage.

Further, the effect of β-estradiol and progesterone on regulation of various cytokines in the female genital tract and their role in clinical outcome of infection was investigated. In women with recurrent infections and FD significant negative correlation was observed between progesterone levels and IL-1β levels. Since IL-1β along with IFN-γ is responsible for clearance of infection as suggested by previous study [Carlin & Weller, 1995] lower progesterone levels in women with recurrent infections or FD suggest that this may cause upregulation of IL-1β and thus contribute to reactivation or persistence of chlamydial infections. A previous study has also demonstrated that a persistent infection can be established in rats by pretreatment with progesterone [Kaushic *et al.*, 1998]. IL-1β, IL-6 and

IL-10 levels showed significant negative correlation with estrogen levels in women with primary infection suggesting that estrogen might modulate both pro and anti-inflammatory mechanisms. Some studies in humans have shown that women are more susceptible to chlamydial infection under β-estradiol influence, since more chlamydial organisms can be isolated during the proliferative part of the cycle [Sweet *et al.*, 1986]. Estrogen was also found to enhance chlamydial adherence or intracellular development of their inclusion [Bose & Goswami, 1986]. However, to exact role of estradiol in modulating immune response towards *Chlamydia* still remains to be elucidated.

A higher prevalence of antibodies to cHSP60 and 10 peptides in cervical washes of women with recurrent infection or FD was found in contrast to antibodies against *C. trachomatis* surface antigens which were significantly higher during primary infections. As postulated previously [Beatty *et al.*, 1993] cells chronically infected with *C. trachomatis* synthesize only low levels of structural components but continue to produce cHSP60 at higher levels. Since bacterial and human HSP share ~50% amino acid sequence homology, it has been proposed that a prolonged exposure of the immune system to cHSP60 may lead to autoantibody formation [Witkin *et al.*, 1997]. Presence of cHSP60 IgA antibody is shown to be considered to reflect an acute immune response [Witkin *et al.*, 1994]. Our study also reported higher prevalence of multiple spontaneous abortions among women with recurrent infections. A study by Witkin *et al.* 1996 implied that cervical IgA antibody to conserved HSP60 epitopes and failure of successful implantation after embryo transfer is interrelated.

We further aimed at evaluating local cervical responses which could act as markers of chronic inflammation and persistent chlamydial infection and could help in prognosis of women who may develop sequelae to chlamydial infection as it is important to screen women

at a higher risk of developing various fertility related complications including infertility or multiple spontaneous abortions. As a single test positive IFN-γ levels were the best predictors of fertility disorders. It is earlier shown that higher levels of IFN-γ in were present in infertile women infected with *Chlamydia trachomatis* [Reddy *et al.*, 2004] and can cause severe inflammation which may be an important factor in deciding the clinical outcome of chlamydial infection.

It was also found that the prevalence of IgA antibodies for synthetic peptides of cHSP 60 and 10 and positive CRP test were significantly higher in patients with fertility disorders as compared to women without such complications. A previous study demonstrated that presence of cervical IgA antibodies to immunodominant epitope of chlamydial hsp60 correlated with embryo loss after transient implantation of embryos in uterus of women undergoing *in vitro* fertilization (IVF) [Witkin *et al.*, 1996]. Studies also suggested that immune sensitization to heat shock proteins probably require prolonged exposure to elevated concentrations of hsp60 and 10 [Witkin *et al.*, 1998] and that chlamydial HSP10 is co-expressed with chlamydial HSP60 [Spandorfer *et al.*, 1999]. It is also demonstrated previously that out of fifty-one of fifty five patients with PID had raised CRP levels. Presence of elevated CRP along with *C. pneumoniae* antibodies showed increased risk of coronary events in another study [Roivainen *et al.*, 2000]. Only few serological studies have been done till now to ascertain the predictive efficacy of elevated CRP levels in fertility related complications during chlamydial infections but no study involving both local markers and CRP was done [Hartog *et al.*, 2005]. The lower OR of hs-CRP test as a single test might be explained by the fact that CRP levels may be elevated due to a number of reasons and thus it may not be a specific marker of *Chlamydia* induced inflammation.

In order to determine if the prognostic value of positive IFN-γ levels can be increased by addition of any other test a forward stepwise logistic regression analysis was performed. Addition of hs-CRP test resulted in a significantly higher OR (37.9) compared to IFN-γ alone OR (15.4). Addition of any other test to positive IFN-γ level did not increase the efficacy of predicting fertility disorders. Further addition of antibodies to peptides of cHSP60 IgA increased the OR (47.7) but the increase was not significant. A combination of all four tests has a specificity and PPV of 100 but the sensitivity and NPV were low. Thus the combination of local IFN-γ levels with systemic CRP levels can help in prognosis and timely treatment of women.

Further, study of correlation of various immune factors with quantitative recovery of chlamydial load was also done. Infectious load was determined by cell culture method which, besides having the advantage of giving counts of viable infecting bacteria also has its own disadvantages as variables including technique and method of specimen collection, the rapidity of transport, the temperature, the methodology used for staining and reading plates can never be completely constant [Eckert *et al.*, 2000]. In view of these facts, we have tried to utilize a standardized and uniform approach and kept the variables as constant as possible. The results showed that maximum numbers of women with MPC have higher inclusion counts whereas maximum women with fertility disorders have lower chlamydial burden. Previous studies have also reported that significantly higher inclusion counts is present in women with cervical mucopus [Hobson *et al.*, 1980; Geisler *et al.*, 2001] and it may lead to increased secretion of inflammatory cytokines thus resulting in acute inflammation. In case of women with FD lower bacterial recovery and persistent chlamydial infection with high suggests that while the host immune defense may not be able to completely remove the

bacteria from the cells, however, there may be an immunological impact on replication leading to lower replication or persistence which ultimately leads to disease sequelae as fertility related disorders [Gomes *et al.*, 2006].

Further, the data suggests that an increase in number of infecting pathogen increases proliferation of protective $CD4^{+}$ T cells to ensure a protective immune response. CD8+ cells on the other hand showed significant negative correlation with chlamydial burden in both asymptomatic women and women with FD however, positive correlation was seen in women with MPC. This shows that during lower chlamydial burden higher number of CD8 cells are present but during acute inflammation, CD8 cell number increases with pathogen burden. This may depict that CD8 cell may have a role in limiting the number of pathogen or in curbing inflammation but the exact mechanism involved needs further elucidation. Significant positive correlation of IL-2, IL-12 and IFN-γ with chlamydial IFUs in asymptomatic women and negative correlation of IL-8 show that inflammatory response is kept at low levels thus leading to absence of symptoms. In women with MPC, IL-1β, IL-8, IL-10 and IFN-γ showed significant correlation with chlamydial burden showing that during inflammation protective cytokines are taken over by inflammatory cytokines and as pathogen load increases the level of regulatory cytokine like IL-10 also increases. In contrast, chlamydial load in women with FD showed significant positive correlation with IL-10 levels and negative correlations with IL-8 and IFN-γ levels showing that higher levels of inflammatory cytokines restrict chlamydial replication leading to their persistence.

A significant positive correlation was observed between chlamydial load and estradiol levels in women with MPC suggesting that higher estradiol levels either help in persistence of chlamydial infection by modulating host immune responses or enhances virulence of

Chlamydia as discussed earlier. Chlamydial MOMP IgA antibodies showed significant positive correlation with chlamydial load in both asymptomatic women and women with FD. In contrast, cervical IgA antibodies to cHSP60 and cHSP10 showed significant negative correlation to chlamydial burden showing that during primary chlamydial infection as the chlamydial burden increases the titers of Major Outer Membrane Protein (MOMP) IgA increased correspondingly but in women with persistent chlamydial infection as in women with FD, as the recovery of *Chlamydia* from the cervix decreases, the titers of chlamydial heat shock protein 60 and 10 increases showing that during recurrent infection more of chlamydial stress proteins are exposed.

Our results overall show that in women where infection occurs without any symptom chlamydial burden correlated with protective immune response factors (CD4 T cells, CD14 cells, IL-2, IL-12, IFN-γ and anti-chlamydial MOMP antibodies). On the other hand in women with no fertility complication but high inflammation (women with MPC) inflammatory factors increased with the pathogen burden (IL-1β, IL-8, IFN-γ). In contrast to these groups in women with FD it was observed that heightened response of both pro and anti-inflammatory factors stops chlamydial replication causing it to persist. Thus, infectious load could prove to be an important marker for type of host immune response and in turn could facilitate pathogenesis research.

The response of primary human cervical cells upon stimulation with *Chlamydia* is largely unknown as most of the studies till date, have been performed with PBMCs. Cervical cells are the actual cells encountering the pathogen and their responses would help in having a much better understanding of immunopathogenesis of chlamydial disease, thus, we evaluated the mucosal immune responses to cHSPs and compared them with peripheral response during

primary and recurrent chlamydial infection. It was found that women with recurrent infections have significant humoral and cell-mediated immune responses towards cHSP10 and 60. Our results correlated with previous studies suggesting that immune sensitization to HSPs probably require prolonged exposure of them at elevated concentrations [Witkin *et al.*, 1998]. Our results demonstrated that cHSP10 induces proliferation of cervical lymphocytes obtained women with recurrent infections more significantly than cHSP60 thus showing that its role can be more important than cHSP60 in pathogenesis of chlamydial infections. A study by LaVerda *et al.*, 1997 demonstrated that women with acute infection and tubal factor infertility (TFI) recognized cHSP10 more frequently, with infertile women having greater seroreactivity to cHSP10 than acutely infected. They also demonstrated that among women with similar exposure to chlamydiae, serologic responses were greater to HSP10 in TFI group than HSP60 or MOMP. It has been reported earlier that under conditions of IFN-γ mediated deprivation of tryptophan, a decrease in MOMP and 60-kDa OMP expression was observed but the synthesis of cHSP60 is maintained as it contains no tryptophan residues, as deduced from nucleotide sequence [Morrison *et al.*, 1990]. Another possibility is that under conditions of stress, mRNA for stress response proteins continues to be transcribed, whereas, that for structural proteins is downregulated. Our results, showed higher levels of IFN-γ during recurrent infections and the levels have significant correlation with cHSP60 antibody titers.

The mucosal immune responses were further evaluated in terms of cytokine secretion during chlamydial infection in women with or without pathological sequelae. Both healthy controls and *C. trachomatis*-positive women (fertile and with fertility disorders) were enrolled, as upon *in vitro* stimulation with chlamydial EBs the cells obtained from controls will act as

primary and recurrent chlamydial infection. It was found that women with recurrent infections have significant humoral and cell-mediated immune responses towards cHSP10 and 60. Our results correlated with previous studies suggesting that immune sensitization to HSPs probably require prolonged exposure of them at elevated concentrations [Witkin *et al.*, 1998]. Our results demonstrated that cHSP10 induces proliferation of cervical lymphocytes obtained women with recurrent infections more significantly than cHSP60 thus showing that its role can be more important than cHSP60 in pathogenesis of chlamydial infections. A study by LaVerda *et al.*, 1997 demonstrated that women with acute infection and tubal factor infertility (TFI) recognized cHSP10 more frequently, with infertile women having greater seroreactivity to cHSP10 than acutely infected. They also demonstrated that among women with similar exposure to chlamydiae, serologic responses were greater to HSP10 in TFI group than HSP60 or MOMP. It has been reported earlier that under conditions of IFN-γ mediated deprivation of tryptophan, a decrease in MOMP and 60-kDa OMP expression was observed but the synthesis of cHSP60 is maintained as it contains no tryptophan residues, as deduced from nucleotide sequence [Morrison *et al.*, 1990]. Another possibility is that under conditions of stress, mRNA for stress response proteins continues to be transcribed, whereas, that for structural proteins is downregulated. Our results, showed higher levels of IFN-γ during recurrent infections and the levels have significant correlation with cHSP60 antibody titers.

The mucosal immune responses were further evaluated in terms of cytokine secretion during chlamydial infection in women with or without pathological sequelae. Both healthy controls and *C. trachomatis*-positive women (fertile and with fertility disorders) were enrolled, as upon *in vitro* stimulation with chlamydial EBs the cells obtained from controls will act as

naive cells mimicking primary immune response and cells from *C. trachomatis*-positive women will give data on secondary immune responses (as they are already exposed to the pathogen). On comparing cytokine levels secreted by cells obtained from *Chlamydia* positive fertile women with those with FD, it was found that cervical cells from women with FD secreted significantly higher levels of IL-1β and IL-8.

Cervical cells obtained from both the *Chlamydia* positive groups secreted significantly higher levels of IL-6 compared to controls. This result suggests that during secondary immune response to chlamydial infection, IL-6 secretion is significantly enhanced. Further, it was seen that this phenomenon is more pronounced in women with FD. Although not as protective as IFN-γ, IL-6 (generated either by epithelial cells or by the interaction of chlamydiae with T lymphocytes) is probably important, together with IL-12, for sustaining the protective T-helper 1 cell mediated immune response [Yu *et al.*, 2003]. However, pathogenic role of IL-6, has also been shown by many studies [Darville *et al.*, 2003; Mpiga *et al.*, 2006]. It has also been shown that IL-10 inhibits high levels of IL-1ß, TNF-α [Enk *et al.*, 1993], IL-2 and IFN γ by downregulating class II MHC molecules [Yang *et al.*, 1996]. Many bacteria or their products have been shown to stimulate production of interleukin 10 by host cells which then acts as a multifunctional cytokine. On the other hand by down-regulating the proinflammatory cytokines, IL-10 can create a favourable environment for persistence of microbes.

ELISPOT analysis and ELISA results show that IFN-γ levels were higher in controls and *Chlamydia* positive fertile women compared to those with FD. This data suggests that either upon primary induction of cells or in secondary stimulation in cases where no apparent clinical pathological symptom is present IFN-γ is the major cytokine to be secreted whereas,

in cases where clinical pathology is present during secondary immune response IFN-γ is down-regulated. This suggests a protective role of IFN-γ during chlamydial infection in contrast to an earlier study by Van Voorhis *et al.*, 1997 which has shown that repeated chlamydial infection in a *Macca nemestrina* model high levels of IFN-γ transcripts were produced. Further, in synovial tissue of patients with *C. trachomatis* associated arthritis, both IL-10 and IFN-γ producing cells were detected and it was suggested that excessive IL-10 production suppresses IFN-γ and mediated persistence [Kotake *et al.*, 1999]. IL-12, on the other hand was significantly higher in *Chlamydia* positive fertile women with compared to controls and women with FD. IL-12 is derived from dendritic cells and monocytes and it induces T_H1 differentiation with induction of IFN-γ [Holland *et al.*, 1996]. Thus secretion of IL-12 upon secondary stimulation may help in providing a protective immune response to chlamydial infection.

In this study significant levels of IL-4 in cervical cell samples were detected at mRNA expression levels but not in cell free supernatants. In an earlier study also, depressed levels of IL-4 were detected using TaqMan™ based real time PCR in patients with a history of HPV infected with *C. trachomatis* [Scott *et al.*, 2006]. These results may be due to the fact that IL-4 secreting cells may be quenched or short lived in peripheral circulation or its secretion was downregulated by other factors in the mixed cell population [Holland *et al.*, 1996].

Increased expression of IFN-γ and IL-12 (pro-inflammatory cytokines) upon secondary stimulation in fertile women and increased expression of IL-10 and IL-4 (anti-inflammatory/regulatory cytokines) transcripts in women with FD suggest that modulation of cytokine expression is the main mechanism which decides whether the infection is cleared or will go for pathological damage. This would however, be hypothetical as there is no

confirmation that in women with fertility related disorders the clinical condition is due to chlamydial infection and not due to any other cause.

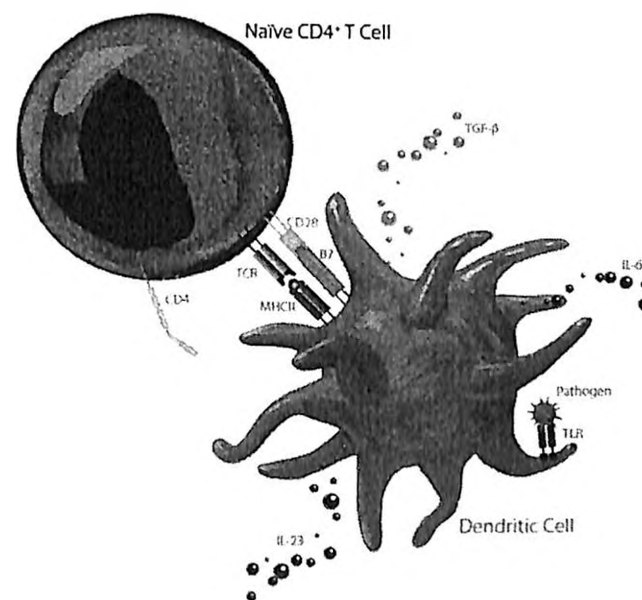
Our study thus revealed that cervical cells play a pivotal role in maintaining a differential cytokine surveillance milieu in women suffering from *C. trachomatis* infection thereby significantly altering the mucosal immune responses. Overall our data suggests that in women who had chlamydial infection for the first time or those which do not have any pathological damage (fertile) secrete higher amounts of IFN-γ and IL-12 but women who possibly develop a sequalae to chlamydial infection, the cells secrete lesser amounts of IFN-γ and IL-12 with higher levels of IL-6, IL-8 and IL-10.

In conclusion, it can be suggested that changes in cytokine patterns can be responsible for development of various pathological consequences to chlamydial infection. Results presented in this study do not provide confirmed evidence whether the cytokine profiles are actually associated with fertility disorders or other factors are responsible. However, since there is an urgent need to develop a vaccine against chlamydial infection, therefore, the basic research challenge is to understand the requirements for inducing and maintaining protective genital mucosal immunity. This study will thus be helpful in demonstrating the association between cytokines and immunopathogenesis and will help in considering new directions and dimensions for therapeutic approaches and strategies for disease prevention.

Chapter 5
Enumeration of cervical dendritic cell subsets and their role in modulation of immune response to Chlamydia trachomatis infection

Observations always involve theory

Edwin Hubble



Enumeration of cervical dendritic cell subsets and their role in modulation of immune response to Chlamydia trachomatis infection

Introduction

Chlamydia trachomatis infections are the most prevalent sexually transmitted bacterial infections worldwide [Gerbase *et al.*, 1998]. Spontaneous clearance of *C. trachomatis* from the lower genital tract occurs in nearly 20% of chlamydial infections without any sequelae [Parks *et al.*, 1997], however, in absence of treatment, these infections often recur, or remain persistent, leading to structural damage to the inflamed tissue and increase the risk of developing a sequelae [Golden *et al.*, 2000; Schachter *et al.*, 1978]. Host factors, such as type of immune response, plays a large role in determining the course and morbidity of *C. trachomatis* infections but the exact immunopathological mechanism leading to *Chlamydia* induced sequelae is still not well understood.

Mucosal surface of the urogenital tract provide a large entry site for various pathogens, the immune response to whom strongly depends on the presence of different infiltrating immune cells. During chlamydial infection [Johnson *et al.*, 2004], T-cell mediated adaptive immune responses, play a major role in the clearance and resolution of infection. T cells are activated by antigen presenting cells as dendritic cells (DCs) which play a crucial role in initiation and maintenance of T-cell immunity [Steinmann *et al.*, 1999]. Besides providing protection, DCs have also been reported to be involved in chronic inflammation [Mellman & Steinmann, 2001].

In peripheral blood, two major DC subsets have been described; the $CD123^{+}$ DCs designated as plasmacytoid DCs (pDCs) and the $CD11c^{+}CD123^{-}$ (-/dim) designated as myeloid DCs (mDCs). Both the subsets express high levels of HLA-DR and lack the lineage markers CD3,

CD14, CD19, CD20, CD16, and CD56, however, functional differences between the two have been described which include T-cell stimulatory activity, production of proinflammatory cytokines and differential expression of co-stimulatory molecules [Reid *et al.*, 2000; Thomas & Lipsky, 1994; Willmann & Dunne, 2000].

In humans, pDCs and mDCs have been shown to be associated with various pathological and disease conditions as viral infections [Azzoni *et al.*, 2005; Gill *et al.*, 2005], bacterial infections [Mendelson *et al.*, 2006], parasitic infections [Breitling *et al.*, 2006], fungal infections [Perruccio *et al.*, 2004], cancerous conditions [Fiore *et al.*, 2006; Brimnes *et al.*, 2006], systemic lupus erythematosus [Robak *et al.*, 2006], rheumatoid arthritis [Jongbloed *et al.*, 2006], coronary artery disease [VanVre *et al.*, 2006] and inflammatory skin disease [Wollenberg *et al.*, 2006].

The cervical mucosa is reported to contain numerous DCs interdigitating between the epithelial cells [Pudney *et al.*, 2005] and these DCs often stain positive for CD1a [Prakash *et al.*, 2004]. A previous study by Bontkes *et al.*, 2005, have shown the presence of both pDCs and mDCs in the female cervix during cervical carcinoma, but no information is available on the mobilization of these two subsets to the cervix during bacterial infections. Enhancement in $CD4^+$ T cells, $CD8^+$ T cells and dendritic cellular phenotypes in cervical samples from *Chlamydia*-positive women has been shown previously [Mittal *et al.*, 2004], but no study on recruitment of these two DC subsets to the cervical mucosa during *C. trachomatis* infection was done. Further upon chlamydial infection, mononuclear cells are triggered to release a number of proinflammatory cytokines including TNF-α, IL-1, IL-6 and IL-8 [Heinemann *et al.*, 1996; Redecke *et al.*, 1998] and many studies have reported association between cytokine profiles and immunopathogenic mechanisms. In Chapter 4, it has been shown that estradiol

levels can modulate immune response in women with chlamydial infection and can have a role in *Chlamydia* induced pathology.

Thus, in the present study, we analyzed the mDC and pDC population in peripheral blood and cervical mucosa of healthy controls and *Chlamydia* positive women with or without Mucopurulent Cervicitis (MPC) and fertility related disorders (multiple spontaneous abortions and infertility (not male related) to study their role in inflammation and development of sequalae. We also correlated the population of these subsets with chlamydial load, C-reactive protein (CRP) levels, sex hormone levels and cytokine levels. We further studied the population of these subsets in *Chlamydia* positive women after full antibiotic therapy in order to understand their role in pathogenesis of chlamydial infection.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plasticware and glassware used in cell culture and immune assay was purchased from Greiner, Germany.

Experimental Methods

Study population and patient classification

After obtaining informed written consent, 261 patients attending the gynecology outpatient department, Safdarjung Hospital, New Delhi, India, were enrolled for the study. Healthy age matched controls attending the family planning department for birth control measures were also enrolled. The study received approval from the hospital's ethics review committee. The procedures followed for sample collection were in accordance with the ethical standards for human experimentation established by the Declaration of Helsinki of 1975 (revised in 1983).

At recruitment, a detailed clinical questionnaire was administered to each patient to collect information on the reasons for referral, gynecology history, including menstruation, symptoms of genital and urinary tract infection, obstetric and medical histories.

Inclusion criteria

The *Chlamydia*-positive patients included in the study comprised of (a) *Chlamydia* positive fertile asymptomatic women attending a family planning clinic, (b) *Chlamydia* positive fertile women with mucopurulent cervicitis (MPC) [thick discharge and inflammation with number of polymorphonuclear leukocytes (PMNLs) > 30] and (c) *Chlamydia* positive women with Fertility Disorders (FD). Inclusion criteria of women in all the groups is done as described in **Chapter 4.**

Exclusion criteria

Factors relating to Exclusion of patients have been described in **Chapter 4.**

Collection of samples and microbiology

Endocervical samples for detection of dendritic cells were collected from patients and detection of *Chlamydia* and other STD pathogens was performed as described previously in **Chapter 4.** Heparinised and non-heparinised venous blood were collected from all the women.

Isolation of endocervical cells

Endocervical cells were isolated from cytobrush samples as described previously in **Chapter 4.**

DC identification

DCs were identified by multiparametric flow cytometry with the following monoclonal antibodies (MoAb): fluorescein isothiocyanate (FITC) conjugated lineage cocktail LIN-1

(anti-CD3, anti-CD14, anti-CD16, anti-CD19, anti-CD20 and anti-CD56), CD123-phycoerythrin, HLA-DR-peridin chlorophyll protein (BD Biosciences). In addition, FITC labeled anti-CD4, anti-CD8, anti-CD19 (for identification of CD4 T cells, CD8 T cells and B cells respectively) and allophycocyanin labeled anti-CD11c were purchased from eBiosciences (San Diego, USA) and PE labeled anti-CD80 and CD86 (costimulatory molecules involved in differential activation of T- helper pathways) CD83 (DC maturation marker) and CD1a (immature DC marker) were obtained from BD Biosciences. To measure expression of CD80, 83, 86 and 1a on $CD123^+$ cells anti-CD123-PE-Cy5 and HLA-DR-APC were purchased from BD Biosciences. For blood dendritic cells, 100µl of whole blood was incubated with antibody cocktail for 20 min at room temperature. Erythrocytes were lysed with FACS Lysing Buffer (BD Biosciences), cells were washed with PBS (with 0.1% (w/v) BSA and 0.1% NaN_3) to remove unbound MoAbs and resuspended in 1%(w/v) paraformaldehyde in PBS. For cervical cells, 100,000 cervical cells/tube were incubated with antibody cocktail for 25 min on ice and subsequently washed and fixed as per the protocol above. Cell preparations were labelled in parallel and included all appropriate isotype control antibodies (BD Biosciences) for establishing the demarcation between negative and positive populations. As cervical specimen exhibit high level of granularity and autofluorescence that could be attributed to many factors. Background fluorescence and presence of lymphocytes was minimized by introduction of an acquisition gate on the forward-scatter (FSC) versus side-scatter (SSC) profile, which included most of the monocytic and dendritic cell fraction and gave reliable differentiation of these cells from epithelial cells, lymphocytes and cell debris. Samples were acquired using a FACS Calibur Cytometer and analyzed with cell quest software (Becton Dickinson). DCs were identified as lineage FITC cocktail negative and

HLA-DR positive population. The gating strategy used to identify and quantify LIN^{-}/DR^{+} cells is described in Figure 4.1.

mDCs and pDCs in cervical secretions were measured as number of events present per 100,000 cells taken for experiment. This number was then adjusted according to the total number of cells obtained in that cervical sample. These were finally presented as number of events per cervical sample. In case of blood, the dendritic cell subsets were counted as events per 100 μl of blood taken and were then calculated and represented as events per milliliter of blood.

Quantification of chlamydial infectious load in cervical samples

Chlamydial infectious load in cervical samples was determined as infection forming units/mL (IFUs/mL) as described in **Chapter 4.**

Quantification of cytokines in cervical washes

Quantification of IL-1β, IL-2, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-α and IFN-γ in cervical washes was done by commercially available ELISA kits (eBiosciences, San Diego, USA and Diaclone, Cedex, France), in accordance with the manufacturer's instructions. Procedure for the same has been described in **Chapter 4.** The sensitivities of cytokine kits were 1 pg/mL.

Determination of C-reactive protein levels

C-reactive protein levels in sera were measured by commercially available hs-CRP ELISA kit (Calbiotech, USA) and levels above 2 microgram per millilter were considered to show higher risk of chronic inflammation as per the manufacturer's instructions.

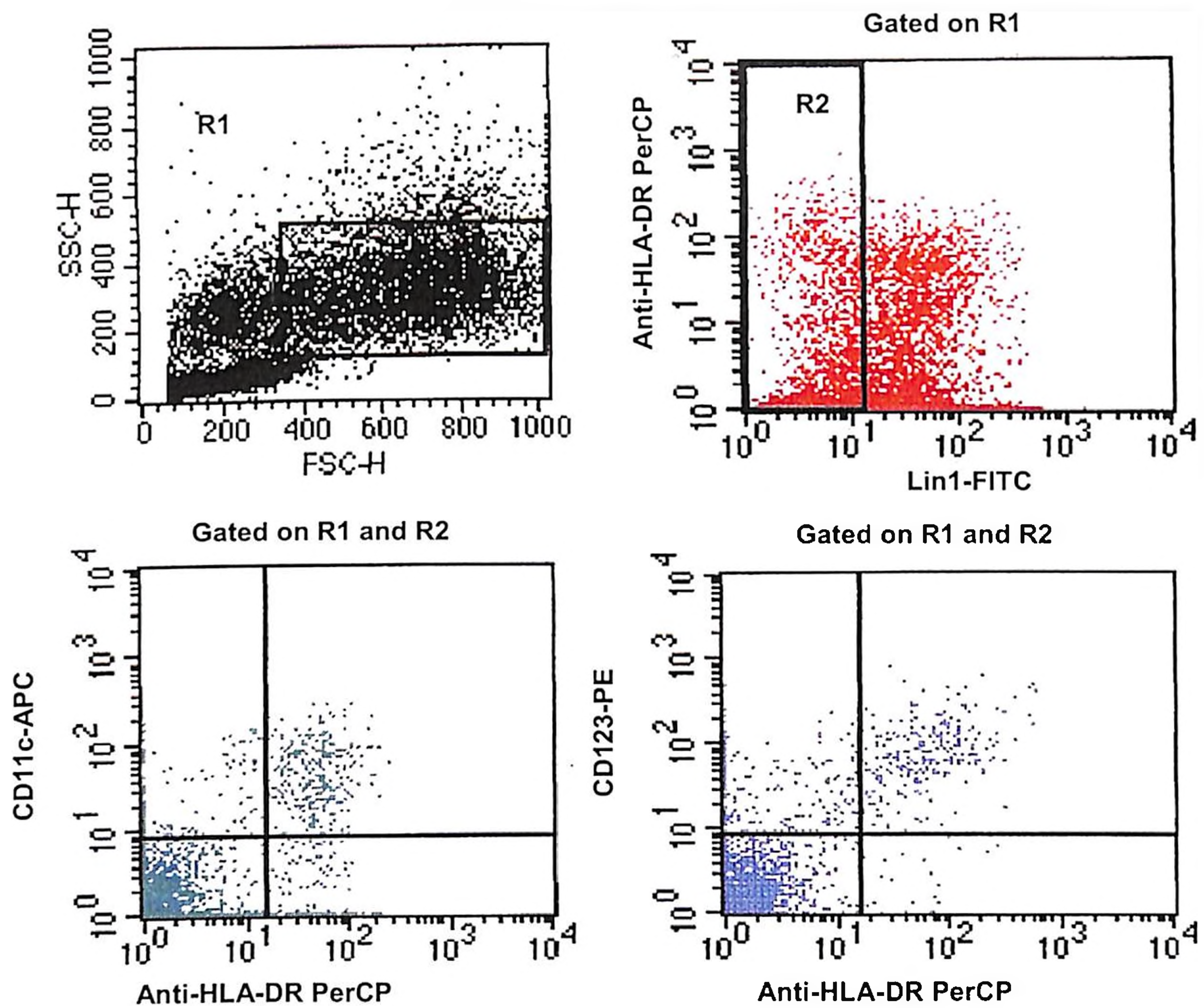


Figure 5.1. Quantification of dendritic cell subsets in cervical mucosa by flow cytometric analysis. (A) Introduction of an acquisition gate (R1) on the forward-scatter (FSC) versus side-scatter (SSC) profile to select mononuclear cell population. (B) cervical DCs were identified within gate R2 as lineage fluorescein isothiocyanate (FITC) cocktail (contains anti-CD3, CD14, CD16, CD19, CD20, and CD56) negative and HLA-DR positive. (C-D) Detection of HLA-DR^{+} CD11c^{+} myeloid DCs and HLA-DR^{+} CD123^{+} plasmacytoid DCs respectively. FSC, forward scatter; SSC, side scatter; PerCP, peridin chlorophyll protein; PE, phycoerythrin; APC, allophycocyanin.

Total cells counted? % of pDCs & mDCs?

Hormonal assay

Levels of β-estradiol and progesterone in sera of patients and controls were measured using commercially available ELISA kits (DRG, International Inc, USA) as per the manufacturer's instructions.

Statistical analysis

The Kruskal-Wallis non parametric test was used to compare continuous variables among multiple groups. The non parametric Mann-Whitney U test was used to compare DC populations and cytokine concentrations. Wilcoxon signed rank test was used to compare DC numbers before and after therapy. Correlation was determined with spearman's correlation coefficient.

Results

Study population

Cervical *C. trachomatis* infection was diagnosed by DFA/PCR in 29 women attending the family planning clinic (asymptomatic) and in 51 women attending the Gynecology Out Patient Department (symptomatic). Nine *Chlamydia* positive women were co-infected either with *Candida* spp., bacterial vaginosis, *T. vaginalis*, *M. hominis*, *U.urealyticum*, or *N. gonorrhoeae* were excluded from the study. Three *Chlamydia* positive patients were excluded, as the mononuclear cell count in the cervical cells was less than 1 million cell/mL. Based on clinical history the *Chlamydia* positive women were divided into four groups: (a) Uninfected healthy controls taken from women attending the family planning clinic (n=28), (b) *Chlamydia* positive fertile asymptomatic women attending a family planning clinic (n=23), (c) *Chlamydia* positive fertile women with MPC (thick discharge and inflammation with number of PMNs>30) (n=25), and (d) *Chlamydia* positive women with fertility

disorders (FD) (n=20). No significant differences in the median ages of the patients (fertile and with fertility disorders) or controls was observed (27, 28, 27 and 28 years respectively).

Immune cell population in the cervix

Flowcytometric analysis demonstrated the presence of mDCs, pDCs, CD14$^+$ monocytes, CD3$^+$CD4$^+$ T cells and CD3$^+$CD8$^+$ T cells in all the *Chlamydia* positive groups and controls. The median range of CD3$^+$CD4$^+$ T cells and CD3$^+$CD8$^+$ T lymphocytes among endocervical leucocytes in women which were included in the study was between 59% and 86% that of B lymphocytes was between 1% and 4%.

mDC and pDC population in cervical mucosa and peripheral blood

Three of 28 healthy controls, 1 of 23 *Chlamydia* positive women asymptomatic women and 2 of 25 *Chlamydia* positive women with MPC did not have mDCs in their cervical samples. Five of 28 healthy controls, 3 of 23 *Chlamydia* positive women without MPC do not have pDCs in their cervical samples. The median and range of absolute numbers of mDCs and pDCs are given in Table 4.1. Healthy controls have significantly lower number of mDCs and pDCs in their cervical samples as compared to *Chlamydia* positive patient groups ($P<0.01$). In comparison to cervical samples there was significant decrease in the absolute numbers of pDCs/mL of blood in women infected with *Chlamydia* when compared with controls.

The decrease in pDC number in blood was found to be more pronounced in case of asymptomatic women and women with fertility disorders. In cervix of both the *Chlamydia* positive groups, the median number of pDCs was found to be higher than mDCs and was significant in case of women with fertility disorders ($P<0.05$) (Table 5.1). In controls the median number of mDCs was higher to pDCs but the difference was not significant (Table 5.1).

Expression of cell surface markers on mDC and pDC in cervical mucosa

In cervical mDCs of *Chlamydia* positive fertile women and on pDCs of *Chlamydia* positive women with fertility disorders, significantly high expression of CD80 was observed compared to the controls (P<0.05). Expression of CD83 was significantly higher on both cervical mDCs and pDCs compared to controls. The expression of CD83 on pDCs obtained from women with fertility disorders was significantly higher than that of pDCs obtained from fertile women. CD86 expression on both mDCs and pDCs was found to be higher in *Chlamydia* positive women with or without fertility disorder, compared to controls but the difference was not significant (Table 5.2). Expression of CD1a on mDCs was significantly higher (P<0.01) in *Chlamydia* positive groups as compared to controls (Table 5.2). The expression on pDCs of CD1a was higher in of *Chlamydia* positive groups compared to controls but was non significant.

Table 5.1. Absolute number of myeloid and plasmacytoid dendritic cells in cervical mucosa and blood samples

		Control	CT positive asymptomatic	CT positive with MPC	CT positive with FD
mDCs	Blood	15834 (3148-57180)	1800 (458-13786)[a]	10800 (1326-27556)	8138 (175-17525)
	Cervix	28 (0-816)	1788 (0-40170)[a]	720 (0-10444)[a,b]	1856 (21-8473)[a]
pDCs	Blood	12857 (3051-45496)	1750 (0-33840)[a]	3890 (1162-80752)[a]	2645 (969-8657)
	Cervix	17 (0- 195)	1216 (637-10430)[a]	2240 (3341 - 22382)[a]	4483 (54-10845)[a]

NOTE: Data represents median values for absolute number of myeloid and plasmacytoid dendritic cells in cervical mucosa (DCs/cervical sample) and blood (DCs/mL blood). Figures in parentheses depict range. CT, *Chlamydia trachomatis*; MPC, mucopurulent cervicitis; FD, Fertility Disorders

[a] $P<0.05$ of individual patient groups compared to controls by Mann Whitney U test.

[b] $P<0.05$ of CT positive group with MPC compared to without MCP by Mann Whitney U test.

Table 5.2. Phenotypic characterization of dendritic cell subsets in cervical mucosa

Co-stimulatory molecules	Percentage expression on mDCs				Percentage expression on pDCs			
	CT positive asymptomatic	CT positive with MPC	CT positive with FD	Controls	CT positive asymptomatic	CT positive with MPC	CT positive with FD	Controls
CD 80	16.0 ± 2.8[a]	26.0 ± 3.4[a]	12.0 ± 2.2	02.0 ± 0.7	03.9 ± 2.4	06.3 ± 3.0	32.5 ± 2.5[a]	02.5 ± 1.3
CD 83	24.0 ± 3.6[a]	30.0 ± 4.2[a]	28.0 ± 1.8[a]	11.0 ± 0.6	14.7 ± 0.9[a]	07.1 ± 2.6[a]	38.0 ± 2.4[c]	03.3 ± 1.2
CD 86	53.0 ± 4.2	65.0 ± 7.2[b]	59.0 ± 2.6	32.0 ± 5.1	02.2 ± 0.4	04.2 ± 2.1	20.0 ± 1.9	01.7 ± 0.7
CD1a	43.0 ± 3.0[a]	26.0 ± 3.8[a]	37.0 ± 4.2	18.0 ± 3.0	45.0 ± 3.0	19.0 ± 2.8	41.0 ± 2.7	28.0 ± 1.0

NOTE: Data represents of costimulatory molecules on myeloid and plasmacytoid dendritic cells in cervical mucosa. CT, *Chlamydia trachomatis*; MPC, mucopurulent cervicitis.

[a] $P<0.05$ of individual patient groups compared to controls by Mann Whitney U test.

[b] $P<0.05$ of CT positive group with MPC compared to controls by Mann Whitney U test.

cP<0.05 of CT positive women with FD compared to other *Chlamydia* positive groups

Concentration of cytokines in cervical washes and their correlation with DC populations

The median levels of IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-α and IFN-γ in cervical washes of controls and *Chlamydia*-positive women with MPC, before and after antibiotic therapy are shown in Table 5.3. The detection limit for all the cytokines was 2 pg/mL, as described by the manufacturers. Before therapy, significantly higher levels of IL-1β and IL-6 ($p < 0.05$) were observed in *Chlamydia*-positive women with MPC than in those without MPC. IL-8 levels were significantly higher ($p < 0.05$) in *Chlamydia*-positive women with MPC than in those without MPC and controls. IL-4 levels were below detection limits in all the samples. After therapy, IL-8 levels in women with MPC showed significant downregulation ($p < 0.01$) (Table 5.3). No other cytokine showed any significant change in levels after therapy. Before therapy, a significant correlation ($r = 0.65$; $p < 0.05$) was observed between the number of mDCs/cervical samples and levels of IL-12 in *Chlamydia*-positive women without MPC. The number of pDCs in *Chlamydia*-positive women with MPC showed a significant correlation with IL-8 levels ($r = 0.58$; $p < 0.05$) and a less apparent correlation with IL-6 ($r = 0.37$). After therapy, no correlation among mDC and pDC populations and cytokine levels was observed.

Table 3. Cytokine concentrations in cervical washes before and after therapy

	Control	CT positive without MPC (Before therapy)	CT positive with MPC (Before therapy)	CT positive without MPC (After therapy)	CT positive with MPC (After therapy)
IL-1β	79.24 (UDL- 350.14)	37.62 (UDL-99.86)	96.90 (8.70-298.0)[a]	54.37 (UDL-296.45)	90.25 (UDL-304.21)
IL-2	04.59 (UDL-30.35)	03.37 (UDL-13.57)	02.98 (UDL-16.42)	03.47 (UDL-28.34)	02.85 (UDL- 15.93)
IL-6	29.46 (UDL-188.70)	09.36 (UDL-39.63)	70.96 (UDL -190.60)[a]	15.41 (UDL-173.93)	43.46 (UDL-201.48)[a]
IL-8	89.9 (UDL -247.57)	137.4 (13.74-375.9)	551.58 (32-1074.0)[b]	112.31 (4.52-295.93)	93.17 (13.69-231.94)
IL-10	07.10 (UDL-22.86)	06.64 (UDL-22.96)	11.68 (UDL-30.91)	08.31 (UDL-24.61)	09.28 (UDL-27.83)
IL-12p40	157.35 (UDL-542.89)	267.5 (24.85-727.56)	166.25 (UDL-532.87)	183.82 (19.27-562.37)	159.74 (2.48-527.17)
IFN-γ	145.78 (19.65-589.73)	173.24 (7.33-566.6)	136.96 (UDL-330.6)	152.75 (5.38-478.59)	141.72 (5.48-394.26)
TNF-α	01.47 (UDL-6.61)	1.45 (UDL-13.73)	01.58 (UDL-6.87)	01.48 (UDL-10.67)	01.51 (UDL-5.85)
IFN-α	20.42 (UDL-74.95)	29.69 (UDL-92.57)	38.57 (UDL-95.31)	23.60 (UDL-77.39)	26.86 (UDL-85.45)

NOTE. Data represent median values. Figures in parentheses depict range. CT, *Chlamydia trachomatis*; MCP, mucopurulent cervicitis; UDL, under detection limit.
[a] $P<0.05$ CT positive women with MCP compared to CT positive women without MCP by Mann Whitney U test.
[b] $P<0.01$ of CT positive group with MPC compared to both the other groups by Mann Whitney U test.

Correlation of mDC and pDC with chlamydial infectious load, C-reactive protein levels and sex hormone levels

The chlamydial infectious load was determined by inoculating cervical samples into HeLa 229 cell line as described in **Chapter 4** and infection forming units/mL (IFU/mL) were quantified. In cervical mucosa, the number of mDCs/cervical sample showed significant correlation with chlamydial IFUs/mL ($r=0.524$; $P<0.05$) in *Chlamydia* positive asymptomatic women. A positive non significant correlation was found between chlamydial IFUs/mL and mDCs/cervical sample in women with MPC ($r=0.312$). In comparison, number of pDCs/cervical sample showed significant correlation with chlamydial IFUs/mL in both women with or without MPC ($r=0.853$, $P<0.001$ and $r=0.724$, $P<0.01$ respectively).

Estradiol or progesterone levels were considered positive if higher than >2SD of the mean obtained from control patients. Number of estradiol positive women was significantly high among women with fertility disorders (65%, 13/20) compared to fertile women (15%, 5/34). Median estradiol levels were also found to be significantly higher in women with fertility disorders (Chapter 4, Table 4.3). Estradiol levels further showed significant correlations with pDC numbers in women with fertility disorders ($r=0.65$; $P<0.05$), with CD80 expression ($r=0.74$ and $r=0.58$ for both *Chlamydia* positive women with or without fertility disorders respectively). IL-6 and IFN-γ levels also showed significant correlation with estradiol levels in women with fertility disorders ($r=0.49$ and 0.73 respectively). No significant difference in the level of progesterone was found among different groups (Chapter 4, Table 4.3) Further, no correlation was observed between progesterone levels and any of parameters studied.

mDCs and pDCs in cervix and blood after resolution of chlamydial infection

Chlamydia positive asymptomatic women and women with MPC were advised full antibiotic therapy and after 4-6 weeks, 8 women from each *Chlamydia* positive group who returned for follow-up were again enrolled. After treatment, none of the CT positive women without MPC was *Chlamydia* positive as revealed by PCR and culture. One patient with MPC (CT18M) was found to be *Chlamydia* positive even after therapy although the number of PMNs was less than 10 with no mucopurulent discharge. Comparing paired measurements of mDCs and pDCs in cervical samples in only the patients with chlamydial infection who returned for follow-up evaluation revealed a significant increase in number of mDCs after therapy in both *Chlamydia* infected groups (Figure 5.2A and B). The median number of pDCs was lower in both the *Chlamydia* infected groups after therapy. The pDC number was significantly lower in women with MPC after therapy (Figure 5.2C and D). The median number of mDCs and pDCs in peripheral blood and cervical samples before and after therapy are given in Table 5.4. The relative frequency of mDCs in cervical samples significantly increased from 0.074% before therapy to 0.19% after therapy in CT positive women without MPC ($P<0.05$) and from 0.068% to 0.11% in women with MPC (non significant). The relative frequency of pDCs in cervical samples of women with MPC significantly decreased from 0.18% before therapy to 0.04% after therapy ($P<0.05$).

Table 5.4. Absolute number of myeloid and plasmacytoid dendritic cells in cervical mucosa and blood samples before and after resolution of infection.

		CT positive without MPC		CT positive with MPC	
		Before therapy	After therapy	Before therapy	After therapy
Cervix	mDCs	1788	5384[a]	720	3917[a]
	pDCs	1750	983[a]	3890	413[a]
Blood	mDCs	1800	5860[a]	10800	18560
	pDCs	7216	13277	6240	17472[a]

NOTE. Data represent median values for all cases before therapy and of the 8 follow-ups after therapy. CT, *Chlamydia trachomatis*; MCP, mucopurulent cervicitis; [a] $P<0.05$ compared paired measurements by Wilcoxon signed ranked test.

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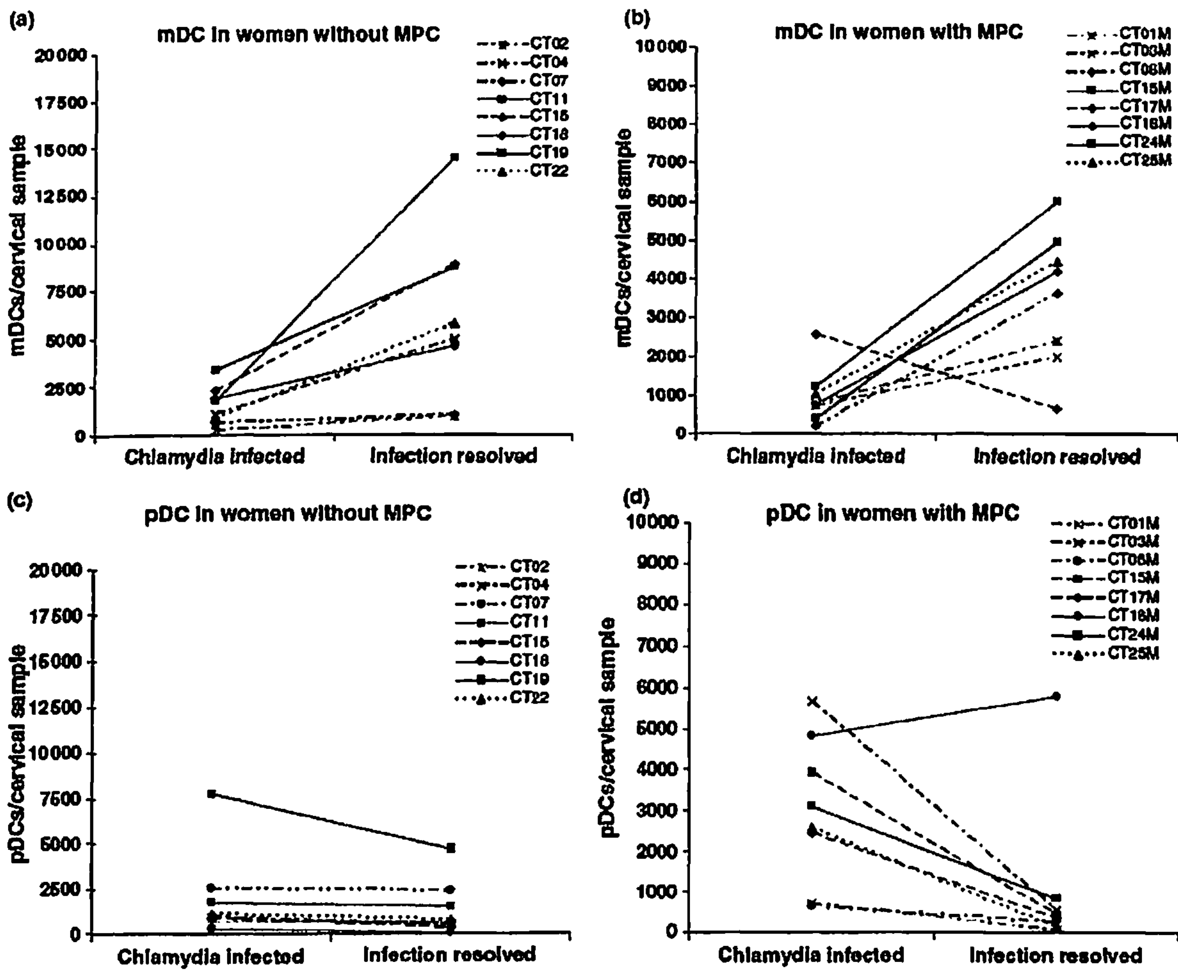


Figure 5.2. Change in number of dendritic cells in cervical samples of *Chlamydia* positive women without or with MCP after resolution of chlamydial infection. (A and B) myeloid DCs (mDCs) in cervical samples of 8 women with or without MCP who returned for follow-up after resolution. (C and D) Similar data for plasmacytoid dendritic cells (pDCs) in cervical samples. MCP, mucopurulent cervicitis.

Discussion

In women, chlamydial infections are often asymptomatic, and subsequent reinfections lead to inflammatory responses with pathological sequelae [Bailey *et al.*, 1995]. In peripheral circulation, the LIN$^-$/DR$^+$ DCs comprise 0.1% to 2% of total PBMCs in healthy individuals and a majority of these cells express either CD11c or CD123 molecules [Savary *et al.*, 1998], however, as knowledge on cervical dendritic cell subsets is almost nil we are prompted to study the DC subsets during *C. trachomatis* infection.

We enrolled both fertile women and women with FD to study differential recruitment of DC subsets during non-inflammatory and inflammatory conditions. Our data showed that during *C. trachomatis* infection both mDCs and pDCs are recruited to the site of infection. The mDC population in cervix was more pronounced during non-inflammatory conditions and pDCs during inflammatory conditions. The number of cervical mDCs was significantly higher in fertile women in contrast to number of pDCs which were much higher in women with fertility disorders. These results depict that different pathological conditions are associated with different phenotype of dendritic cells and in women having sequelae to chlamydial infection, pDCs outnumber mDCs in the cervix. A previous study has also demonstrated enhanced number of pDC count in bronchoalveolar lavage fluid of immunocompetent patients with pneumonia [Lommatzsch *et al.*, 2007]. Another study by Hartmann *et al.*, 2006, have also shown high number of pDCs in patients with upper respiratory tract infection. In comparison to mDCs, high number of pDCs were reported to be present in synovial fluid from patients with spondyloarthropathy [Van Krinks *et al.*, 2004]. Beulens *et al.*, 1995, have originally described mDCs as functionally mature DCs with a strong T cell stimulatory capacity and have been reported to induce protective immune

response [O'Doherty *et al.*, 1994], whereas, pDCs are generally defined as tolerogenic dendritic cells. Thus, these results collectively suggests that mDCs provide protective response while pDCs are involved in pathogenesis but how they help in development of a disorder is not well understood.

Compared to controls in both the *Chlamydia* positive groups significant upregulation of CD83 and CD86 markers (data not shown) on mDCs was observed, with higher upregulation in women with MPC, suggesting the presence of more number of matured mDCs during inflammation of cervix. The lower numbers of mDCs/cervical sample in women with MPC may be a possible cause for the incomplete clearance of *Chlamydia* and therefore the pathogenesis of chlamydial infection. As for immune modulation by costimulatory molecules, previous studies have demonstrated that CD80/CD86 co-stimulatory molecules differentially activate T_H1/T_H2 type pathway and act as co-stimulatory signals for generation of T_H1/T_H2 cells $CD4^+$ T cells [Buelens *et al.*, 1995; Kuchroo *et al.*, 1995]. It has been shown previously that silencing of CD80 expression on dendritic cells significantly decreases IFN-γ secretion while that of CD86 decreases IL-4 secretion [Suzuki *et al.*, 2007]. CD86 has also been shown to stimulate IL-10 production in CD4+ T cells [Nakajima *et al.*, 1997]. Significantly high expression of CD80 molecules on pDCs obtained from women with fertility disorders and on mDCs from fertile women was observed. This suggests that significantly high expression of CD80 may lead to increased activation of T_H1 cells during chlamydial infection. As no difference in expression levels of CD86 among different groups was observed it suggests that CD86 may not be involved in induction of immune response to *C. trachomatis*.

CD83 expression is taken as a marker for phenotypic maturation of dendritic cells and higher percentage of DCs expressing them in both *Chlamydia* positive cases show that during chlamydial infection high number of both mDC and pDC take up antigen for processing and turn into mature DCs. This data suggests that although both mDCs and pDCs are present in the cervix of women with fertility disorders, but pDCs are the ones which are more matured and hence activated.

No apparent correlation of mDCs was observed with chlamydial infectious load in women with MPC, thereby, suggesting that *C. trachomatis* infection attracts more pDCs than mDCs during infection. An inverse correlation of C-reactive protein levels with mDC numbers, have been shown previously [Jongbloed *et al.*, 2006], but no correlation was found among them in this study. pDCs were also found to be more pronounced in extensive coronary artery disease with the authors suggesting their role in plaque progression [Van vre *et al.*, 2006]. A recent study by Rey-ladino *et al.*, 2008, have shown the persistence of *Chlamydia* inside dendritic cells and this study when applied to our results suggests that pDCs are not efficient in presenting chlamydial antigens to T cells and can be used by *C. trachomatis* for their survival and persistence, leading to inflammation, but further studies are required to prove this hypothesis.

We then measured the cervical cytokines and found significantly higher levels of IL-1β, IL-6 and IL-8 in *Chlamydia* positive women with IL-1β and IL-8 considerably higher in women with MPC. Number of mDCs/cervical sample showed significant correlations with cervical IL-12 levels in women without MPC and pDCs correlated with IL-8 levels in women with MPC. As for cytokine production by these subsets previous studies have shown production of interferon-α by pDCs on viral challenge [Lichtner *et al.*, 2006; Liu, 2001; Shortman & Liu,

2007], and production of IL-12 by mDCs and IL-12, IL-10 and IFN-α by pDCs in response to fungal infections [Perruccio *et al.,* 2004]. pDCs are reported to express TLR9 and upon stimulation with CpG motifs they are shown to express IFN-α, IL-6 [Faith *et al.,* 2007], and IL-12p70 [Boonstra *et al.,* 2006] in mouse, but the cytokine secretion pattern of pDCs upon bacterial challenge in humans is yet to be ascertained fully. Secretion of IL-12 has been shown by mDCs after incubation with mycobacteria [Mendelson *et al.*, 2006] and that of IL-10 and IL-23 after incubation with *H. pylori* [Mitchell *et al.,* 2007]. Only, few studies have been done where these DCs were stimulated with LPS to know cytokine secretion patterns [Hellman & Eriksson, 2007]. These studies revealed that upon LPS stimulation mDC produce high amounts of IL-8. This is contrary to the results of this study where significant correlation of IL-8 with number of pDCs was observed. In this study, we also found no significant increase in levels of IFN-α during chlamydial infection. This result was somewhat in concordance with the results of Dai *et al.,* 2004 who have also shown no induction of IFN-α production from pDCs when they were stimulated with LPS. The results we got above may shed a new light on chlamydial pathogenesis but are still hypothetical due to two limitations in our study; (1) scarcity of cell numbers making it impossible for us to separate these subsets and then see the expression of cytokines upon stimulation with *C. trachomatis* and (2) the fact that cytokines like IL-12 are secreted by other cell populations like macrophages which outnumber DCs in the cervix.

Chlamydia positive women with fertility disorders significantly higher levels of IL-6, IL-8, IL-10 and IFN-γ were present compared to the other groups. In comparison to this IL-12 levels were significantly higher in *Chlamydia* positive fertile women. Although not as protective as IFN-γ, IL-6 (generated either by epithelial cells or by the interaction of

chlamydiae with T lymphocytes) is probably important, together with IL-12, for sustaining the protective T-helper 1 cell mediated immune response [Yu *et al.*, 2003]. As for IL-8, it has also been shown previously that synovial tissues from chronic arthritis patients with synovial *C. pneumoniae* infection have significant levels of mRNA for IL-8 [Gerard *et al.*, 2002]. These results suggest that IL-6 and IL-8 may be involved in chlamydial pathology but their actual role and mechanism is yet to be ascertained.

IL-10 was found to be up-regulated in cervical washes obtained from *Chlamydia* positive women with fertility disorders. IL-10 can create a favourable environment for persistence of microbes by down-regulating the proinflammatory cytokines. In case of chlamydial infection IL-10 has been reported to be associated with typical pathological changes like fibrosis and granuloma formation [Conti *et al.*, 2003]. Association of increased frequency of IL-10 detection in endocervical secretions of women with non-ulcerative STDs, like *C. trachomatis* have suggested that this may be a potential mechanism through which these infections may alter susceptibility to HIV-1 infection [Cohen *et al.*, 1999]. These results along with ours, suggests, that excessive secretion of IL-10 may lead to either incomplete clearance of bacteria or may develop a state of anergy resulting in development of fertility related disorders. IFN-γ levels were found to be significantly higher in women with fertility disorders as compared to other groups. An earlier study by Van Voorhis *et al.*, 1997 has also shown that repeated chlamydial infection in a *Macca nemestrina* model high levels of IFN-γ transcripts were produced.

After resolution of infection by antibiotic treatment we found increased levels of mDCs in both the cervix and blood of *Chlamydia* positive women. pDCs on the other hand were lower in both the groups after infection is resolved except for the one patient (CT18M) in group III

(MCP) where the infection was not resolved confirming our hypothesis that *C. trachomatis* mobilizes pDCs in the cervix in accordance with its load. Many studies have been previously carried out to study population of DCs before and after therapy in blood samples but very few studies have been done to enumerate DCs at mucosal surfaces after therapy [Gill *et al.*, 2005]. The increase in number of DCs in blood can be explained by the fact that since the infection is resolved the DCs are not migrating from the blood to the cervix. In our study, we propose that lower numbers of mDCs in cervix before therapy can be due to migration of these DCs to the lymph nodes for initiation of immune responses; however, after resolution of infection these mature DCs again came to reside in the cervix, thereby increasing the numbers.

Further, median CRP levels were found to be significantly higher in women with fertility disorders and showed significant correlation with pDCs. CRP levels are considered marker for inflammation and its association with pDCs in women with fertility disorders suggests that pDCs are involved either in induction or in maintenance of inflammatory responses.

We detected significantly high levels of estradiol in women with fertility disorders, which correlated significantly with pDC levels, CD80 expression and IL-6 and IFN-γ secretion. In a previous study in humans it has been shown that women are more susceptible to chlamydial infection under β-estradiol influence, since more chlamydial organisms can be isolated during the proliferative part of the cycle [Sweet *et al.*, 1986]. Estrogen was also found to enhance chlamydial adherence or intracellular development of their inclusion [Bose & Goswami, 1986].This suggests that estradiol may enhance inflammation by helping in increased secretion of proinflammatory cytokines either by up-regulating CD80 expression or by modulating the cytokine secretion profile of other cell. A previous study by Dimayuga *et*

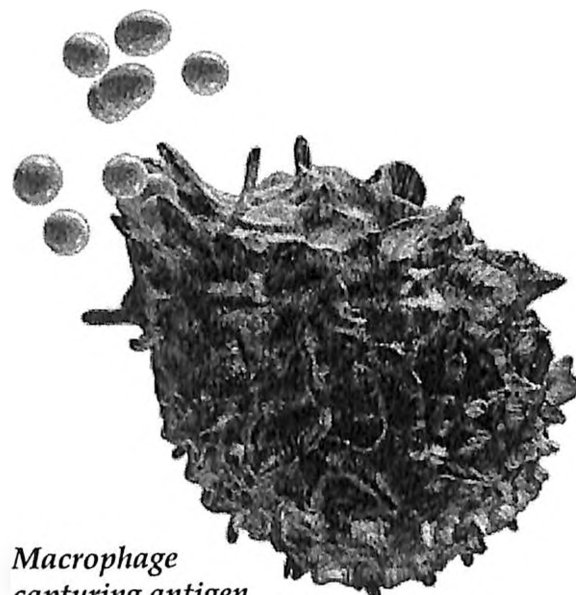
al., 2005 has also shown up-regulation of CD80 expression in LPS stimulated microglial cells by estrogen treatment. Overall these results show that high estradiol levels may help in persistence of *Chlamydia* directly by helping in its intracellular development or indirectly by modulation of cytokine secretion profile of immune cells.

In conclusion, we have shown that both the DC subsets are attracted to site of chlamydial infection with more prevalence of pDCs in inflammatory condition. pDCs correlated with chlamydial load and after resolution of infection a sudden drop in number of pDCs was observed. This suggests a possible role of pDCs in immunopathogenesis of chlamydial infection. A possible hypothesis that emerges from the above results is that pDCs are not able to clear the infection and somehow allow the persistence of *Chlamydia* inside them, but further studies are required as this study has limitations in form of lower number of cells obtained from the cervix which hindered the *in vitro* analysis of these two subsets. Whether the mobilization is due to chlamydial infection or other infections also mobilize DCs to the cervix to the same extent was also a limitation of this study. Regardless of the limitations faced, the results of this study are quite encouraging and with the fact in mind that a vaccine against *C. trachomatis* is still unavailable, this study will facilitate the understanding of interplay between *Chlamydia* and dendritic cells. As dendritic cells are recently being targeted as efficient adjuvant for delivery chlamydial antigens this study can provide newer insights leading to production of an efficient vaccine for *Chlamydi*a.

Chapter 6
Innate immune response (TLRs and nitric oxide) to Chlamydia trachomatis in protection against infection

The true method of knowledge is experiment

William Blake



Macrophage capturing antigen

Innate immune response (TLRs and nitric oxide) to Chlamydia trachomatis in protection against infection

Introduction

In the realm of infectious diseases, it has often been observed that an overly aggressive inflammatory host response can be more problematic than the infection that initiated it. This is certainly true in the case of genital tract infection with *Chlamydia trachomatis*, where the pathology that leads to fallopian tube inflammation, scarring, and infertility is the result of a robust host inflammatory response. *C. trachomatis* is a Gram-negative obligate intracellular bacterium that can initiate a variety of immune system responses from infected hosts. Data obtained from both human studies and animal models point to $CD4^+$ Th1 cells as essential to recovery from infection and resistance to reinfection [Kimani *et al.*, 1996; Holland *et al.*, 1996; Faal *et al.*, 2005]. However, the immunopathogenesis of chlamydial disease is not clear. Both cellular [Stephens, 2003] and immunological hypotheses [Brunham & Peeling, 1994] have been proposed. Evidence that the innate immune response may be the primary mediator of pathology comes from the mouse model of genital tract infection, in which the presence of neutrophils that release and activate proteolytic molecules during acute infection correlates directly with development of fibrotic occlusion of the oviduct [Ramsey *et al.*, 2005; Shah *et al.*, 2005].

One of the most important arm of innate immune response are pattern recognition receptors (PRRs) found on innate immune cells which enable the recognition of signature structures of pathogens, called pathogen-associated molecular patterns (PAMPs). Engagement of PRRs, which include Toll Like Receptors (TLRs), by pathogen-associated molecular patterns leads to activation of innate immune cells that stimulate and direct the activity of pathogen-specific

T cells [Medzhitov, 2000]. TLRs are a family of proteins that share homology with the Toll antimicrobial proteins of *Drosophila* [Rock *et al.*, 1998]. These receptors are found primarily on mammalian innate immune cells, such as macrophages and dendritic cells, but are also expressed on many epithelial cells. Engagement of these receptors can lead to activation of phagocytes and, through activation of the transcription factor nF-κB, production of inflammatory cytokines such as TNF-α and IL-6 [Aderem, 2001; Medzhitov & Janeway, 2000; Kopp & Medzhitov, 1999]. Of the 13 currently described TLRs, TLR2 and TLR4, along with their ligands, are perhaps the best understood in terms of innate responses to bacteria including *Chlamydia*. TLR4 is the signal-transducing receptor for LPS [Poltoraket *et al.*, 1998]; it is aided by two accessory proteins, CD14 and MD-2. TLR2 is involved in the recognition of a broad range of microbial products, including peptidoglycan (PGN) from Grampositive bacteria [Takeuchi *et al.*, 1999], bacterial lipoproteins [Brightbill *et al.*, 1999], mycobacterial cell wall lipoarabinomannan [Means *et al.*, 1999], and yeast cell walls [Underhill *et al.*, 1999]. *Chlamydia* has several cell wall and outer membrane components that may serve as PAMPs recognized by TLRs. Chlamydial LPS [Prebeck *et al.*, 2003] and chlamydial heat shock proteins (HSPs) [Bulut *et al.*, 2002; Ohashi *et al.*, 2000; Kol *et al.*, 2000] are ligands for the TLR4 receptor. However, recent papers describe TLR4-independent cytokine production from inflammatory cells exposed to live chlamydial elementary bodies (EBs) [Prebeck *et al.*, 2002; Netea *et al.*, 2002].

TLR expression in the female reproductive tract has recently been examined. There is constitutive TLR1 to TLR6 mRNA and protein expression as well as MyD88 and CD14 mRNA expression in the fallopian tubes, uterine endometrium, cervix, and ectocervix [Pioli *et al.*, 2004; Fazeli *et al.*, 2005]. TLRs are increasingly documented to play a role in female

reproductive tract physiology. Stimulation of TLR2, TLR3, TLR4, and TLR5 with their ligands has been shown to induce proinflammatory cytokine release in uterine epithelial cells [Schaefer *et al.*, 2004; Fichorova *et al.*, 2002; Schaefer *et al.*, 2005]. In placenta, binding of TLR2 with its ligand Gram-positive bacterial cell wall component peptidoglycan induces trophoblast apoptosis [Abrahams *et al.*, 2004]. Both maternal and fetal polymorphisms of the *tlr4* gene have been associated with spontaneous preterm labor and preterm birth in certain populations [Varner & Esplin, 2005].

It has been shown that TLR2-deficient mice do not develop oviduct pathology after chlamydial infection, demonstrating that TLR2 signaling is directly involved in disease development [Darville *et al.*, 2003]. Recently, it has also been documented that TLR4 deficient mice are more immune to re-infection from *Chlamydia* compared to normal mice [den Hartog *et al.*, 2009].

Another arm of innate immune response is production of nitric oxide by macrophages. Inducible nitric oxide synthase (iNOS) is regulated by cytokines especially IFN-γ, and it generates nitric oxide and other toxic nitrogen radicals that are lethal to a variety of intracellular pathogens [Chan *et al.*, 1995; Jacobs *et al.*, 1996]. *In vivo* studies have shown that iNOS is not essential for the clearance of microbiologically detected infection in mice, but it may provide protection against chronic *C. trachomatis* infection and the associated complications [Ramsey *et al.*, 2001]. It has been reported that the courses of genital chlamydial infection in $iNOS^{-/-}$ and $iNOS^{+/+}$ mice were identical, and both models resolved *C. trachomatis* infection in a similar fashion [Hosseinzadeh *et al.*, 2004; Ramsey *et al.*, 2001]. However, $iNOS^{-/-}$ mice sustained greater pathological outcome subsequent to infection than $iNOS^{+/+}$ controls [Ramsey *et al.*, 2001]. A strong positive correlation between

elevated iNOS activity and protection from chronic disease sequelae (e.g., hydrosalpinx formation and infertility) [Ramsey *et al.*, 2001; Ramsey *et al.*, 2003] and prevention of chlamydial systemic spread has also been reported [Igietsme *et al.*, 1998]

Thus, in the present study we at first analyzed the Toll like receptor expression patterns in cervical monocytes upon *C. trachomatis* infection. Further, Toll like receptor signalling pathway molecules involved during chlamydial recognition were also studied. Secondly, we studied induction of *inos* gene expression and release of nitric oxide in human macrophages upon chlamydial infection and its role in clearance of *Chlamydia* to gain insight into the role of innate immune mechanisms in chlamydial pathology.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plasticware and glassware used in cell culture and immune assay was purchased from Greiner, Germany.

Experimental Methods

Cell line

THP-1 (a human monocytic cell line), was procured from National Centre for Cell Sciences (NCCS), Pune, India. THP-1 cells are non adherent cells and were grown at 37°C under 5% CO_2 in RPMI-1640 medium with 2 nmol per liter glutamine adjusted to contain 1.5g/L sodium bicarbonate, 10 mM HEPES and supplemented with 10% fetal bovine serum (FBS) and 100μg/mL penicillin and streptomycin. To differentiate monocytes into macrophages, cells were plated at 4 x 10^5 cells/ml and stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) for 24 h. Cells were washed twice with PBS before adding fresh medium and growing for upto 5 days. For growing *Chlamydia* stocks, HeLa 229 (a human cervical

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epithelial cell line), was procured from NCCS, Pune, India. HeLa cells were grown at 37°C under 5% CO_2 in minimal essential medium (MEM) with 2nM/L glutamine, supplemented with 10% fetal bovine serum (FBS) and 10 μg of gentamicin/ml.

***Chlamydia* culture**

HeLa cells were pretreated with diethylaminoethyl-dextran (DEAE-D). Thereafter, HeLa cell monolayers were infected with *C. trachomatis* serovar D (a human clinical isolate) by centrifugation at 2000 rpm for 1 hr. Infected HeLa cells were cultured at 35^0C and 5% CO_2. *Chlamydiae* were harvested from infected cells after 66 hrs postinfection and chlamydial EBs were purified by differential renograffin gradient centrifugation. For harvesting cells were removed by agitation with 4mm glass beads and 2 mL of cold PBS. The cell suspensions were pooled and the cells were ruptured by sonication. This suspension was centrifuged at 500 x g for 15 min at 4^0C. EBs were harvested by removing debris in the supernatant by centrifugation at 30,000 x g for 1 hr at 4^0C. The pellet was resuspended in 10mM sodium phosphate, 250mM sucrose, 5mM L-glutamine and 150 mM sodium chloride buffer (SPGS). Cell line and bacterial stocks were tested for *Mycoplasma* contamination by PCR prior to use and were found to be negative.

Study population and patient classification

After obtaining informed written consent, patients attending the gynecology outpatient department, Safdarjung Hospital, New Delhi, India, were enrolled to study the expression of TLR2, TLR4 and iNOS on cervical mononuclear cells. Control women were enrolled for obtaining healthy cervical cells from which $CD14^+$ monocytes were isolated as described in **Chapter 4**. The inclusion and exclusion criteria for the patients are as described in **Chapter 4.**

Determination of Multiplicity of infection by cytotoxicity assay

Multiplicity of infection for chlamydial EBs to be used in various experiments so as the concentration is not toxic to the monocytes/macrophages was determined by CytoTox 96 Non-radioactive cytotoxicity assay kit (Promega, USA) as per the manufacturer's instructions. Briefly, in a 96 well plate effector cells (EBs) and target cells (monocytes/macrophages) were placed and the plate was centrifuged at 250xg for 5 minutes. The plate was then incubated at 37^0C for 4 hrs. Forty five minutes prior to supernatant harvest lysis solution is added to all wells. The plate is further incubated at 37^0C for 1 hr. 50μl of supernatant was transferred to each well of a flat bottom 96 well plate. To each well 50μl of substrate mix was added and the plate was incubated in dark for 30 minutes. To each well 50μl of stop solution is added and the absorbance is read at 490nm.

Real Time PCR for TLR and iNOS gene expression

THP-1 cells and cervical monocytes were seeded in 24-well plates at a density of 2.5×10^5 cells/well. THP-1 macrophages and cervical monocytes were infected with live, heat inactivated and UV-inactivated chlamydial EBs for 4h, 8h, 12h, 18h, 24 hrs and 48hrs with mock-infected cells taken as control. After exposure, total RNA was extracted from the cells using the RNAeasy Mini Kit (Qiagen, Valencia, USA) according to manufacturer's instructions. cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, USA). SYBR Green reactions were conducted with the Dynamo SYBR Green mix (Finnzymes, Espoo, Finland). Cycling conditions were as follows: initial denaturation at 94° for 2 min, 35 cycles each of denaturation at 94° for 30 s, annealing for 1 min, extension at 68° for 1 min with final extension at 68° for 7 min. Reactions were run on using the Real-Time PCR Detection System (Eppendorf). The relative quantities of the gene

napthylethylenediamine dihydrochloride, 2·5% phosphoric acid) at room temperature for 10 min Absorbance was read at 540 nm. The concentration of nitrite (NO_2) was determined by using sodium nitrite as standard. Nitrite release was reported as μM/l × 10^6 cells per well. Cell-free medium was used as blank for the assay.

Quantification of cytokines by ELISA

Quantification of cytokines (IL-1β, IL-6, IL-12, and IFN-γ) in culture supernatants was done by using commercially available ELISA kits (eBiosciennces, San Diego, USA), in accordance with manufacturer's instructions and as described in **Chapter 4**.

Statistical analysis

The data was presented as mean plus minus standard deviation of three individual experiments. Statistical analysis was determined using two tailed student t-test and a P value less than 0.5 was considered significant.

Results

TLR and iNOS gene expression on cervical mononuclear cells

RNA was isolated from endocervical cells of *Chlamydia* positive asymptomatic women, women with MPC and women with FD and gene expression of TLR2, TLR4 and iNOS was studied to assess the role of these molecules in pathology of chlamydial infection. The fold increase in mRNA expression is shown in Figure 6.1 as compared to gene expression levels in cells obtained from control women. TLR2 expression was found to be significantly ($P<0.05$) higher in women with fertility disorders as compared to the other two groups. In comparison to it expression of TLR4 was significantly higher in women with MPC and that of iNOS in asymptomatic women compared to the other groups.

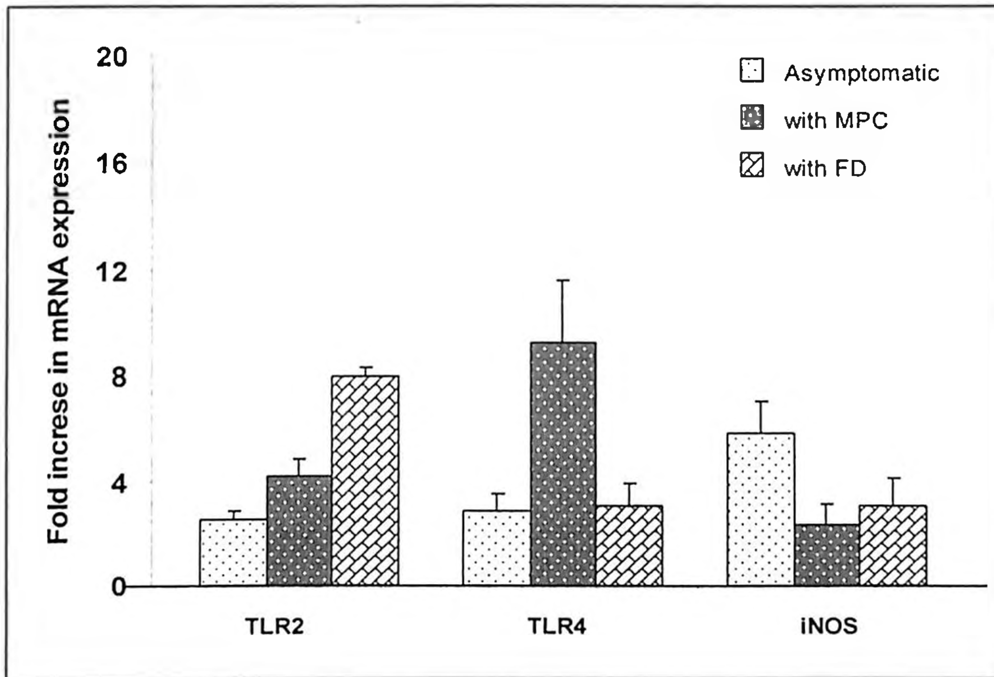


Figure 6.1. mRNA expression of TLR2, TLR4 and iNOS gene in endocervical monocuclear cells obtained from *Chlamydia* positive women.

Determination of Multiplicity of infection (MOI)

Multiplicity of infection to be used for all experiments was determined by cytotoxicity assay as number of EBs/number of monocytes giving minimum cytotoxic response and maximum induction. It was observed that an MOI of 2 or more was highly toxic to monocytes leading to death of more than 40% of infected cells (Figure 6.2). An MOI was 10 or more was completely lethal to cells with ~99% of cells dying at that concentration of EBs. However, an MOI of 0.1 to 1 did not have any severe effect on the viability of cervical monocytes. To further assess the best MOI to be used, cervical monocytes were stimulated with an MOI of 0.25 to 2 and TLR gene expression was studied (Figure 6.3). It was found that an MOI of 1 gives maximum increase in expression of TLRs on cervical monocytes. Increasing the MOI reduces TLR gene expression. Therefore, for all further experiments an MOI of 1 was used.

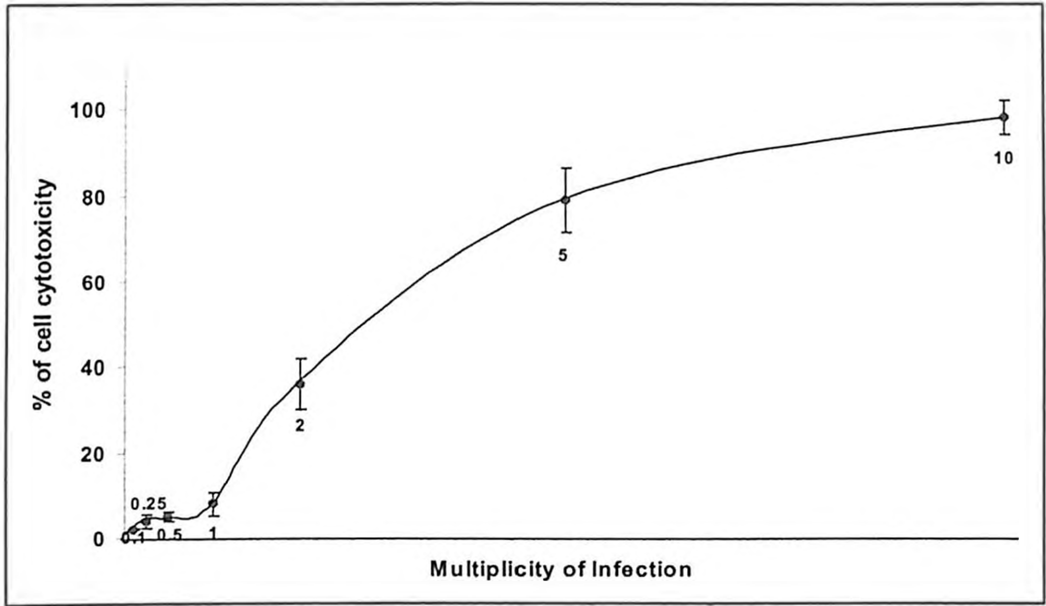


Figure 6.2. Determination of Multiplicity of Infection (MOI). Figures below the error bars are MOI used for induction. All data are expressed as mean of three individual experiments

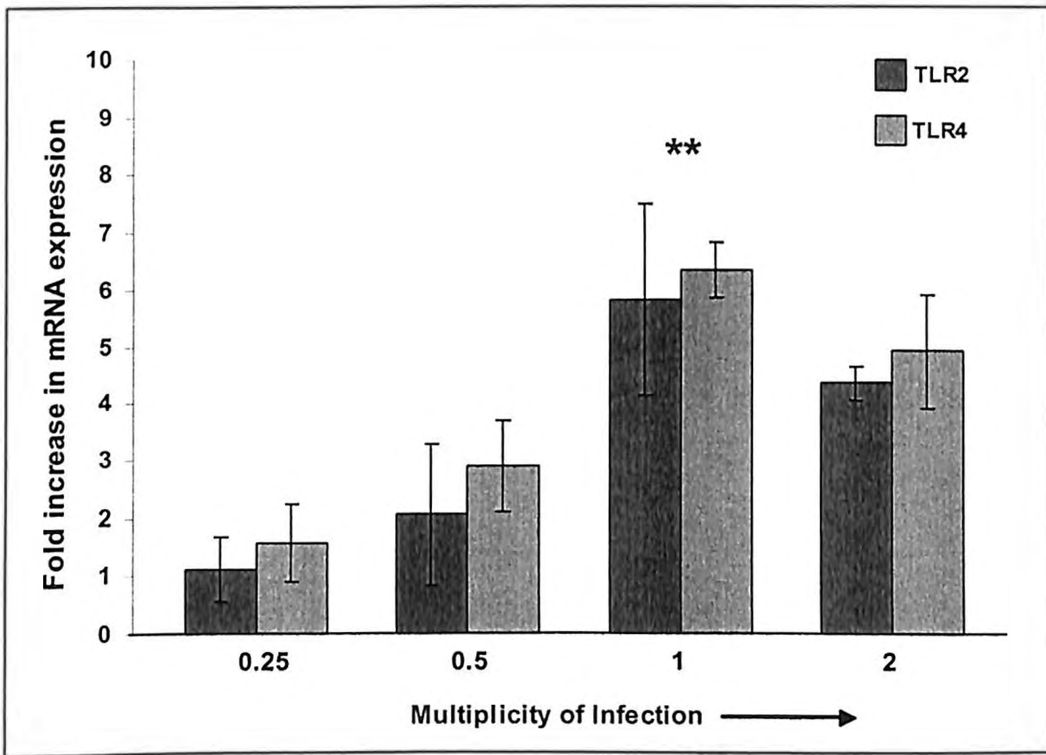


Figure 6.3. Effect of different MOI on TLR2 and TLR4 gene expression.

Expression of TLRs and iNOS in cervical monocytes and THP-1 cells

Expression of TLR and iNOS genes were studied at different time points post infection and the results are as shown in Figure 6.4 and 6.5 respectively. Expression of TLR 4 in cervical monocytes was significantly higher than TLR 2 just after infection. Maximum expression of both TLR2 and TLR4 was observed at 8 hrs post-infection. Expression of iNOS gene was found to be significantly high after 12 hrs post infection and addition of iNOS inhibitor L-NMMA to the medium after infection significantly reduced the expression of iNOS after 8 hrs post infection (Figure 6.5).

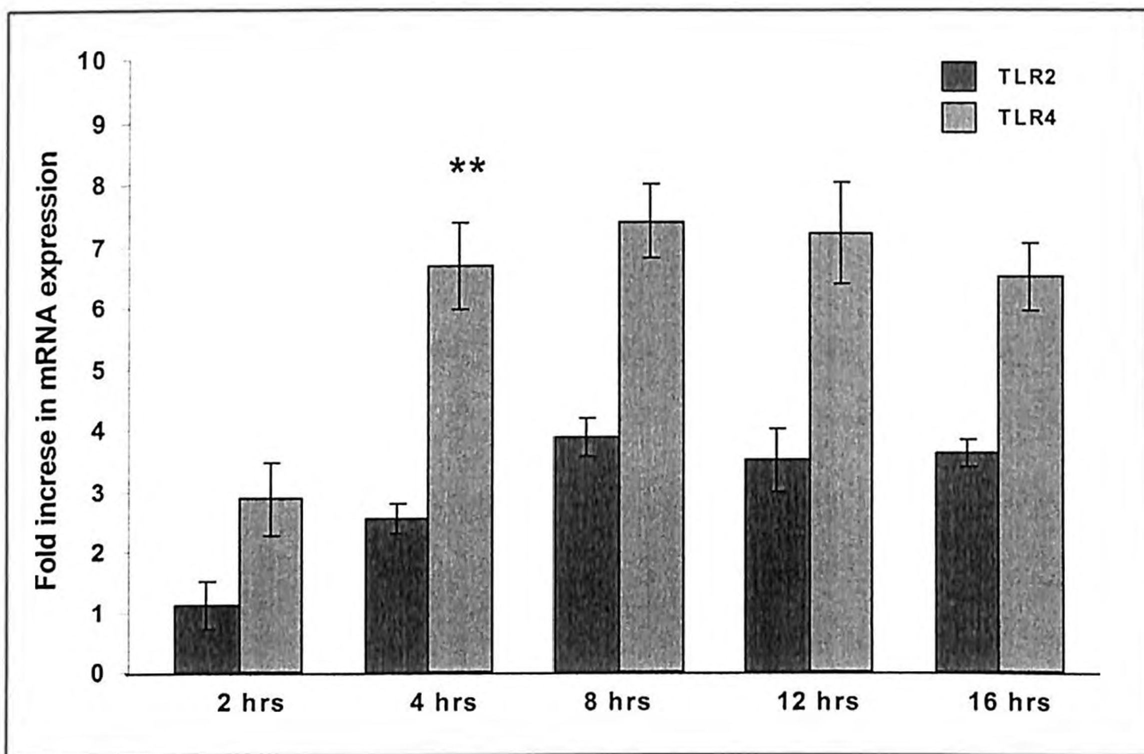


Figure 6.4. Expression of TLR2 and TLR4 at different time intervals post infection.

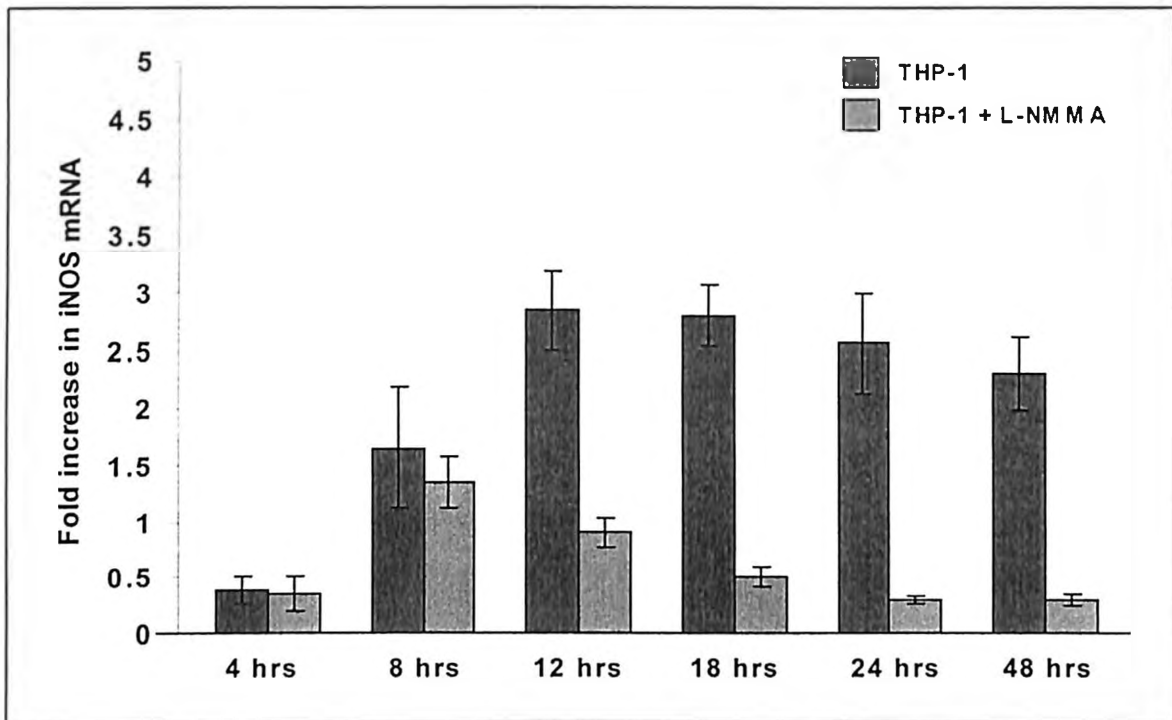


Figure 6.5. Expression of iNOS gene at different time intervals post infection. Addition of iNOS inhibitor L-NMMA significantly reduced iNOS expression

Nitric oxide production by THP-1 macrophages

Nitric oxide production by THP-1 macrophages upon EB induction was studied at 24 and 48 hrs post-infection. Level of nitric oxide produced by infected cells was significantly higher at 48 hrs post-infection as compared to 24 hrs (Figure 6.6). Addition of iNOS inhibitor L-NMMA to cells after induction significantly reduced nitric oxide production.

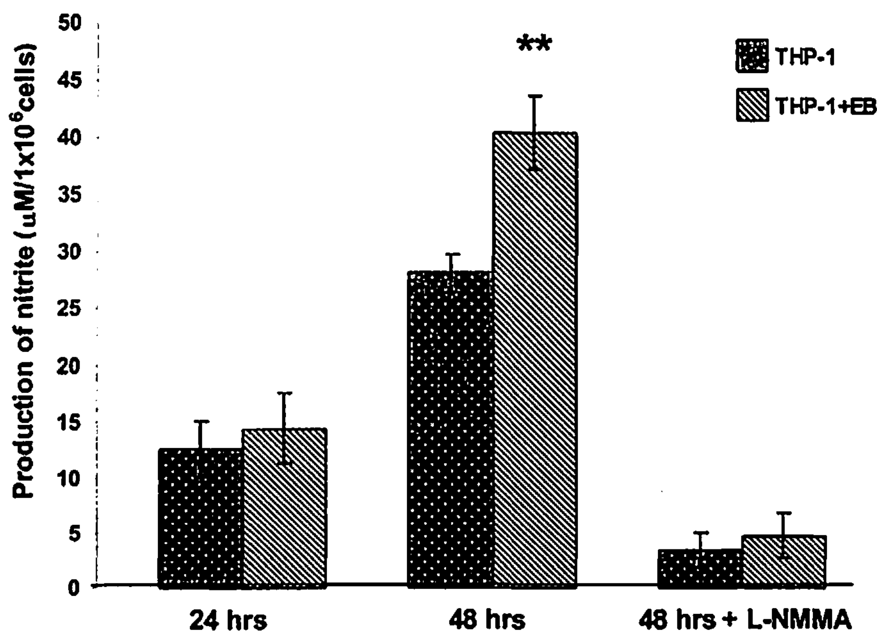


Figure 6.6. Nitric oxide production by THP-1 macrophages upon infection with chlamydial EBs.

Effect of Toll like receptor blocking on cytokine secretion by cervical monocytes

Cytokine levels were quantified in culture supernatants of induced cervical monocytes at 48 hrs post infection. Significantly higher levels of IL-1β, IL-6, IL-12 and IFN-γ were observed upon stimulation of cervical monocytes as compared to mock infected controls (Figure 6.7). Upon blockage of TLR2 significant reduction in level of secreted IL-1β, IL-6 and IFN-γ was observed. In contrast, TLR4 blockage significantly reduced secretion of all cytokines by cervical monocytes. Blockage of both TLR2 and 4 completely inhibited secretion of all cytokines.

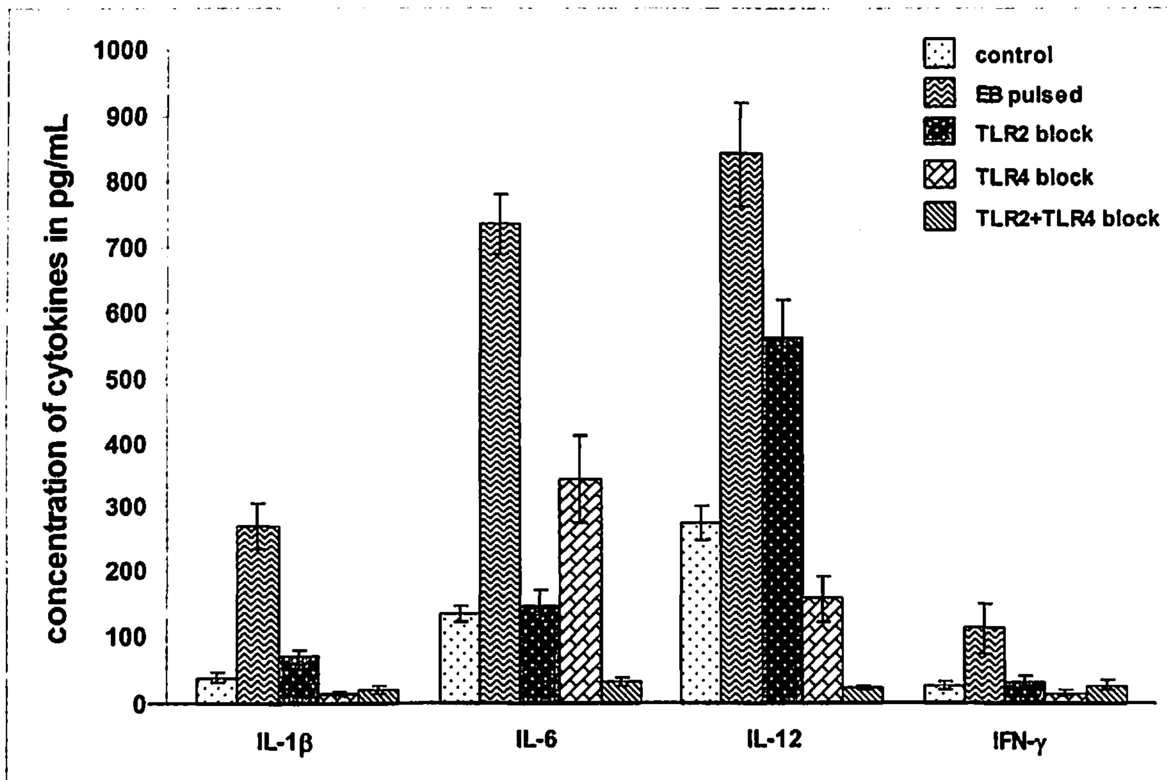


Figure 6.7. Cytokine secretion pattern of cervical monocytes pulsed with live chlamydial EBs.

Toll like receptor signalling

To study Toll like receptor signalling in cervical monocytes, RNA was isolated from infected cells 2 hrs post infection and the human TLR signaling pathway RT^2-PCR-Profiler PCR Array (SA Biosciences) was run using Real Time PCR. The array consists of a panel of 84 genes involved in TLR signalling pathway. Chlamydial infection significantly upregulates CD86, TLR4, MyD88, IRAK2, NF-κB, IL-1β and IL-12 genes, however, significant reduction in expression level of TLR6, TLR9 and MAPK8 genes was observed. As infection with chlamydial EBs significantly upregulates TLR4 and blocking of TLR4 caused significant reduction of secreted IL-12, TLR4 signalling pathway was studied. It was

observed that inhibition of TLR4 adapter molecule MyD88 decreases IL-12 secretion but not significantly, thus showing the presence of a MyD88 independent mechanism (Figure 6.8). Inhibition of other TLR4 adapter molecule TRIF also decreased IL-12 secretion, but not significantly. Inhibition of both MyD88 and TRIF significantly decreased IL-12 production thus confirming the presence of both MyD88 and TRIF dependent recognition of *Chlamydia* by TLR4.

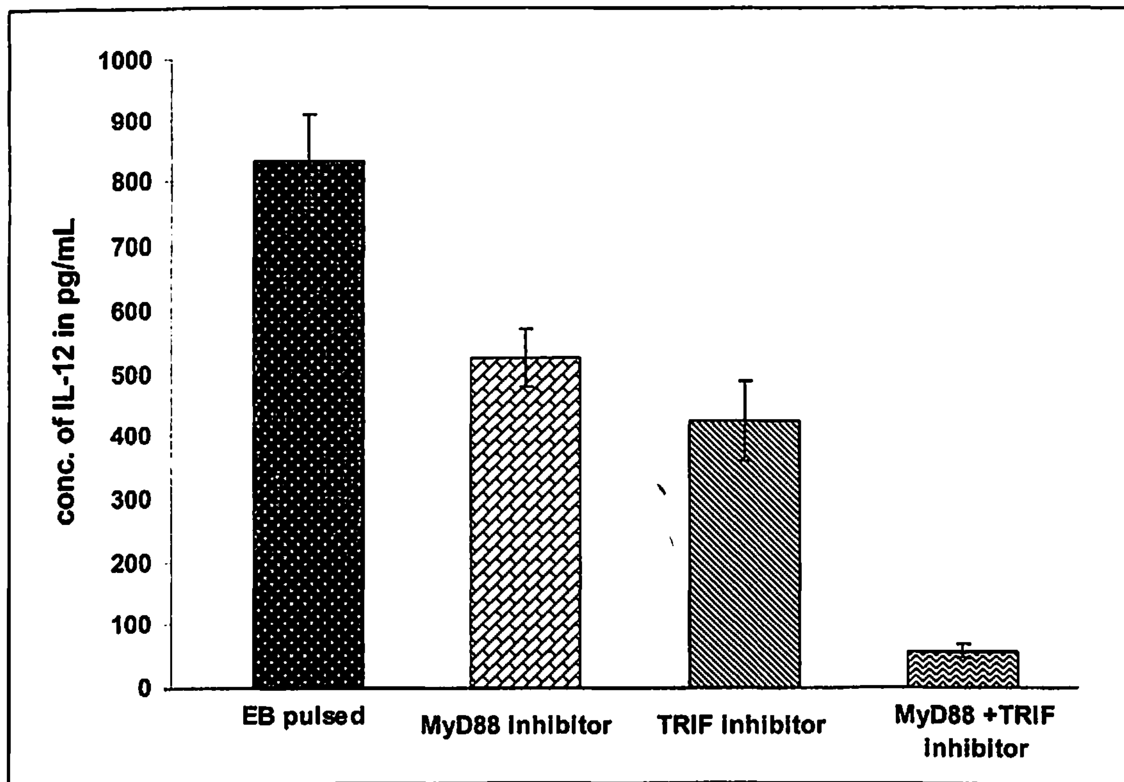


Figure 6.8. Effect of TLR4 adapter molecule inhibition on secretion of IL-12 from cervical monocytes.

Discussion

Resolution of a primary chlamydial genital tract infection in the host ultimately relies on a Th1 cell-mediated immune response [Cain & Rank, 1995; Ramsey & Rank, 1991; Rank *et al.*, 1992; Perry *et al.*, 1997; Ramsey & Rank, 1990; Igietsme *et al.*, 1993; Su & Caldwell, 1995]. This adaptive immunity in turn is initiated by the innate immune system. It is likely that *C. trachomatis* initially invades genital tract epithelial cells as well as resident tissue macrophages and dendritic cells. Infected cells respond by releasing inflammatory mediators and chemokines that induce an influx of NK cells and neutrophils into the area, activate nearby phagocytes and APCs, and ultimately dictate the ensuing acquired response. This is the first study wherein role of TLRs in cervical monocytes has been demonstrated. We demonstrated the presence of both TLR2 and TLR4 in the cervical monocytes of noninfected and infected females in our initial experiments, which allowed us to proceed with an examination of their relative importance, if any, in chlamydial infection. We observed that TLR2 expression was significantly higher in women with fertility disorders while expression of TLR4 was significantly higher in women with MPC. A study by Darville *et al.*, 2003 has also shown that TLR2 has an important role in production of inflammatory cytokines in response to active chlamydial infection and TLR2 knockout mice have less RNA for IL-1 and IL-6. In infection with *C. pneumoniae* has also been shown that early immune responses partially depend on TLR2 and TLR2 deficient mice attracted fewer polymorphonuclear neutrophils into the lung [Rodriguez *et al.*, 2006]. In *Chlamydia* infected women iNOS expression was significantly higher in asymptomatic women as compared to the other groups. This data suggests that while TLRs are involved in initiating inflammatory responses

iNOS induced production of nitric oxide helps in clearance of *Chlamydia* leading to non-inflammatory conditions and thereby, no symptoms.

When cervical monocytes from control uninfected women were pulsed with chlamydial EBs significant expression of TLR4 as compared to TLR2 was observed just after infection. This suggests that initial recognition of *Chlamydia* by monocytic cells is through TLR4 and at a later stage by TLR2. iNOS expression in infected THP-1 macrophages show that significant expression of iNOS and production of nitric oxide takes place at a later stage of infection and thus the role of reactive nitrogen species can be assigned as to kill the growing bacterium inside the host cell rather than recruiting outside mediators.

TLRs are key in translating microbial recognition into expression of specific subsets of chemokines and cytokines from macrophages and dendritic cells [Sielin & Modlin, 2002; Prebeck *et al.*, 2001; Brightbill *et al.*, 1999; Sielin & Modlin, 2001; Kaisho & Akira, 2003]. Furthermore, TLR2 and TLR4 have been shown to induce different sets of chemokines and cytokines, implying that the innate immune response can be customized for different pathogens [Dabbagh & Lewis, 2003; Dabbagh *et al.*, 2002; Qi *et al.*, 2003; Edwards *et al.*, 2002]. It is therefore vital to elucidate the role of these two receptors in the expression of inflammatory mediators by infected cells in response to *C. trachomatis*. The results of the *in vitro* experiments wherein TLR2 and 4 were blocked with blocking antibodies demonstrated an important contribution of TLR2 in macrophage production of IL-6 and IFN-γ. In case of blockage of this receptor, cervical monocytes showed a statistically significant reduction in expression of these cytokines. However, despite significant decreases, inhibition was not complete. This finding underscores an important redundancy in the TLR system whereby inflammatory mediators can still be produced, albeit in smaller amounts, by cells lacking

only one of these two receptors. We further found that a significant dependence on TLR4 for production of IL-12 in response to infection with *Chlamydia*. As IL-12 secretion by antigen presenting cells pave way for a Th1 immune response, TLR4 can be considered indispensable for initial immune response to *Chlamydia trachomatis*. It has been shown by Prebeck *et al.*, 2001 that secretion of IL-12 is TLR4 dependent and in absence of a functional TLR4 IL-12 secretion is attenuated.

We further observed that inhibition of MyD88 reduces IL-12 production, but not significantly suggesting that both MyD88 dependent and independent mechanism operate. Downstream signalling by TLR4 is mediated by both MyD88 and TRIF. Inhibition of TRIF also reduced secretion of IL-12 and combined inhibition of both these molecules completely blocks IL-12 secretion.

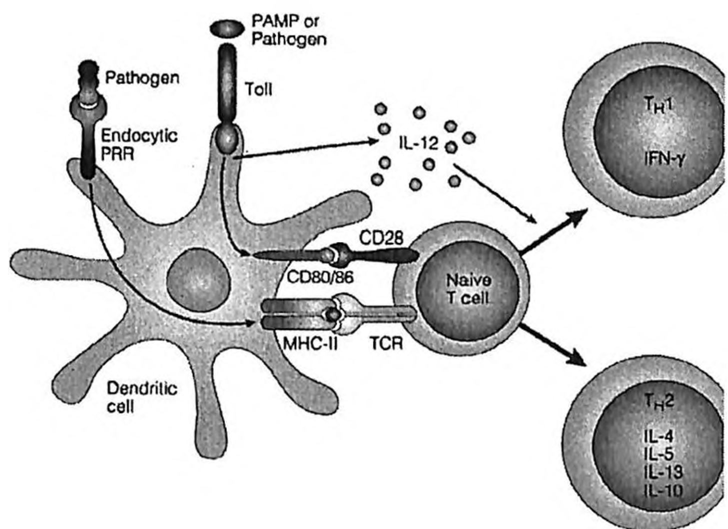
In conclusion, this study revealed important information regarding the interaction of *C. trachomatis* with TLRs. *Chlamydia* stimulates innate immune cells by both activation of TLR2 and TLR4. TLR2 plays a significant role as a pattern recognition receptor for and is involved in early secretion of pro-inflammatory mediators. Non significant reduction in secretion of key cytokines by MyD88 and TRIF inhibition, point to the redundancy of the immune response, and the number of collateral pathways available for innate responses to infections. Importantly, the in vitro data indicate a protective role for TLR4 while a inflammatory and thus pathogenic role of TLR2.

Chapter 7
In vitro presentation of chlamydial elementary bodies to monocyte derived dendritic cells to autologous CD4$^+$ T cells

by

A theory can be proved by experiment; but no path leads from experiment to the birth of a theory

Manfred Eigen



In vitro presentation of chlamydial antigens by monocyte derived dendritic cells to autologous CD4⁺ T cells

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Introduction

Chlamydia trachomatis is an obligate intracellular bacterium with a unique biphasic life cycle, causing various human diseases. *Chlamydia* induced sexually transmitted diseases in women include chronic pelvic pain, ectopic pregnancy and pelvic inflammatory disease, which often results in infertility. Consistent with the intracellular localization of *Chlamydia*, cell-mediated immune responses against it are primarily mediated by T-cells which play a major role in clearance and resolution of infection. It is well-recognized that $CD4^+$ T cells dominate the lymphocytic infiltrate [Kelly & Rank, 1997; Morrison *et al.*, 2000] during chlamydial infection. Furthermore, $CD4^+$ T helper type 1 (Th1) cells are necessary for eradication of *Chlamydia* from the genital mucosa.

Dendritic cells are the most efficient antigen presenting cells (APCs), especially in priming naïve T cells [Banchereau & Steinmann, 1998] and consequently, playing a central role in the induction of T-cell and B-cell immunity *in vivo* [Steinmann, 1999]. DCs also contributes to the innate resistance against microbial pathogens [Mellman & Steinmann, 2001]. DCs are found throughout the body as immature precursors and after microbial encounter, DC loses their phagocytic and endocytic ability, migrate to the draining lymph nodes, and increase their capacity for antigen processing and presentation [Bhardwaj, 2001]. Their ability to prime $CD4^+$ T cells, to modulate the type of T-cell responses (Th1/Th2), and to contribute to the inflammatory response largely depends on the up-regulation of costimulatory and adhesion molecules and on the secretion of inflammatory cytokines [Mellman & Steinmann, 2001; Sallusto & Lanzavecchia, 1999; Pulendran *et al.*, 2001]

As generation of large quantities of relatively pure DCs became possible [Inaba *et al.*, 1992], DCs have been successfully used to induce antigen-specific immune responses and protective immunity against various cancers and infectious diseases [Ludewig *et al.*, 1998; Ossevoort *et al.*, 1995; Schuler *et al.*, 1997], including chlamydial infections [Su *et al.*, 1998]. In many of these studies, DCs were pulsed ex vivo with either peptide, or whole-protein antigens, and are delivered *in vivo* to syngeneic hosts. DCs not only possess the ability to prime an antigen-specific response but also are able to direct a protective immune response against infections. It has been shown that for several intracellular pathogens including *Chlamydia* [Morrison & Caldwell, 2002], protective immunity requires the induction of Th1 cytokines, notably IFN-γ, TNF-α, and IL-2; however, pathology develops if Th2 cytokines (i.e., IL-4, IL-5, and IL-10) are induced [Fresno *et al.*, 1997; Abbas *et al.*, 1991]. The pattern and interaction of Th1 and Th2 cytokines elicited could therefore affect the outcome of an infection. Furthermore, since several pathogens are preferentially susceptible to either Th1 or Th2 response, the identification of immunobiologic factors that selectively regulate Th1 or Th2 response against a given pathogen could aid in skewing protective immune responses to the desired route as part of vaccine design strategy.

Many current chlamydial vaccine efforts centre on the interaction between *Chlamydia* and dendritic cells, to produce specific effector and memory responses. The current immunological paradigm maintains that antigen presentation by immature (non-activated) DCs leads to tolerance, whereas mature, antigen-loaded DCs promote the development of antigen-specific protective immunity [Dubsky *et al.*, 2005]. Thus, factors that influence the maturation of DCs and their ability to generate a protective Th1 immune response against

the interaction between DC and *Chlamydia* spp. may contribute to the understanding of the role DC play either in control of chlamydial infection or in *Chlamydia*-induced pathology.

Materials

Unless otherwise noted, all fine chemicals used in the study were purchased from Sigma Aldrich (St. Louis, USA). Plasticware and glassware used in cell culture and immune assay was purchased from Greiner, Germany.

Experimental Methods

Cell line

HeLa 229 (a human cervical epithelial cell line), was procured from National Centre for Cell Sciences (NCCS), Pune, India. HeLa cells were grown at 37°C under 5% CO_2 in minimal essential medium (MEM) with 2 nmol per liter glutamine, supplemented with 10% fetal bovine serum (FBS) and 10 μg of gentamicin/ml. For growing *Chlamydia* stocks, McCoy cell line (NCCS, Pune, India), were propagated at 37°C in MEM containing Hanks' salts and glutamine and supplemented with 10% FBS. Cell lines were allowed to reach 80% confluency after which they were split following trypsinisation.

***Chlamydia* culture**

Chlamydia culture was done as described in Chapter 6.

Culture of human Monocyte Derived Dendritic Cells (MDDCs)

Peripheral blood mononuclear cells (PBMCs) were prepared by ficoll-Hypaque density gradient centrifugation and were washed three times with RPMI 1640 medium. PBMCs were then stained with anti-CD14 antibodies labeled with magnetic microbeads and were then separated from other leukocytes using a magnetic cell sorter (Miltenyi Biotec, Germany). The $CD14^+$ cells were inoculated (1-2 x 10^6/ml) into each well of a six well plate and were

cultured in RPMI 1640 supplemented with 10% heat inactivated Fetal Calf Serum (FCS), 2mM L-glutamine, 25mM HEPES, 0.02M 2-mercaptoethanol, 10μg/ml Gentamycin and 2μg/ml Amphotericin B at 37^0C in a 5% CO_2 incubator, in the presence of 50 ng/ml Granulocyte Macrophage Colony Stimulating factor (GM-CSF) and 20 ng/ml IL-4 for 6-7 days. GM-CSF and IL-4 were supplemented every 2 days. After 6-7 days immature MDDC's were washed and analyzed for CD14 and CD1a expression (marker for immature DC's) by Flow cytometry and Confocal Laser Scanning Microscopy.

Flow Cytometry

Quantification of immature MDDCS was performed by standard flow cytometry technology. Immature MDDCs obtained were stained with Phycoerythrin (PE) conjugated anti-CD14, and –CD1a antibodies, and FITC conjugated anti-HLA-DR (Becton Dickinson, San Jose, USA) for 25 min on ice. Appropriate isotype-matched control antibodies were also used to rule out nonspecific fluorescence. Preparations were then washed with stain buffer (PBS supplemented with 0.1% NaN_3 and 2% Fetal Bovine Serum) and acquired using Fluorescence-activated cell sorter (FACS) Caliber (BD Biosciences, San Jose, USA). A total of 10,000 events were acquired and analysis was done using CELLQUEST software (Becton Dickinson).

Pulsing of immature MDDCs with chlamydial EBs and estradiol treatment

After 5 to 6 days of culture, 10^5 cells/mL immature MDDCs in 24 well cell culture plates were infected with live chlamydial EBs at an MOI of 2: 1 (DC: EB) for 6 hrs. In parallel experiments DCs were pretreated with estradiol (Sigma) at concentrations of 10μg/mL, 1μg/mL and 0.1μg/ml for 24 hrs and were then pulsed with chlamydial EBs. *E. coli* LPS was used as positive control in all the experiments.

PCR array analysis for TLR signalling pathway:

Dendritic cells were seeded in 6-well plates at a density of 2.5 X 10^5 cells/well. DCs were infected with chlamydial EBs as described earlier for 12 h and mock-infected cells were taken as control. After exposure, total RNA was extracted from the cells and TLR signalling pathway genes are studied as described in Chapter 6.

Western blot analysis

Dendritic cells were grown and infected with chlamydial EBs, lysed with 1X lysis buffer (50nM Tris-Hcl, 100 mM DTT, 2% SDS, 10% glycerol) and protein concentration were determined using Bradford reagent (Sigma-Aldrich, USA) as described in Chapter 4. Twenty micrograms of lysate protein per lane was boiled and separated by SDS-PAGE under reducing conditions and blotted on to the nitrocellulose membranes. Membranes were then incubated overnight at 4^0C with primary antibodies specific for phosphorylated or total signaling pathway members (IκBα monoclonal antibody and nF-κB antibody) along with and β-actin antibody (internal control) (Cell Signalling, Danvers, USA). Membranes were then incubated with 0.2μg/ml peroxidase-conjugated anti-rabbit immunoglobulin G (Cell Signalling, California, USA) for 2 h at room temperature and were then visualized on X-ray film using enhanced chemiluminescence reagents (Cell Signalling, Danvers, USA).

Coculture of Dendritic cells with autologous CD4 T lymphocytes

Immature MDDCs were infected with live *C. trachomatis* EB's and cultured in RPMI 1640 for 24 hrs at 37^0C. Autologous $CD4^+$ T cells were separated from PBMCs using a magnetic cell sorter and were then cocultured with the EB pulsed dendritic cells for further 4 days at 37^0C. Proliferation of CD4 T cells was studied by Cell proliferation kit (Promega) in accordance with the manufacturer's instructions. The culture supernatants were harvested

and analyzed for production of various cytokines. In parallel experiments culture of dendritic cells with autologous T cells was performed in presence of estradiol.

Quantification of cytokines by ELISA

Quantification of cytokines (IL-6, IL-12, IL-4, IL-10, TNF-α and IFN-γ) in culture supernatants was done by using commercially available ELISA kits (eBiosciennces, San Diego, USA), as described in **Chapter 4**.

Statistical analysis

The data was presented as mean plus minus standard deviation of three individual experiments. Statistical analysis was determined using two tailed student t-test and a P value less than 0.5 was considered significant.

Results

Culture of Monocyte Derived Dendritic Cells

After 5-7 days of culture in recombinant GM-CSF and IL-4, monocytes transform into immature dendritic cells as confirmed by flow cytometry (Fig 7.1) and Confocal laser scanning microscopy (Fig 7.2). Immature DCs were identified and differentiated from monocytes by analysis of cell surface markers. Flow cytometry results show that at day 0 the percentage of monocytes ($CD14^+$ cells) in cell population was 92.7 % compared to percentage of immature DCs ($CD1a^+$ cells) which was 1.3%. On day 7, the percentage of immature DCs was 77.4% compared to 2.9% of monocytes.

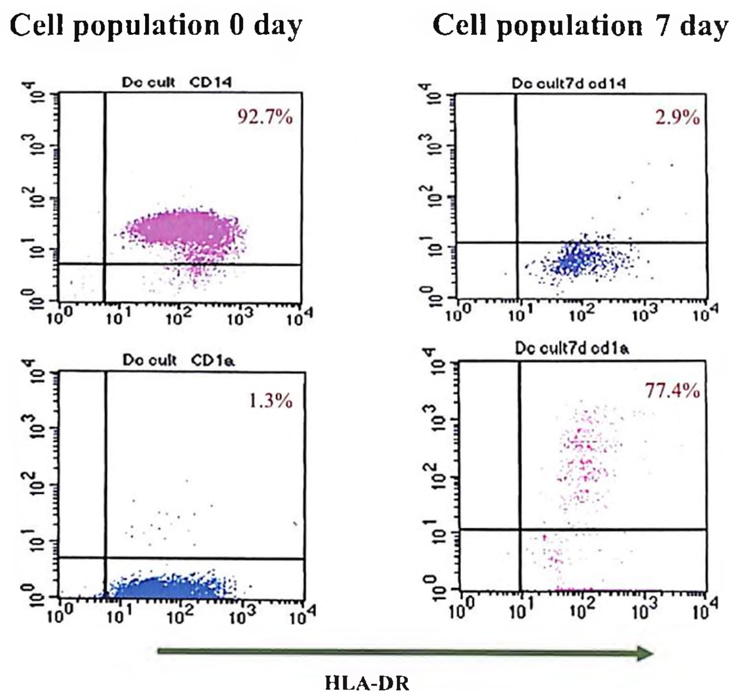


Figure 7.1 Flow cytometric analysis for purity of cell population at 0 day and 7 day of culture. After 7 day culture $CD14^+$ monocytes lose the expression of this marker and converts to $CD1a^+$ immature DCs .

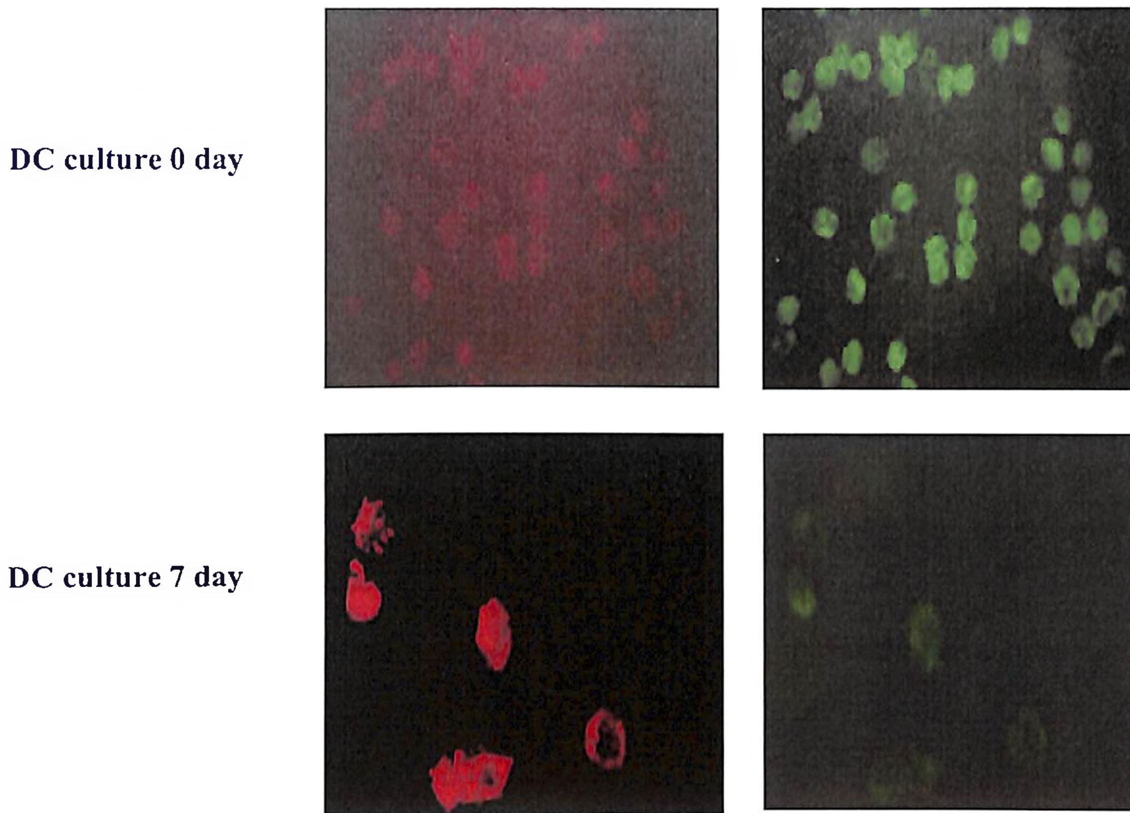


Figure 7.2 Confocal Laser Scanning micrographs of monocytes stained with FITC conjugated CD14 MoAb (green) and PE conjugated CD1a MoAB (red)

Expression of costimulatory molecules

For analysis of maturation of immature MDDCS upon pulsing with chlamydial EBs flow cytometry was done. Flow cytometric analysis of EB pulsed immature dendritic cells revealed upregulation in expression of MHC-II, CD83 (DC maturation marker) and CD86 (Figure 7.3) compared to unstimulated dendritic cells. However, no significant difference in expression levels of any other costimulatory molecule was observed.

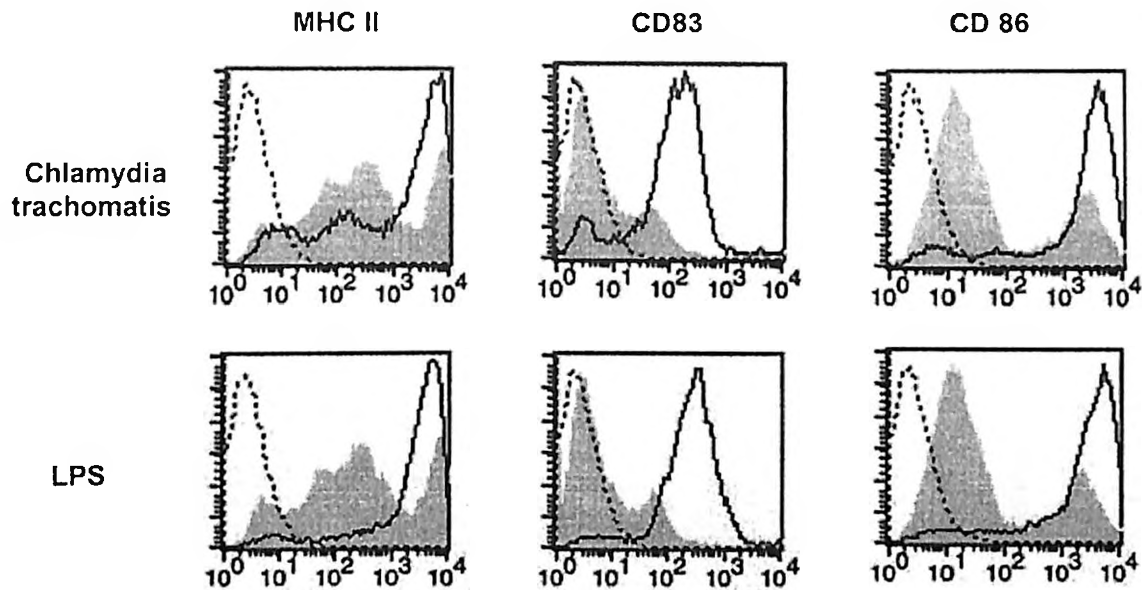


Figure 7.3 Flow cytometric analysis showing expression of co-stimulatory molecules on dendritic cells pulsed with chlamydial EBs and LPS (positive control).

Estradiol downregulates Toll like receptor 4 and associated signalling *in vitro*.

To study the mechanism by which estradiol enhances susceptibility to chlamydial infection, immature dendritic cells were generated and evaluated for purity by flow cytometry after 5 to 6 days. The cells were pretreated for 24 hrs with estradiol and were then pulsed with live chlamydial EBs in presence of estradiol. RNA was extracted after 3 hrs post infection and toll like receptor signalling pathway gene expression was studied by Real Time PCR array. The expression of a number of Toll like receptor signalling genes such as IRAK4, MyD88 and nF-κB were upregulated following chlamydial infection. Gene expression of significantly expressed genes in estradiol treated and untreated conditions are shown in Figure 7.5. Estradiol pretreatment significantly reduced expression of TLR4 as compared to untreated cells. Gene expression of TLR4 associated downstream signalling molecules as IRAK4 and nF-κB was also found to be downregulated. Gene expression of Th1 associated

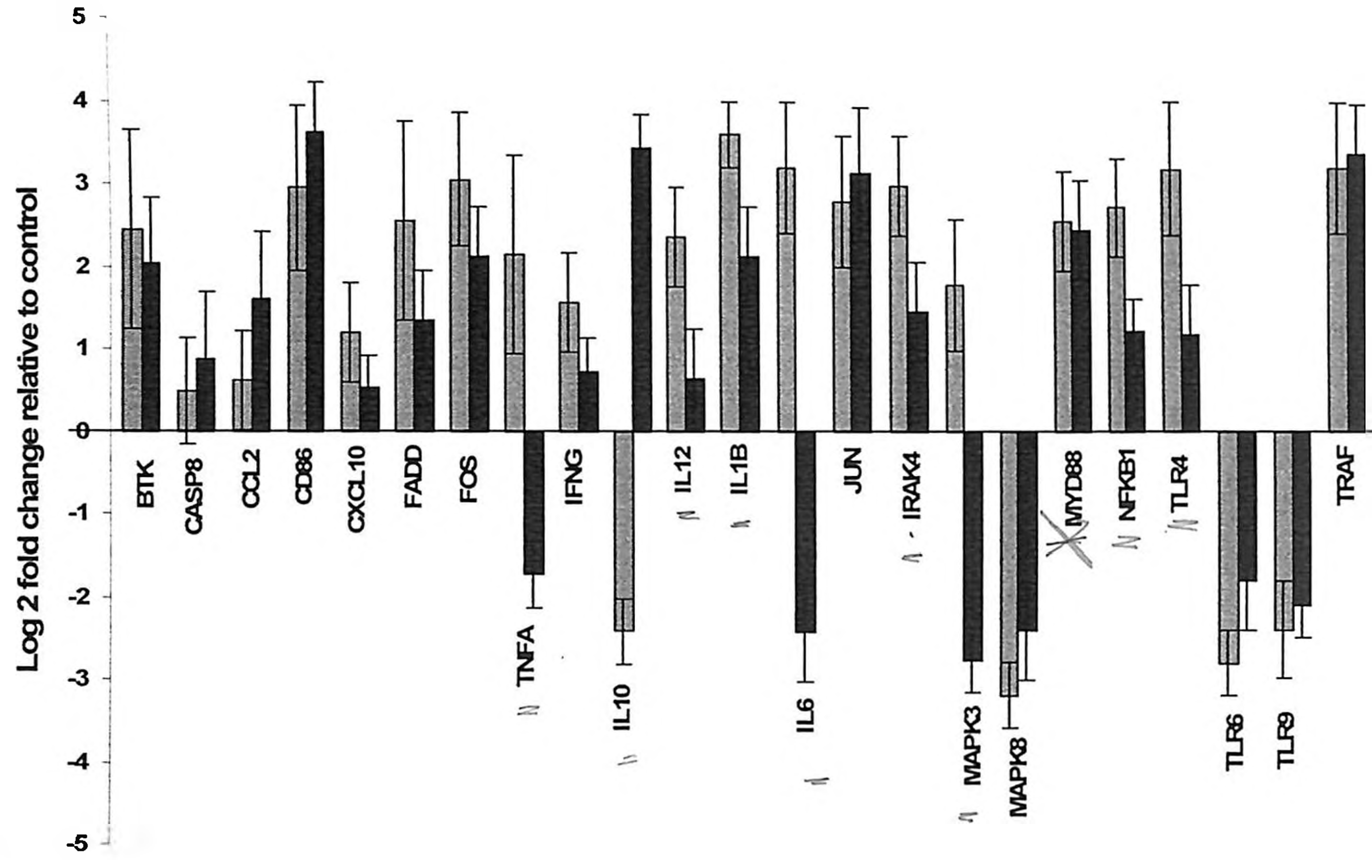


Figure 7.5. Effect of estradiol pretreatment on Toll like receptor signalling pathway. Results are shown as log 2 – transformed means of three experiments. Dark columns indicate gene expression in dendritic cells untreated with estradiol. Light coloured columns show gene expression in estradiol pretreated dendritic cells. Estradiol pretreated cells show significant decrease in gene expression of TLR4.

cytokines IL-12, IL-6, TNF-α and IFN-γ was also reduced, however, expression of IL-10 by dendritic cells increased significantly after estradiol pretreatment.

Protein expression by western blotting

To see degradation of IκBα and nF-κB activation western blot was done (Figure 7.5). Estradiol pretreatment significantly reduced nuclear nF-κB p65 production, thereby, reducing the secretion of proinflammatory cytokines. However, IκBα degradation was reduced during E2 treatment but not significantly. In E2 treated dendritic cells pulsed with *E. coli* LPS, no significant change in IκBα and nF-κB activity was seen compared to untreated cells.

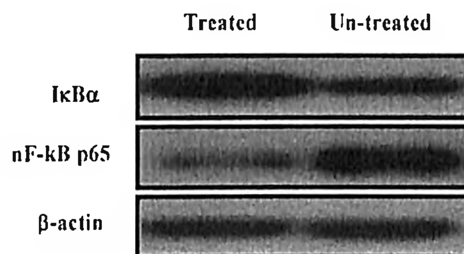


Figure 7.5. Effect of estradiol treatment on IκBα and nf-kB activation. A representative Western Blot of treated and untreated cytosolic and nuclear extracts of dendritic cells showing IκBα and nF-κB.

Secretion of cytokines by dendritic cells upon estradiol pretreatment

To confirm the effect of estradiol pretreatment on TLR signalling on cytokine secretion from infected dendritic cells, supernatants were harvested 24 hrs post-infection and tested for secreted cytokines by ELISA (Figure 7.6). Estradiol treatment significantly reduced the release of proinflammatory cytokines IL-12 and TNF-α at the highest concentration, however, a non-significant decrease in levels of IL-6 was observed. A significant increase in

secretion of IL-10 was observed with highest E2 concentration. IL-4 was below detection limit in all the experiments.

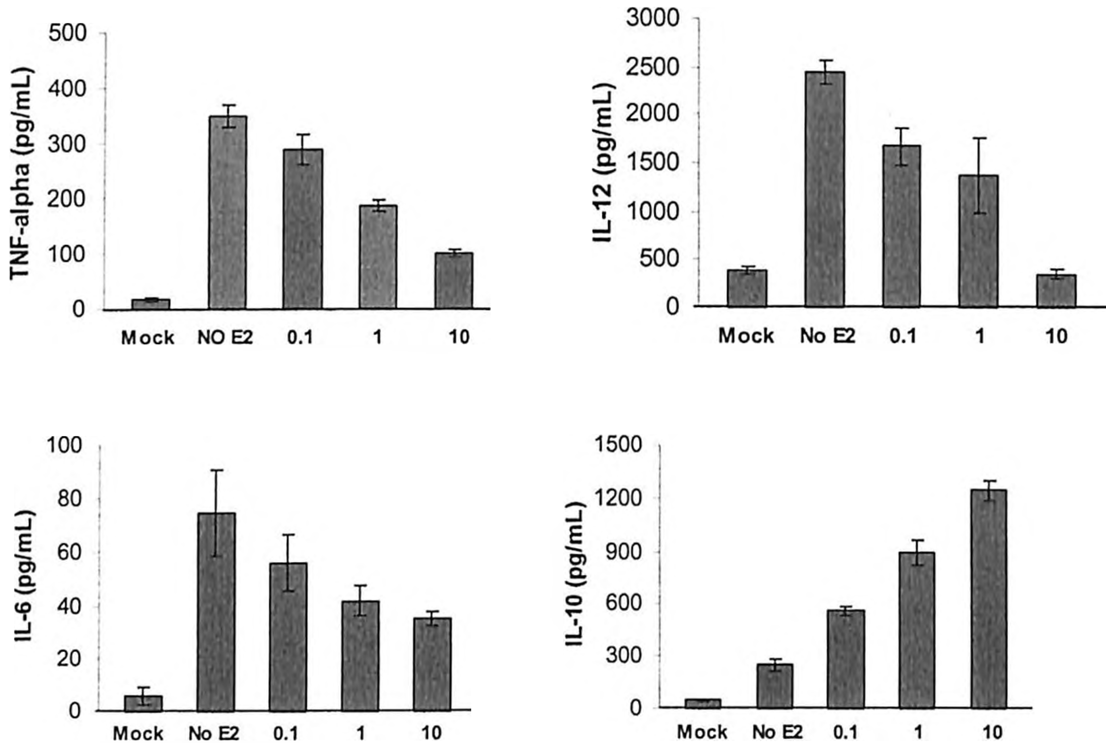


Figure 7.6. Cytokine levels in supernatant of dendritic cells pulsed with *Chlamydia*. Significant reduction in secretion of TNF-α and IL-12 was observed. IL-10 levels increased upon increasing estradiol concentrations. No E2: estradiol untreated dendritic cells

Culture of EB pulsed dendritic cells with autologous T cells

Interaction of DCs with autologous $CD4^+$ T cells was observed in phase contrast microscopy (Fig 7.7). To confirm antigen presentation by dendritic cells to autologous T cells, cell proliferation assay was done to study proliferation of activated T cells. A significant increase in numbers of $CD4^+$ T cells was observed when they were cocultured with antigen pulsed dendritic cells compared to $CD4^+$ T cells cocultured with monocytes which showed

proliferation of $CD4^+$ T cells but to a lower extent. These results show that DC are more potent antigen presenting cells as compared to monocytes (Fig 7.8).

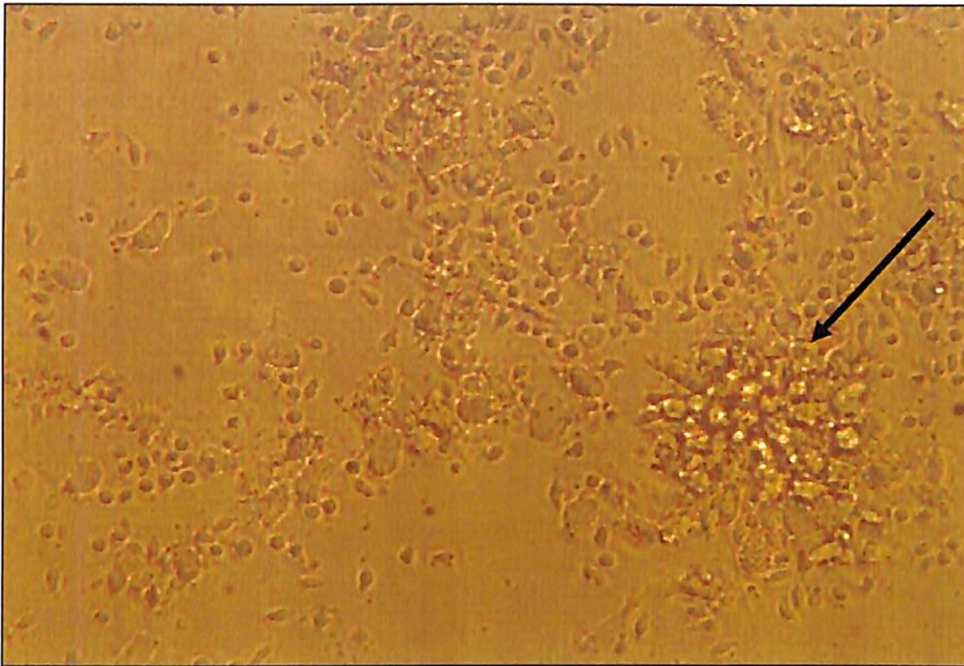


Figure 7.7 Phase contrast micrograph showing interaction of EB pulsed DCs with autologous CD4 T cells.

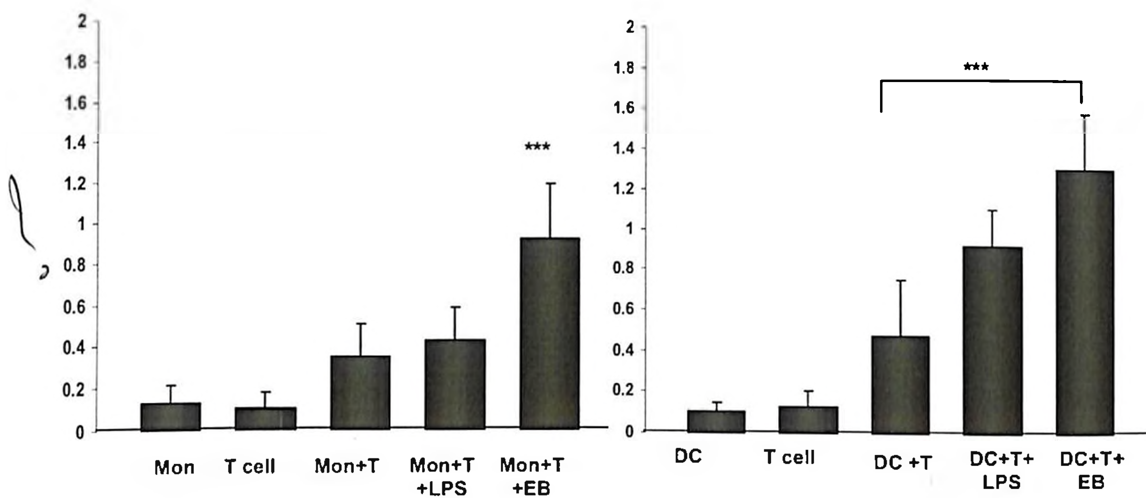
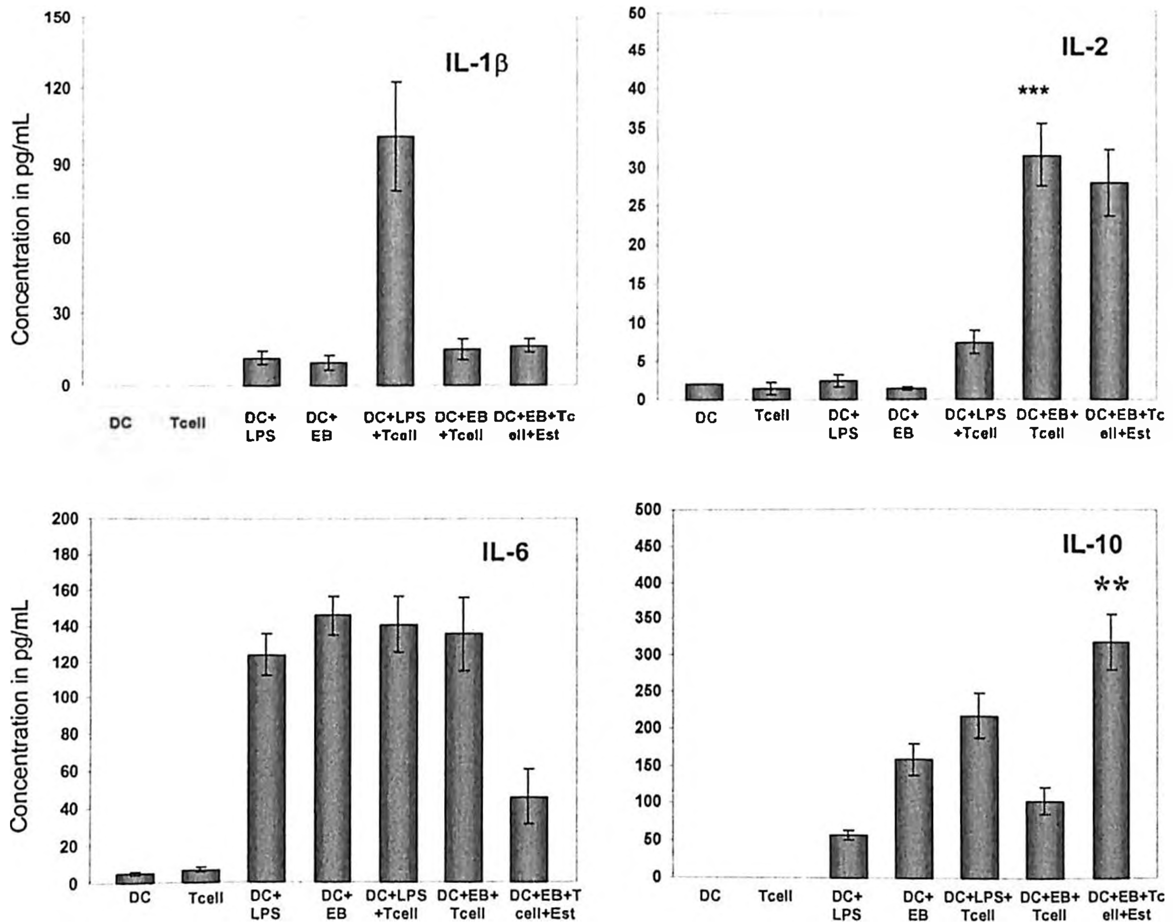


Figure 7.8. Significant proliferation of autologous $CD4^+$ T cells upon interaction with chlamydial EB-pulsed monocytes and dendritic cells.

Cytokine secretion by activated autologous CD4$^+$ T cells and effect of estradiol

The mean levels of IL-1β, IL-2, IL-6, IL-10, IL-12 and IFN-γ in culture supernatants of DC activated autologous CD4$^+$ T cells along with controls are shown in Fig. 7.9. IL-2 and IL-12 levels were significantly higher ($P<0.05$) in supernatants of activated autologous CD4$^+$ T cells compared to other conditions. Pretreatment of dendritic cells with estradiol significantly reduces secretion of IL-6 and IL-12 ($P<0.05$) by CD4 T cells, however, estradiol increases secretion of IL-10 from activated CD4 T cells.



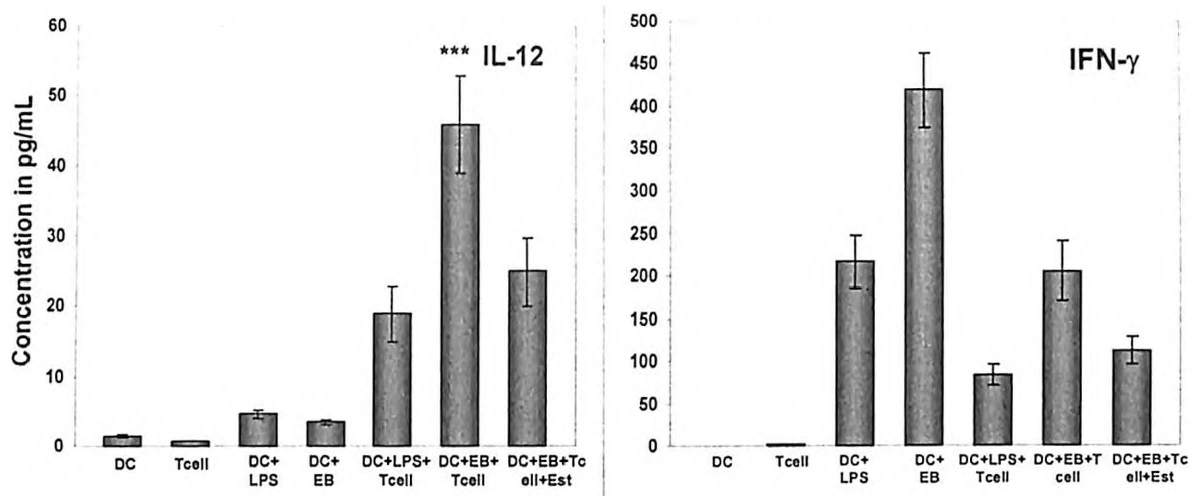


Figure 7.9. Cytokine levels in supernatants of CD4$^+$ T cells activated in presence of dendritic cells pulsed with *Chlamydia*.

Discussion

Dendritic cells are likely to be the first professional antigen presenting cells that encounter *C. trachomatis* following genital and ocular chlamydial infections. DC are important for priming cellular mediated immune responses and innate inflammatory processes and therefore potentially play important roles in both host immunity and chronic inflammation, the hallmark of chlamydial disease. The goal of this study was to examine the interaction between human DC and *C. trachomatis* with respect to DC and CD4$^+$ T cells activation and induction of proinflammatory immune responses along with role of estradiol in modulating dendritic cell function and thus increasing susceptibility of host to *C. trachomatis*.

In the present study, viable chlamydial EB were used to induced the activation of DC, as they would give a real insight into how DCs process and present chlamydial antigens to T cells. In this study it was observed that pulsing of DC with live chlamydial EBs was characterized by the production of inflammatory cytokines IL-6, IL-10 and TNF-α along with the up-regulation of cell surface markers MHC-II, CD83 and B7-2 (CD86). Protective immunity has

been shown to be achieved with adoptive transfer of DCs pulsed with inactivated chlamydial organisms, and this protection correlates with the induction of a Th1 immune response [Igietseme *et al.*, 2000; Lu & Zhong, 1999; Shaw *et al.*, 2001; Su *et al.*, 1998]. The protective ability of the *Chlamydia*-pulsed DC correlates with their ability to secrete IL-12 [Lu & Zhong, 1999; Shaw *et al.*, 2001; Zhang *et al.*, 1999] and induce a protective Th1 type of response when transferred *in vivo*. However, adoptive transfer of DC pulsed with recombinant chlamydial MOMP induced a nonprotective Th2 response [Shaw *et al.*, 2002]. Thus, there is a need to understand the mechanism involved in activation of DCs to induce a protective Th1 immune response. As the expression of DC costimulatory molecules is critical for effective DC-mediated T-cell activation [Hackstein & Thomson, 2004], it stands to reason that EB-pulsed DC would be more effective at stimulating a T-cell response due to increased surface expression of MHC-II and CD86. Stimulated DCs secrete low levels of IL-10, which has been found to suppress protective immunity against chlamydial infections [Igietseme *et al.*, 2000], however, this small amount of IL-10 may actually be necessary for curbing excessive pro-inflammatory response which may lead to inflammation and thus scarring of tissue. IL-12 secretion is required to effectively control chlamydial infections [Lu *et al.*, 2000], and TNF-α is known to promote DC migration [Stoitzner *et al.*, 1999]. Thus, this cytokine profile and maturation phenotypes is consistent with live EB-pulsed DC being immunogenic and therefore effective at promoting an antichlamydial immune response.

When *Chlamydia* pulsed dendritic cells were co-cultured with autologous $CD4^+$ T cells it was found that dendritic cells efficiently presented chlamydial antigens to $CD4^+$ T cells as evident by significant proliferation of $CD4^+$ T cells. It was further observed that these activated T cells secrete large amounts of IL-2, IL-12 and IFN-γ. IL-2 is an important

cytokine secreted by T cells which acts in an autocrine manner and leads to proliferation of T cells into a large number of effector T cells. IL-12 induces Th1 differentiation with induction of IFN-γ [Holland *et al.,* 1996]. Thus secretion of IL-12 upon stimulation of $CD4^+$ T cells may help in providing a protective immune response to chlamydial infection.

Sex hormones like estradiol and progestins have been shown to exert a powerful effect on various immune system functions. In the present study we determined the effect of estradiol on chlamydial recognition and downstream signalling in dendritic cells. Because DCs are key cells involved in the initiation, and control of adaptive immune responses, any impact on their functions might have profound effects on immune responsiveness. While there is no definitive information on the mechanism by which hormones affect the immune system, several reports have demonstrated some degree of regulation of DC function by estradiol [Hughes & Clark, 2007]. Our data shows that estradiol reduces TLR4 expression and its downstream signalling which may lead to improper recognition of *Chlamydia* by dendritic cells causing an increase in susceptibility to chlamydial infection.

Estradiol has also been shown to attenuate LPS mediated IL-8 production in human peripheral monocytes and in polarized human uterine epithelial cells [Pioli *et al.,* 2007; Fahey *et al.,* 2008]. It has been shown to reduce TLR4 expression and thus inflammatory response in epidermal keratinocytes [Moeinpour *et al.,* 2007]. We also found that estradiol reduces activation of nF-kB p65 which has a central role in control of genes involved in inflammation. The exact mechanism by which estradiol did so is attributed either to direct interaction of estradiol with nF-kB or by indirect effect of heat shock proteins [Stice & Knowlton, 2008]. In our study, as LPS stimulation has not shown any significant reduction in nF-kB p65 activity compared to stimulation by EBs, a heat shock protein based mechanism

can be suggested. Cytokine secretion pattern of CD4$^+$ T cells also confirmed the observations that estradiol reduces protective Th1 response and increases anti-inflammatory response leading to incomplete clearance of *Chlamydia* and thus enhanced pathogenesis.

In conclusion it can be suggested than pulsing of dendritic cells with live chlamydial EBs induces a Th1 immune response by secretion of IL-12 by dendritic cells and IFN-γ by activated CD4$^+$ T cells. However, addition of estradiol leads to improper recognition of *Chlamydia* by down-regulating TLR4 and its associated pathway and also by up-regulating secretion of IL-10 a Th2 cytokine from CD4$^+$ T cells. The conclusions can be summarized as shown in figure 7.10. Since DCs are now being used as adjuvants for vaccine studies, these results will enhance the knowledge of *Chlamydia* and DC interaction and will help in better designing of a chlamydial vaccine which could induce protective responses even in women with higher than normal cytokine levels (contraceptives, pregnancy and disorders).

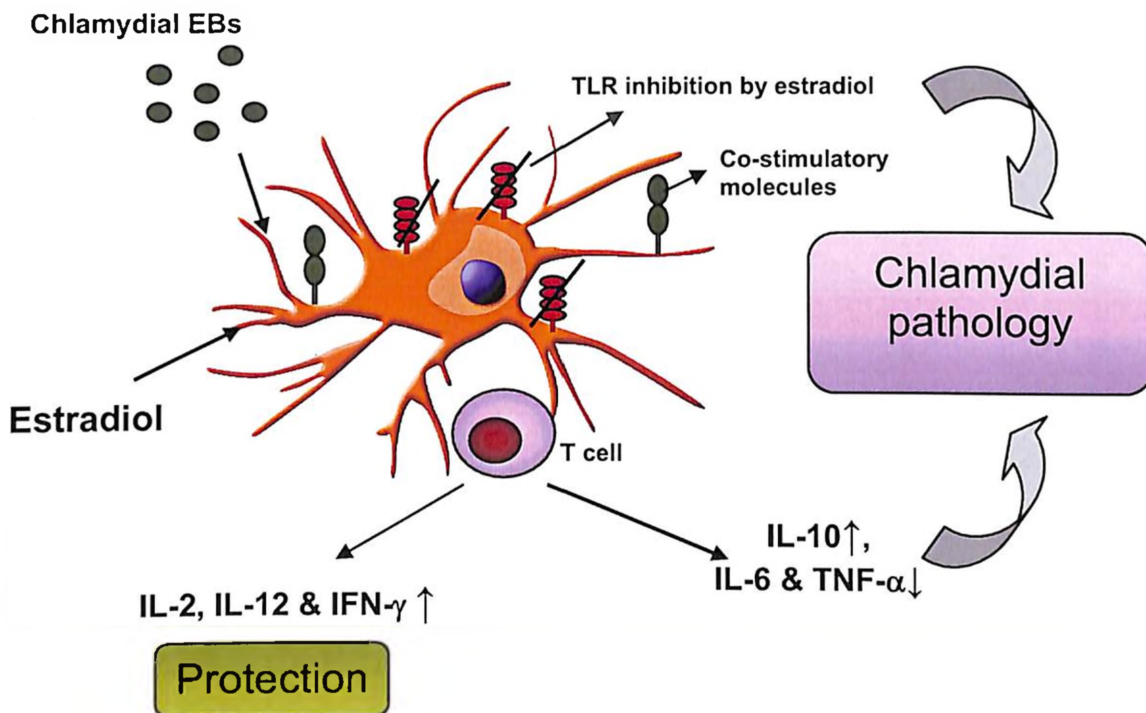
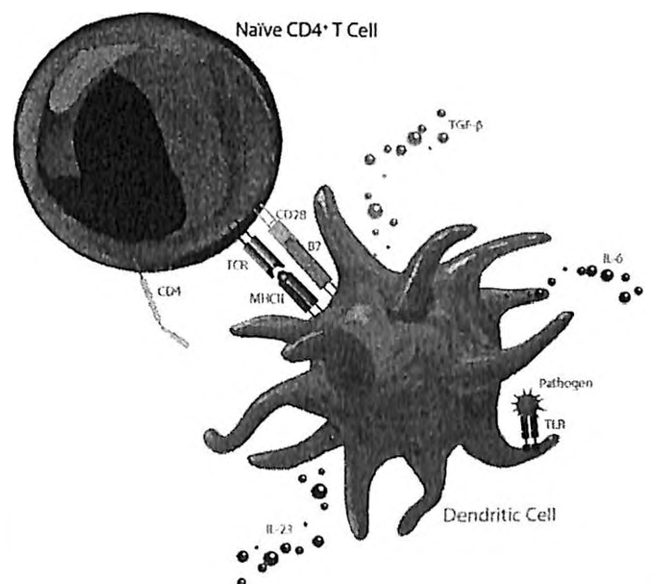


Figure 7.10. Effect of estradiol on dendritic cell function and cytokine secretion.

Chapter 8
Conclusions and future scope of work

Reasoning draws a conclusion, but does not make the conclusion certain, unless the mind discovers it by the path of experience.

Roger Bacon



Conclusions and Future scope of work

In the present study host-pathogen interactions during *Chlamydia trachomatis* infection in terms of both the innate and the adaptive immune responses was assessed. Since cervical mucosal cells are the actual cells encountering the pathogen and their response would help in having a much better understanding the immunopathogenesis of chlamydial disease, these cells were selected to study the immune response generated during chlamydial infection.

1. The study revealed that IFN-γ levels were significantly high in cervical washes of women with recurrent infection and correlated strongly with cHSP60 antibody titers. Thus, it can be suggested that IFN-γ could be involved in modulation of the immune responses towards chlamydial infection directly by causing acute inflammation or indirectly through modulation of heat shock proteins expression. Further, high chlamydial infectious load was observed in women with MPC and lower infectious burden in women with FD. Chlamydial burden have positive correlation with number of $CD4^+$ T cells, IL-2, IL-12 and IFN-γ levels in asymptomatic women. In contrast, in women with inflammation, chlamydial burden showed correlation with IL-1β and IL-8 levels. In women with FD chlamydial burden correlated with both pro and anti-inflammatory factors which may lead to incomplete clearance of bacteria and thus persistence. When cervical cells were stimulated with chlamydial antigens it was observed that cells from women with recurrent infection responded more to cHSPs rather than MOMP. It was observed that cells from *Chlamydia* positive women without any pathological condition secrete higher amounts of IFN-γ and IL-12 upon secondary stimulation while cells from women with sequelae secrete IL-6, IL-8 and IL-10. These results

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suggest that changes in cytokine pattern can be responsible for various pathological consequences.

2. Cervical dendritic cell subsets were enumerated to understand their role in immune response to *Chlamydia.* It was found that *C. trachomatis* infection mobilizes both mDC and pDC to the cervical mucosa. pDCs correlated significantly with chlamydial load, C-reactive protein levels, β-estradiol levels, cervical IL-8 and IFN-γ levels in women with fertility disorders. In contrast, mDCs correlated significantly with IL-12 levels in *Chlamydia* positive fertile women. Median numbers of mDCs was significantly higher in cervix of women who received antibiotic therapy. Median numbers of pDCs were, however, found to be lower in the cervix after therapy. This suggests that mDCs provide protective immune response while pDCs are involved in pathogenesis and can be used by *Chlamydia* for their survival and persistence.

3. As for innate immune responses presence of both TLR2 and TLR4 was detected on cervical monocytes. TLR2 expression was higher in women with fertility disorders, TLR4 in women with MPC while that of iNOS in asymptomatic women. This suggests role of TLRs in inflammatory response while that of iNOS in clearance. On pulsing with chlamydial EBs, cervical monocytes upregulate both TLR2 and TLR4, suggesting the role of both in recognition of *Chlamydia*. Late induction of iNOS expression with nitric oxide production suggests its involvement in direct killing of bacteria rather than initiation of adaptive immunity. Production of IL-12 was found to be dependent on TLR4 while that of IL-6 on TLR2 showing the role of TLR4 in initiation Th1 immune response. To deduce the downstream signalling pathway, inhibition of MyD88 was done which reduced IL-12

production by monocytes but not completely. This suggests the presence of both MyD88 dependent and independent downstream signalling.

4. In this study role of dendritic cells in processing and presentation of chlamydial antigen and induction of immune response was studied. EB pulsed dendritic cells showed induction of a protective type Th1 immune response with upregulation of TLR4 expression, secretion of IL-6, IL-12 and IFN-γ along with up-regulation of MHC-II, CD83 and CD86. When co-cultured with autologous $CD4^+$ T cells dendritic cells efficiently presented chlamydial antigens as shown by proliferation of T cells. The activated T cells secrete further IL-2 and IFN-γ which provide a protective immune response to chlamydial infection. However, pretreatment of cells with estradiol significantly reduces TLR4 expression and up-regulates IL-10 secretion modulating the Th1 type immune response to Th2 type which may lead to pathogenesis as in case of women with high estradiol levels.

Future scope of work

The role of intracellular recognition molecules like NODs expressed by cells of the female reproductive tract (e.g. Fallopian tube epithelium) in the response to chlamydial infection, or any STI, remains poorly understood. A suboptimal innate immune response may result in a permissive environment for pathogen colonization, whereas an over-exuberant response will cause excessive inflammation and tissue damage. Modulation of the host response to infection is an attractive alternative or adjuvant approach to antibiotic therapies in treatment of genital tract infections. Genome sequence analysis has revealed that *Chlamydia* possesses numerous novel genes that might be involved in the manipulation of the host cells. The infected cells often display altered metabolic, immunological and cell biological characteristics, however, at the same time the microbes have to maintain the integrity and

viability of host cells before completing their own intracellular replication. To achieve this goal chlamydiae have evolved the ability to both prevent the infected cells from undergoing apoptosis induced by intracellular stress and to protect these cells from recognition and attack by lymphocytes. Analysis of *C. trachomatis* genome had identified more than two dozens of open reading frames encoding proteins with potential proteolytic activity. Some of these proteases may be used to target host cell proteins because some proteins are cleaved and/or degraded in infected cells. The attacked host proteins include transcriptional factors, pro and anti apoptoytic proteins, DNA repairing enzymes, cyclins and cytoskeletal protein. However, survival strategies of *Chlamydia* at the tissue level and the relevance of these findings in disease pathogenesis have yet to be determined. Since till date a *Chlamydia* vaccine is unavailable, a better definition of human immune response along with Chlamydial survival strategies needs to remain an important research priority if we are to develop a vaccine against *C. trachomatis* infection that has protective and not deleterious effects.

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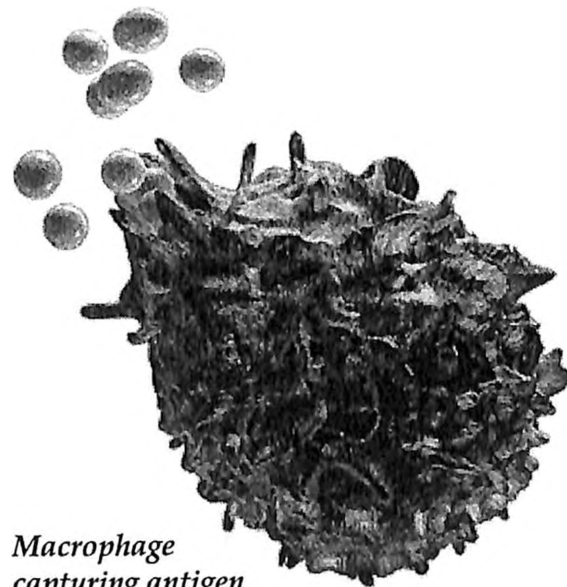
A goal is not always meant to be reached; it often serves simply as something to aim at.

Bruce Lee



Macrophage capturing antigen

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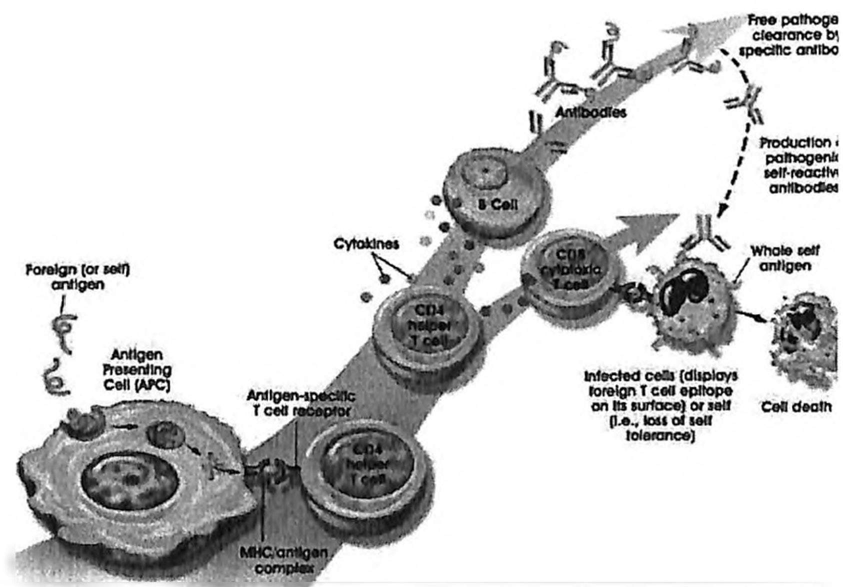
APPENDIX

Heaven is not reached by a single bound. But we build the ladder by which we rise.

J.C. Holland



Appendix

Preparation of reagents (Stock solution of commonly used reagents)

Cell Viability assay: MTT stock solution: 5mg/ml MTT (Sigma Aldrich, USA) in EMEM (Sigma Aldrich, USA) without phenol red.This solution is filtered through a 0.2 μm filter and stored at 2-8^{0}C.MTT working solution:1:10 dilution of the 5mg/ml stock (MTT in EMEM without phenol red).

1M Tris

121.1gm of Tris base was dissolved in 800ml of double distilled water and pH set (6.8, 7.4, 8.0) with concentrated HCl. Volume was made up to 1liter and autoclaved.

0.5M EDTA

186.1gm of disodium EDTA.-2H2O was added in 800ml of double distilled water, stirred vigorously on a stirrer, pH set to 8.0 with NaOH (~20 gm of NaOH pellets) and volume made up to1liter and autoclaved.

10% SDS

10gm of electrophoresis grade SDS was dissolved in 70ml of ddH2O, heated at 60°C to dissolve and the volume made up to 100ml.

Calcium Chloride (0.1 M)

1.47gm of CaCl2.2H2O was dissolved in 100ml of ddH2O and sterilized by autoclaving.

.DEPC water

0.1% diethylpyrocarbonate was added to 1ltr double distilled water in a fume hood and mixed well. After incubating it for 1hr at 37°C it was autoclaved.

Phosphate Buffer Saline (PBS)

8gm of NaCl, 2gm of KCl, 1.44gm of Na_2HPO_4 and 0.2gm of KH_2PO_4 were dissolved in 800ml of dw. pH was set to 7.4 with HCl. Final volume was made up to 1 liter and sterilized by autoclaving at 15lb/ sq.in for 20 minutes and stored at room temperature.

Ammonium persulfate (10%)

To 1gm of ammonium persulfate, 10ml of dw was added and the solution stored for several weeks at 4°C.

10 X TAE buffer (Tris acetate, EDTA)

4.84gm of Tris base in 80ml of dw was dissolved and 1.2ml of glacial acetic acid and 2ml of 0.5 EDTA pH 8.0 were added. Final volume was made up to 100ml.

10X TBE buffers (Tris borate, EDTA)

8 gm of Tris base, 55 gm of boric acid and 9.3gm $Na_2EDTA.H_2O$ were dissolved in 700ml dw and the final volume made up to 1 liter.

SDS-PAGE electrophoresis buffer

3gm of Tris base, 14.4gm of glycine and 1gm of SDS were dissolved in 1 liter of dw.

Protein transfer buffer

5.8gm of Tris base, 2.9gm of glycine and 0.33gm of SDS were dissolved in 500ml of dw. 200 ml of ethanol was added and the final volume was made up to 1 liter.

Sodium Phosphate (1M)

Monobasic

138gm of $NaH_2HPO_4.H_2O$ was dissolved in 800ml of ddH_2O and volume made up to 1liter.

Dibasic

268gm of $Na_2HPO_4.7H_2O$ was dissolved in 700ml of ddH_2O and volume made up to 1liter.

2X SDS-PAGE sample buffer

Tris-Cl (pH6.8) 100mM
DTT 200mM
SDS 4%
Bromophenol blue 0.2%
Glycerol 20%
β-mercaptoethanol 10%

SDS-PAGE reagents

Composition of resolving gel (12%) 10 ml

30% acrlyamide solution 4.0 ml
1.5M Tris-Cl pH 8.8 2.5 ml
dw 3.3ml
10% SDS 100ml
10% APS 100ml
TEMED 10μl

Composition of stacking gel (5%) (5.0 ml)

30% acrylamide solution 0.83ml
1.0M Tris.Cl pH 6.8 0.68 ml
dw 3.4ml
10% SDS 50ml
10% APS 50ml
TEMED 5μl

Staining solution

1gm of coomassie blue was dissolved in 450ml of methanol. 100ml of glacial acetic acid was added and the volume made up to 1liter by double distilled water, filtered through Whatman no.1 filter and stored at room temperature.

Destaining solution

Methanol: water: acetic Acid were mixed in the ratio of 45:45:10 and stored at room temperature.

Cytoplamic/Nuclear Extraction Buffer:

Buffer 'A'

HEPES (pH 7.9)	10mM
MgCl2	1.5mM
KCl	10mM
DTT	0.5mM
PMSF	0.5mM

Buffer 'B'

HEPES (pH 7.9)	20mM
MgCl	1.5mM
Nacl	420mM
EDTA	0.2mM
Glycerol	25% V/V
PMSF	0.5mM

Appropriate amount of protease inhibitor cocktail.

Binding Buffer RNA/DNA-Protein

Gentamicin (stock solution):

100mg/ml Gentamicin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at –20^{0}C.

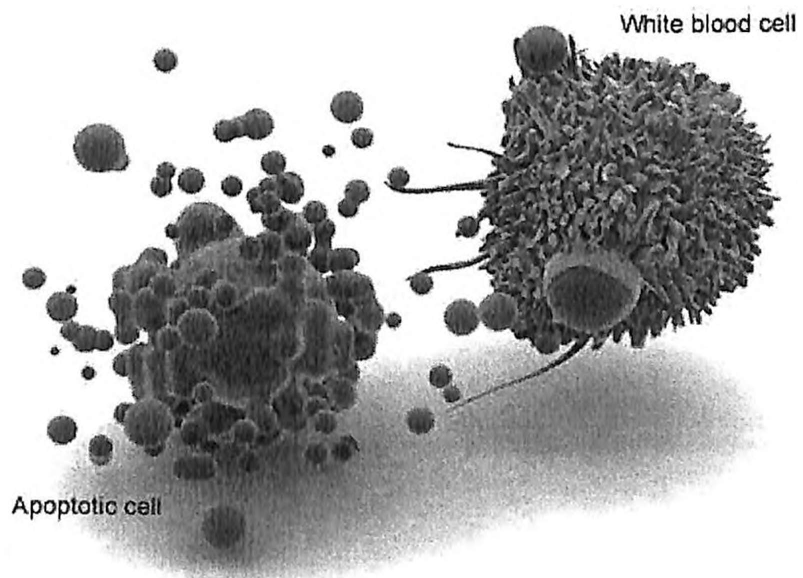
Amphotericin (stock solution):

100mg/ml Amphotericin stock was prepared in autoclaved distilled water and sterilized by filtration through 0.22µm filter. 100µl aliquot was stored by freezing at –20^{0}C.

LIST OF PUBLICATIONS AND PRESENTATIONS

In the field of observation, chance favours only prepared minds.

Louis Pasteur



List of Publications

Publications

1. Vats, V., **Agrawal, T.**, Salhan, S. and Mittal, A. (2007) Primary and secondary immune response of mucosal and peripheral lymphocytes during *Chlamydia trachomatis* infection. *FEMS Immunology and Medical Microbiology* 49, 280-287.

2. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. (2007) Local markers for prediction of women at higher risk of developing sequalae to *C. trachomatis* infection. *American Journal of Reproductive Immunology* 57, 153-159.

3. **Agrawal, T.**, Vats, V., Wallace, P.K., Salhan, S. and Mittal, A. (2007) Cervical cytokine responses in women with primary or recurrent chlamydial infection. *Journal of Interferon and Cytokine Research* 27, 221-226.

4. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. (2007) Mucosal and peripheral immune responses to chlamydial heat shock proteins in women infected with *Chlamydia trachomatis. Clinical Experimental Immunology* 148, 461-467.

5. **Agrawal**, T., Vats, V., Salhan, S. and Mittal, A. (2008) Role of cervical dendritic cell subsets, costimulatory molecules, cytokine secretion profile and beta-estradiol in development of sequalae to *Chlamydia trachomatis* infected women. *Reproductive Biology & Endocrinology* 6, 46.

6. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. (2009) Determination of chlamydial load and immune parameters in asymptomatic, symptomatic and infertile women. *FEMS Immunology and Medical Microbiology* 55, 250-257.

7. **Agrawal, T.**, Vats, V., Wallace, P.K., Singh, A., Salhan, S. and Mittal, A. (2009) Recruitment of myeloid and plasmacytoid dendritic cells in cervical mucosa during *C. trachomatis* infection. *Clinical Microbiology Infection* 15, 50-59.

8. **Agrawal, T.**, Gupta, R., Dutta, R., Srivastava, P., Bhengraj, A.R., Salhan, S. and Mittal, A. (2009) Protective or pathogenic immune response to genital chlamydial infection in women-A possible role of cytokine secretion profile of cervical mucosal cells. *Clinical Immunology* 130, 347-354.

9. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. (2009) Human mucosal immune response to *Chlamydia trachomatis* infection of the female reproductive tract. *Journal of Reproductive Immunology* 83, 173-178.

10. Vats, V., **Agrawal, T.**, Salhan, S. and Mittal, A. (2010) Characterization of apoptotic activities during *Chlamydia trachomatis* infection in primary cervical epithelial cells. *Immunological Investigations* (In press)

Manuscripts in Preparation

1. **Agrawal, T.**, Vats, V. and Mittal A. (2010) Beta-estradiol downregulates TLR-4 mediated recognition of *Chlamydia trachomatis* and cytokine secretion by dendritic cells. (In preparation)

Patent filed

1. Primary epithelial cell line from cervical lavage (44/DEL/2010).

Presentations

1. Gave poster and oral presentation at 33rd Annual Indian Immunology Society Conference, organized by Department of Biochemistry, All India Institute of Medical Sciences, New Delhi and held during 28th-31st Jan 2007.

2. Gave poster presentations at International congress on Bio-immunoregulatory mechanisms associated with reproductive organs: Relevance in fertility and in Sexually transmitted infections organized by National Institute of Immunology, New Delhi and held during Feb 9-13, 2009.

3. Gave oral presentation at National conference on emerging trends in life sciences research organized by Birla Institute of Technology and Science, Pilani and held during March 6-7, 2009.

4. Gave oral presentation 11th International Union against Sexually Transmitted Infection (IUSTI) World Congress and held during 9th-12th November 2009, Cape Town, South Africa.

Publications in proceedings

1. Vats, V., **Agrawal, T.**, Salhan, S. and Mittal, A. *Chlamydia* induces caspase activity and apoptosis signaling pathways in cervical epithelial cells. IMMUNOCON 2009, NIMHANS, Bangalore, Dec 16-19, 2009.

2. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. Roll of toll like receptors on human cervical monocytes in providing protective immune response to chlamydial infection. In proceedings of 11th IUSTI World Congress, Cape Town, South Africa, Nov 9-12, 2009.

3. Vats, V., **Agrawal, T.**, Salhan, S. and Mittal, A.. Toll-like receptor (TLR) and inflammatory cytokine gene expression by human cervical epithelial cells upon *C. trachomatis* infection. In proceedings of 11th IUSTI World Congress, Cape Town, South Africa, Nov 9-12, 2009.

4. **Agrawal, T.** and Mittal, A. Regulation of cytokine secretion and pathogen recognition by human dendritic and $CD4^+$ T cells by β-estradiol upon *Chlamydia trachomatis* infection. In proceedings of National conference on emerging trends in life sciences research. B.I.T.S, Pilani, March 6-7, 2009

5. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. ß- Estradiol regulates cytokine secretion and pathogen recognition by human dendritic and CD4 + T cells upon *C. trachomatis* infection. International congress on Bio-immunoregulatory mechanisms associated with reproductive organs: Relevance in fertility and in sexually transmitted infections. NII, New Delhi, Feb 9- 13, 2009.

6. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. Regulation of chlamydial infectious load in the cervix of infected women -various immunological aspects. Sixth European Chlamydia meeting, July 1-4, Aarhus, Denmark, 2008.

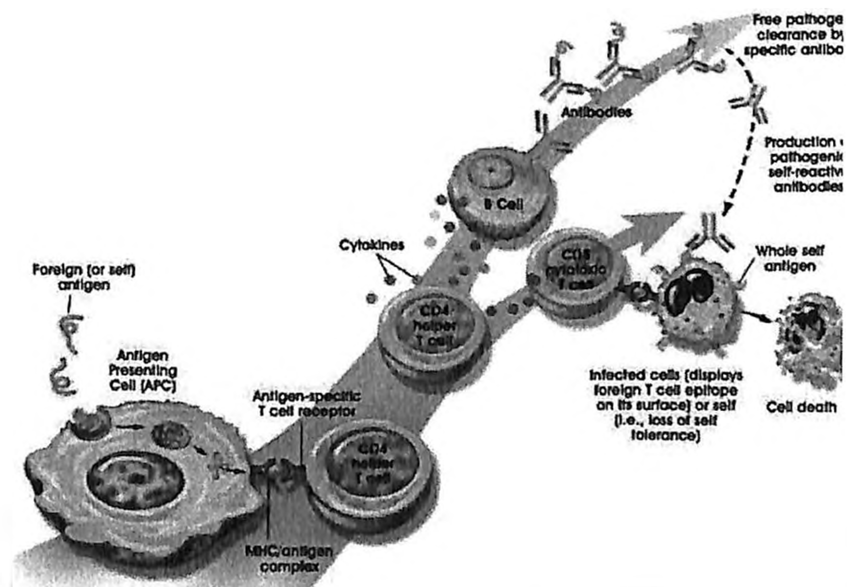
7. Mittal, A., **Agrawal, T.**, Vats, V., Wallace, P. and Salhan, S.: Mobilization of dendritic cells subsets to cervical mucosa during Chlamydia *trachomatis* infection. Proceeding World congress on International against sexually transmitted infections and International society for STD research at Washington Seattle USA, July, 2007. 49.

8. **Agrawal, T.**, Vats, V., Salhan, S. and Mittal, A. Role of IFN gamma in modulating the mucosal immune responses during Chlamydia trachomatis infection In proceedings 33rd Indian Immunology Society conference, AIIMS, New Delhi, Jan 28-31,2007.

BIOGRAPHY OF CANDIDATE AND SUPERVISOR

Science is simply common sense at its best, that is, rigidly accurate, and merciless to fallacy in knowledge.

T.H. Huxley



Brief Biography of the Candidate

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Education

2004-till date ....Birla Institute of Technology and Science, Pilani, Doctoral student in Biological Science (Ph.D.), thesis entitled Role of Antigen Presenting Cells (APC's) and Toll like receptors in providing a protective immune response during *C. trachomatis* infection

2001-2003......................G.B. Pant University of Agriculture and Technology, Pantnagar, Master of Science in Microbiology **(81.64%).**

1998-2001.......................University of Allahabad, Allahabad, Bachelor of Science **(78.5%)**

Employment

August 2009 – Feb 2010............Senior Research Fellow, Institute of Pathology (ICMR), New Delhi.

August 2006 – August 2009.........CSIR-UGC Senior Research Fellow, Institute of Pathology (ICMR), New Delhi.

August 2004– Aug. 2006...........CSIR-UGC Junior Research Fellow, Institute of Pathology (ICMR), New Delhi.

January 2002 – June 2003......... Part time Graduate Teaching Assistant, GB Pant University of Agriculture and Technology, Pantnagar.

Awards and Fellowships

2001 ....Shanti Devi Srivastava Gold Medal for proficiency in botany by University of Allahabad

2003................................CSIR-UGC Junior Research Fellowship

2003................................GATE 2003 (95.83)

2007................................Best Poster award at 33rd Indian Immunology Society Conference, New Delhi.

2009................................Full scholarship for attending 11th IUSTI World congress, Cape Town, November.

2010................................Awarded ICMR postdoctoral fellowship.

Skills and Expertise

Cell Culture..........................Culture of Monocyte derived Dendritic cell, maintenance of McCoy, HeLa and THP-cell lines, *Chlamydia trachomatis* culture in cervical epithelial cell line.

Immunology........................... Multicolor flowcytometry, Lymphocyte proliferation assays, Cell cytotoxicity assays, ELISA, ELISPOT and Cell separation using MACS, Western Blotting,

Proteomics ....Protein separation by Native and SDS PAGE, Inhibition assays to detect downstream signaling

Microscopy............................Phase-contrast, Fluorescent and Confocal Laser Scanning Microscope

Microarray............................cDNA microarray on glass slides.

Molecular Biology.....................Isolation of DNA, RNA, PCR, Real Time RT-PCR, Gene expression profile using real time PCR based arrays.

Computational.........................Software such as sigma-plot, SPSS and MS office

Additional............................Planning /organization skills to support & maintain the laboratory's system

Patent filed

1. Primary epithelial cell line from cervical lavage (44/DEL/2010).

Conference attended

1. Attended National Symposium on "Improving crop productivity in an ecofriendly environment: Physiological and molecular approaches" organized by Department of Plant Physiology, Pantnagar, October 2003 and presented a poster.

2. Attended 21st annual conference of Indian Association of Pathologists and Microbiologists, Delhi Chapter organized by Institute of Pathology (ICMR) and Safdarjung Hospital and V.M.M. College held on 16 April 2006.

3. Attended 33rd Annual Indian Immunology Society Conference, organized by Department of Biochemistry, All India Institute of Medical Sciences, New Delhi, held during 28th-31st Jan 2007 and gave a poster and an oral presentation.

4. Attended International congress on Bio-immunoregulatory mechanisms associated with reproductive organs: Relevance in fertility and in Sexually transmitted infections organized by National Institute of Immunology, New Delhi, held during Feb 9- 13, 2009 and gave a poster presentation.

5. Attended National conference on emerging trends in life sciences research organized by Birla Institute of Technology and Science, Pilani, held during March 6-7, 2009 and gave an oral presentation.

6. Attended 11th International Union against Sexually Transmitted Infection (IUSTI) World Congress, held during 9th-12th November 2009, Cape Town, South Africa and gave an oral presentation.

Publication in peer reviewed journals

Published:

1. Vats V, **Agrawal T**, Mittal A. Characterization of apoptotic activities during *Chlamydia trachomatis* infection in cervical epithelial cells. **Immunological Investigations**, 2010 **(In press)**

2. **Agrawal T**, Vats V, Salhan S, Mittal A. Human mucosal immune response to *Chlamydia trachomatis* infection of the female reproductive tract. **Journal of Reproductive Immunology**, 2009, 83:173-178. **(Impact Factor – 2.778)**

3. **Agrawal T**, Gupta R, Dutta R, Srivastava P, Bhengraj AR, Salhan S, Mittal A. Protective or pathogenic immune response to genital chlamydial infection in women-A possible role of cytokine secretion profile of cervical mucosal cells. **Clinical Immunology**, 2009, 130:347-354. **(Impact Factor- 3.606)**

4. **Agrawal T**, Vats V, Wallace PK, Singh A, Salhan S, Mittal A. Recruitment of myeloid and plasmacytoid dendritic cells in cervical mucosa during *C. trachomatis* infection. **Clinical Microbiology Infection.** 2009, 15:50-59. **(Impact Factor- 3.554)**

5. **Agrawal T**, Vats V, Salhan S, Mittal A. Determination of chlamydial load and immune parameters in asymptomatic, symptomatic and infertile women. **FEMS Immunology and Medical Microbiology**, 2009, 55:250-257. **(Impact Factor- 1.97)**

6. **Agrawal T**, Vats V, Salhan S, Mittal A. Role of cervical dendritic cell subsets, costimulatory molecules, cytokine secretion profile and beta-estradiol in development of sequalae to *Chlamydia trachomatis* infected women. **Reproductive Biology & Endocrinology**, 2008, 6:46 **(Impact Factor- 2.64)**

7. **Agrawal T**, Vats V, Salhan S, Mittal A. Mucosal and peripheral immune responses to chlamydial heat shock proteins in women infected with *Chlamydia trachomatis*. **Clinical Experimental Immunology.** 2007, 148:461-467 **(Impact Factor- 2.853)**

8. **Agrawal T**, Vats V, Wallace PK, Salhan S, Mittal A. Cervical cytokine responses in women with primary or recurrent chlamydial infection. **Journal of Interferon and Cytokine Research**. 2007, 27:221-226. **(Impact Factor- 2.667)**

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10. Vats V, **Agrawal T**, Salhan S, Mittal A. Primary and secondary immune response of mucosal and peripheral lymphocytes during *Chlamydia trachomatis* infection. **FEMS Immunology and Medical Microbiology**. 2007, 49:280-287. **(Impact Factor- 1.97)**

In preparation:

1. **Agrawal T**, Vats V, Mittal A. Beta-estradiol downregulates TLR-4 mediated recognition of *Chlamydia trachomatis* and cytokine secretion by dendritic cells. (In preparation)

Publication in proceedings

1. Vats V, **Agrawal T**, Salhan S, Mittal A. Chlamydia induces caspase activity and apoptosis signaling pathways in cervical epithelial cells. IMMUNOCON 2009, NIMHANS, Bangalore, Dec 16-19, 2009.

2. **Agrawal T**, Vats V, Salhan S, Mittal. Roll of toll like receptors on human cervical monocytes in providing protective immune response to chlamydial infection. In proceedings of 11th IUSTI World Congress, Cape Town, South Africa, Nov 9-12, 2009.

3. Vats V, **Agrawal T**, Salhan S, Mittal. Toll-like receptor (TLR) and inflammatory cytokine gene expression by human cervical epithelial cells upon *C. trachomatis* infection. In proceedings of 11th IUSTI World Congress, Cape Town, South Africa, Nov 9-12, 2009.

4. **Agrawal T**, Mittal A. Regulation of cytokine secretion and pathogen recognition by human dendritic and $CD4^{+}$ T cells by β-estradiol upon *Chlamydia trachomatis* infection. In proceedings of National conference on emerging trends in life sciences research. B.I.T.S, Pilani, March 6-7, 2009

5. **Agrawal T**, Vats V, Salhan S, Mittal A. ß- Estradiol regulates cytokine secretion and pathogen recognition by human dendritic and CD4 + T cells upon *C.trachomatis* infection. International congress on Bio-immunoregulatory mechanisms associated with reproductive organs: Relevance in fertility and in Sexually transmitted infections. NII, New Delhi, Feb 9- 13, 2009.

6. **Agrawal T**, Vats V, Salhan S. Mittal A. Regulation of chlamydial infectious load in the cervix of infected women -various immunological aspects. Sixth European Chlamydia meeting, July 1-4, Aarhus, Denmark, 2008.

7. Mittal A, **Agrawal T**. Vats V. Wallace P. Salhan S: Mobilization of dendritic cells subsets to cervical mucosa during Chlamydia *trachomatis* infection. Proceeding World congress on International against sexually transmitted infections and International society for STD research at Washington Seattle USA, July, 2007. 49.

8. **Agrawal T**, Vats V. Salhan S, Mittal A. Role of IFN gamma in modulating the mucosal immune responses during Chlamydia trachomatis infection In proceedings 33rd Indian Immunology Society conference, AIIMS, New Delhi, Jan 28-31,2007.

9. Kaur P, **Agrawal T**, Kumar A, Tewari L. Plant growth promontory mechanism of the biocontrol agent *Trichoderma harzianum*. In proceedings of the National Symposium on Improving crop productivity in an ecofriendly environment: Physiological and molecular approaches, Pantnagar, 2003.

10. Tewari L, **Agrawal T**, Kaur P, Singh T, Tandon SM. Influence of mycorrhizal inoculation on growth and development of fingermillet (*Eleucine coracana*). In proceedings of the National Symposium on Improving crop productivity in an ecofriendly environment: Physiological and molecular approaches, Pantnagar, 2003.

Biography of supervisor

Name in Full: **Dr. Aruna Singh nee Mittal**

Designation: **Scientist F, Institute of Pathology (ICMR)**

Educational Qualifications: **M.Sc, PhD**

Email: **amittal_iop@yahoo.com**

Area(s) of Research: **Immunology/Chlamydia**

Awards/Special recognitions:

- **'Shakuntala Amir Chand Prize for Young Scientists' by ICMR for miniaturizing radiometric assay for *M.Leprae* viability and drug resistance in 1984.**
- **ICMR Kshanika award for research work on Immunobiology of Chlamydia for the year 2003.**
- **ICMR Lala Ram Chand Kandhari award for research work for the year 2006**
- **Title of '*Chlamydia* Farmer' by Center for Disease Control, Atlanta, USA, 1989.**
- **Awarded 'Biotechnology Overseas *long-term* Associateship' by Department of Biotechnology, Ministry of Science & Technology and worked at Center for Disease Control, Atlanta, USA, 1988-89.**
- **Selected for taking advance training at Rockfeller University, New York, USA under 'Indo-US SSP Program', 1984.**

Patent filed: 3

Technology Transfer: 1

Membership of National/International bodies:

i) **Member-** International Union against sexually transmitted infections(Asia-Pacific)

ii) **Fellow-**Indian college of Allergy & Applied Immunology

ii) **Life member-** Indian Immunology society

iii) **Member-** Indian Association of Pathologists and Microbiologists

Extramural Projects awarded:

National: 7

International: 2

Supervisor:

Ph. D students =10,

MSc. Students Dissertation done/completed=11,

MD Thesis=1

Joint supervisor for Ph. D student= 1

Complete list of books, monographs etc. published. (Chapters):

1. Nath I and Mittal A. Subpopulation of lymphocytes. In manual of symposium-workshop 'New Directions in foetal liver transplantation', 20: 1986.
2. Mittal A. Rapid radiometric in vitro assay for evolution of M. leprae viability, drug screening and drug resistance: In Handbook of Practical Immunology. ed. G.P. Talwar, 288, 1992.
3. Talwar GP, Upadhyay SK, Garg S, Kaushik C, Kaur R, Dhawan S, Mittal A. Introduction of cell-mediated immunity in genital tract. In book: 'Neem Research and Development', eds. N.S. Randhawa and B.S. Parmar, 227-234, 1993.
4. Mittal A. Diagnostic methods for Chlamydia trachomatis-a chapter in manual---- National workshop on Cytology and Immunohistochemistry of the Female Genital Tract, 19, 1994.
5. Kapur S, Singh R, Mittal A. Correlation of HIV infection with Hepatitis B and syphilis. In HIV/AIDS research in India, eds. Aggarwal, O.P., Sharma, A.K. Indrayan, 1997, pp. 465-468.
6. Mittal A. Laboratory Diagnosis of C. trachomatis Infection. Chapter in book: 'Chlamydial Infections', eds. V. Talib, 43-67, 1999.

No. of publications in peer reviewed journals: 76

Recent publications:

1. Agrawal T, Vats V, Wallace P, Singh A, Salhan S and **Mittal A.**: Recruitment of myeloid and plasmacytoid Dendritic cells in cervical mucosa during *C. trachomatis* infection. *Clinical Micobiology Infection* (2008)15:50-59.
2. Dutta R, Jha R, Salhan S and **Mittal A.** *Chlamydia* specific heat shock protein 60 antibodies can serve as prognostic marker for chronic chlamydial infection. *Infection.* (2008) 36, 374-378.
3. Jha HC, Prasad J and **Mittal A.** High IgA seropositivity for combined *Chlamydia pneumoniae*, *Helicobacter pylori* infection and high sensitive C-

reactive protein in Coronary Artery Disease patients in India can serve as atherosclerotic marker. *Heart and Vessels*. (2008) 23:390-396.

4. Srivastava P, Jha R, Salhan S and **Mittal A.** In Infertile women, cells from *Chlamydia trachomatis* infected site release higher levels of interferon- gamma, interleukin-10 and tumor necrosis factor-alpha upon heat shock protein stimulation than fertile women. *Reproductive Biology and Endocrinology* (2008) 6, 20.
5. Srivastava P, Gupta R, Jha HC, Bhengraj AR, Jha R, Salhan S and **Mittal A.** Serovar specific immune responses to peptides of variable regions of *Chlamydia trachomatis* Major Outer Membrane Protein in serovar D infected women. *Clinical and Experimental Medicine* (2008) 8, 207-15.
6. Jha H, Prasad J, Srivastava P, Sarkar R and **Mittal A.** *Chlamydia pneumoniae IgA* and elevated level of IL-6 may synergize to accelerate coronary artery disease outcome. *Journal of Cardiology* (2008) 52, 140-45.
7. Agrawal T, Vats V, Salhan S and **Mittal A.** Role of cervical dendritic cell subsets, costimulatory molecules, cytokine secretion profile and beta estradiol in development of sequelae to *Chlamydia trachomatis* infected women. *Reproductive Biology &Endocrinology* (2008) 6, 46.
8. Gupta R, Jha R, Salhan S, Eickhoff M, Krupp G and **Mittal A.** Existence of plasmid less clinical isolate of *Chlamydia trachomatis* in India is a cause of concern. ***International Journal Microbiology*** (2008) 5(2), 1-8.
9. Agrawal T, Gupta R, Dutta R, Srivastava P, Bhengraj R, Salhan S and **Mittal A.** Protective or pathogenic immune response to genital chlamydial infection in women-a possible role of cytokine secretion profile of cervical mucosal cells. *Clinical Immunology* (2009) 130, 347-354.
10. Srivastava P, Jha HC, Salhan S and **Mittal, A.** Azithomycin treatment modulates cytokine production in *Chlamydia trachomatis* infected women. *Basic and Clinical Pharmacology & Toxicology* (2009)104, 478-482.
11. Agrawal T, Vats V, Salhan S and **Mittal, A.** Determination of chlamydial infectious load and immune parameters in asymptomatic, symptomatic and infertile women. *FEMS Immunology and Medical Microbiology* (2009) 55, 250-257.
12. Jha H, Prasad J and **Mittal A.** Why first degree relatives of coronary artery disease patient's have *Chlamydia pneumoniae* infection? *International Journal Cardiology* (2009) doi:10.1016/j.ijcard.2008.12.062.
13. Jha, H. Prasad,J. and **Mittal, A.** Association of plasma circulatory markers, chlamydia pneumoniae and high sensitive C-reactive protein. *Mediators of Inflammation* (2009) 2009, 561532.
14. Gupta R, Salhan S and **Mittal A.** Seroprevalance of antibodies against *C. trachomatis* inclusion protein B & C in infected women. *Journal of Infection in developing countries* (2009) 3, 3.
15. Jha R, Bas S, Salhan S, and **Mittal A.** In infertile women, cervical epithelial cells from *Chlamydia trachomatis* infected site co-express higher level of chlamydial heat shock protein 60 and 10 than in fertile women. *Gynaecologic and Obstetric Investigation* (2009) 68, 160-166.

16. Gupta R, Salhan S and Mittal A. Host immune responses to chlamydial inclusion membrane proteins B and C in *Chlamydia trachomatis* infected women with or without fertility disorders. *Reproductive Biology and Endocrinology* (2009) 7, 38.
17. Vardhan H, Dutta R, Vats V, Gupta R, Jha H, Jha R, Srivastava P, Bhengraj A and **Mittal A.** Persistently elevated level of IL-8 in *Chlamydia trachomatis* infected HeLa 229 cells is dependent on intracellular available iron. *Mediators of Inflammation.* (2009) doi:10.1155/2009/417658 .
18. Vardhan H, Bhengraj A, Jha R and **Mittal A.** *Chlamydia trachomatis* alters iron regulatory protein-1 binding capacity and modulates cellular iron homeostasis in Hela-229 cells. *Journal of Biomedicine Biotechnology* (2009) doi:10.1155/2009/342032.
19. Sachdeva P, Patel A, Sachdev D, **Mittal A** and Saluja D. Evaluation of indigenous PCR assay for detection of *Chlamydia trachomatis* using direct fluorescent assay and Roche amplicor detection kit. *Journal of Medical Microbiology* (2009) 58, 867-873.
20. Bhengraj AR, Goyel A, Talwar GP and **Mittal A.** Assesment of antichlamydial effects of a novel polyherbal tablet "BASANT". *Sexually transmitted infection* (2009) (Accepted for publication).
21. Jha H. Srivastava P, Prasad J and **Mittal A.** Evaluation of plasma circulatory markers, *Chlamydia pneumoniae* and high sensitive C-reactive protein in coronary artery disease patients. *Atherosclerosis Supplements* (2009)10, (issue2)
22. Jha, H.Srivastava, P Prasad,J. and **Mittal,A:** Coronary artery disease: Biomarkers in first degree relatives. **Atherosclerosis Supplements.** 2009;10(issue2)
23. Gupta R, Salhan S and **Mittal A.** Modulation of cytokines and transcription factors (T Bet and GATA3)) in CD4 enriched cervical cells of *Chlamydia trachomatis* infected fertile and infertile women upon stimulation with chlamydial inclusion membrane proteins B and C. *Reproductive Biology and Endocrinology* (2009) 7, 84.
24. Jha H, Prasad J and **Mittal A.** Sequencing of *Chlamydia pneumoniae* in coronary artery disease patients attending tertiary hospital in New Delhi, India. *American Journal of Infection Control.* doi.101016/j-ajic200909007.
25. Jha H, Prasad J and **Mittal, A.** The plasma circulatory markers in male and female coronary artery disease patients. *Heart and Lung* doi. 10.1016/j.hrtlng.2009.10.005.
26. Jha H, Prasad J and **Mittal A.** Prevalence of *Chlamydophila pneumoniae* is higher in aorta than in carotid artery in coronary artery disease patients. *Acta Pathologica Microbiologica Immunologica Scandinavia* (2009) 117, 905–911.
27. Agrawal T, Vats V, Salhan S, **Mittal A.** Human mucosal immune response to Chlamydia trachomatis infection of the female reproductive tract. Journal of Reproductive Immunology, 2009, 83: 173-179.
28. Vats V, Agrawal T, Salhan S, **Mittal A.** Characterization of apoptotic activities during *Chlamydia trachomatis* infection in Primary cervical epithelial cells. Immunological Investigations. 2010 (*In Press).*