Role of Iron in Pathogenesis of *Chlamydia trachomatis* Infection *in vitro*

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

BY
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UNDER THE SUPERVISION OF
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PILANI (RAJASTHAN) INDIA

2010

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CERTIFICATE

This is to certify that the thesis entitled "**Role of Iron in Pathogenesis of *Chlamydia trachomatis* Infection *in vitro***" which is submitted by **Mr. Harsh Vardhan** (ID- 2004 PHXF 429P) for the award of Ph.D. Degree of the Institute embodies original work done by him under my supervision.

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Date : 17.03.2010

Place: New Delhi

Acknowledgements

Gratitude to whom, who helped me to realize the intuition as a compilation. It has become a reality under visionary guidance of Dr. Aruna Singh.

In the first place I would like to record my gratitude to Dr. Aruna Singh for her supervision, advice, and guidance from the very early stage of this research as well as giving me extraordinary experiences throughout the work. Above all and the most needed, she provided me unflinching encouragement and support in various ways. Her involvement with her originality has triggered and nourished my intellectual maturity that I will benefit from, for a long time to come. Her truly scientist intuition has made her as a constant oasis of ideas and passions in science, which exceptionally inspire and enrich my growth as a student, a researcher and a scientist want to be. I am indebted to her more than she knows.

I am deeply grateful to our Director, Dr. Sunita Saxena who let me join this esteemed institute and allowed me to pursue this research work. I extend my gratitude to fellowship granting agency ICMR and especially to Dr. Shandya Divaker, an administrator with great acumen of research. I sincerely thank you for this help.

Gratitude is accorded to B.I.T.S., Pilani for providing all the necessary facilities to complete the research work. I am immensely thankful to Prof. L. K. Maheshwari, Vice-Chancellor, B.I.T.S, Pilani for providing me this opportunity to pursue the off-campus PhD of the Institute. I express my gratitude to Prof. Ravi Prakash, Dean, Research and Consultancy Division (RCD) and Dr. A. K. Das, Group leader, Biological Sciences group, BITS, Pilani for his constant official support, encouragement and making the organization of my research work through the past few years easy. I also thank my Doctoral Advisory Committee (DAC) members, Dr. Uma Dubey and Dr Prabhat Nath Jha. I thank Dr. Hemanth Jadav, Mr. Dinesh Kumar, Ms. Monica Sharma, Mr. Sharad Shrivastava, Mr. Gunjan Soni, Mr. Amit Kumar and Ms. Sunita Bansal, nucleus members of RCD and all office staff of RCD B.I.T.S, Pilani, for their cooperation and guidance in all official procedures associated with the doctoral program.

I am thankful to seniors, who make a *déjà vu* experience for me from first day at lab and institute. Thanks are due to my seniors Dr. Amit Kaushik , Dr. Raini Dutta, Dr. Bharat Bhushan, Dr. Nasim Aktar Ansari and Dr. B. V. Subba Raju.

I am thankful to Madhu Ma'am, Asha Ma'am, Rosi Ma'am, Ajitji and Kamalji for the help they provided for hassle less pursuance of research. I wish to express my gratitude to the staff of the Administrative section, Library and store section at Institute of Pathology for all the support they provided me.

I also take this opportunity to thank my labmates Rishien, Rajneesh, Hem, Rashmi, Pragya, Vikas, Tanvi and Rakesh. A mere thought of these people always brings a smile to my face and belief to tackle problem of any extent. I am thankful to Rishein for raising my intellectual limit on turf of realty, Rajneesh' for in depth intellectual exchange of thought in various arena, Hem for infecting enthusiasm, Rashmi for encouragement and help to touch the best of myself, Pragya's for keeping me awaken towards

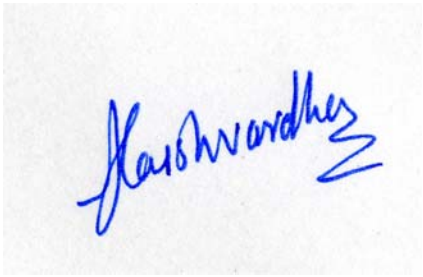
accomplishment of work, Vikas for selfless help, Tanvi for creative and intellectual supports and Rakesh for keeping fun environment afresh. All I can say to you all is-thank you for standing by me always, and for everything that you all have done for me. I have been really fortunate to have great friends like you.

I would also like to thank Shashikant, Dhiraj, Paresh, Pradeep, Srividya, Abha, Regina, and Gajender for supporting me when the going was tough. You guys have been true friends to me. A special thank you to all members of the Institute's cricket team for making me part of the great fun times that we all had during matches and practice sessions. It really helped me unwind during the stress times.

I am very grateful to DJCC (Club of social activities) for re-energizing me to start every week with highest potency. My special thanks to Anurag, Umakant, Vikas, Manish, , Sanjay, Neeraj, Rahul ……..and all of you for understanding me at the depth and extended support before I ask for!

Where would I be without my family? My parents deserve special mention for their inseparable support and prayers. My Father, in the first place is the person who put the fundament my learning character, showing me the joy of intellectual pursuit ever since I was a child. My Mother is the one who sincerely raised me with her caring and gently love. I was extraordinarily fortunate in having Pappu Bhaiya and Neelabh as supportive and caring siblings.

Thanks God, the merciful and the passionate, for providing me the opportunity to step in the excellent world of science.



Date 10.03.201 (Harsh Vardhan)

Place New Delhi

Abstract

Chlamydia trachomatis (CT) is a gram-negative obligate intracellular parasite responsible for myriad of diseases including trachoma, pelvic inflammatory diseases and infertility. Chlamydial biphasic developmental cycle is closely interwoven with host metabolic and regulatory pathways for its requirements. Virtually all cells and organisms utilize iron as a cofactor in a multitude of biochemical activities. Iron, as an essential nutrient and potential toxin poses an exquisite regulatory problem in biology and medicine. Availability of iron determines the course and disease outcome of chlamydial infection. In the present study CT-host interaction was assessed in HeLa 229 cells under iron depleted and supplemented conditions.

It was observed that at critical concentration of 25-50uM deferoxamine (DFO), CT infection lead to greater sustenance of cells and delayed chlamydial developmental cycle as observed in cell viability assay and direct fluorescence assays. Further irrespective of iron depletion and supplementation CT infected HeLa cells showed decrease in surface expression of transferrin receptor (TfR) (flow cytometry), however level of ferritin heavy chain (FHC) increased as observed in immunoblotting. Intracellular ROS and mitochondrial membrane potential was analysed using DCFH-DA and JC-1 probes respectively. Level of ROS was dampened, moreover mitochondrial membrane potential remained stable in iron depleted and supplemented conditions in CT infected cells. Increase in level of anti-apoptotic Bcl-2, Trx, PPAR-γ and decrease in pro-apoptotic cytoplasmic cytochrome 'c', active caspase-3/8/9 and Bax was observed in CT infected cells. These results suggested that critical concentration (25-50uM) of DFO was synergistic to anti-apoptotic effect of CT.

Chlamydial infection attenuated binding activity of iron regulatory protein (IRP-1) to iron responsive element as inferred from RNA-gel shift assay. In CT infected HeLa 229 cells, the

outcome of attenuation in IRP-IRE binding resulted in decrease in TfR and increase in FHC levels as visualized in immunoblotting. Further activation of NF-kB and hypoxia inducible factor (HIF)-1α was retarded in CT infected cells as observed in gel shift assay. These results suggest blockage of alternate regulatory pathway for transferrin receptor expression. Further increased level of prolyl hydroxylase (PHD)-1 observed in CT infected cells. This might be responsible for inactivation of HIF-1α by degrading it. This study further showed that in CT infected cells, levels of labile iron pool (LIP) is associated with secretion of interleukin-8 (IL-8) in converse order. Further it was observed that iron dependent regulation of IL-8 is mediated through nitric oxide and not by ROS in CT infected cells. Increased expression of FHC was associated with level of IL-10 in CT infected cells in direct order. These findings suggest immunomodulatory effect of Chlamydial *trachomatis* is also mediated through iron, and proteins involved in iron acquisition and storage.

Conclusively these results inferred that chlamydia infection leads to greater sustenance of cells, wherein DFO act synergistically in anti-apoptotic mechanism. Decline in intracellular free iron either by chlamydia or increased FHC results in dampening of ROS levels and stabilization of MMP (Mitochondrial membrane potential), helpful in establishment of infection. Further higher expression of FHC (Intracellular iron storage protein) in CT infection resulted due to attenuated binding activity of IRP-IRE. Thereby decline in LIP was apparent CT infected HeLa cells as observed in calcein staining for divalent iron, which might be resulted in higher secretion of IL-8 and IL-10. Extrapolation of these *in vitro* results will help in development of effective strategy to eradicate chronic chlamydial infection *in vivo*.

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Abbreviations

α	Alpha
β	Beta
BSA	Bovine Serum Albumin
cDNA	Complementary DNA
cm	Centimeter
CMI	Cell-mediated immunity
CT	*Chlamydia trachomatis*
CP	*Chlamydia pneumoniae*
DAB	3, 3'- Diamino Benzidine
DC	Dendritic cell
DCFH-DA	2',7'-dichlorofluorescein diacetate
DFA	Direct fluorescence assay
DFO	Deferoxamine
DEAE	Diethylaminoethyl
ddH_2O	Double Distilled water
DNA/RNA	Deoxyribose/Ribose nucleic acid
DMEM	Delbecco's Minimum Essential Media
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EBs	Elementary Bodies
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immunosorbent assay
EMEM	Earle's Minimum Essential Media
EMSA	Electrophoretic mobility shift assay
FCS/FBS	Fetal calf/bovine serum
HBSS	Hank's balanced salt solution
HEPES	N-2-hydroxyethgylpiperazine-N-2 ethane sulfonic acid
HIF	
H/h	Hour/hours
Hpi	Hours post infection
γ	Gamma

GM-CSF	Granulocyte-macrophage colony-stimulating factor
IDO	2',3'-indolamine dioxygenase
IFN	Interferon
IL	Interleukin
IRE	Iron response element
IRP	Iron regulatory protein
Kb	Kilo base
LGV	Lymphogranuloma venereum
LIP	Labile iron pool
M	Molarity
mg	Milligram
MHC	Major histocompatibility complex
MMP	Matrix metalloprotease
MOMP	Major Outer membrane protein
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide
min/mins	Minute/Minutes
ml	Milliliter
mm	Millimeter
mM	Mill molar
N	Normality
$NaHCO_3$	Sodium bicarbonate
ng	Nanogram
NO	Nitric oxide
^{o}C	Degree Celsius
OD	Optical Density
ORF	Open reading frame
PAGE	Poly Acryl amide Gel Electrophoresis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate Buffered Saline
PBS-T	Phosphate buffered Saline with Tween-20
PCR	Polymerase chain reaction
pg	Pico gram
PID	Pelvic inflammatory disease

PPAR	Peroxisomes proliferator-activated receptor
PVDF	Polyvinyl Difluoride
RBs	Reticulate Bodies
RNase	Ribonuclease
ROS	Reactive oxygen species
rpm	Revolution per minute
RT-PCR	Reverse transcription- PCR
s/ secs	Second/ Seconds
SD	Standard deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly acryl amide Gel Electrophoresis
SE	Standard error
SOD	Superoxide dismutase
SPG	Sucrose phosphate glutamate
STD	Sexually transmitted diseases
TAE	Tris acetate EDTA
TEMED	N,N,N',N' tetramethyl ethylene diamine
TfR	Transferrin receptor
TGF	Tumor growth factor
Th	T-helper
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tris	Tris (hydroxymethyl) amino acid
TrX	
U	Unit
UV	Ultra Violet
µm/ µM	Micromole / Micro molar

Chapter 1
Introduction

The process of scientific discovery is, in effect, a continual flight from wonder.
Albert Einstein

Chapter 1

Introduction

Chlamydiae are gram-negative obligate intracellular parasites which preferentially target columnar epithelial cells of mucosal surface and replicate within specialized parasitophorous vacuoles. The pathogenic chlamydia species includes *Chlamydia trachomatis* (CT), an established agent of infectious blindness (serovars A–C), sexually transmitted disease (serovars D–K), and systemic disease (lymphogranuloma venereum; LGV1, 2, and 3) and the respiratory pathogen *Chlamydia pneumoniae* (CP) (Schachter, 1988). In addition to these human pathogens, Chlamydial species and Chlamydia-like organisms have been found associated with other vertebrates, invertebrates, and even protozoa (Horn, 2008). This impressive diversity and seemingly ubiquitous distribution implies long co-evolution with host species leading to an exquisite degree of adaptation to eukaryotic systems (Horn *et al.*, 2004).

World-wide *CT* is annually responsible for >92 million sexually transmitted infections and 85 million ocular infections (World Health Oranization report, 2001). The global prevalence and incidence have awakened public health policymakers to target *CT* as a major problem, because the organism causes long-term sequelae such as infertility, ectopic pregnancy, and blindness. Sexually transmitted *CT* is also a potent cofactor facilitating the transmission of human immunodeficiency virus (HIV) (Plummer *et al.*, 1991) and interacts with oncogenic human papilloma virus in the pathogenesis of cervical neoplasia (Koskela *et al.*, 2000, Smith *et al.*, 2009).

According to Centre for disease control (CDC) report, first *CT* infection was frequent among 15 and 34 years of age, representing peak reproductive years and abundance of these cases

remain asymptomatic or untreated (Gray *et al.*, 2009). Underneath these statistics are physiological conditions, showing its possible role. Source and concentration of iron in the female reproductive tract fluctuate due to the hormonal influence of estrogen and progesterone throughout menstruation (Cohen, 1987, Andrews, 2000). Thus infection of CT in young women remains for longer period. Phase variable hemoglobin receptor on *Neisseria gonorrhoeae* is selected in vivo only during the first half of the female menstrual cycle. It is strongly supporting the notion that the female reproductive tract is a dynamic environment for iron sources and concentrations (Anderson, 2001). The study conducted by Murrey *et al*, in 1991 on *Chlamydia psittaci* has shown that there was no change in number of EBs observed, however alteration in form of chlamydiae was reported after iron depletion (Murray *et al.*, 1991). Influence of iron deprivation was distinctively observed, when these EBs were used for reinfecting the cells as significant decrease in infectivity was observed by Raulston *et al* in 1997 (Raulston, 1997). This study was further authenticated by other studies showing detrimental effect of iron deprivation and cell type on *CP* and *CT*. In HEp-2 cells, *CP* showed higher sensitivity than LGV to iron deprivation (Al-Younes *et al.*, 2001). Iron is essential for both host and pathogen and complex systems of acquisition and utilization have evolved in competition. Our increasing knowledge of the basic mechanisms of homeostasis and their adaptation during deficiency, overload, and infection indicate that iron is a key regulator of host pathogen interactions. The redox potential of the Fe^{2+}/Fe^{3+} switch is utilized in key biological systems of both eukaryotes and prokaryotes. This utility, however, is balanced with a ready capacity to participate in Fenton-type reactions with hydrogen or lipid peroxides that lead to the production of highly toxic free radicals (Kell, 2009). Cells invest in complex systems to control iron reactivity, availability, and flux to prevent free radical

damage to proteins, ribonucleic acids, and cell membranes. Iron is essential and iron deficiency significantly impairs cell proliferation and immune function (Weiss *et al.*, 1995). Chlamydia brings on host cells to sustain longer or avoid apoptosis, thereby provide time window for completion of biphasic developmental cycle and productive infection. Apoptosis, or programmed cell death, is an evolutionarily conserved, strictly regulated, genetic and biochemical program that plays critical roles during development and tissue homeostasis in multicellular organisms (Zakeri *et al.*, 2008). Cells undergoing apoptosis manifest profound changes in cellular architecture characterized by a distinct morphology (Cohen, 1993).Apoptosis plays an important role in modulating the pathogenesis of a variety of infectious diseases (Bhavsar *et al.*, 2007). The chlamydial infection protects cells against different forms of apoptosis : extrinsic, intrinsic and granzyme B mediated by various stimuli including staurosporine ,TNF-α, etoposide, granzyme B/perforin, and UV light (Fan *et al.*, 1998, Fischer *et al.*, 2004a). Chlamydiae, on the other hand, presumably secrete protein(s) that results in blocking the release of cytochrome *c* from the mitochondria and inhibition of the activation of caspase-3 (Byrne *et al.*, 2004). This implied that there was a block in the mitochondrial permeabilization, which in turn is controlled by Bax and Bak. Later studies showed that the chlamydial-infected cells failed to achieve activation of these regulators of mitochondrial permeabilization (Fischer *et al.*, 2004b, Xiao *et al.*, 2004). Redox reactions are the central to life and death decision of cells and pathogens and balancing act have been played by iron. There is an intimate relationship between oxidative stress and iron metabolism. Iron is both an essential cofactor and a potentially hazardous metal participating in the production of ROS (Pantopoulos *et al.*, 1996). Lack of iron regulation may impose

oxidative stress upon cells, and many microorganisms have evolved systems that couples control of iron homeostasis to protection against ROS (Ricci *et al.*, 2002).

Iron homeostasis of cells has been regulated post-transcriptionally by binding of iron regulatory protein (IRP) 1/2 to iron response element (IRE). In iron-starved cells, IRE/IRP interaction stabilizes transferrin receptor (TfR) mRNA and inhibit translation of mRNAs encoding ferritin heavy (H) and light (L) chains, thereby promoting cellular iron uptake and preventing iron sequestration (Hentze *et al.*, 2004). Virtually all cells and organisms utilize iron as a cofactor in a multitude of biochemical activities. Chlamydiales are an evolutionary distinct group of human pathogenic bacteria and chlamydia-related symbionts sharing a biphasic developmental cycle in which the intracellular phase tethers the pathogen to its specific host (Horn *et al.*, 2004). *CT* and *CP* which are known pathogenic species for humans, take advantage of metabolic and biosynthetic pathways of infected host cells (Belland *et al.*, 2003a, Fischer *et al.*, 2001). Deprivation of certain amino acids or glucose prevents the implementation of replicative chlamydial infection and may result in chlamydial persistence, characterized by reduced infectivity and metabolism (Harper *et al.*, 2000, Nelson *et al.*, 2005). The biphasic life cycle of chlamydia provides various opportunities for cross-communication with host regulatory network. Cells sequester iron to avoid iron acquisition by invading organisms by reducing expression of principal iron uptake protein transferrin receptor-1 (TfR1) (Jabado *et al.*, 2000), increasing synthesis of iron storage protein ferritin (Lieu *et al.*, 2001) or by increasing expression of iron release gene ferroportin (Dill *et al.*, 2007). The Cells also need to protect iron from intracellular pathogens to maintain their own homeostasis. However, smarter pathogens always find the way to modulate host system for their own favour like *Mycobaterium tuberculosis* take control of IRPs (Banerjee *et al.*, 2007)

and *CP* over HIF-1 (Rupp *et al.*, 2007). Mechanism employed by chlamydia to acquire and transport iron is still in infancy and its genome analysis has shown the essentiality of iron for survival. Annotated genome shows the presence of iron transporter ExbB/D analog, but identifiable Ton B is absent (Stephens *et al.*, 1998). Further studies illustrated close association of Tf – containing endosome and early chlamydial inclusion. It is established that iron is essential for chlamydial growth and viability. Intracellular developmental cycle of chlamydia meets its iron requirement from host resources but how, it is yet not clear. *CT* may obtain its required iron from ferritin, the large macromolecular protein complexes used by eukaryote cells to store iron and protect itself from rampant production of oxygen radicals (Raulston, 2006).

Cellular iron availability alters the proliferation and activation of immune cells, and is able to modulate immune effector pathways and cytokine activities (Weiss *et al.*, 1995). Moreover, iron is directly involved in cytotoxic immune defense mechanisms, where iron is needed to catalyze the formation of the hydroxyl radical (OH^{o}) via Fenton reaction (Gray *et al.*, 2001). During microbial infection, competition for iron between the host and microorganisms is therefore inevitable. Given the potential physiological importance of iron in immune function, iron chelators have been implicated to modulate certain inflammatory mediators and regulate inflammatory processes (Marx *et al.*, 2002, Choi *et al.*, 2004). It is reported that patients receiving higher doses of IL-10 developed anemia and presented with a dose-dependent increase in ferritin and soluble TfR levels, an indicator of iron restriction to erythroid progenitor cells (Gray *et al.*, 2002). Hyper-ferritinemia results from direct stimulation of ferritin translation by IL-10 in activated monocytic cells, most likely by cytokine-mediated reduction of the binding affinity of translational repressor, iron-regulatory

proteins, to the 5' untranslated region of ferritin mRNA (Weiss *et al.*, 1995). In addition, the metal also down-regulates cellular immune effector mechanisms by inhibiting the activity of interferon-γ (IFN-γ) toward macrophages (Weiss *et al.*, 1992). Macrophages that have been loaded with iron lose their ability to kill intracellular pathogens such as *CT, Legionella pneumophila*, *Ehrlichia chaffeensis,* or *Listeria monocytogenes* by IFN-γ mediated pathway (Alford *et al.*, 1991, Byrd *et al.*, 1991, Krausse-Opatz *et al.*, 2009)

Bacterial iron chelator (desferal, deferoxamine mesylate) triggers inflammatory signals, including the production of CXC chemokine IL-8 in human intestinal epithelial cells (IECs) by activating ERK1/2 and p38 kinase pathways (Choi *et al.*, 2004). Chlamydia enters into persistence stage in presence of iron-chelating drug (Desferal), thereby showing its dependence on iron for completion of developmental cycle (Dill *et al.*, 2007). Persistence can also be induced by antibiosis and tryptophan starvation induced by penicillin G and IFN-γ respectively. Earlier model of *CP* persistence showed that after IFN-γ and penicillin treatment chlamydia-induced IL-8 expression was inhibited, while it stayed up regulated in iron-depletion (Peters *et al.*, 2005).

Gap in existing research:

Existing research have demonstrated that chlamydia are heavily dependent on iron to complete its developmental cycle and disseminate infection. Chlamydial persistence is phenomenal on iron deprivation through certain iron chelator and persistently infected cells have longer viability. These studies thereby indirectly indicate towards association of iron, persistence and cell death. Further pathology of chlamydia is not direct and it is conferred to immunological reaction generated due to chronic infection.

Earlier studies showed the importance of iron in establishment, maintenance and immuno-pathology of CT infection. However, lacunae exist for explaining role of iron in avoidance of apoptosis, prerequisite for completion of chlamydial developmental cycle. Intracellular developmental cycle of chlamydia is dependent on host for nutritional requirements and iron is the one of most vital component for host as well as chlamydia. How Chlamydiae regulate the iron homeostasis in cells for survival is not clear? Pathological consequences in chlamydial infection are mainly dependent on inflammatory reactions and these reactions are modulated by iron at greater extent. However, there is no study till date regarding the modulatory effect of iron on inflammation and the effect of anti and proinflammatory cytokines on regulatory mechanism of iron in chlamydial infected cells.

Chapter 2
Review of Literature

Scientific thought, then, is not momentary; it is not a static instance; it is a process.
Jean Piaget

Chapter 2

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).

1. History, taxonomy and evolution

1907 in Java, Indonesia, on a research expedition to find the causative agent of syphilis, the German radiologist Ludwig Halberst Adter and the Austrian zoologist Stanislaus Von Prowazek discovered a conspicuous agent they considered responsible for trachoma, which was a global disease (Haferkamp *et al.*, 2006). They found irregularly blue-stained inclusions with small, dense particles in giemsa-stained conjunctival epithelial cells of trachoma patients, which they called "Chlamydozoa" (from the Greek word χλαμυσ, meaning mantle or cloak) (Byrne, 2003). It was originally considered neither protozoa nor bacteria and then regarded as viruses. In the 1960s they were recognized as bacteria later, these unique microorganisms were found to be among the most important bacterial pathogens of humankind (Moulder, 1966b). Halberst adter's and Prowazek's chlamydozoa are now called

Chlamydia trachomatis, and it is the most prominent representative of a small group of closely related bacteria, the chlamydiae (Horn *et al.*, 2004).

Chlamydial taxonomy evolves with the evolution of scientific techniques and computing ability. The availability of cell culture techniques and electron microscopy unequivocally demonstrated the bacterial nature of chlamydiae (Moulder, 1966a). Subsequently the genus *Chlamydia* was established and divided into two species, *Chlamydia trachomatis* and *Chlamydia psittaci* (Page, 1966). Earlier ocular isolates of *C. psittaci* taken from diseased human later proved to be common in human respiratory disease. Later, these organisms were designated as *Chlamydia pneumoniae* (Grayston *et al.*, 1990). Similarly *Chlamydia pecorum* was established as a group of ruminant-infecting *C. psittaci* isolates that had been distinguished from other *C. psittaci* strains by bio-typing and immuno-typing (Perez-Martinez *et al.*, 1985).

2. Chlamydial Genomes: Divergence and without Difference

The research in chlamydial arena has been drove deep within a research framework immersed in genomics. There are complete genome sequences representing the entire range of chlamydial organisms providing unprecedented depth and breadth for comparison (Rockey *et al.*, 2000). Phylogenetic analyses based on 16S rRNA gene it was proposed to assign chlamydial strains in the single genus, Chlamydia, to two genera, Chlamydia and Chlamydophila (Everett *et al.*, 1999). However, protein distribution based on complete chlamydial genome sequences, the distribution of proteins was not consistent with this early separation (Gupta *et al.*, 2006). Nevertheless, with several exceptions, they are all > 97% similar, which is typically used for bacterial speciation, not genus-level demarcation. Because the separation of strains was not robust and lacked other consistent biological

markers, the proposal was rejected by many scientific communities (Schachter, 2001). The deception against bifurcated clustering of chlamydial species by 16S rRNA gene sequence comparison is apparent. However, this is merely a biased outcome of their early separation in geologic time, their isolation in geographic host islands, and the evolutionary constraints imposed by the chlamydial developmental cycle. Genomic comparison reveals the true evolution of chlamydiae as they began their separation into different hosts 60–100 million years ago (Stephens *et al.*, 2009). It is confirmed now chlamydia have one genus;Chlamydia and nine species (Draghi *et al.*, 2007). Thereby showing that spectrum of divergence among Chlamydiae is without generic difference.

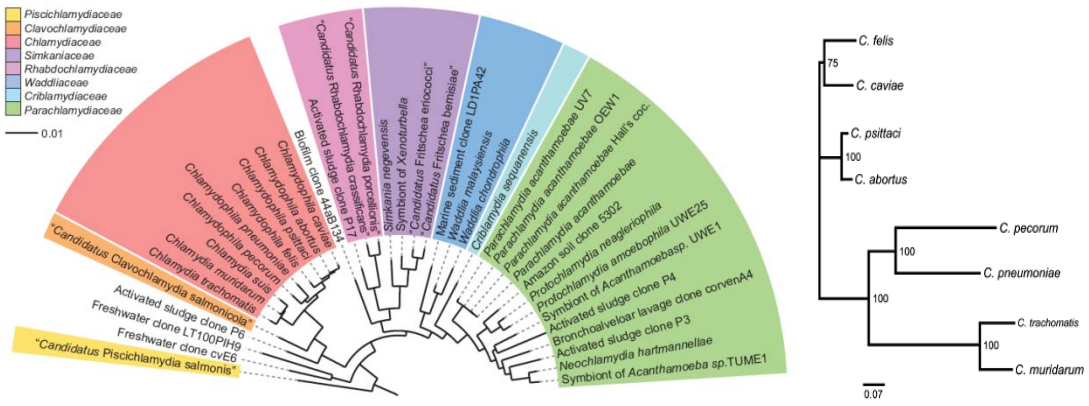


Fig 2.1: Divergence of chlamydiae.

3. Molecular Biology:

Intracellular niche of chlamydia prevented genetic evolution in time and manipulation by researchers. Intracellular lifestyle in turn has complicated the functional characterisation of the molecular biology of chlamydiae. The constraints are underlying to understand and decide effector in molecular mechanism leading to disease pathology of chlamydia encourage the researcher to develop ingenious technique of significance. Present understanding is intuitively guided by comparative genomics and has predicted chlamydial

genes based on sequence homologies between chlamydial genomes and those of other organisms. The Major breakthrough in understanding chlamydial biology come through comparative approach proved its worth. This approach has yielded major, often surprising, insights into chlamydial biology. However a recent development a construct variants by homologous recombination was investigated in *C. psittaci* 6BC (Binet *et al.*, 2009). Similarly, expression studies of the chlamydial transcriptome and proteome under various conditions are of vital importance in the elucidation of the molecular mechanisms underlying chlamydial pathogenesis.

4. Chlamydia : Host and Diseases

Ubiquitous pathogen Chlamydiae are widely distributed in the animal kingdom (Table), affecting both invertebrates and vertebrates (Horn, 2008). In mammals, these infections is responsible for a wide array of clinical diseases, including abortion, pneumonia, conjunctivitis, encephalomyelitis and polyarthritis (Debattista *et al.*, 2003, Brunham *et al.*, 2009). Severe and systemic Chlamydial infections are apparent in animals but can also be clinically unapparent (Coonrod, 2002).

Chlamydial infections in human shows diverse range of complications from acute, readily resolved infections to chronic, long-term infections that evade immune clearance while resulting in inflammation and scarring. *CT* has been grouped into 21 serovars responsible for the myriad of human disease syndromes that result from infection. Serovars A to C are the agents of trachoma leading cause of preventable blindness (Grayston *et al.*, 1985). It has been estimated that about 500 million people have had the disease. In the developing countries, about 7 to 9 million people are estimated to be blind because of *CT* infection [WHO, 2001]. Serovars D to K can also infect the eyes, causing neonatal conjunctivitis, but are better known as the world's most common sexually transmitted bacterial infections (Gerbase *et al.*, 1998).

According to the World Health Organization, 92 million new cases occur each year worldwide [WHO, 2001]. In India, the occurrence of *CT* serovar D, E, F, G and I in infected women has been reported (Mittal, 1998, Singh *et al.*, 2003).

Women suffer disproportionately from the effects of unresolved genital infection, which often ascends to the upper genital tract and can result in ectopic pregnancy or tubal infertility (Rastogi *et al.*, 2003, Cates *et al.*, 1991). The serovars D and E are the predominant *CT* serovars in urogenital infections (Mittal, 1998). Symptomatic genital tract infections in women have been suggested to be related to the chlamydial serovar G, whereas asymptomatic infections are often caused by the chlamydial serovars D and F (Lan *et al.*, 1995). Serovar E has been found in both symptomatic and asymptomatic women. Furthermore, it has also been shown that almost all patients with repeated chronic or recurrent *CT* infections are infected with uncommon complex C serovars (Dean *et al.*, 2000). Genital *CT*, in particular serovar K, can also disseminate to the joints and has been found in association with reactive arthritis (Taylor-Robinson *et al.*, 1992). The clinical significance of *CT* in non-gonococcal urethritis (NGU) and accessory sexual gland infection in men has been established during the past two decades (Paavonen *et al.*, 1999). NGU is the most common clinical genital syndrome seen in males, and *CT* is the most important etiological agent in it. Urethral *C trachomatis* infection has been found in 18- 24.7 % symptomatic male NGU patients in India (Vats *et al.*, 2004). Finally, serovars L1 to L3 cause lymphogranuloma venereum (LGV), a relatively rare but particularly severe sexually transmitted infection characterised by invasion of lymphatic tissue and systemic spread (Schachter *et al.*, 1983). Respiratory infection with *C. pneumoniae* is ubiquitous (Thom *et al.*, 1994). Estimates of the proportion of acute episodes of respiratory disease caused by *C. pneumoniae* have been as high as 25 percent (Jha *et al.*, 2007).

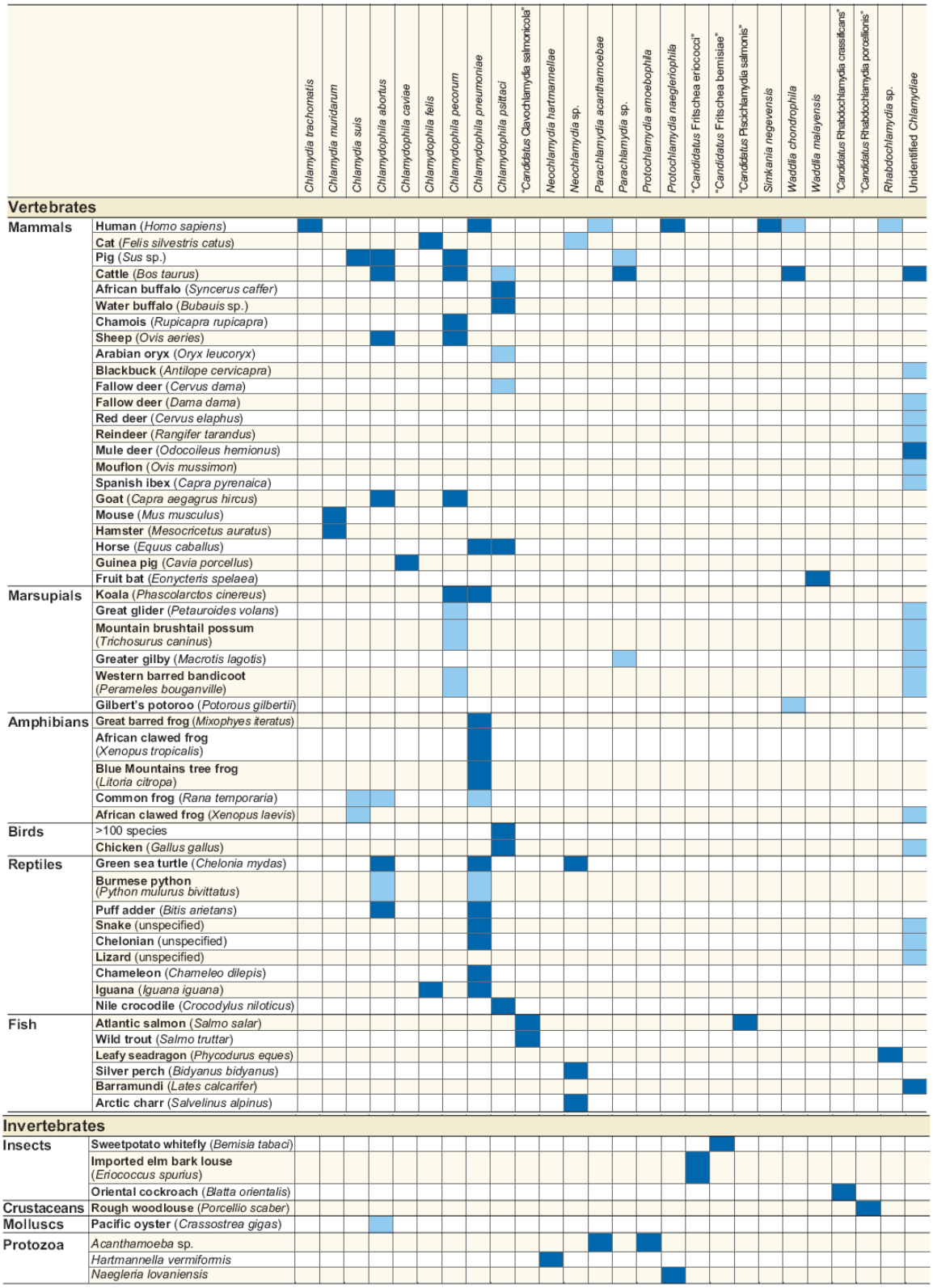


Table 2.1: Host and diseases of Chlamydiae.

5. Chlamydial developmental cycle

Chlamydiae are solely oblique on host to complete its intracellular biphasic developmental cycle. Developmental cycle of chlamydia is embedded with different kind of stealth, intricate and active strategies to cope the adverse conditions posed by host and environment. Discovering the traversal of chlamydiae through host in alternating form attracted the researcher since its emergence as pathogen.

5.1 History

In 1932, Bedson and Bland in a light microscopic study described the developmental cycle of *C. psittaci* (Barwell *et al.*, 1971). It had put the base for the future studies employing light and electron microscopy revealing similar developmental changes for *CT* (Kramer *et al.*, 1971, Higashi, 1964). In latter studies using fast growing LGV and TRIC from the infected human conjunctiva and genital tract has been described in various cell culture systems (Gordon *et al.*, 1969, Richmond, 1976).

5.2 Entry

Complex infectious cycle of Chlamydiae starts with attachment of a metabolically inactive 'spore-like' form [elementary body (EB)] of the bacteria, to the surface of epithelial cells (Kumar *et al.*, 2008). An initial electrostatic-mediated cell association induces the localized activation of the Rho-GTPase Rac1 and is followed by irreversible secondary binding, during which actin is rapidly recruited to the site of EB resulting in filamentous actin reorganization (Carabeo *et al.*, 2004). These signaling cascades translate in to transient microvillar reorganization and the formation of pedestal-like structures beneath the attached EB and culminate in EBs internalization (Kumar *et al.*, 2006). RacGTPase facilitate the activation of Arp2/3 complex by recruiting WAVE2 and Abi-1 for actin reorganization. However this

process is augmented by at least one chlamydial effector protein translocated actin recruiting phosphoprotein Tarp (Jewett *et al.*, 2006). Tarp is present in all pathogenic strains of Chlamydia, is translocated early in the invasion process, and plays a key role in actin recruitment and remodeling via modulation of cytoskeletal signaling (Dautry-Varsat *et al.*, 2004). In spite of modern development it is likely that Tarp represents only stepping stone towards unraveling the chlamydial entry conundrum and that additional bacterial and host factors are involved in modulation of the host cytoskeleton to facilitate chlamydial entry. Indeed, it is likely that multiple pathways are equally capable of promoting chlamydial entry. For example, host receptor-mediated initiation of actin recruitment subsequent to chlamydial attachment represents one alternative (Derre *et al.*, 2007). Given the absolute dependence of chlamydial development on gaining entry it is not surprising that redundant mechanisms may exist.

5.3 Establishment of Niche:

As the process termed as 'parasite-specific phagocytosis' completed intracellular growth of Chlamydia spp. is initiated by differentiation of EBs into the metabolically active reticulate bodies (RB) (Byrne *et al.*, 1978). Phagocytic vacuole containing RBs bypasses normal endosomal maturation pathways and generates a membrane-bound parasitophorous vacuole termed an 'inclusion'.The plasma membrane markers are shed from the nascent inclusion within 30 min after entry, however inclusion intimately associates with recycling endosomes and recruits the minus-end-directed motor dynein to migrate along microtubules to the Microtubule Organizing Center (MTOC) (Grieshaber *et al.*, 2003).

Chlamydial infective units EBs are approximately 300 nm in diameter and its surface is ornamented with hexagonally arranged projections. These ''supramolecular structures''

extend approximately 30 nm from the EB surface and have a rotational symmetry corresponding to a 9-subunit composition (Abdelrahman *et al.*, 2005). Earlier it has speculated that these spike-like projections correspond to Type III secretion system (TTSS) ‘‘needle’’ structures, similar to those seen in *Salmonella enterica* serovar typhimurium. This may play a role in invasion of the host cell (Bavoil *et al.*, 1998). The chlamydial outer membrane complex (OMC) consists of dominant structural molecule major outer membrane protein (MOMP) (Caldwell *et al.*, 1981). Further, In EBs, OMC strengthens by highly cysteine-rich proteins (CRPs) that are tightly locked together by disulfide bonds (Hatch *et al.*, 1984). The DNA within EB is supercoiled and it is maintained by histone-like DNA-binding proteins, Hc1 and Hc2, thereby inhibiting transcription (Hackstadt *et al.*, 1991). Within 2 h following internalization, EB begin to convert to larger (1000nm), metabolically active reticulate bodies (RB). The highly expressed early upstream open reading frame (EUO) gene product help in chromosome dispersal by degrading Hc1 and contributes to OMC relaxation by repressing transcription of the genes encoding CRPs (Kaul *et al.*, 1997). Chlamydial histone–DNA interactions are disrupted upon germination by a small metabolite in the non-mevalonate pathway (MEP) pathway of isoprenoid biosynthesis. The metabolite is thought to be 2-C-methylerythritol 2,4-cyclodiphosphate and is involved in functional antagonism of HctA (Belland *et al.*, 2003b). The RB multiply by binary fission, localising to the inclusion membrane, which itself expands. The predicted TTS projections protrude through the inclusion membrane and are therefore likely to inject virulence-related effector proteins into the host cell cytoplasm (Hueck, 1998). After 8 to 12 rounds of multiplication, RB reverts asynchronously to EB, disengaging from the inclusion membrane. Expression of Hc1, Hc2 and the CRPs is specific to these late stages of the developmental cycle. At 30 to 84 hour

post infection (hpi), depending primarily on the infecting species, infectious EB progeny are released from the host cell to initiate another cycle (Wolf *et al.*, 2000).

5.4 Exit:

In spite of significant advances that have been made for understanding the unique strategies used by bacteria to invade cells, however little is known for exit mechanisms. The specific pathways of pathogen exit are of immense importance to understand microbial pathogenesis because of its intimate association with dissemination, transmission and inflammation (Hybiske *et al.*, 2007). Exit of pathogens is an organized and directed process mediated by both bacterial and cellular factors. This is best exemplified by *Shigella* and *Listeria*, which promote their escape from phagosomes through the action of pore-forming cytolysins (Lianou *et al.*, 2007, High *et al.*, 1992). Cellular release then occurs as these bacteria use actin polymerization to protrude out of the cells, although additional unknown mechanisms are likely involved. In contrast, it has been found that phospholipase activity mediates the release of *Rickettsia prowazekii* from phagosomes (Winkler *et al.*, 1989), and a hemolysin is responsible for the phagosomal exit of *Trypanosoma cruzi* (Rosenthal, 2004). However, little information exists concerning the mechanisms that mediate the release of chlamydia from either the inclusion or cell. As for most pathogens, it has assumed that chlamydia are released by lysing their host cell , although it has also been proposed that chlamydia may exit by exocytosis or apoptotic pathways (Todd *et al.*, 1975, Hackstadt *et al.*, 1996). It have been shown that chlamydia release occurred by two mutually exclusive pathways. The first is lysis pathway consisted of an ordered sequence of membrane permeabilizations: inclusion, nucleus and plasma membrane rupture. The second release pathway- packaged release mechanism, called as extrusion (Hybiske *et al.*, 2007). Chlamydia release is a consequence of

a biological interaction between the inclusion and cell established late in the developmental cycle and is not due to physical stress imposed on the cell simply by a massive inclusion (Clifton *et al.*, 2004). In addition, very late (30 h after infection) gene expression patterns have been reported, thus supporting the view that a late-expressed chlamydial protein could induce release (Nicholson *et al.*, 2003). The dual strategies used by intracellular bacteria to escape cells are poorly understood. However, Chlamydia induced lysis and its regulation by cysteine proteases are analogous to what has been described for the exit of *Plasmodiam falciparum* from erythrocytes (Rosenthal, 2004). Given the disparity between organisms, this important similarity suggests that protease mediated membrane lysis might be a fundamental strategy used by intracellular pathogens. In contrast, the extrusion phenomenon characterized for *Chlamydia* represents an unusual escape mechanism for intracellular bacteria.

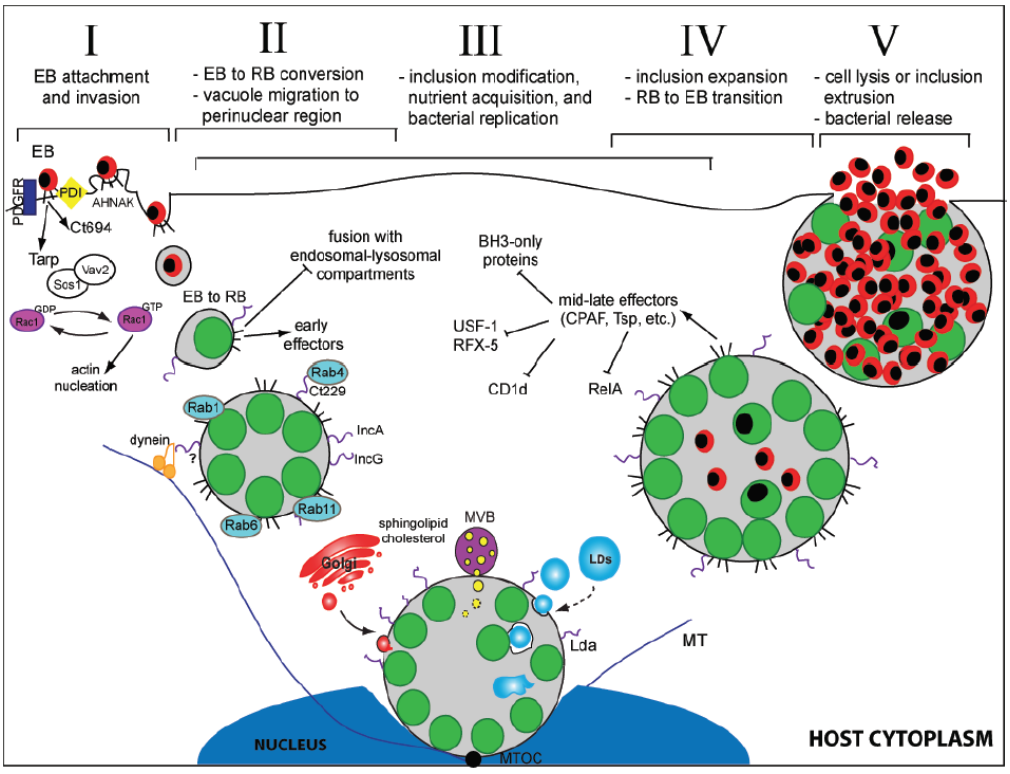


Fig 2.2: Chlamydial developmental cycle: Molecular mechanism of entry, multiplication and exit.

6. Cell Biology of *Chlamydia trachomatis*: Modulation of cell death

Chlamydiae are most evolved pathogen in disguising host system to gain advantage for their survival(Ying *et al.*, 2007). The molecular mechanisms by which chlamydiae manipulate the mammalian host are difficult to explore because they are inflexible to genetic manipulation. However, large set of chlamydial effector proteins have been revealed due to emergence of heterologous expression systems. These effector proteins translocated into the host cytoplasm and are able to manipulate mammalian cellular processes including the cell cycle, innate immunity, and lipid and membrane transport (Fischer *et al.*, 2004a, Fischer *et al.*, 2001, Miyairi *et al.*, 2007, Ying *et al.*, 2006).

Chlamydiae have evolved a pathogenic strategy to promote their survival by modulation of programmed cell death pathways of infected host cells. The chlamydiae determine the live and death of host cell according to circumstances (Byrne *et al.*, 2004). This subtle pathogenic mechanism highlights the manner in which these highly successful pathogens take control of infected cells to promote their own survival — even under the most adverse circumstances.

Apoptosis is mainly induced through two pathways – the extrinsic or the death receptor pathway and the intrinsic or the mitochondrial pathway. A third route involves cytotoxic T-cell-mediated apoptosis initiated by perforin/granzyme. Effectors in the different pathways of apoptosis are proteases of the caspase (cysteine-dependent aspartate-specific proteases) family (Hengartner, 2000). Mitochondria play a central role in apoptosis regulation. Besides being essential for the intrinsic pathway, mitochondria are also involved in apoptosis induction by the extrinsic pathway in the so-called 'Type II cells,' which require signal amplification by the mitochondrial pathway (Neise *et al.*, 2008). The mitochondrial membrane becomes permeabilized, apoptogenic factors – cytochrome c, Smac and AIF – are

released into the cytosol, where they can initiate the caspase activation cascade (Lum *et al.*, 2003). Mitochondrial membrane permeabilization is under the control of members of the Bcl-2 family of proteins, characterized by the presence of at least one of the four conserved Bcl-2 homology domains, BH 1-4 (Cory *et al.*, 2003). A subset of this family, the BH3-only proteins, acts as a damage sensor in the cells, becoming activated in response to specific apoptotic stimuli (Strasser *et al.*, 2000). Activated BH 3-only proteins can activate the proapoptotic Bax and/or Bak, eventually leading to mitochondrial membrane permeabilization (Adams *et al.*, 2001).

Early in infection, CT prevents pro-apoptotic phosphorylated BAD and atypical PKC-delta from binding to mitochondria by sequestering their binding partners 14-3-3 proteins and diacylglycerols respectively (Levites *et al.*, 2002). At least one chlamydial protein, CADD, can induce cell death when ectopically expressed in mammalian cells by interacting with death domains of TNF family of receptors (Stenner-Liewen *et al.*, 2002), but the significance of this association is unknown. Later in infection, the secreted chlamydial protease CPAF degrades BH3-only domain pro-apoptotic protein, ensuring a complete shutdown of the infected cell's ability to undergo apoptosis in response to intrinsic and extrinsic stimuli (Paschen *et al.*, 2008). CPAF further disables adaptive immune responses by degrading factors required for MHC expression (RFX-5 and USF-1) and lipid antigen presentation (CD1d) (Dong *et al.*, 2004, Kawana *et al.*, 2007, Valdivia, 2008). During early stages of infection, chlamydial infection leads to a MAPK dependent increase of the anti-apoptotic proteins Mcl-1 and cIAP-2. Mcl-1 upregulation inhibits mitochondrial outer membrane permeabilization, thus blocking the release of Smac and cytochrome c into the cytosol (Sharma *et al.*, 2009). Upregulation of cIAP-2 leads to the stabilization of the IAP–IAP

complex, which would directly block the activation of caspase-3. The role of Mcl-1 in sustaining bacterial infection has also been shown for Helicobacter pylori infections, indicating that the activation of MAPK pathways for Mcl-1 upregulation could be a more common strategy for resisting host apoptotic pathways during bacterial infection (Mimuro *et al.*, 2007).

In addition to the modulation of apoptosis by productive infections, persistent chlamydia are seem to prolong the inhibition of apoptosis in their host cells (Dean *et al.*, 2001). Another mechanism which may help to perpetuate chronic chlamydial infection *in vivo* is the induction of T cell apoptosis by tumor necrosis factor alpha (TNF-) derived from *CT* infected macrophages (Jendro *et al.*, 2004).

Finally, Chlamydial infections significantly impact the cell cycle of infected cells, with evidence for cleavage of the mitotic cyclin B1 (Balsara *et al.*, 2006), delays in cytokinesis and centrosome supernumeracy (Johnson *et al.*, 2009). Interestingly, all these later functions can lead to genomic instability, which in conjunction with the strong anti-apoptotic effect of chlamydial infection may explain the epidemiological association between CT infections and cervical cancers (Gopalkrishna *et al.*, 2000).

7. Immunobiology of *Chlamydia trachomatis*:

Endosymbiont chlamydiae are inheriting the potential to escape or divert the immunological response and causing the immunopathological changes to bystander periphery leading to various disease syndromes. *CT* is an obligate intracellular bacterium that causes several sexually transmitted diseases in humans. *CT* normally infects the single-cell columnar layer of the epithelium in the endocervix of women and the urethra of men (Ward, 1995). At the site of mucosal infection, intense inflammation that is characterized by redness, oedema and

discharge can occur, resulting in the clinical syndrome of mucopurulent cervicitis in women and non-gonococcal urethritis in men (Hafner *et al.*, 2008). However, despite initiating local inflammation, *CT* infection remains subclinical in a high proportion of infected individuals (70–90% of women and 30–50% of men). Symptomatically infected women show signs of disease: in general, mucopurulent endocervical discharge hypertrophic cervical ectopy and friability. Clinical symptoms include dysuria, abnormal vaginal discharge, abnormal menstrual bleeding, postcoital bleeding and lower abdominal pain (Mascellino *et al.*, 2008). In some untreated women (20–40%), infection ascends the endometrial epithelium to the fallopian tubes, where *CT* can establish persistent infection and cause PID (Cohen *et al.*, 2005, Agrawal *et al.*, 2009b). The protective immune response to infection with *Chlamydia* spp. is highly dynamic and involves both innate and adaptive immune responses. Infection of mice with *C. muridarum* has shown that CD4+ T cells, and possibly CD8+ T cells, producing IFN-γ, as well as B cells, are required to clear infection and to prevent re-infection. However, immune responses that are associated with persistent infection with *CT* seem to induce pathology as a result of chronic inflammation and tissue damage (Morrison *et al.*, 2000). So, a fine balance between protective immunity and immune-associated disease pathogenesis characterizes the host response to infection with *CT*.

7.1 Adaptive immunity

Disease outcome in chlamydial infection is dictated by the immune responses posed by host. In human ocular *CT* infection, local secretory IgA anti-chlamydial antibody responses are associated with limited protection (Bailey *et al.*, 1993). However, individuals who mount a T-helper 2 (Th2), antibody-dominated response are more likely to develop severe trachomatous scarring than those who mount an effective T-helper 1 (Th1), cytotoxic T

lymphocyte response (Holland *et al.*, 1996). Moore et al reported in an *in vitro* experiment that anti-chlamydial antibodies increases the rate of Th1 activation by FcR + / + but not FcR − / − antigen-presenting cells and it provide a mechanistic basis for the need for both T-cell and humoral immune responses in protective immunity to chlamydial reinfection (Moore *et al.*, 2003). These finding reveals the major role for antibodies in chlamydial immunity in the enhancement of Th1 activation via FcR-mediated processes involving DCs (Bilenki *et al.*, 2006). Antibodies are induced and present in the genital tract secretions and serum following *CT* infection, and the immuno-protective role of antibody-mediated immunity has been well reported (and debated) in the literature (Beagley *et al.*, 2009). Antibody has been shown to contribute to a protective response against genital tract reinfection with *CT.* Indeed, antibody has demonstrated many beneficial effects against many infectious agents, including chlamydia , in which CMI would be assumed to represent the key protective mechanism (Igietseme *et al.*, 2004). However, owing to the many chlamydial challenge doses that are used by different investigators, the exact role of antibody in protection against chlamydial infection is difficult to assess.

The protective CMI response is strongly associated with the production of IFN- γ either by CD4 + or by CD8 + T cells (Igietseme, 1996). Infected DCs efficiently present chlamydial antigens to CD4 + T cells and expanded *CT* -specific CD8 +T cells (Matyszak *et al.*, 2004, Agrawal *et al.*, 2009b). The form of chlamydia initially encountered by DCs and the hormonal status at the time of this encounter may influence the type of adaptive immune response (protective vs. inflammatory) that is elicited by infection (Gervassi *et al.*, 2004, Gray *et al.*, 2009, Agrawal *et al.*, 2009c). Indeed, most recent evidence from gene knock-out animal models indicates that the Th1 response and strong production of gamma interferon

(IFN-γ), the major Th1 effector is critical for the eradication of established intracellular infection (Joyee *et al.*, 2008). An increase in the endocervical production of the Th1-associated cytokine IL-12 and a decrease in IL-2 concentrations in endocervical secretions coincided closely with genital *CT* infection (Johansson *et al.*, 1997). Decreased local T-cell response and IFN- γ production may promote latent *CT* infection and inflammation or immunopathology. It has recently been hypothesized that IL-12 acts with other host factors to influence the recruitment of effector T lymphocytes to the infected female reproductive tract (Agrawal *et al.*, 2009a, Gupta *et al.*, 2009). Therefore, host genotypes contributing to the types of immune response that prevail are likely to play important role in disease outcome. Another possible factor influencing the outcome of a chlamydial infection is the potential for self-induced damage arising from chlamydial antigens that are similar to, or mimic, host components (Bas *et al.*, 2003). Various chlamydial antigens have been implicated in the hypersensitivity response, most notably chlamydial heat shock protein 60 (cHSP60), which is a stress response chaperone (Morrison *et al.*, 1989, Jha *et al.*, 2009). Many studies have shown an association between antibodies to cHSP60 and the chronic sequelae of chlamydial infection (Bachmaier *et al.*, 2005). However, a role for autoimmune responses in the pathogenesis of chlamydial disease remains to be proven. Antibody responses to *Chlamydia*-specific or human-reactive components on cHSP60 might simply arise by chance as a result of long-term exposure to chlamydial infection (Stephens, 2003). Innate immune responses were proved to be key determinant of disease immunopathology in CT infection.

7.2 Innate Immunity:

The innate immune system has evolved to recognize molecular patterns (rather than antigens) on microbial invaders that are not normally found on the host. It relies on receptors that

recognize conserved pathogen-associated molecular patterns found in certain microorganisms (Bauer *et al.*, 2009). Toll Like Receptors (TLR) are one group of pattern recognition receptors that are expressed on macrophages, dendritic cells (DCs), neutrophils, NK cells, and epithelial cells. TLR2 has been identified as the principal TLR responsible for the secretion of Interleukin (IL)-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) in a MyD88-dependent manner in *Chlamydia muridarum* –infected cloned murine oviduct epithelial cell lines (Darville *et al.*, 2003). TLR2 and MyD88 are colocalized within the chlamydial inclusion and that expression of TLR2 was required for IL-8 secretion from infected cells (O'Connell *et al.*, 2006).

Establishment of infection is depending heavily on availability of key nutritional recourses, thereby host tends to limit these recourses to restrict advancement of pathogens, and this hypothesis is popularly known as 'Nutritional Immunity Hypothesis.'

7.4 Nutritional Immunity

The host cell by necessity must contain all of the nutrients required for its own metabolism; however, these stores also are a rich source for bacterial pathogens to exploit. Thus, another major strategy for innate resistance to intracellular infection is to sequester key nutrients such as iron from the invading bacteria. Iron is essential for many vital processes of the host and bacterium, including metabolism and protection from oxidative stress. Although iron is present in host cells, it is predominantly bound to proteins and is virtually unavailable in free soluble form.

Upon phagocytosis of a bacterium, iron can be removed from the phagosome via multiple mechanisms. First, ferroportin, Nramp1 and Nramp2 (DMT1) act as iron transporters to move ferrous iron from the lumen of the phagosome into the cytosol (Chlosta *et al.*, 2006).

Nramp1 also acts as a manganese efflux pump; both manganese and iron can be used as co-ordinating metal centres for bacterial proteins involved in protection against oxidative stress such as superoxide dismutase (Jabado *et al.*, 2000). Second, expression of transferrin receptor, a high affinity receptor for iron that allows iron uptake from plasma, is downregulated in response to IFN-γ which decreases the amount of iron in the phagosome (Byrd *et al.*, 1993). *Coxiella burnetii* overcomes the iron deficit by stimulating upregulation of transferrin receptor (Howe *et al.*, 1999). Third, lactoferrin, an iron chelator found in neutrophil granules, can be taken up into the macrophage endocytic pathway from the extracellular milieu and thereby decrease the concentration of available iron in the phagosome (Byrd *et al.*, 1991). Hepcidin, a small anti microbial peptide which is essential for iron homeostasis during normal host metabolism, also acts to increase iron efflux in macrophages (Peyssonnaux *et al.*, 2006). Both Fe^{2+} and Mn^{2+} are critical for virulence of many pathogens, such as *S. typhimurium* (Boyer *et al.*, 2002). To enable replication, many intracellular bacteria produce high affinity iron chelators, called siderophores, which are able to steal iron from host proteins (Parent *et al.*, 2002, Fischbach *et al.*, 2006). Host cells have in turn evolved innate immune mechanisms to protect iron acquisition by siderophores. Lipocalin-2, which is upregulated by TLR signalling, binds siderophores of the enterobactin family produced by Gram negative bacteria like *S. typhimurium* (Flo *et al.*, 2004). Thus, regulation of iron availability is central in determining the balance of the host–pathogen interaction. Intracellular bacteria must also acquire many other nutrients to promote survival and replication. Global gene expression analysis has revealed that intracellular bacteria upregulate genes characteristic of nutritional stress such as iron, magnesium, glucose, amino acid and phosphate limitation when compared with extracellular counterparts. Further it is

reasonable to speculate that nutrients may be sequestered by the host cell during infection, but few relevant mechanisms have been described (Passalacqua *et al.*, 2007). One mechanism of nutrient deprivation has been well established –tryptophan catabolism by indoleamine 2,3-dioxygenase (IDO). IDO has two functions in innate immunity – a direct role in nutrient deprivation during intracellular infection and a role in negative regulation of T cell activation (Thomas *et al.*, 1993, Beatty *et al.*, 1994, Krausse-Opatz *et al.*, 2009). Upregulation of IDO by IFN-γ restricted growth of *CT*; the bacteriostatic effect was abrogated by inhibition or deficiency of IDO or by addition of excess tryptophan. Interfering with bacterial metabolism is a powerful strategy to restrict bacterial growth. Since it has been established fact that the ability of a microorganism to attach colonize, invade and spread within mammalian hosts depends on the sequestration of iron and other micronutrients at each step of the infectious process; only the most successful will emerged as pathogens.

8. Iron and Chlamydial infection

Study heading towards revelation of intricate relationship of chlamydial pathogenesis and iron is still in infancy. Investigating iron and micronutrients in chlamydial infections represents reasonable avenue for pursuing answers to specific questions on how these agents cause disease. Since the study conducted by Murrey et al, in 1991 on *C. psittaci* shown that there was no change in number of EBs observed (Murray *et al.*, 1991), however alteration in form was reported. Influence of iron deprivation was distinctively observed, when these EBs were used for reinfecting the cell, significant decrease in infectivity observed (Raulston, 1997). This study was further authenticated by other studies showing detrimental effect of iron deprivation and cell type on *CP* and *CT* (Wyllie *et al.*, 2001). In HEp-2 cells CP shows higher sensitivity than LGV to iron deprivation (Al-Younes *et al.*, 2001). According to CDC

first *CT* infection was frequent among 15 and 34 years of age, representing peak reproductive years and abundance of these cases remain asymptomatic or untreated (Schachter, 1998). Underneath these statistics, physiological conditions showing its possible role. Source and concentration of iron in the female reproductive tract fluctuate due to the hormonal influence of estrogen and progesterone throughout menstruation (Cohen, 1987, Andrew *et al.*, 2000). Thus infection of CT in young women remains for longer period. Phase variable hemoglobin receptor on *Neisseria gonorrhoeae* is selected for in vivo only during the first half of the female menstrual cycle strongly supports notion that the female reproductive tract is a dynamic environment for iron sources and concentrations (Anderson, 2001).

Desferroxamine mesylate (DFO) is a pharmaceutical compound that specifically chelates intracellular iron (Robbins *et al.*, 1972) and is frequently used to study host-iron-pathogens studies and have shown its effectiveness against several pathogens. Although there are no significant differences in the actual number of chlamydial inclusions observed, however size appears much smaller and infectivity was lost after sub-passaging on desferal treatment (Raulston, 1997). Infectivity decline in dose dependent manner relative to increasing concentration of desferal and similar response was seen in CP (Al-Younes *et al.*, 2001). Activity of DFO on chlamydia is indirect and by interruption of host metabolic processes. Desferal inhibits the eukryotic ribonucleotide reductase because iron is required for its activity (Cooper *et al.*, 1996). The infectivity potential of CT is resumed after supplementation with iron loaded transferrin, thereby augmenting that desferal act in direct manner by withholding the iron required by the chlamydiae (Raulston, 1997).

Mechanism employed by chlamydia to acquire and transport iron is still in infancy; however, its genome analysis shows the essentiality of iron for survival. Annotated genome shows the

presence of iron transporter ExbB/D analog, but identifiable Ton B is absent (Stephens *et al.*, 1998). Acquisition of radiolabeled iron by chlamydiae from holotransferrin was demonstrated. Further studies illustrated close association of Tf containing endosome and early chlamydial inclusion. Now it is established that iron is essential for chlamydial growth and viability. Intracellular developmental cycle of chlamydia meets its iron requirement from host resources but how yet not clear. *CT* may obtain its required iron from ferritin, the large macromolecular protein complexes used by eukaryote cells to store iron and protect itself from rampant production of oxygen radicals (Raulston *et al.*, 2007). *N. gonorrhoeae* (NG) utilizes host iron complexes by outer membrane receptors. These receptor do not appear to undergoes the phase or/ and antigenic variation events, proving its utilities as vaccine. NG is heavily dependent of tranferrin for its iron requirement and it is transported through Tf-binding proteins (Tbp) (Cornelissen *et al.*, 1998). Unfortunately; there is no obvious Tbp ortholog in chlamydial genome (Stephens *et al.*, 1998). Alternatively, hydrated complex of iron, such as ferric citrate, can be transported across bacterial envelopes. In an anaerobic environment, soluble ferrous iron freely diffuses across the outer membrane of gram-negative bacteria (Koster, 2001). Siderophore receptors and iron- complex receptors on gram- negative bacterial outer membranes require energy from the proton motive force to mobilize iron into the periplasm; this energy is generally supplied through a transmembrane complex composed of TonB, ExbB and ExbD (Cornelissen *et al.*, 1994). Iron is ultimately mobilized across the Cytoplasmic membrane of both Gram- negative and Gram- positive bacteria by ATP- binding cassette (ABC) transporters (Agranoff *et al.*, 1998). Chlamydial HSP60, proteins involved in metabolism, type III secretion system, envelope structure, and cell division, and several hypothetical candidates have emerged in the arena of iron

regulation. Among these Ytg A- a periplasmic metal binding protein of an ABC transporter is emerging as a frontrunner (Dill *et al.*, 2009). Recent studies recognize YtgA as an iron-binding protein that is influenced specifically by iron levels. Secondary structure analysis and the *CT* genome strongly suggests that YtgA is a part of an ABC transporter system due to its location within an ABC transporter operon (Stephens *et al.*, 1998). YtgA preferentially binds iron over other metals, consistent with the specificity of metal transporting ABC systems. YtgA localizes to the periplasm and membrane region of *CT*, consistent with the area of interest for metal acquisition and ABC transporter function (Raulston *et al.*, 2007). In other pathogens Ytg A homologs function in iron uptake pathways in Yersinia (Bearden *et al.*, 1998, Perry *et al.*, 1999, Saken *et al.*, 2000) zinc, manganese and iron transport that is essential for virulence in streptococcus pneumoniae (Brown *et al.*, 2001), iron transport in staphylococcus (Cockayne *et al.*, 1998) and transport of micronutrient in treponema (Weinstock *et al.*, 1998). The chlamydial macromolecular surface structure should also involve in the acquisition of micronutrients. *CT* infection have shown that iron limitation causes (i) a significant decrease in chlamydial infectivity, (ii) a delay in chlamydial development, (iii) the formation of abnormal morphological chlamydial forms, (iv) an increased quantity of membranous blebs within chlamydial inclusions, and (v) quantitative changes in the expression levels of specific chlamydial proteins (Timms *et al.*, 2009). Effect of iron restriction has global effect on chlamydiae and leading to the various reversible changes, indicating towards the consensus regulators.

8.1 Iron Acquisition and Transport: Regulatory Mechanism

Iron is the most essential element due to its bioenergetics chemistry, involved in myriad of biochemical events required for growth and development (Groves, 2003). The reversible Fe

(II)/ Fe (III) redox pair is best suited to catalyze a broad spectrum of redox reactions and to mediate electron chain transfer (Miethke *et al.*, 2007). Furthermore, several transcriptional (e.g., bacterial Fur and PerR) and posttranscriptional (e.g., mammalian iron regulatory proteins [IRPs]) regulators interact with iron to sense its intracellular level or the current status of oxidative stress in order to efficiently control the expression of a broad array of genes involved mainly in iron acquisition or reactive oxygen species (ROS) protection (Hantke, 1983). The cellular uptake of iron is restricted to its physiologically most relevant species, Fe^{2+} (ferrous iron) and Fe^{3+} (ferric iron). Fe (II) is soluble in aqueous solutions at neutral pH and is hence sufficiently available for living cells if the reductive state is maintained. Generally, Fe^{2+} can be taken up by ubiquitous divalent metal transporters. Systems for specific Fe^{2+} uptake are known in bacteria and yeast. However, in most microbial habitats, Fe^{2+} is oxidized to Fe^{3+} either spontaneously by reacting with molecular oxygen or enzymatically during assimilation and circulation in host organisms. In the environment, Fe^{3+} forms ferric oxide hydrate complexes in the presence of oxygen and water at neutral to basic pH. These complexes are very stable, leading to a free Fe^{3+} concentration of 10^{-9} to 10^{-18} M (Kell, 2009). In mammalian hosts, the assimilated iron is tightly bound to various proteins. Hemoproteins such as hemoglobin contain about two-thirds of the body iron in the heme-bound state. Ferritin, the intracellular iron storage protein, is able to store up to 4,500 Fe^{3+} ions per oligomer and contains about 30% of the iron pool (Pantopoulos *et al.*, 1995a). The iron of the circulating exchangeable pool that comprises only several milligrams is bound to transport proteins such as transferrin in the plasma delivering iron into the cells via transferrin receptor-mediated endocytosis or innate defense proteins such as lactoferrin in various body fluids. Both transferrin and lactoferrin contain two Fe^{3+} binding sites per

molecule. This strict iron homeostasis leads to a free serum iron concentration of about 10^{-24} M (Raymond *et al.*, 2003). Thus, a plethora of microorganisms, among them important human and animal pathogens, are severely restricted in iron acquisition. During evolution, this restriction made life advantageous for those microbes that developed skills for highly selective iron uptake, which basically include mechanisms for the utilization of iron sources by either direct or indirect contacts. Direct mechanisms comprise the uptake of various iron sources such as lactoferrin, transferrin, ferritin, heme, and/or hemoproteins (Wandersman *et al.*, 2004).

8.2 Regulation of Iron metabolism in pathogens:

Virtually all the organism has micronutrient-responsive DNA/RNA-binding regulatory elements. These molecules mediates the pathogen response to iron deprivation, or other trace metals (Hantke, 2001), The regulators have also been used as tools to locate and identify proteins, antigens and virulence factors that are produced in response to stress in the microenvironment. A system termed as FURTA, for Fur titration assay was developed to recognize stress response protein following the above lead. Post- chlamydial genome era, each open reading frames (ORFs) was aligned against 13 known iron-dependent repressors and there was no high homology found (Stephens *et al.*, 1998). The five CT ORFs having limited homologies were expressed and shown higher cross reactivity with anti-sera of Fur-like protein (Wyllie *et al.*, 2001). Protein CT296 showed specific binding to a synthetic E. coli Fur Box and is able to functionally compliment Fur activity in an E. coli mutant strain. Protein CT296 was assigned the name ***dcr*** A, for divalent cation-dependent regulator A (Wyllie *et al.*, 2001). Fur- like repressor in other bacterial genera exhibit binding features uniquely tailored for their native chromosomal features (Sebastian *et al.*, 2002). However the

chlamydial Fur-like regulator, DcrA, may be only one of the several such regulatory elements and DcrA may function at different level (transcriptional and post transcriptional) and/or use several cations as co- factors. Despite the small size of the chlamydial genome, it would seem imperative to keep an open perspective for the possibility of more than one "micronutrient-responsive" regulatory element and/or mechanism (Wehrl *et al.*, 2004). Because the chlamydiae are evolutionarily isolated (Stephens, 1998) and DcrA is a considerably distant relative of Fur, substrate recognition will likely show significant differences from the Fur boxes described in E. coli.

For most bacteria, the orchestration of an iron stress response involves regulators related to *E. coli* Fur (ferric uptake regulator) or *C. diphtheriae* DtxR (Diptheria toxin regulator). Fur like proteins are predominantly found in Gram- negative (Hantke, 2001) and DtxR like in Gram- positive bacteria (Tao *et al.*, 1994). Conceptually, the Fur-like and DtxR-like families of proteins both function as negative regulators, but they do not share primary amino acid sequence homology. Growing family of Fur- like regulators includes, for example, Zur, dependent on zinc (Patzer *et al.*, 1998), and PerR, an oxidative stress-response regulator with a high affinity for manganese (Bsat *et al.*, 1998). Traditionally Fur is a 17-18 kDa repressor that, when iron levels are sufficient for the bacterium, forms a dimmer (using iron as a cofactor) and binds to a 19 bp consensus sequence, termed a Fur box (GATAATGATAATCATTATC) (Escolar *et al.*, 1999) and silence the gene expression in iron dependent manner. Fur also contributes to post-transcriptional control of protein expression by the stabilization of specific mRNA species (Dubrac *et al.*, 2002).

8.3 Regulation of iron metabolism in mammalian cells:

More than 15 years ago, elegant studies by Hentze, Rouault, Klausner, and their associates established the existence of RNA motifs called iron responsive elements (IRE) in numerous transcripts of genes involved in iron metabolism and homeostasis. These motifs are bound by iron regulatory proteins 1 and 2 (IRP1 and IRP2) depending on cellular iron levels (Rouault, 2002, Rouault *et al.*, 1997, Hentze, 1996). When these proteins bind to IRE motifs in the 5'-untranslated region of, for example, the ferritin mRNA transcript, translation of the transcript is blocked and synthesis of ferritin is halted. In contrast, when IRP1 and IRP2 bind to the IRE in the 3'-untranslated region of, for example, the transferrin receptor transcript, the transcript is stabilized, translation proceeds, and the transferrin receptor is synthesized. Evolution has generously provided two IRPs, both of which bind to IREs but sense iron in very different ways (Beutler *et al.*, 2004). IRP1 is a bifunctional cytosolic protein that contains an iron-sulfur cluster. In the presence of iron, IRP1 acts as an aconitase (interconverting citrate and isocitrate), but in the absence of iron, IRP1 binds to the IREs of various iron homeostasis transcripts with high affinity. By contrast, IRP2 undergoes iron-dependent degradation in iron-replete cells and therefore is not available to bind to the IREs. But things are a little more complicated than this. IRP2 is also sensitive to degradation in the presence of nitric oxide (NO), whereas IRP1 is activated by NO (Meyron-Holtz *et al.*, 2004). It had been presumed that IRP1 is the principal iron sensor and a major player in iron homeostasis, yet mice deficient in IRP1 appear normal. In contrast, mice deficient in IRP2 show pronounced misregulation of iron metabolism and nerve damage. In the new work, Rouault's group provides some answers. These investigators show that cells from IRP2-deficient mice are unable to regulate iron when cultured in 3 to 6% oxygen (the physiological

oxygen concentration in tissue), but could do so in 21% oxygen because IRP1 operates as an iron sensor only in a high-oxygen environment (Rouault, 2002). Thus, IRP2 is the predominant regulator of iron homeostasis in mammalian cells at physiological oxygen tensions, and IRP1 acts as both an oxygen sensor and an iron sensor. These authors suggest that destabilization of IRP1 when bound to iron also occurs during inflammation through the action of oxygen free radicals. This interaction results in increased expression of the transferrin receptor and decreased expression of ferritin. Although tantalizing, other recent observations suggest that this model does not tell the whole story. A mouse in which the 5'-IRE, a negative regulatory element, is deleted does not manifest an increased iron burden, but surprisingly exhibits a complex phenotype: iron deficiency at birth with elevation of body iron levels only later in life and eventual normalization of iron stores(Hentze *et al.*, 2004). Mice heterozygous for deletion of the 5'- IRE manifest a marked increase in red blood cell production 7 weeks after birth due to increased transcription of the gene encoding the hematopoietic growth factor erythropoietin. These findings imply strong links among the pathways that regulate red blood cell production and iron homeostasis. For example, it would not be surprising if HIF-1α transcription factor that switches on genes including the erythropoietin gene in response to hypoxia— is involved in iron homeostasis (Rivera *et al.*, 2005)(Nemeth, E, 2004).

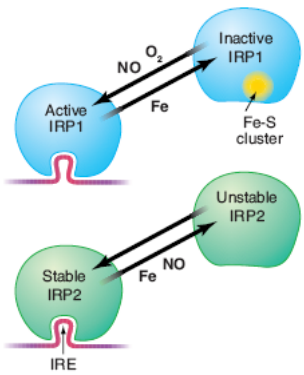


Fig 2.3: IRP-IRE interaction regulates iron homeostasis.

8.5 Host-Pathogen interaction in perspective to iron:

Intriguingly, infection of epithelial cells with *N. meningitides* appears to interfere with eukaryotic IRP activity and leads to destabilization of TfR1 mRNA for reduced expression of TfR1 receptors (Bonnah *et al.*, 2004). In recent study using RNA interference, CT LGV showed strong dependence on iron delivered by Tf to complete the developmental cycle.The mobilization of iron into eukryotic cells involves multiple pathways, but the endocytosis of iron-Tf-receptor complexes is best known and clearly represents a major pathway. Following endocytosis, insoluble ferric iron is reduced to soluble ferrous iron for release into the eukryotic cell cytoplasm; this step involves the activity of a reductase as well as a lowering of the pH during endosomal acidification. Iron does not float free in the cytoplasm; it is either used immediately (Enzymes) or stored within ferritin. Determination of mechanism by which elemental iron is mobilized into cytoplasm of chlamydia constitutes a considerable experimental and intellectual challenge.

Chapter 3
Aims and Objectives

Science, in the very act of solving problems, creates more of them.
Abraham Flexner

Chapter 3

Aim and Objectives

The aim of the study was to decipher the mechanisms involved in the establishment, maintenance and immuno-modulation of *CT* infection under iron stress conditions. The study will give the impetus to understand the pathogenesis of CT under iron stress conditions and help to develop comprehensive regimen to get rid out of chronic chlamydial infection. The specific objectives of the present study have been defined as under.

(1). Establishment of chlamydial infection *in vitro* under iron stress condition

Apoptosis of the host cell constitute a defense mechanism to confine the infection by bacterial pathogens. Chlamydiae have developed elegant mechanisms to modulate the fate of the host cell, which include induction or blockage of apoptosis. In this study the modulatory effect of chlamydia on apoptosis was studied under iron stress condition by examining level of pro and anti-apoptotic proteins, redox status (ROS) of cells, changes in mitochondrial membrane potential and cytochrome 'c' release. Surface expression of iron transport protein was examined to ascertain the role played by iron in cellular and chlamydial viability. The above mentioned parameters were examined by using various techniques including western blotting, flow cytometry, fluorescence microscopy, and colorimetric assays.

(2). Host-chlamydial interaction at interface of iron: *in vitro* regulation of iron acquisition and storage in CT infected cells

The struggle for iron between the pathogen and the host is the key determinant of progression or elimination of disease. Most pathogens evolve various mechanisms to get hold of iron as a result of several constraints made by the host. In this study we examined the modulatory effect of *CT* on host iron regulatory mechanism which involves interaction of iron regulatory

protein and iron response element and also cross talk between transcription factors. Further proteins involved in iron acquisition and intracellular storage, level of intracellular iron and other regulatory proteins were examined. Experimental techniques used for the study included DNA- Gel shift assay, In vitro transcription, RNA- Gel shift assay, western blotting, and fluorescence microscopy.

(3). *Chlamydia trachomatis* : Iron and cytokines

Most of pathological consequences associated with chlamydial infection are conferred by host immunological inflammatory responses to pathogens. In this study we examined involvement of iron in regulation of proinflammatory cytokine IL-8 and anti-inflammatory cytokine IL-10. For this study detection of in vitro cytokines (IL-8, IL-1b, TNF-α and IL-10) concentration in culture supernatants in chlamydia infected cells was done. Promoter (IRE) analysis by GFP fluorescence, intracellular level of free iron and associated proteins, effect of antioxidant and signaling components was also evaluated. Techniques involved in experimentation were ELISA, Flow cytometry, transfection of cell line, transformation and plasmid preparation, fluorescence microscopy and western blotting.

Chapter 4

Establishment of Chlamydial Infection in vitro under iron stress condition

The same thing that makes you live can kill you in the end.

Neil Young

Chapter 4

Establishment of Chlamydial Infection in vitro under Iron Stress Condition

Introduction

Pathogens have developed elegant mechanisms to modulate the fate of the host cell, which include induction or blockage of apoptosis (Gao *et al.*, 2000), thereby, able to maintain a long-term relationship inside the host cell. In case of chlamydia, this could help the bacteria to complete their replication cycle inside the cell, at the end of which numerous elementary bodies are produced and released to infect other cells. Chlamydia can sometimes develop long-term chronic infections that could contribute to diseases such as atherosclerosis and pelvic inflammatory disease. Protection of the host cell against apoptosis would acquire greater significance for the bacteria under such conditions. It is not surprising, therefore, that persistently infected cells have also been shown to be potently resistant to various forms of apoptosis (Dean *et al.*, 2001).

Apoptosis, or programmed cell death, is an evolutionarily conserved, strictly regulated, genetic and biochemical program that plays critical roles during development and tissue homeostasis in multi-cellular organisms (Zakeri *et al.*, 2008). Cells undergoing apoptosis manifest profound changes in cellular architecture characterized by a distinct morphology and, at a biochemical level, by cleavage of chromosomal DNA into the internucleosomal fragments (Cohen, 1993). In contrast to necrosis, apoptosis in multicellular organisms allows elimination of unnecessary or damaged cells in an immunologically silent manner, in which apoptotic cells expressing specific surface molecules are scavenged by phagocytic cells, thus

avoiding spillage of intracellular contents that could cause inflammation and tissue damage (Savill, 1996).

Apoptosis can be induced through both extrinsic (death receptor pathway) and intrinsic (mitochondria pathway) pathways. Death receptor pathways are initiated by ligand binding to cell-surface receptors (for example, Fas ligand binding to Fas). Mitochondrial pathway signaling involves the activation of the proteins Bax and Bak that can permeabilize mitochondrial membranes (Danial *et al.*, 2004). In most cases, both of these pathways require the release of mitochondrial cytochrome *c* (Newmeyer *et al.*, 2003) and the subsequent activation of a group of cytosolic proteases, called caspases (Shi, 2004). Cell death can also be induced in the absence of caspases, but authors disagree whether or not this should be called apoptosis (Kurokawa *et al.*, 2009, Martinvalet *et al.*, 2008). Usually, the family of caspase proteases causes the death of the cell and the appearance of typical morphological changes such as chromosomal fragmentation and nuclear condensation. Caspase activity has been described as a cascade, with the activation of upstream caspases leading directly to the cleavage and subsequent activation of downstream caspases. Activation of caspase-3, for example, is mediated either by activated caspase 8 during death receptor signaling, or by caspase-9 in the case of signals transmitted through mitochondria (Green, 1998). The central step of mitochondrial apoptosis is the release of cytochrome *c* into the cytosol, which in turn binds to Apaf-1 and caspase-9 as part of a complex known as the apoptosome, the caspase-activating signaling complex (Chan, 2006).

Numerous pro-apoptotic signal transduction and damage pathways converge on mitochondrial membranes to induce their permeabilization. Mitochondrial membrane potential (MMP) differentially affects the outer membrane, which becomes protein-

permeable, and the inner membrane, which continues to retain matrix proteins yet, can dissipate the mitochondrial transmembrane potential ($\psi\Delta m$) (Jourdain *et al.*, 2009). The temporary order of outer and inner membrane permeabilization, as well as their relative contributions to cell death, are a matter of debate and probably depend on the MMP-initiating stimulus. MMP triggers the activation of catabolic hydrolases, mainly caspases and nucleases. The release of cytochrome *c* is governed by Bcl-2 family proteins. This family consists of three groups of proteins: anti-apoptotic Bcl-2 like proteins, pro-apoptotic BH3-only proteins, and the multidomain Bax/Bak groups (Kuwana *et al.*, 2002). The anti-apoptotic members of the Bcl-2 family (such as Bcl-2 and Bcl-XL) reside mainly but not exclusively in mitochondrial membranes, where they locally inhibit MMP. Pro-apoptotic members of the Bcl-2 family such as Bax can translocate from other cellular localizations to mitochondria while undergoing a conformational change; they then oligomerize within mitochondrial membranes and facilitate MMP (Shore *et al.*, 2008). This translocation-oligomerization- permeabilization reaction is inhibited by anti-apoptotic members of the Bcl-2 family and is stimulated by pro-apoptotic BH3-only members of the Bcl-2 family (such as Bid). The Bax homologue Bak constitutively resides in the outer mitochondrial membrane and also undergoes a Bid-stimulated allosteric activation leading to its oligomerization within the membrane (Shelton *et al.*, 2009). Membrane potential is also sensitive to redox state of cells, which primarily governed by reactive oxygen and nitrogen species (Waster *et al.*, 2009).

The redox environment of the cell is currently thought to be extremely important to control either apoptosis or autophagy as many redox-sensitive proteins characterize these networks (Kubota *et al.*, 2009). There are two major forms of free radicals, reactive oxygen species

(ROS) and nitric oxide (NO), which are formed from oxygen and nitrogen. Under normal conditions, ROS are cleared by antioxidant enzymes including catalase (CAT), superoxide dismutase (SOD); upon stress stimuli, an imbalance of the redox milieu develops and leads to the accumulation of ROS, which results in oxidative stress (Boveris *et al.*, 1973). Another gaseous free radical, NO, has been identified as a fundamental molecule that interplays with ROS in a variety of ways, either as a crucial partner in regulating the redox status of the cell, determining cell fate or in signalling in response to a number of physiological and stress-related conditions (Thannickal *et al.*, 2000). The particular outcome varies depending on cell types as well as the redox status. Several enzymes generate ROS including NAD(P)H oxidase, xanthine oxidase, cytochrome P450 and mitochondrial electron transport chain complexes (Buttke *et al.*, 1995). Mitochondria are the primary site of ROS generation within the cell and are also sensitive targets for ROS because of their phospholipid- containing membranes and the vulnerable mitochondrial DNA [Wei, YH, 2002 (Thannickal *et al.*, 2000, Wei *et al.*, 2002). Many studies suggested that ROS participated in the apoptotic process through inducing MMP, which led to the release of cytochrome c into the cytosol and thus triggered the apoptotic cascade (Newmeyer *et al.*, 2003). The mechanism that can regulate MMP involves the Bcl-2 family of proteins such as Bax, Bcl-2 and Bcl-xL, which act on the outer mitochondrial membrane to either promote or prevent MMP. The mitochondrial apoptotic signalling was found to be regulated by ROS/NO via inducing MMP, which is related to the degradation of Bcl-xL and Bcl-2, an antioxidant protein considered to detoxify the intracellular ROS (Gottlieb *et al.*, 2000). Moreover, a caspase-dependent apoptosis was also found to be regulated by ROS/NO, as demonstrated by the obvious inductive effects of ROS/NO on caspase-3 truncation. This role was probably related to the reported sensitivity

of caspase-3 to the alterations of intracellular redox status (Yang *et al.*, 2009). Many studies suggested that ROS participated in the apoptotic process through inducing MMP, which led to the release of cytochrome c into the cytosol and thus triggered the apoptotic cascade (Li *et al.*, 2004).

There is an intimate relationship between oxidative stress and iron metabolism. Iron is both an essential cofactor and a potentially hazardous metal participating in the production of ROS (Kell, 2009). Lack of iron regulation may impose oxidative stress upon cells and many microorganisms have evolved systems that couples control of iron homeostasis and protection against ROS (Ricci *et al.*, 2002). The unique abilities of iron to change its oxidation state and redox potential in response to changes of liganding environment enable it to play an essential role in many biological reactions. However, the same physiochemical properties that make iron essential for most living organisms also make it very dangerous. Iron in transit (Labile iron pool) play central role in free radical generation (Kruszewski, 2003) and the most important reaction of hydrogen peroxide with (free or poorly liganded) Fe(II) is the Fenton reaction (Wardman *et al.*, 1996), leading to the very reactive and damaging hydroxyl radical (Kruszewski, 2003).

(OH•) $Fe^{2+} + H_2O_2 = Fe^{3+} + OH^- + OH•$

Superoxide can also react with ferric iron in the Haber-Weiss reaction (Kehrer, 2000) to produce Fe(II) again, thereby effecting redox cycling:

$O_2•^- + Fe^{3+} = O_2 + Fe^{2+}$

Trace amounts of “free” iron may catalyze generation of a highly toxic hydroxyl radical *via* Fenton/Haber–Weiss reaction cycle. Living organisms try to avoid an excess of “free” iron by tightly controling iron homeostasis. In most cells iron homeostasis consists of iron uptake,

utilization and storage. The principal effectors of these processes are transferrin receptor, a protein involved in iron uptake, and ferritin, an iron-sequestering protein. Since uptake and storage of iron is carried out by different proteins, there is a pool of accessible iron ions, called labile iron pool (LIP) that constitutes crossroads of the metabolic pathways of iron containing compounds. LIP is a low-molecular-mass pool of weakly chelated iron that rapidly passes through the cell. LIP level is midway between the cellular need for iron and the hazard of excessive generation of hydroxyl radical, as it has been proposed that LIP is a cellular source of iron ions available for Fenton reaction (Breuer *et al.*, 1997).

Redox reactions are the central to life and death decision of cells and pathogens and balancing act have been played by anti-oxidant properties of well known functional proteins. The well known anti-apoptotic protein Bcl-2 also plays to increase cellular redox capacity (Hockenbery *et al.*, 1993). Another unique anti-oxidant Thioredoxin (Trx) is a redox protein that is found in several species, such as bacteria, plants and mammals, and contains a conserved active site consisting of Trp-Cys-Gly-Pro-Cys. Trx has several biological functions (Junn *et al.*, 2000). It acts as a hydrogen donor for ribonucleotide reductase, which is critical for DNA synthesis, and modulates the DNA-binding activity of several transcription factors, including NFκB, AP-1, p53, TFIIIC and glucocorticoid receptor. Trx also stimulates cell growth, is an inhibitor of apoptosis and plays a role in the protection against oxidative stress (Tanaka *et al.*, 2000). Drugs that inhibit Trx have antitumor activity, suggesting that thioredoxin is involved in a variety of human diseases, including cancer (Berggren *et al.*, 2001). Additionally peroxisome proliferator-activated receptor- gamma (PPAR-γ) also plays important role in regulation of MMP and apoptosis. PPAR-γ is a ligand-activated nuclear receptor implicated in several significant human pathologies, including

cancer, atherosclerosis, and inflammation (Kersten *et al.*, 2000). PPAR-γ inactivates components of the cell death machinery, and up-regulate anti-apoptotic proteins, such as Bcl-2, which increases mitochondrial membrane potential, thereby, protecting cells against apoptosis (Fuenzalida *et al.*, 2007). Bcl-2-overexpressing neural cells display a reduced redox state, low levels of reactive oxygen species (ROS), and a resistance to mitochondrial injury and cell death induced by oxidative stress (Soane *et al.*, 2005).

Apoptosis also plays an important role in modulating the pathogenesis of a variety of infectious diseases. A number of studies show that apoptotic death of the host cell may not necessarily be a quiescent one, but rather may contribute to an antimicrobial immune response. On other hand many pathogens have been shown to possess a plethora of strategies to hijack the control over the fate of the host cell. These pathogens are either directly or indirectly engaged in the host cell apoptotic pathways (Bhavsar *et al.*, 2007). Intracellular shigella and salmonella secrete factors into the cytoplasm, where they directly bind to and activate a pro-apoptotic protein, the IL-1β converting enzyme (or caspase-1) (Hilbi *et al.*, 1998, Hersh *et al.*, 1999). Pathogenic *Mycobacterium tuberculosis* promotes secretion by infected macrophages of soluble tumor necrosis factor (TNF) receptor 2 (sTNFR2), which neutralizes the proapoptotic activity of TNF-α (Balcewicz-Sablinska *et al.*, 1998, Kornfeld *et al.*, 1999). Chlamydia, on the other hand, presumably secretes a protein(s) that results in blocking the release of cytochrome *c* from the mitochondria and inhibition of the activation of caspase-3 (Fan *et al.*, 1998). This implied that there was a block in the mitochondrial permeabilization, which in turn is controlled by Bax and Bak. Later studies showed that the chlamydial-infected cells failed to achieve activation of these regulators of mitochondrial permeabilization (Xiao *et al.*, 2004). It was further shown that the infected cells are not

resistant to apoptosis in Type I cells, which do not require the mitochondrial pathway for activation of the effector caspases (Fischer *et al.*, 2004b). The chlamydial infection protects cells against different forms of apoptosis : extrinsic, intrinsic and granzyme B mediated by various stimuli including staurosporine,TNF-a, etoposide, granzyme B/perforin, and UV light (Rajalingam *et al.*, 2001). However till date there is no study explaining involvement of iron controlled redox environment in apoptosis of CT infected cells. Therefore in this study pro and anti-apoptotic factor was assessed under iron stress conditions to ascertain role of iron in establishment of Chlamydia *trachomatis* infection *in vitro*.

Experimental Methods

Materials: Unless otherwise stated, all the reagents were purchased from Sigma Aldrich (Saint Louis, USA) and antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Plastic and glassware's for tissue culture were obtained from Greiner, Germany.

***CT* culture :** *CT* laboratory reference serovar D (D/UW-3/Cx) was propagated in HeLa 229 cells as described previously (Schachter *et al.*, 1994), purified and stored at -80ºC in sucrose phosphate glutamate medium (SPG, pH 7.0). *CT* inoculum was confirmed to be free from mycoplasma contamination by mycoplasma detection kit (Takara, Madison, USA).

Infection and treatment protocol: HeLa 229 cells were grown in 6 well tissue culture plates (with cover slip) with cell density of 1×10^6 cells/well. On reaching the sub-confluence, cells monolayer were washed twice with hank's balanced salt solution (HBSS) and infected with chlamydial EBs at multiplicity of infection (moi) of 2. Further, tissue culture plates were placed on a rocker for 2 hour at 35ºC to obtain a homogenous infectivity. Media containing unbound EBs were aspirated and supplemented with complete Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Infected HeLa 229 cells were

incubated at 37^{o}C with 5% CO_2 in a humid environment for 24 hours. Deferroxamine (DFO) (50 μM), ferric ammonium citrate (FAC) (1mM) and H_2O_2 (1uM) was added after 16 hours post infection (hpi) to respective wells. On completion of 24 h.p.i cells were taken out for analysis. Cover slips were taken out for quantification of chlamydial inclusion. Infected cells were fixed in methanol for 10min, and then FITC conjugated MOMP monoclonal antibody or geimsa stain was applied following manufacturer protocol. After staining, cells were observed under fluorescent microscope equipped with 488 excitation and 525 emission spectra. All the experiments were performed twice in triplicate with their respective controls using mock infected cells.

MTT cell viability assay:

Cell proliferation was assessed by monitoring the conversion of MTT to formazan. The reduction of MTT is catalysed by mitochondrial dehydrogenase enzymes and is therefore a measure for cell viability (Mosmann, 1983).

Cultured cells in 24 well plate were washed with warm phenol red free EMEM. MTT working solution was added into wells being assayed, 0.1ml for each well. Incubate at 37°C for 2 hours. At the end of the incubation period, the medium was aspirated. The converted dye is solubilized with 0.2ml acidic isopropanol (0.04 M HCl in absolute isopropanol). Pipette up and down several times to make sure the converted dye dissolves completely. Transfer the dye solution with the cells into a 1.5 ml eppendorf tube and centrifuge at 13,000 rpm for 2 min. Transfer the supernatant into a new eppendorf tube. Absorbance of the converted dye is measured at a wavelength of 570nm with background subtraction at 620nm on ELISA plate reader (Biotech, USA).

Determination of intracellular hydrogen peroxide and superoxide:

Intracellular hydrogen peroxide and superoxide were determined by flow cytometry according to Robinson *et al.* (Robinson *et al.*, 1988). The principle of the assay is that a probe, the nonfluorescent molecule 2', 7'-dichlorofluorescin diacetate (DCFH-DA), is loaded into the cells to provide a readily oxidizable substrate. DCFH-DA is lipophilic and easily crosses cell membranes. Inside the cell, cytosolic enzymes (esterases) deacetylate the DCFH-DA to form polar, nonfluorescent 2', 7'-dichlorofluorescin (DCFH) which, due to its polarity, is trapped either within the cytoplasm or in myeloperoxidase (MPO)-positive intracellular granules. The oxidative potentials of H_2O_2 together with peroxidases are able to oxidize the trapped DCFH to 2',7'M dichlorofluorescein (DCF), whose green fluorescence at 525 nm is easily measurable on the flow cytometer in a manner similar to the detection of fluorescein. The amount of DCF formed is proportional to the cellular oxidant production. The fluorescence intensity becomes a measure of the oxidants produced by the cells, in particular, H_2O_{2+} and $O^{\mathbf{0}}$.

For ROS determination, HeLa cella was incubated with 10 μM DCFH-DA at 37°C for 15 min, and then the cell was treated with DMSO in the presence of reagents to be tested. The cells were incubated at 37°C for 15 min, then cell-associated fluorescence (DCF) was monitored on FACaliber (Becton Dickinson, USA) with the excitation wavelength at 488 nm and the emission wavelength at 525 nm. Experiments were repeated at least twice and each experiment was done in triplicate. Data were acquired and analyzed by CELL QuestPro / FCS Express V3 (Becton Dickinson, USA/ DeNOVO), and the differences in the mean fluorescence between stimulated and unstimulated cells were calculated as fluorescence

increase. To confirm that the cell-associated fluorescence was the intracellular fluorescence, not the cell surface fluorescence, we observed the cell with a Carl Zeiss LSM5 confocal laser scanning microscope.

Determination of mitochondrial membrane potential using flow cytometer and microscopy

The mitochondrial respiratory chain produces energy which is stored as an electro-chemical gradient and consists of a trans-membrane electrical potential (negative inside of about 180-200 mV) and a proton gradient of about 1 unit. This energy is then able to drive the synthesis of ATP, a crucial molecule for a consistent variety of intracellular processes (Galluzzi *et al.*, 2007b). To detect variations in $\Delta \psi$ at the single cell or at the single organelle level, cytofluorimetric (FCM) technique is used by using the lipophilic cation 5,5', 6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1). JC-1 is capable of entering selectively into mitochondria, since it changes reversibly its color from green to orange as membrane potentials increase (over values of about 80-100 mV). This property is due to the reversible formation of JC-1 aggregates upon membrane polarization that causes shifts in emitted light from 525 nm (*i.e.*, emission of JC-1 monomeric form) to 590 nm (*i.e.*, emission of J-aggregate) when excited at 488 nm; the color of the dye changes reversibly from green to greenish orange as the mitochondrial membrane becomes more polarized. Both colors can be detected using the filters commonly mounted in all flow cytometers, so that green emission can be analyzed in fluorescence channel 1 (FL1) and greenish orange emission in channel 2 (FL2). The main advantage of the use of JC-1 is that it can be both qualitative, considering the shift from green to orange fluorescence emission, and quantitative, considering the pure fluorescence intensity, which can be detected in both FL1 and FL2

channels. Cells were harvested (at least $2x10^5$) from 6-well culture plate and total volume was made up to 1 mL with fresh complete medium. Cells were then stained (2.5 μg/mL JC-1) and were kept in a dark place at room temperature for 15-20 minutes. Stained cells were washed twice by centrifuging at 500 g for 5 min with a double volume of PBS. Then cells were resuspended in 0.3 mL of PBS, and analyzed immediately with the flow cytometer, typically equipped with a 488 nm argon laser. Set the value of photomultiplier (PMT) detecting the signal in FL1 at about 390 V, and FL2 PMT at 320 V; FL1-FL2 compensation was 4.0%, while FL2-FL1 compensation was 10.6%. To confirm that the cell-associated fluorescence was the intracellular fluorescence, we observed the cell with a Carl Zeiss LSM5 confocal laser scanning microscope. The cells were cultured on cover slip in 24 well plates and labeled with JC-1 for determination of MMP in similar condition as for flow cytometer. Further cover slip with respective cells were further analysed microscopically (Olympus BX51, Japan) with the excitation wavelength at 488 nm and the emission wavelength longer than 525 nm.

Immunobloting of proteins

CT infected HeLa 229 cells were washed with PBS and subsequently treated with lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4 and 1 μM PMSF) supplemented with a complete protease inhibitors cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by Bradford protein assay (Bio-Rad Laboratories, USA) with BSA as standard. Extracted proteins (40 μg) were electrophoresed on 8-12% SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad, USA) that reversibly stained with Ponceau S (Sigma Aldrich, St Louis, USA) to confirm complete transfer following standard protocol (Miniatis vol. 3, edition 3).

Membranes were blocked with 5% nonfat dry milk in PBS-Tween-20 and incubated with rabbit anti- FHC (1:1000), Bcl-2 (1:1000), Bax (1:500), Trx (1:2000), PPAR-γ (1:500), Caspase-3 (1:1000), Caspase-8 (1:1000), and Caspase-3 (1:2000). Membranes were further incubated with the goat anti-rabbit IgG conjugated with horseradish peroxidase (1:3000) and subsequently developed using diaminobenzamide (DAB) as a detection system. Images were analyzed with the help of Image J software (NIH, Bethesda, USA).

Statistical analysis:

The GraphPad Prizm software was used for statistical analyses (Student t-test). Values are expressed as Mean ±SD. Values of $p \leq 0.05$ are considered to be statistically significant.

Results:

Critical balance of cell death and chlamydia developmental cycle at interface of iron:

CT infected cells resist the cytotoxic effect of iron chelator DFO and Iron supplement FAC. Mock infected cells were more susceptible than chlamydia infected cells to increasing concentration of DFO, starting from 100µm till 1mM. However count of chlamydial inclusion started to decline after 50µm concentration of DFO. DFO at concentration range 25-50µm conferred stability to host cells and chlamydia inclusion, however mock showed less viability. Surface plot showed plateau at abovementioned concentration till 96hpi, however at other concentrations slope was imminent. Upon DFO (50µM) treatment CT infected cells showed smaller inclusion in comparison to control.

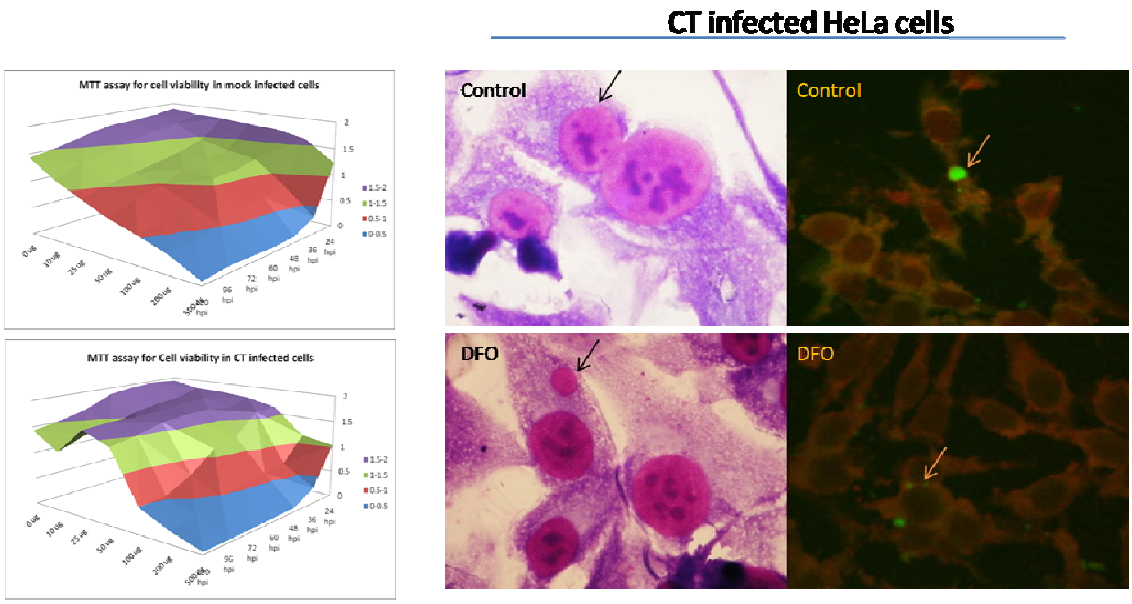


Fig 4.1: Cellular sustenance increases in CT infected cells at lower concentration of DFO **(A)** Surface plot showing OD values (570/620) obtained in MTT assays. Area under purple

colour showing sustained cellular viability in CT infected cells. **(B)** DFA and giemsa stained cells showing smaller inclusion in presence of DFO (50µM).

Decrease in surface expression of transferrin receptor in CT infected cells:

Surface expression of TfR was determined by mean fluorescence intensity (MFI) in flow cytometric analysis. Decrease in TfR level was observed in CT infected cells in comparison to mock infected cells. DFO treated (50µm) mock infected cells showed significant increase in TfR level in comparison to untreated mock, whereas in DFO treated CT infected cells degree of change was lesser in comparison to untreated CT. On addition of FAC (1mM), evident decrease in TfR was observed in mock (treated) in comparison to untreated mock, in contrast CT infected cells did not record significant change.

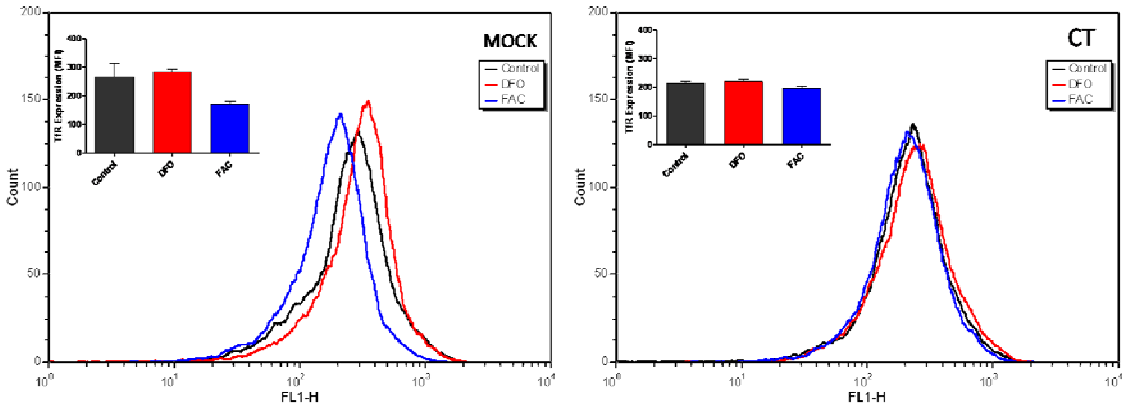


Fig 4.2: Surface expression of TfR decreases in CT infected cells: Flow cytometric histogram with quantitative data represented in bar diagram in insat.

Level of ROS dampens in CT infected cells:

Declined ROS level was observed in CT infected cells in comparison to mock infected cells. On addition of DFO, significant decrease in ROS production was observed in both CT and mock in comparison to untreated CT and mock respectively. However degree of change was

higher in CT than mock. FAC treated mock cells showed increase in ROS level in comparison to untreated mock, whereas in CT infected cells lesser degree of increase was observed in comparison to untreated CT.

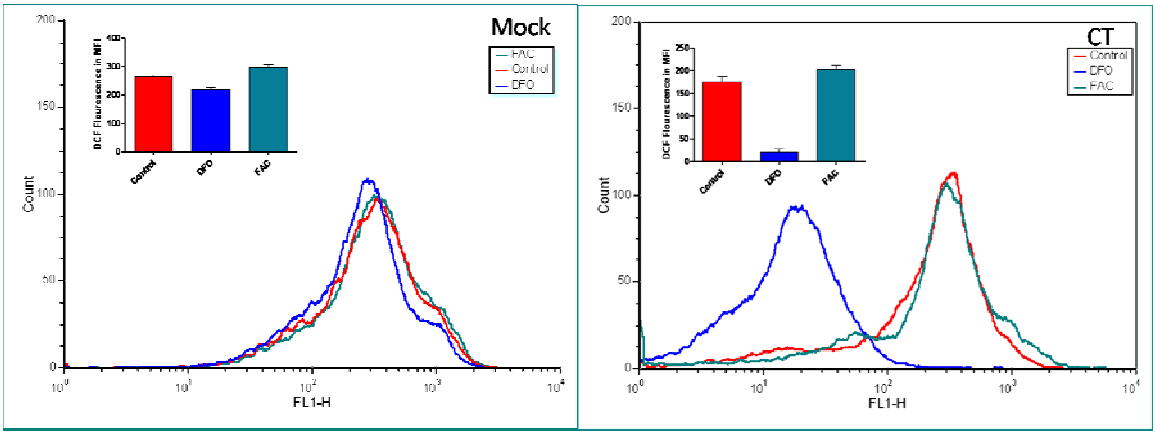


Fig 4.3: Level of ROS dampens in CT infected cells: Flow cytometric histogram with quantitative data represented in bar diagram in insat.

gp91PHOX decreased in CT infected cells:

There was decrease in expression of gp91Phox observed in CT infected cells in comparison to mock infected cells. Under iron strived condition, decrease in the level of gp91Phox was observed in mock infected cells, however CT infected cells further it was dampened in relative to untreated mock and CT infected cells. In access iron supplementation with FAC, no change in gp91Phox expression was observed in both, CT as well mock infected cells in comparison to their untreated control.

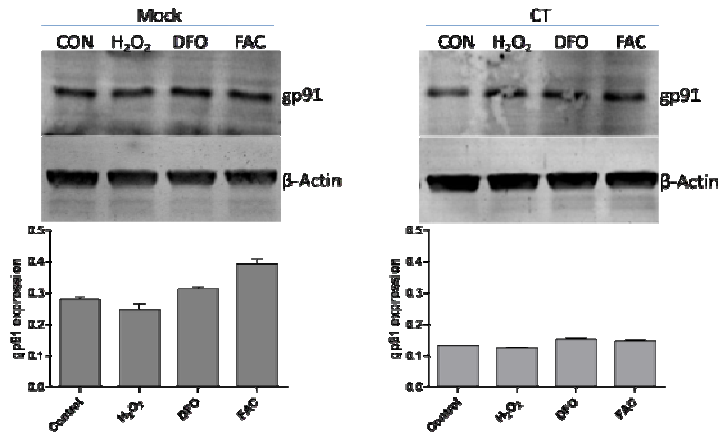


Fig 4.4: Expression of gp91Phox decreases in CT infected cells: Immunoblot showing band representing gp91Phox and actin normalized semi-quantitative data depicted in bar diagram.

Level of Ferritin increases in CT infected HeLa cell:

Western blot analysis was performed to know the intracellular level of ferritin and that showed level of FHC increased significantly in CT infected cells than mock infected cells. On addition of apoptotic inducer H_2O_2 (10μM), level of FHC further increases in CT infected cells, however no significant change was observed in mock infected cells. Under apoptotic condition higher OD value of MTT was observed in CT infected cell, however mock infected cell not able to resist apoptotic stimuli, cell death occurs as H_2O_2 was added. Addition of DFO in Mock and CT infected cells resulted in differential degree expression of FHC significant decrease in earlier and non-significant decreases in later. Moreover, apparent increase in FHC expression was observed in mock infected cells on addition of FAC, in contrary unapparent increase observed in CT infected cells.

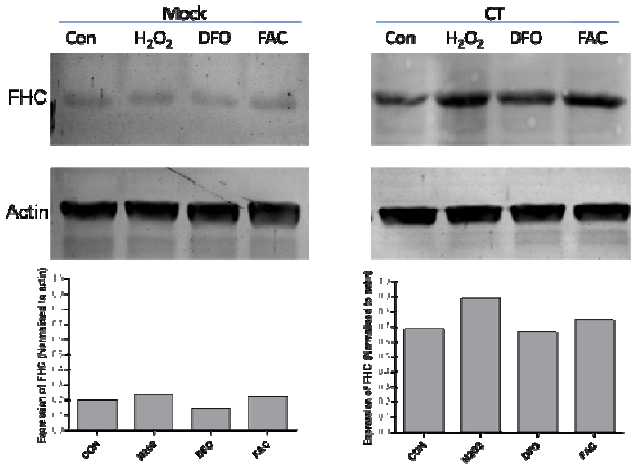


Fig 4.5 Level of FHC increases in CT infected cells: Immunoblot showing band representing FHC and actin normalized semi-quantitative data depicted in bar diagram.

CT infected cells changes redox state of infected cells by decreasing Trx expression:

Expression level of Trx was increased in CT infected cells in comparison to mock infected cells. On addition of DFO, significant increase in Trx was observed in CT infected cells than mock infected cells. In contrary on addition of FAC, increase was observed in both the conditions CT and mock in comparison to untreated CT and mock.

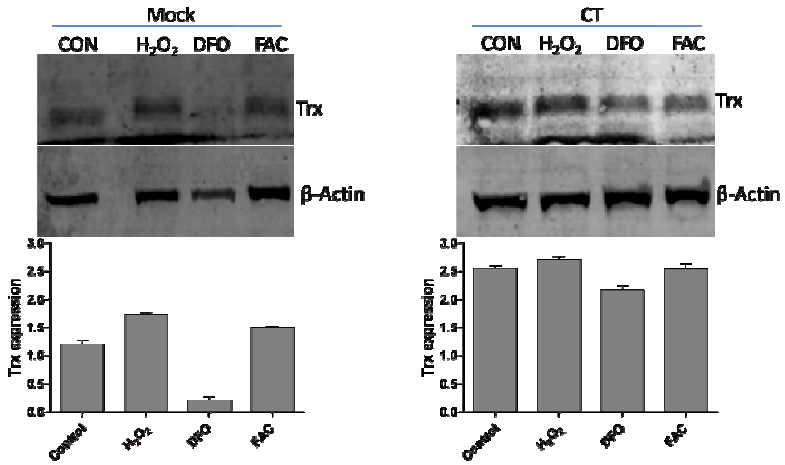


Fig 4.6: Expression of Trx-1 increases in CT infected cells: Immunoblot showing band representing Trx-1 and actin normalized semi-quantitative data depicted in bar diagram.

CT infection stabilizes mitochondrial membrane potential under iron stress condition:

Mitochondrial membrane potential was analysed by using JC-1 dual colour voltage sensitive stain. Ratiometric analysis was performed to determine the change in membrane potential. CT infected cell showed higher ratio in JC-1 analysis than mock infected cells. DFO treatment resulted in the dissipation of membrane potential in mock infected cells observed as decrease in FL-2/FL-1 ratio. In contrary ratio in CT infected cells remains stable upon treatment with DFO. On addition of FAC ratio decline in mock infected cells, however lesser decline was observed with CT infected cells. On addition of H_2O_2, complete loss of membrane potential was observed, however CT infected cells showed retention of potential.

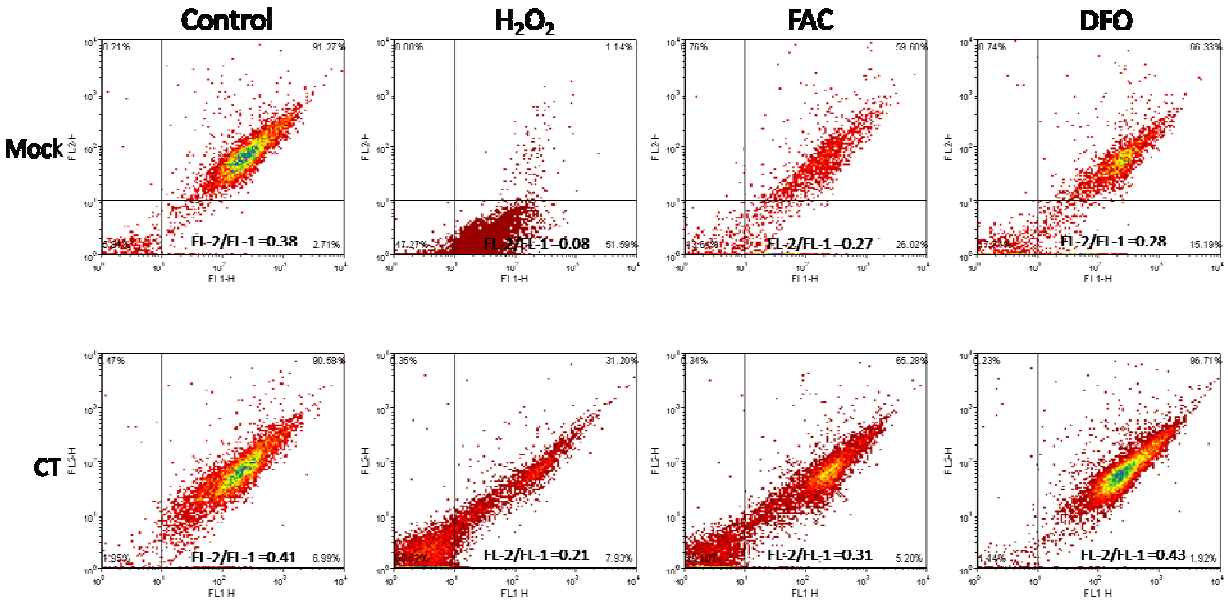


Fig 4.7 Mitochondrial membrane potential stabilizes in CT infected cells: representative Dot plot showing increased ratio (± 0.02) of FL-2/FL-1 in CT infected cells.

Ratio of Bcl-2 and Bax remains higher on iron deprivation in chlamydia infected cells:

Ratio of Bcl-2 and Bax is a marker of intact mitochondrial membrane and viability of cells. Higher Bcl-2/Bax ratio was observed in CT infected cell in comparison to mock infected cells on stimulation with 10μM H_2O_2. On addition 50μM DFO it remains higher in CT

infected cells, however mock showed abolished Bcl-2/Bax ratio in comparison to untreated CT and mock infected cells respectively. Ratio decline sharply in mock infected cells on addition of 1mM FAC, in contrary chlamydia infected cells showed constantly higher ratio in comparison to untreated mock and CT infected cells respectively.

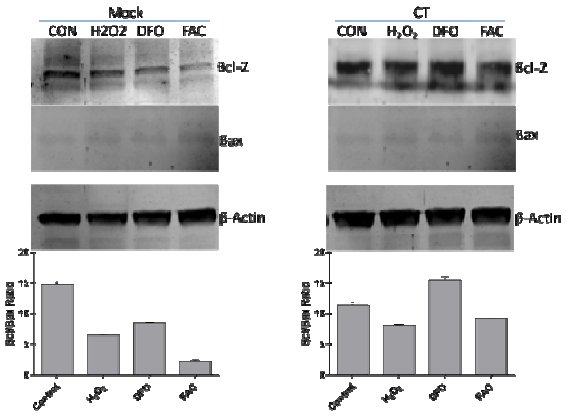


Fig 4.8: Ratio of Bcl-2/Bax increases in CT infected cells: Immunoblot showing bands representing Bcl-2 and Bax and actin normalized semi-quantitative data showing ratio depicted in bar diagram.

Higher expression of PPAR-γ in CT infected cells:

PPAR-γ is multifunction protein and plays an important function in stabilization of MMP and energy metabolism. CT infected cell showed higher expression of PPAR-γ than mock infected cells. Further increase in expression was evident in CT infected cells on addition of DFO, however mock remains at basal level. In contrast there was decline in expression on addition of FAC in mock at greater degree than CT. On addition of H_2O_2 increase in PPAR-γ expression was evident in both mock and CT, however at higher extent in later.

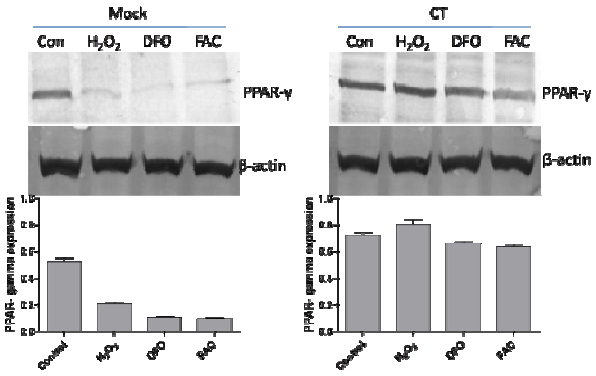


Fig 4.9: Immuno-blot showing bands representing PPAR-γ and actin normalized semi-quantitative data depicted in bar diagram.

Release of Cytochrome 'C' blocked in CT infected cells under iron restricted condition:

Release of cytochrome 'C' in cytoplasm is central to mitochondria mediated apoptosis, so cytoplasmic level of cyt 'c' was analysed after induction with H_2O_2, DFO and FAC. Induction of apoptosis by H_2O_2, CT infected showed lesser release of cyt 'c' than mock infected cells. Addition of DFO resulted in cyt 'c' release from mitochondria in mock infected cells however it was blocked in CT infected cells. On addition of access FAC, cyt 'C' release was observed in both the condition CT and mock, however it remained very low in CT in comparison to mock.

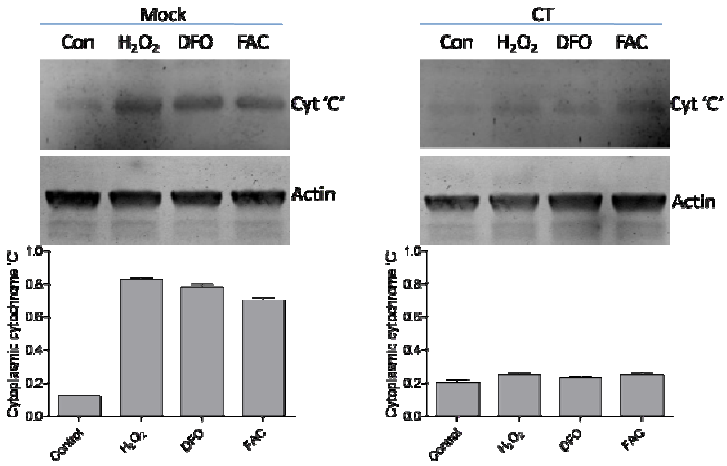


Fig 4.10 Release of cytochrome 'C' in cytoplasm is inhibited in CT infected cells: Immunoblot showing bands representing cytochrome 'C' and actin normalized semi-quantitative data depicted in bar diagram.

Level of active caspases altered in CT infected cells:

Protein fragment representing active domain was analysed by western blotting. CT infected cells showed low level of caspases 3 and 9 activation than mock infected cells on induction with H_2O_2 as detected in western blot. On addition of DFO, activation of caspases 3, 9 and 8 was observed in mock infected cells, contrastingly CT infected cells oppose activation. In comparison to H_2O_2 treated mock, CT infected cells resist activation of caspases 3 and 9, however low level of caspase 8 activation was observed on addition of excess FAC.

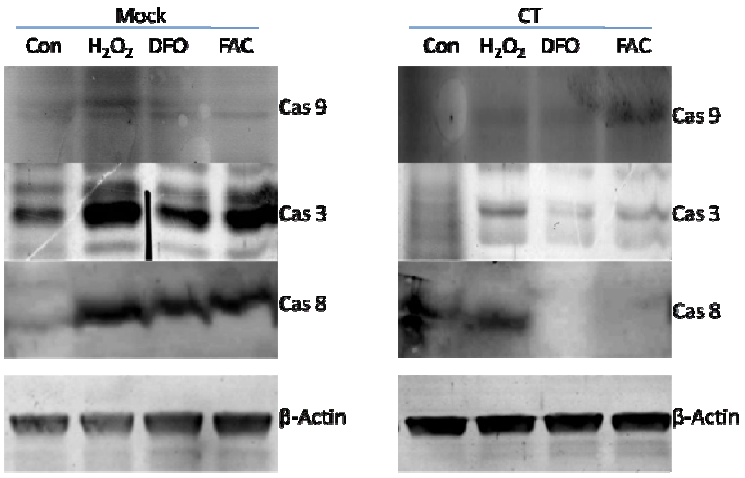


Fig 4.11 Caspase activation is regressed in CT infected cells: Immunoblot showing bands representing Caspase 9, 8 and 3.

Discussion:

Apoptosis is a cellular response to a multitude of triggers. Apoptosis plays an important role in modulating the pathogenesis of a variety of infectious diseases (Thompson, 1995, Weinrauch *et al.*, 1999). A number of studies show that apoptotic death of the host cell may

not necessarily be a quiescent one, but rather may contribute to an antimicrobial immune response. Given the plethora of known apoptosis-inducing factors and stimuli, it appears almost inevitable that infectious agents can also cause apoptosis. In viral infections, modulation of apoptosis is already an old theme. Induction and inhibition of apoptosis have been described in infections with protozoa, and apoptosis has undoubtedly the potential to shape the outcome of a bacterial infection (Hacker *et al.*, 2002). Chlamydia has evolved various strategies to survive intra-(Dean *et al.*, 2001)cellularly, whereby it not only prevents the detection of the host cell by the immune system but also protects it from destruction by the immune system in case of recognition. Across the time numerous studies indicated that Chlamydia–host interaction in particular shifted to apoptosis modulation. The organism appears to confer resistance to apoptosis upon actively infected cells, which prolongs the life of the infected cell, allowing the organism to complete its complex intracellular life cycle within 48 to 72 h (Dean *et al.*, 2001).

Here we were able to show that under iron stress condition chlamydia infected HeLa cells survive longer at varying concentration of DFO and FAC than their respective control as observed in colorimetric cell viability assay. However extensive survival of CT was especially observed at critical concentration of 25-50μM DFO, in contrast mock infected cells moves towards death. Thus indicating towards involvement of iron chelation in increased survival of CT infected HeLa cell. These results augment the earlier finding showing induction of persistence and increased survival of host cells under iron deprivation imposed by DFO (Dill *et al.*, 2007). Microbial success in a host requires the ability to grow and overcome the host's defences. The microbe must be able to access sufficient nutrients, overcome physical forces and thwart innate or adaptive host defense molecules; these are

host 'signals' to which the microbe must adapt. Conversely, microbial metabolites, toxins and anti-defence molecules, and physical adherence to host cells are microbial 'signals' to the host. The host and microbial derived signals may be either unlinked or linked. In the unlinked model, when the host wins, the microbe is eliminated, but if the microbe wins, the host dies. An alternative model, based on linked signals between microbe and host, implies selective pressure favouring co-evolved phenotypes (Kirschner *et al.*, 1995) and is most applicable to persistent organisms. In such a model, the host sequesters the bacterium into a discrete compartment that is surrounded by responding host cells that do not permit the microbe to extend into adjacent tissues (Biedzka-Sarek *et al.*, 2006). A linkage between host and microbial signals and the achievement of persistence implies that equilibrium (homeostasis) has been reached (Blaser *et al.*, 2007).

Iron starvation has been observed to induce persistence in chlamydiae. Upon depletion of intracellular iron stores with the iron-chelating drug Desferal (DFO), chlamydiae demonstrate delayed development, decreased infectivity of progeny EB, and the appearance of enlarged RB, which is reversible upon supplementing the medium with an iron source (Al-Younes *et al.*, 2001). The reliance of chlamydiae on iron availability is not surprising, as iron is the most important metal in biological systems and is required by nearly all organisms studied. This ubiquitous reliance of life on iron has evolved because of the chemical properties of iron allow it to function as a biocatalyst and electron carrier when incorporated into proteins. While life depends on iron, iron in an aerobic environment poses some difficulties. Ferric iron (the form predominating in an aerobic environment) has a solubility of only 10^{-18} M at pH 7.0, and free iron can generate damaging free radicals via the Fenton reaction (Kell, 2009). Thus, organisms tightly control transport and sequester iron with high-

affinity binding proteins. Major iron-binding proteins in mammals include transferrin, lactoferrin, heme, and ferritin and transport mainly through transferrin receptor. It is clear that iron availability fluctuates in the endometrium of menstruating women (Andrews, 2000) and thus, chlamydiae inhabiting this environment experience a modulation in iron availability. Thereby surface expression of transferrin receptor provides the chance to understand the chlamydial involvement in iron transport.

Surface expression of transferrin receptor level was analysed by flow cytometre. Mean fluorescence intensity was decreased in CT infected cell in comparison to mock infected cells. In contrast to mock, there was no significant change occurred in TfR level in CT infected cells on addition of iron chelator DFO and supplement FAC in comparison to untreated CT infected cells. Thus these results show independent regulation of iron transport irrespective of iron stress conditions in CT infected cells. Sequestering iron uptake by infected cells inhibits the growth of these pathogens. To access host-derived iron sources, these bacteria exploit specific intracellular niches in the host cell (Al-Younes *et al.*, 2001). Host cells acquire iron via the transferrin receptor (TfR) which binds the plasma protein transferrin (Tf) which has a high affinity for ferric iron. Following binding, the Tf/TfR complex is internalized via the classical endocytic pathway into an early endosomal compartment. The acidic pH of this vacuole facilitates the release of iron from Tf and the empty Tf molecule and the Tfr recycle to the cell surface (Ganz *et al.*, 2006). The intracellular iron is transported across the endosomal membrane to enter the intracellular labile pool. The iron is then utilised where required and any surplus is stored bound to ferritin, the iron storage molecules (Lieu *et al.*, 2001). Thus, although the transferrin receptor has no direct contact with the iron, its expression controls most of the iron uptake within the

host. Therefore, modulation of the expression of this receptor is one way in which host cells can regulate the amount of iron transported into the cell. Indeed, the exposure of macrophages to IFN-γ results in downregulation of the TfR (Hamilton *et al.*, 1984). As IFN-γ is critical for the control of intracellular bacteria, this may reflect a strategy to prevent the pathogen from accessing the iron it requires for its intracellular survival. Iron is required for various innate host defence mechanisms, including the respiratory burst and iNOS mediated reactive nitrogen species (RNI) production (Raupach *et al.*, 2001). Constitutive production of ROI, as described in patients and experimental animals with iron overload, might downregulate NO production (Bartfay *et al.*, 2000). In contrast, depleting mice of iron using DFO exacerbated *S. typhimurium* infection through the inhibition of ROI production (Collins *et al.*, 2002). Similarly, iron depletion of listeria infected macrophages blocked ROI production and enhanced listerial growth (Alford *et al.*, 1991).

Ferritin is the major iron storage protein and have considerable amount of potency to works as anti-oxidant. Here, in this study higher expression of FHC was observed in CT infected cells, which remained higher irrespective external microenvironment. Living organisms try to avoid an excess of "free" iron by tightly controling iron homeostasis (Kell, 2009). In most cells iron homeostasis consists of iron uptake, utilization and storage. The principal effectors of these processes are transferrin receptor, a protein involved in iron uptake, and ferritin, an iron-sequestering protein. There exist intrinsic defense systems that cancel ROS toxicity in mammalian cells (Aung *et al.*, 2007). A major iron storage protein ferritin is one of the examples. The protein is a 24 subunits protein composed of two subunit types, termed heavy chain and light chain. Ferritin has enzymatic properties, converting Fe^{2+} to Fe^{3+}, as iron is internalized and sequestered in the ferritin mineral core. This function is an inherent feature

of ferritin heavy chain (FHC) subunit which exhibits ferroxidase activity that is required for iron sequestration (Torti *et al.*, 2002, Arosio *et al.*, 2002). It has been shown that FHC regulates the intracellular iron which catalyses the formation of toxic ROS (Cozzi *et al.*, 2000). Lack of iron regulation may impose oxidative stress upon cells, and many microorganisms have evolved systems that couples control of iron homeostasis to protection against ROS (Ricci *et al.*, 2002). Indeed, ferritin levels increase as a direct response to oxidative stress. Other factors that regulate ferritin expression include intracellular iron concentration, gamma interferon (IFN-γ), proinflammatory cytokines, growth factors, and differentiation (Torti *et al.*, 2002). In an elegant study, Pham et al. reported the key role of FHC in regulating apoptosis during inflammation. They showed that FHC is required to prevent sustained c-Jun N-terminal kinase (JNK) cascade activation, thus inhibiting apoptosis induced by tumor necrosis factor (TNF)-α. FHC-driven inhibition of JNK signaling depends on suppressing ROS generation and is achieved through its ability to sequester iron (Pham *et al.*, 2004). Involvement of various pathogens in up-regulation of FHC is well documented in literature and this may represent a common theme among intracellular pathogens, as *Legionella pneumophila* (Byrd *et al.*, 1991), *Mycobacterium tuberculosis* (Olakanmi *et al.*, 2002), *Anaplasma phagocytophilum* (Carlyon *et al.*, 2005) and *Listeria monocytogenes* (Schaible *et al.*, 2004). Pathogens have developed elegant mechanisms to modulate the fate of the host cell, which include induction or blockage of apoptosis (Gao *et al.*, 2000), thereby, able to maintain a long-term relationship inside the host cell. In case of chlamydia, this could help the bacteria to complete their replication cycle inside the cell, at the end of which numerous elementary bodies are produced and released to infect other cells.

Further we were able to show that ROS level decline in CT infected cells and level further dampen in presence of iron chelator DFO. The production of peroxide and superoxide is an inevitable consequence of aerobic metabolism, and while these particular 'reactive oxygen species' can exhibit a number of biological effects, themselves they are not excessively reactive and thus they are not especially damaging at physiological concentrations. However, their reactions with poorly liganded iron species can lead to the catalytic production of the highly reactive and dangerous hydroxyl radical, which is exceptionally damaging, and a major cause of chronic inflammation (Kell, 2009). Hydroxyl radicals directly interact with many biological macro- and small molecules, including DNA, proteins and unsaturated lipids. Thus Fe^{2+} and certain Fe^{2+} chelates react with lipid hydroperoxides (ROOH split the O–O bond and generates RO•, an alkoxyl radical, which can also abstract H• from polyunsaturated fatty acids and from hydroperoxides. The resulting peroxyl radicals ROO• can continue propagation of lipid peroxidation. Oxidative stress also leads to considerable DNA damage and to the polymerisation and denaturation of proteins (Welch *et al.*, 2002). Thus, these results show global downregulation of ROS production mediated through iron are a consequence of lower surface expression of TfR. Iron is needed for basic metabolic activity but is also required as a co-factor for superoxide dismutase, which protects the pathogens against toxic oxygen species generated by the phagocyte NADPH oxidase (Asayama *et al.*, 2007). Our findings suggest that chlamydia downregulates TfR expression, upregulates FHC expression and dampen ROS production to successfully complete their life cycle in their niche.

Further we analysed the level of gp91-Phox major component of mitochondrial ROS production machinery. In CT infected cells downregulation of gp91-Phox was observed,

which further declined on addition of DFO, however revert to normal on addition of FAC. It is recognized in a variety of cells that reactive oxygen species (ROS) are produced not only as by-products in aerobic metabolism (Fukuzawa *et al.*, 2005) but also as true products by specialized enzymes to play roles in various events such as host defense, oxygen sensing, and signal transduction (Cheng *et al.*, 2004). Such ROS-generating enzymes include members of the superoxide-producing NADPH oxidase (Nox) family, which contain heme-binding sites on the membrane-spanning region in the N-terminal half and the NADPH- and FAD-binding domains in the C-terminal half, thereby forming a complete electron-transporting apparatus from NADPH to molecular oxygen (Quinn *et al.*, 2004). The NADPH oxidase is a multicomponent enzyme complex that consists of the membrane-bound cytochrome b558, which contains gp91phox and p22phox, the cytosolic regulatory subunits p47phox and p67Phox, and the small GTP-binding protein Rac. Activation of NADPH oxidase leads to superoxide generation (Vignais, 2002). NADPH oxidase is a major source of ROS in the endothelial cells (Murdoch *et al.*, 2006). Increased ROS induces apoptosis in endothelial and fibrous tissue (Zhao, 2004) and inhibition of NADPH oxidase blocks ROS production and inhibits apoptosis (Qin *et al.*, 2007).The gp91phox, is homologous to the yeast iron reductase subunit FRE I, and these two proteins share many structural and functional characteristics and regulated through iron (Frank *et al.*, 1999). Thus evidences suggest that chlamydia effectively reduce availability of free iron in cytoplasm by downregulating the gp91Phox, which help to increase bioavailability of iron. Secondarily it also reduces the production of superoxide radical, potential threat for chlamydia and harboring cells. Further it was interesting to know that level of superoxide scavenger thioredoxin, which accompany NADPH system.

Thioredoxin (Trx)-1 level was analysed to ascertain the ROS buffering capacity of cells under stress condition. CT infected cells showed higher expression of Trx which increased on addition of FAC, contrastingly mock showed no such changes. Thioredoxin (Trx) is a 12 kDa protein ubiquitously expressed in all living cells, which has a variety of biological functions related to cell proliferation and apoptosis(Nakamura *et al.*, 1997). It is characterized by the reduction/oxidation (redox) active site sequence Trp-Cys-Gly-Pro-Cys-Lys, which is conserved through evolution. The two cysteine residues within the redox active center provide the sulfhydryl groups involved in Trx-dependent reducing activity. The oxidized form (Trx-S2) contains a disulfide bridge in the active site that is reduced to a dithiol by NADPH and the flavoprotein Trx reductase. Thus, the Trx system is composed of Trx, Trx reductase and NADPH *in vivo* (Buchanan *et al.*, 1994) . The reduced form [Trx-$(SH)_2$] acts as a potent protein disulfide oxido-reductase. Trx has also been found to act as a powerful antioxidant by reducing ROS, and protects against hydrogen peroxide (H_2O_2), TNF-α and *cis*-diaminedichloroplatinum (II) (CDDP)-induced cytotoxicity (Sasada *et al.*, 1996), in which the generation of intracellular ROS is thought to participate. Therefore chlamydial infection might be involved in upregulation of Trx-1 in iron dependent way and avoiding apoptosis. Mitochondria are the central target and source apoptosis initiation in most of the condition. Mitochondria maintain its integrity from physiological and physical force by maintaining its voltage gated potential.

Mitochondrial membrane potential was analysed for changes taking place during chlamydial infection under iron stress condition. Voltage sensitive dichromatic dye JC-1 was used to analyze changes in mitochondrial membrane potential in live cell by flow cytometric techniques. Chlamydia infected cells showed greater membrane integrity than mock infected

cells under normal and iron stress conditions. Further Expression of PPAR-γ increases in *CT* infected cells and remains increased at 50 μM concentration DFO, however it decreases in case of FAC treatment. The fate of cells succumbing to the intrinsic pathway of apoptosis is sealed by mitochondrial-membrane permeabilization (MMP), and this also applies to some cases of necrosis (Kroemer *et al.*, 2007). MMP eventually culminates in the loss of barrier function in both mitochondrial membranes, thus leading to the dissipation of the mitochondrial transmembrane potential, the cessation of ATP production and the release of several cytotoxic proteins into the cytosol that normally reside in the mitochondrial intermembrane space (Galluzzi *et al.*, 2007a).The MMP activation started with formation of multiprotein complex built up at the contact sites between the outer and inner membranes, the permeability transition pore complex (PTPC). The backbone of the PTPC consists of two proteins, namely the voltage-dependent anion channel (VDAC, outer membrane) and the adenine nucleotide translocase (ANT, inner membrane), both of which can form non-specific pores, either alone or in collaboration with Bax-like proteins (Zamzami *et al.*, 2001).Variety of second messengers can induce MMP including Ca2+, oxygen radicals, nitric oxide, ganglioside GD3, arachidonic acid and peroxynitrate, as well as fatty acids and their oxidation products (such as hydroxynonenal), many of which have been shown to act on ANT and to convert it into a non-specific pore (Brenner *et al.*, 2000). Several experimental chemotherapeutic agents and toxic xenobiotics also induce MMP, presumably through an action on the PTPC, although their exact mode of action is not understood (Costantini *et al.*, 2000). In addition, proteins encoded by infectious pathogens directly induce MMP, through unknown mechanisms (as in vacuolating cytotoxin A (VacA) from *Helicobacter pylori*), through an interaction with VDAC (as in porin B of *Neisseria gonorrhoeae* and hepatitis B

virus X-protein) or through an interaction with ANT (as in Vpr from HIV-1) (Boya *et al.*, 2003). Obviously, inhibitors of the respiratory chain can induce apoptosis via direct mitochondrial effect. Various infections enhance free radical production and induce the release of Fe from Fe-S clusters (Akaike *et al.*, 2000, Cinatl *et al.*, 1995). A majority of these Fe proteins are located in mitochondria and, in particular, in the cytochromes and Fe-S proteins of the electron transport chain (Atamna *et al.*, 2002). In contrary, chlamydial infection lead to stabilization of membrane potential therefore survival of host cell increase and gives enough time for completion of developmental cycle. However, in case of iron starved condition chlamydia turn to alternate persistent form and might induce expression of MMP stabilizing proteins. Therefore we assessed the PPAR-γ, which is involved in retention of mitochondrial membrane potential. The recent report has shown that ligand-activated PPAR-γ protects N2-A cells against H-R damage by enhancing Bcl-2/Bcl-x and maintaining p-Bad via preservation of p-Akt (Wu *et al.*, 2009).

In this study we observed that ratio of Bcl-2/ Bax was increased significantly in comparison to mock infected cell after apoptotic induction with H_2O_2. Bcl/Bax ratio further increase in CT infected cells on addition of DFO thereby showing its cytoprotective effect in association with CT. Further release of cytochrome 'c' in cytoplasm was lowered in CT infected cells. Decrease in level of active fragment of caspase 3, caspase 9 and caspase 8 was observed in CT infected cells as well as in DFO treated CT infected cells. It has been reported that during chlamydial infection, there is a broad-scale degradation of the different BH3-only proteins in the cell. The mRNA levels of these proteins were not affected, indicating that the downregulation was at the protein level. The chlamydial protease-like activity factor (CPAF) was shown to be responsible for targeting those active proteins with an exposed BH3

domain, for degradation (Paschen *et al.*, 2008), implying that Chlamydia destroy most, if not all, active BH3-only proteins. In the absence of the BH3-only proteins, death signals cannot be transmitted to the mitochondria, and this could account for the block in apoptosis upstream of the mitochondria. In recent study no noticeable degradation of the BH3-only proteins during CT infection of HeLa cells was observed (Rajalingam *et al.*, 2001). However, the infected cells were still able to strongly resist apoptosis induced by various stimuli including TNF-α, staurosporine and granzyme B. These contradictory results could have been because of differences in the experimental conditions (different cell types, Chlamydia serovars, etc.). However, it becomes clear that the degradation of the BH3-only proteins cannot be the only mechanism used by chlamydia to achieve the strong and widespread inhibition to apoptosis induced by various stimuli. In an elegant experiment analysing the extent of apoptosis inhibition in the infected cells, it was observed that the cytosolic extract from chlamydial-infected cells resisted the activation of caspase-3 even on treatment with cytochrome c (Fischer *et al.*, 2004a). This indicated that Chlamydia infected cells block apoptosis downstream of the mitochondria as well, which would prevent the activation of caspases in spite of cytochrome c release. Evidently, chlamydia interfere with the host apoptotic machinery at different levels – not only at the mitochondria but also downstream of it. Genetic studies in the infected cells showed that chlamydia indeed widely interfere with the host protein synthesis. Infection with CT led to the upregulation of some genes including certain antiapoptotic genes (Xia *et al.*, 2003). Prominent among the antiapoptotic proteins transcriptionally upregulated by chlamydia was cIAP-2, a member of the IAP family (Rajalingam *et al.*, 2006). It was also observed that although their levels were not increased upon infection, the silencing of XIAP and cIAP-1 also sensitized the host cells to apoptosis.

This gives credence to the growing opinion that the IAPs work together in a concerted mechanism in a complex to block the activation of caspase-3. Chlamydial infection stabilized the IAP–IAP complexes. Silencing of any of the components of the complex – XIAP, cIAP-1 or cIAP-2 – causes disruption of the complex, which in turn affect the stability of the remaining components. Thus, the IAP-mediated block on caspase-3 was abrogated, and the infected cells could undergo apoptosis. During apoptosis induction in an uninfected cell, the inhibition of caspase-3 processing and activation by IAPs is countered by the release of Smac from the mitochondria (Rajalingam *et al.*, 2007). In the presence of chlamydia, there is no mitochondrial outer membrane permeabilization and therefore Smac is not released into the cytosol. Indeed, the incomplete processing of caspase-3 observed in the infected cells upon apoptosis induction is similar to that seen after apoptosis induction in cells with Smac knockdown (Rajalingam et al., 2006, 2007). Anti-apoptotic protein Mcl-1 is prominent among the targets whose ablation lead to the sensitization of infected cells to apoptosis by TNF-α (Valdivia, 2008). Chlamydial infection leads to the activation of the Raf/MEK/ERK and the PI3/AKT survival pathways in the host cells by an as yet unknown mechanism (Su *et al.*, 2004). Mcl-1 depletion failed to sensitize the cells in the late stages of infection (around 48 h in case of CT), suggesting that different mechanisms were at play at different stages of infection (Rajalingam *et al.*, 2008). The activation of the PI3K pathway in the infected cells has also been shown to sequester the BH3-only protein BAD away from the mitochondria. It was seen that activation of the PI3K pathway led to the phosphorylation of BAD, after which it was recruited at the surface of the chlamydial inclusion (Verbeke *et al.*, 2006).

Conclusively here we are able to show that chlamydia would be able to manipulate redox state of host cell to avoid earlier cell death by apoptosis. It is also demonstrated that modulatory effect on redox status was controlled by iron metabolism of host and chlamydia.

Chapter 5

Regulation of Iron acquisition and storage in Chlamydia infected cells

There are grounds for cautious optimism that we may now be near the end of search for the ultimate laws of nature.
Stephen W. Hawking

Chapter 5

Regulation of Iron acquisition and storage in Chlamydia trachomatis infected cells

Introduction

The struggle for iron between the parasite and the host organism can be regarded as a most important scenario of co-evolution. The pathogen responds to iron scarcity by developing systems for high-affinity iron acquisition (Miethke *et al.*, 2007). Notably, most pathogens possess an arsenal of systems for iron acquisition via direct and/or indirect contacts with host iron sources, which provides an advantage for multiplication in different compartments with changing iron source composition and pH conditions (Kurz *et al.*, 2008). The broad spectrum of iron acquisition mechanisms is the result of several constraints made by the host to suppress pathogen multiplication using passive and active strategies. In general, the withholding of iron is mediated by several iron binding proteins such as ovalbumin, lactoferrin, ferritin, and transferrin (Appelberg, 2006). Unoccupied iron binding sites of these proteins serve as buffer to sequester a surplus of iron entering into circulation, thus keeping levels of free iron constantly low (Koorts *et al.*, 2007) .

Virtually all cells and organisms utilize iron as a cofactor in a multitude of biochemical activities. Chlamydiales are an evolutionary distinct group of human pathogenic bacteria and chlamydia-related symbionts sharing a biphasic developmental cycle in which the intracellular phase tethers the pathogen to its specific host (Horn *et al.*, 2004), *CT* and *CP* which are known pathogenic species for humans, take advantage of metabolic and biosynthetic pathways of infected host cells (Belland *et al.*, 2003b). Deprivation of certain amino acids or glucose prevents the implementation of replicative chlamydial infection and

may result in chlamydial persistence, characterized by reduced infectivity and metabolism (Harper *et al.*, 2000, Nelson *et al.*, 2005). *CT* EBs harvested from deferroxamine-exposed polarized epithelial cells reduces infective potential that is restored on replacement with iron-saturated transferrin medium confirming chlamydial dependence on host for its requirement of iron (Wyrick *et al.*, 1997). Most of the bacterial species secrete low molecular weight compounds called siderophores that complex iron and then internalized by specific receptors on the outer membrane of the bacterium. Intracellular bacterium *N. meningitidis* is not believed to synthesize siderophores, but produces several surface receptors that are able to bind and remove the iron or haem from host iron binding proteins (Tf, Lf, Hb, and Hb-Hp complexes) (Gail *et al.*, 2001, Murray *et al.*, 1991). Chlamydial genomic annotation gives an idea about iron transporter analog of ExbB/D, but an identifiable TonB is absent(Giles *et al.*, 2006). However recent study have reported iron transporter YtgA in chlamydia (Miller *et al.*, 2009). The *CT* divalent cation-dependent regulator (DcrA) is a distant relative of the ferric uptake regulator (Fur) family of iron-responsive regulators (Rau *et al.*, 2005).

The redox properties and coordination chemistry of iron make it ideally suited for a variety of biological functions (Liao *et al.*, 2005); these same properties make it potentially dangerous, by virtue of its ability to generate reactive oxygen species (Gutteridge *et al.*, 2000). Excess iron promotes the generation of reactive radicals, which channelise pathogenic cascade damaging cells and tissues. Iron, as an essential nutrient and potential toxin poses an exquisite regulatory problem in biology and medicine (Pantopoulos, 2004).

Iron homeostasis of cells has been regulated post-transcriptionally by binding of iron regulatory protein (IRP) 1/2 to iron response element (IRE). In iron-starved cells, IRE/IRP interaction stabilizes transferrin receptor (TfR) mRNA and inhibit translation of mRNAs

encoding ferritin heavy (H) and light (L) chains, thereby promoting cellular iron uptake and preventing iron sequestration (Hentze *et al.*, 2004). IRP-1, formerly referred to as IRE-BP, FRP, IRF, or IRP, has two mutually exclusive activities, which are switched by changes in an iron-sulfur cluster (Constable *et al.*, 1992). With a fully assembled 4Fe-4S cluster in iron-replete cells, IRP-1 is a cytoplasmic aconitase, whereas in its apoprotein form in iron-deficient cells, IRP-1 binds with high affinity to IREs (Kennedy *et al.*, 1992). Post-translational interconversion between the 4Fe-4S- and apoprotein forms thus constitutes the basis for the regulation of IRP-1 activities by iron (Lauble *et al.*, 1992). IRP-2, formerly also known as IRE-BP2, IRFB, or IRPB, is less well characterized. IRP-2 activity was originally identified in murine cells (Guo *et al.*, 1994) and appears to be present in almost all species and cell types expressing IRP-1 (Hershko, 1994). Rat IRP-2 exhibits 61% amino acid identity and 79% similarity with rat IRP-1(Iwai *et al.*, 1995). The two proteins differ most significantly in their amino termini: IRP-2 contains a 73-amino-acid insertion encoded by a unique exon (Iwai *et al.*, 1995). In contrast to IRP-1, IRP-2 lacks aconitase activity , despite the conservation of 16 of the 18 aconitase active-site residues(Guo et al. 1995). The three cysteine residues that coordinate the Fe-S cluster in IRP-1 are conserved in IRP-2 (Guo *et al.*, 1994). However, IRP-2 regulation does not appear to require the formation of an IRP-1-like cubane iron-sulfur cluster (Iwai *et al.*, 1995). IRP-2 is degraded in iron-replete cells and accumulates in iron-deficient cells requiring de novo protein synthesis (Pantopoulos *et al.*, 1995a). The 73-amino-acid insertion contains a cysteine-rich element which mediates iron-dependent degradation of IRP-2, possibly by the proteasome pathway (Guo *et al.*, 1994). The IREs have a six nucleotide apical loop with the consensus sequence 5'-CAGUGN-3' on a stem of five paired bases, a small asymmetrical bulge with an unpaired cytokine on the 5'

strand, and an additional stem of variable length (Muckenthaler *et al.*, 1998). The nucleotide making up the two stem segments may vary considerably.

The interaction of IRPs and IRE is sensitive to reactive oxygen and nitrogen species and hypoxia other than iron (Pantopoulos, 2004, Pantopoulos *et al.*, 1996). TfR1 expression is also regulated at the transcriptional level and is up-regulated by the hypoxia-inducible factor (HIF1) (Lee *et al.*, 2006, Robach *et al.*, 2007, Bianchi *et al.*, 1999), which is typically activated under hypoxic conditions but can also be turned on by a number of non-hypoxic stimuli, including inflammatory signals such as NO and LPS (Dery *et al.*, 2005). The inflammatory signals induce NF-kB activation and lead to HIF-1-dependent and IRP-independent TfR1 expression and the uptake of transferrin-bound iron (Tacchini *et al.*, 2008). The hypoxia-inducible factor-1 is a ubiquitously and constitutively produced transcription factor composed of an oxygen-labile **a** subunit and an oxygen-resistant **b** subunit (Gradin *et al.*, 1996, Wood *et al.*, 1996). HIF-1α possesses two central oxygen dependent degradation domains (ODDs) and two transactivation domains. In the presence of O_2, human HIF-1α is hydroxylated at the proline residues Pro-402 and Pro-564 within the ODDs (Ivan *et al.*, 2001, Masson *et al.*, 2001). This reaction is catalysed by specific prolyl hydroxylase domain (PHD)-containing enzymes that are members of the 2-oxoglutarate dependent and Fe^{2+}-dependent dioxygenases (Epstein *et al.*, 2001). Prolyl-hydroxylated HIF-1 is captured immediately by the von Hippel–Lindau protein (Jaakkola *et al.*, 2001), which is the substrate recognizing subunit of an E3 ubiquitin ligase (Maxwell *et al.*, 1999). Once polyubiquitinated, HIF-1α is degraded by the 26S proteasome (Salceda *et al.*, 1997).

The biphasic life cycle of chlamydia provides various opportunities for cross-communication with host regulatory network. Cells sequester iron to avoid iron acquisition by invading

organisms by reducing expression of principal iron uptake protein transferrin receptor-1 (TfR1) (Scidmore, 2005), increasing synthesis of iron storage protein ferritin (Gail *et al.*, 2001) or by increasing expression of iron release gene ferroportin (Torti *et al.*, 2002). The Cells also need to protect iron from intracellular pathogens to maintain their own homeostasis. However smarter pathogens always find the way to modulate host system for their own favour like *Mycobaterium tuberculosis* take control of IRPs (Banerjee *et al.*, 2007) and *C. pneumoniae* over HIF-1 (Rupp *et al.*, 2007).

There is a lacuna in explaining CT interaction with host regulatory mechanism- controlling iron acquisition and storage. Therefore this was undertaken in order to provide a clue to understand chlamydial modulatory effect on host cells at the interface of iron acquisition and its commensal utilization.

Experimental methods

Unless otherwise stated, all the reagents were purchased from Sigma Aldrich (Saint Louis, USA) and antibodies from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Plastic and glasswares for tissue culture were obtained from Greiner, Germany.

***CT* culture**

CT laboratory reference serovar D (D/UW-3/Cx) was propagated in HeLa 229 cells as described previously (Schachter *et al.*, 1994), purified and stored at -80ºC in sucrose phosphate glutamate medium (SPG, pH 7.0). *CT* inoculum was confirmed to be free from mycoplasma contamination by mycoplasma detection kit (Takara, Madison, USA).

Infection and treatment protocol

HeLa 229 cells were grown in 6 well tissue culture plates with cell density of 1×10^6 cells/well. On reaching the sub-confluence, cells monolayer were washed twice with hank's

balanced salt solution (HBSS) and infected with chlamydial EBs at multiplicity of infection (moi) of 2. Further, tissue culture plates were placed on a rocker for 2 hour at 35ºC to obtain a homogenous infectivity. Media containing unbound EBs were aspirated and supplemented with complete EMEM containing 10% fetal calf serum (FCS). Infected HeLa 229 cells were incubated at 37^{o}C with 5% CO_2 in a humid environment for 24 hours. Deferoxamine (50 μM) and ferric ammonium citrate (1mM) was added after 16 hours post infection (h.p.i.) to respective wells. On completion of 24 hpi cells were taken out for analysis. All the experiments were performed in triplicate with their respective controls using mock infected cells.

Cytosolic extract preparation for RNA gel shift assay

cells (2×10^6) were lysed in 140 μL of lysis buffer (20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 25mM KCl, 0.5% NP-40, and 1mM DTT) for 10 minutes at 4^0C. Subsequently, the lysates were centrifuged (Platocraft, Rota 4R-V, Mumbai, India) at 15, 000 × g for 15 minutes at 4^0C to remove the cell debris from nucleus. Protein concentrations were determined by the Bradford protein assay (BioRad, Laboratories, USA), with bovine serum albumin (BSA) as the standard.

RNA Gel Mobility Shift Assay

Radio-labeled probe of iron response element (IRE) was transcribed by *in vitro* transcription method. IRE sequence 5'GAGTTCCGTCCAAGCACUTGAAGCAGGAAACTCTCTCCCTATAGTGAGTCGTATTA3' was used as a template and T7 sequence 5'TATAGTGAGTCGTATTA 3' as primer for the amplification using *in vitro* transcription kit (Roche Applied Science, Penzberg, Germany). Probe preparation was treated with RNase-free DNase I to remove DNA

contamination subsequently extracted by phenol-chloroform (1:1, v/v) method. Further, 20µg of cytoplasmic protein was mixed with 10 ng of [^{32}P] CTP-labeled IRE in binding buffer (10 mM HEPES, pH 7.6, 40 mM KCl, 3 mM $MgCl_2$ and 5% glycerol) and incubated at room temperature for 20 min. After incubation, proteins were run on non denaturing 6% acrylamide gel prepared in TBE buffer. In a parallel experiment, samples were treated with 2-mercaptoethanol (2-ME) at a final concentration of 2% prior to addition of IRE probe to allow full expression of IRE binding activity. Gels were dried and exposed to Amersham phosphoscreen. The IRP-IRE complex was analyzed with a PhosphorImager (Typhoon 9700, Amersham, uppasala, Sweden).

Nuclear Extract Preparation:

100µl of buffer 'A' [HEPES 10mM, $MgCl_2$ 1.5 mM, KCl 10mM, DTT 0.5mM, PMSF 0.5mM and protease inhibitor cocktail (Sigma-Aldrich, USA) 10µl] was added to $2X10^6$ cells and incubated on ice for 15 min with intermittent tapping. Triton X-100 was added immediately after completion of incubation and vortexed for 10 sec followed by centrifugation at 6500 X g for 1 minute at 4^{o}C. Further supernatant (Cytoplasmic fraction) was collected in 0.5 ml tube and pellet was resuspended in 50µl of buffer 'C'

[HEPES 20mM, $MgCl_2$ 1.5 mM, NaCl 420mM, EDTA 0.2mM, Glycerol 25%, PMSF 0.5mM and protease inhibitor cocktail (Sigma-Aldrich, USA)10µl] for 30 min on ice. After completion of incubation it was centrifuged at 12000X g for 10 min at 4^{o}C and supernatant representing nuclear fraction was stored at -80^{o}C.

Radio labeling of NF-kB/HIF-1α probe:

Probe procured from Santa Cruz were activated by heating at 95^{o}C for 5 min on heated block (Eppendorf, Germany) and subsequent cooling at -20^{o}C. After activation, nucleic acid probes

were radio labeled with γ-^{32}P ATP using T4 polynucleotide kinase. After completion of reaction, 40µl of deionised water was added and unbounded radioactivity removed by quick spin column (Qiagen). Radiolabeled oligo were stored at -80^{o}C till use.

Electrophoresis Mobility Shift Assay for NF-kB and HIF-1α:

The nuclear extracts were incubated with [γ-^{32}P] ATP-labeled oligonucleotides corresponding to the sequence of binding sites for HIF-1α and NF-kB (SantaCruze, USA). These ingredients were incubated for 20 min at room temperature (25^{0}C) , thereafter 2 µl of loading dye was added to stop reaction. Complexes were run on non denaturing 6% acrylamide gel prepared in TBE buffer. Gels were dried and exposed to Amersham phosphoscreen. The complex was analyzed with a PhosphorImager (Typhoon 9700, Amersham, uppasala, Sweden).

Immunobloting of proteins

CT infected HeLa 229 cells were washed with PBS and subsequently treated with lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4 and 1 mM PMSF) supplemented with a complete protease inhibitors cocktail (Sigma Aldrich, St Louis, USA) were determined by Bradford protein assay (Bio-Rad Laboratories, USA) with BSA as standard. Extracted proteins (40 µg) were electrophoresed on 8-12% SDS-PAGE and transferred to polyvinyl difluoride (PVDF) membrane (Bio-Rad, USA) that reversibly stained with Ponceau S (Sigma Aldrich, St Louis, USA) to confirm complete transfer following standard protocol (Meniatis, Vol-3, 3rd Edition). Membranes were blocked with 5% nonfat dry milk in PBS-Tween-20 and incubated with rabbit anti TfR-1, FHC, IRP-1, IRP-2, and PHD-1 IgG. Membranes were further incubated with the goat anti-rabbit IgG conjugated

with horseradish peroxidase and subsequently developed using diaminobenzamide (DAB) as a detection system. Images were analyzed with the help of Image J software.

Calcein quenching for labile iron pool:

Level of labile iron pool was determined by decrease in fluorescence of intracellular calcein-AM. Higher fluorescence is inversely co-related with lower free labile iron and control was adjusted by using iron chelator DFO. Cells were cultured of cover slip and loaded with 1nM calcein just before analysis by fluorescence microscopy.

Statistical analysis:

Statistical analyses were performed with GraphPad Prism software (version 5.0). Differences were tested for statistical significance by one way analysis of variance (ANOVA) followed by Bonferroni post-test. Each experiment was done in triplicate.

Results

Lower expression of transferrin receptor in *CT* infected HeLa cells

Transferrin receptor expression was analyzed by western blotting to assess the intracellular intake of iron. Expression of TfR was decreased in *CT* infected HeLa cells in comparison to mock infected cells. Expression of TfR was not induced on addition of intracellular iron chelator deferoxamine mesylate (DFO), whereas mock showed significant increase. Even the Ferric ammonium citrate (FAC) supplementation to *CT* infected HeLa cells did not lead to the change in TfR level in comparison to respective controls (Fig 5.1). These results suggest that expression of transferrin receptor in *CT* infected HeLa cells do not respond to inducers like DFO.

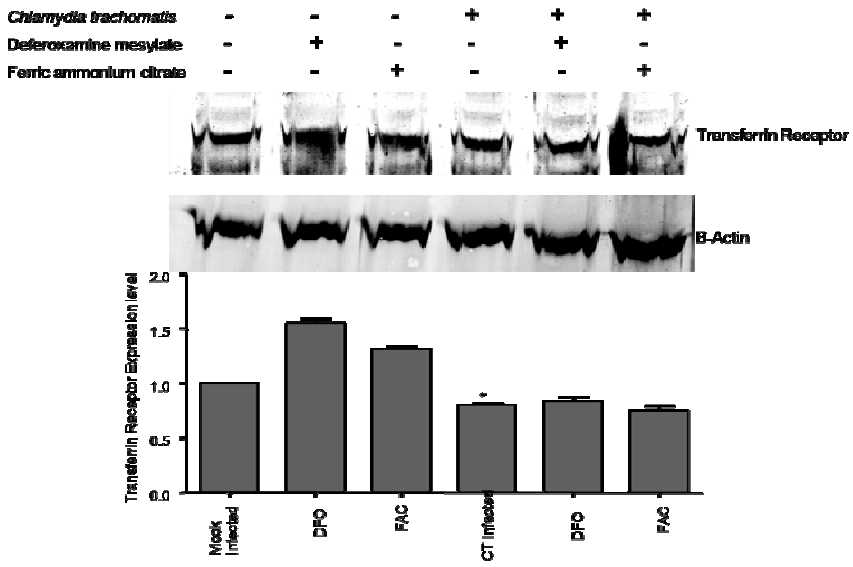


Fig 5.1: Expression of transferrin receptor (TfR) was down–regulated in *CT* infected HeLa 229 cells and did not change on addition of DFO and FAC.

Higher expression of Ferritin heavy chain in *CT* infected HeLa cells

Iron storage is controlled by ferritin comprised of Heavy and light chain indicator of intracellular iron. Chlamydia infected HeLa229 cells showed higher expression of ferritin Heavy Chain (FHC) in comparison to mock infected cells. Upon addition of DFO, FHC expressions remain above the basal (Mock) level as was observed in immunoblotting. On addition of FAC, up-regulation of FHC was observed in infected and mock infected HeLa cells (Fig 5.2). These results showing that regulation of iron homeostasis was modulated in chlamydia infected cells.

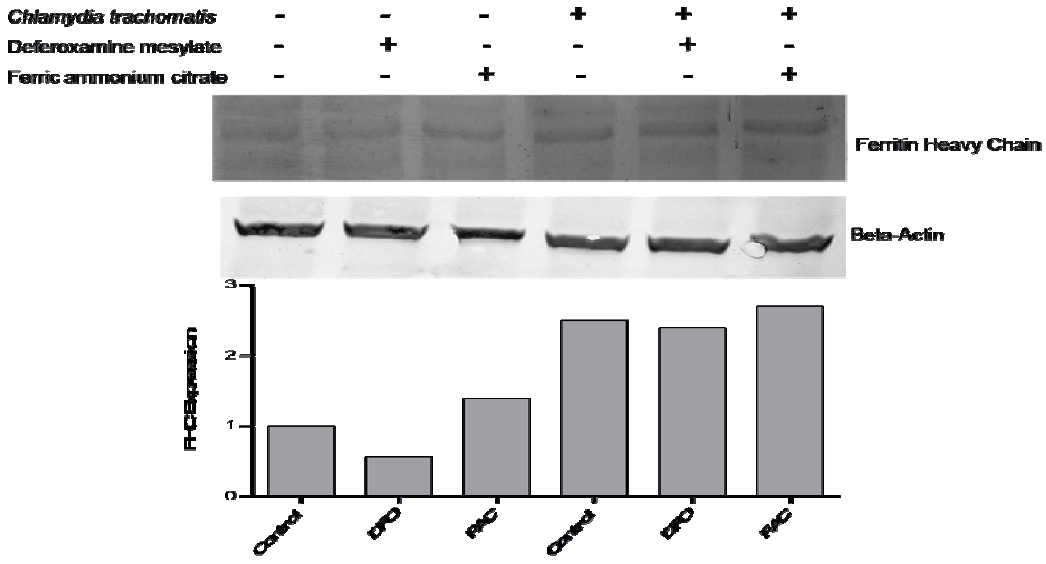


Fig 5.2: Expression of FHC was detected in *CT* infected HeLa cells in comparison to control. FHC level remains above the basal level (Mock) in *CT* infected cells on addition of DFO. Conversely additions of FAC lead to higher degree of induction in FHC level *CT* infected cells than control.

Level of Iron response protein 1 and 2 (IRP-1 and 2)

Iron response protein 1 and 2 are the central regulator of iron metabolism and play important role in iron homeostasis. In chlamydia infected HeLa cells level of IRP 1 is higher in comparison to mock infected cells (Fig. 5.3A), whereas IRP2 did not show significant difference. On addition of Intracellular iron chelator DFO, level of IRP1 increased significantly whereas IRP2 did not show such change in *CT* infected cells in comparison to mock infected cells (Fig.5.3B). Addition of iron supplement leads to significant changes in both infected as well as in mock infected HeLa 229 cells (Fig 5.3A). These results suggest that IRP1 may play a major role in iron homeostasis in chlamydia infected HeLa cells.

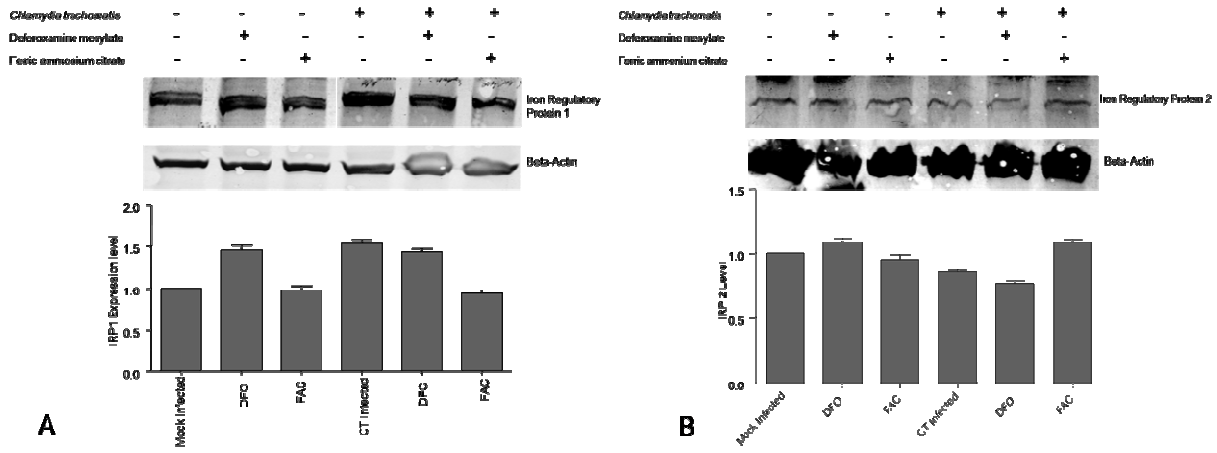


Fig 5.3: (A) *CT* infected HeLa cells showed higher expression of IRP-1 which decline on addition of DFO. (B) Decline in expression of IRP-2 level was observed in *CT* infected HeLa cells and there was further decline on addition of DFO. No significant change was observed in FAC treated control and infected cells.

Attenuated Binding activity of IRE- IRP (RNA electrophoretic mobility shift assay)

Stability of TfR mRNA and translational blockage of Ferritin expression in iron-deprived condition is defined by kinetics of IRE-IRP binding activity. The binding activity of EMSA was determined by comparison of maximum activity (b-ME treated cytoplasmic

extract show maximum activity) with actual activity. Cytoplasmic extract of *CT* infected HeLa cells showed significant decrease in binding activity relative to mock infected cells. Deferoxamine treatment leads to increased binding activity in both the conditions; although signal was relatively low in *CT* infected cells (Fig.5.4 A).Prior incubation of cytosolic extract with anti-IRP-1 and IRP-2 antibodies showed loss of binding only in case of IRP-1 (Fig 5.4 B). Resumption of binding activity was observed after chloramphenicol treatment of CT infected HeLa cells (Fig. 5.4 C). These results showed that *CT* infection modulates the iron homeostatic machinery of host cell by attenuating the binding activity of IRPs to IRE.

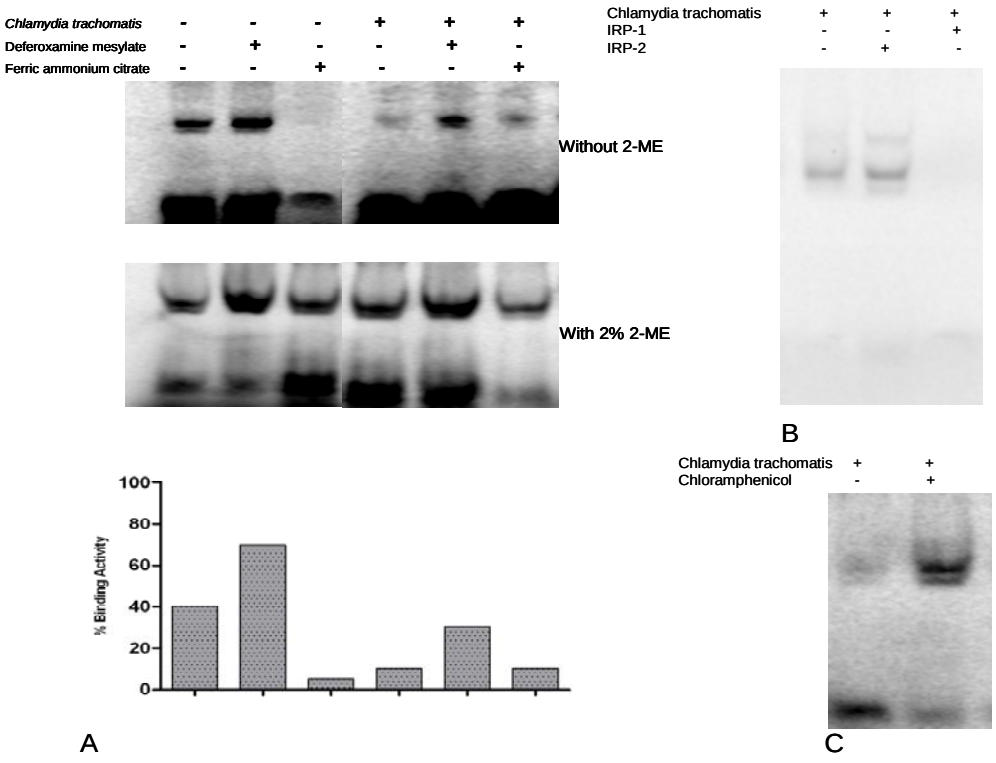


Fig 5.4: Effect of *CT* infection on interaction of IRP-IRE:

[A] Binding activity of IRP-1/IRE was attenuated in *CT* infected HeLa cells as observed in electrophoresis mobility shift assay.

[B] Inhibition of binding was observed with IRP-1 antibodies in *CT* infected HeLa cells.

[C] Attenuation of binding activity reverted to normal after treatment with chloramphenicol.

Suppression of NF-kB and HIF-1α activation in CT infected cells:

Nuclear extract of cells infected with CT showed no binding NFkB with radiolabelled NF-kB probe in gel shift assay, after stimulation with H_2O_2. On inhibition of chlamydial growth by using chloramphenicol, binding of NF-kB and NF-kB probe was re-achieved. Further, transcriptional activation HIF-1α was ascertained by incubating nuclear extract with radiolabelled HIF-1 α, and no binding was observed in CT infected cells. On addition of chloramphenicol CT infected cells showed binding to the HIF-1 α probe (Fig 5.5).

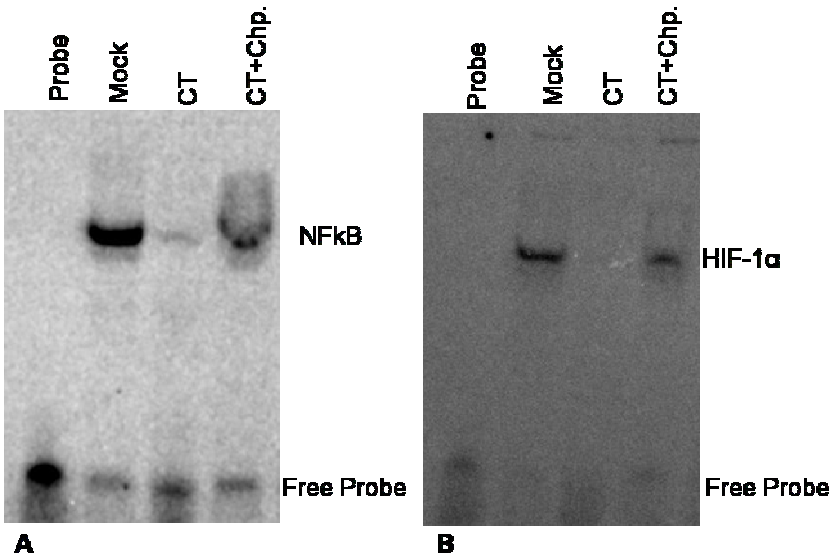


Fig 5.5: Effect of CT on NF-kB and HIF-1α activation:

(A) Activation of NFkB is not observed in CT infected cells on H_2O_2 stimulation.

(B) Activation of HIF-1α is not observed in CT infected cells on DFO stimulation.

Up-regulation of PHD-1 and down regulation of TfR in CT infected cells:

The Cells were treated with DFO to mimic hypoxic condition and Cytoplasmic extract was resolved on SDS page and transferred to PVDF membrane for immunobloting. Expression of PHD-1 increased in CT infected cells in comparison to mock infected and chloramphenicol treated CT infected cells. (Fig 5.6).

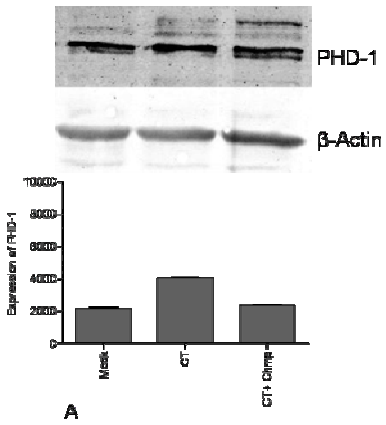


Fig 5.6: Up-regulation of PHD-1 in CT infected cells.

Decreased level of intracellular calcein chelatable iron (labile iron pool):

Cells were stained in calcein- green stain for determination of labile iron pool and decrease in green fluorescence is indicator of labile iron pool. CT infected cells showed evident decrease in fluorescence intensity in comparison to DFO treated cells; however fluorescence is higher than mock infected cells. The labile iron pool was dampened not vanished in comparison to mock infected cells (Fig 5.7).

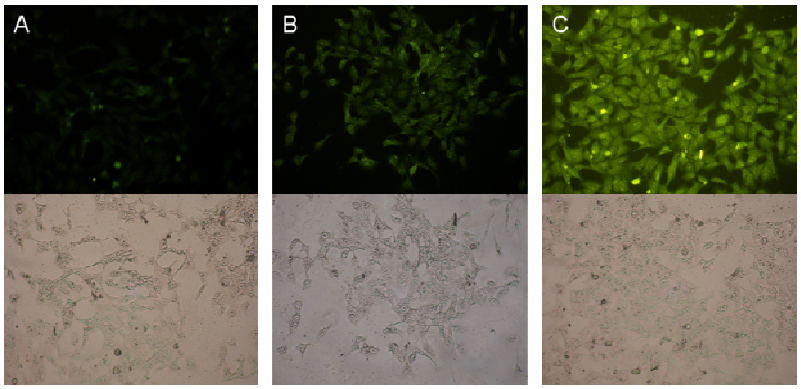


Fig 5.7: Calcein fluorescence quenching showing labile iron pool: (A) Mock infected cells. (B) CT infected cells (C) DFO treated cell.

Discussion

CT is one of the most successful bacterial pathogen in terms of intracellular survival due to its ability to modulate the host resources towards its own development. Both host as well as the pathogens has higher affinity for the Iron, most essential element required for survival.

In accordance with previous study our results showed decline in the level of transferrin receptor in *CT* infected HeLa cells (Murray *et al.*, 1991). Transferrin receptor mediated iron acquisition has been reported as important mode for iron uptake contributing major part of iron requirement in various cell types (Hentze *et al.*, 2004). TfR regulation is sensitive to various intracellular physiological conditions and secretory factors. In addition it is well documented that IFN-γ, tumor necrosis factor-α, interleukin-1, and interleukin-6 induced cells down regulates TfR level in various cell types (Kang, DK, 2004). Experimental models of chlamydial infection showed up-regulation in the levels of IFN-γ (Devitt *et al.*, 1996). The reduction in epithelial cell transferrin uptake triggered by CT may be a general aspect of cellular immunity such as that seen in interferon (IFN)g- and lipopolysaccharide (LPS)-activated macrophages (Ludwiczek *et al*., 2003). This macrophage response has been shown to limit transferrin iron availability to intracellular pathogens and may result in mobilization of ferritin iron to meet cellular metabolic needs. Pathogens with diverse iron-scavenging tactics such as *N. meningitidis* are likely to exploit this process by tapping into ferritin iron reserves and bypassing the need for transferrin iron within cells. Therefore, these results may suggest the involvement of secretory factors in the down-regulation of TfR in chlamydia infected cells. Furthermore, the present study showed that TfR expression did not change

significantly after addition of DFO and FAC. This result deciphers the modulation of the iron-regulatory machinery of cells infected with *CT*.

In this study, up-regulation of FHC was observed in *CT* infected cells, which did not alter significantly after addition of the iron chelator DFO. Physiological responses reported for TNF-α, IL-1, IL-6, and IFN-γ include stimulation of H-ferritin expression and inhibition of TfR1 expression (Torti *et al.*, 2002). A major function of ferritin is to limit Fe^{2+} available to participate in the generation of ROS. Oxidative stress activates transcriptional and posttranscriptional pathways of ferritin regulation (Torti, FM, 2002). HL-60 cells demonstrate a marked increase in *fhc* mRNA synthesis during the course of *Anaplasma phagocytophilum* infection (Carlyon, GA *et al*, 2005). A similar result was noted for THP-1 cells infected with *Mycobacterium bovis* BCG (Lim, JS, 1997). Thus, an increase in *fhc* expression may exemplify a common theme among host expression profiles associated with intracellular bacterial infection. At one side induction of FHC expression is suggestive of activation of acute host response against pathogens, by depriving cytoplasm from essential element iron. However on other side higher FHC also able create a favourable microenvironment for pathogens by attenuating oxidative defence of host. These results further explain the involvement of CT in the up-regulation of FHC. However, non-responsiveness of the *CT*-infected cells to the iron chelator DFO might indicate modulation of the iron regulatory system.

Chlamydia-infected HeLa cells showed the predominant expression of IRP-1 rather than IRP-2, in contrast to mock infected cells. In iron-depleted condition, IRP-1/2 binding to IRE blocks ferritin-mRNA translation and simultaneously stabilizes the TfR mRNA, thus regulating the iron uptake and storage (Hentze *et al.*, 2004). Because of the additional 73

amino-acid peptide sequence, IRP-2 is prone to proteosomal degradation in the presence of nitric oxide and iron (Rouault, 2002). Chlamydial infection induces the production of both nitric oxide and IFN-*γ/α* in different cell types (Devitt *et al.*, 1996). In response to the same stimulus, IRP-1 and -2 are regulated conversely (Bouton *et al.*, 1998). Hence, it may suggest that lower levels of IRP-2 might be due to the proteosomal degradation induced by nitric oxide or converse regulation by IFN-*γ*.

In this study, Chlamydia-infected HeLa cells showed attenuation in IRP-IRE binding activity; however, the binding activity resumed after addition of chloramphenicol. Levels of TfR and ferritin are principally regulated through the binding of IRPs with IREs, and the binding activity of IRP-IRE is dependent on both the free reactive iron (labile iron pool) and the intracellular redox state (Hentze *et al.*, 2004). The labile iron pool regulates the binding of IRP-1/2 to IREs through distinct mechanisms. In the presence of high levels of iron, IRP-1 assembles in a cubane [4Fe-4S] cluster, inhibiting the IRE-binding activity, and converting IRP-1 to an aconitase. When cellular iron level is low, IRP-1 binds to IRE targets as an apoprotein (Eisenstein *et al.*, 2003, Brown *et al.*, 1998). IRPs are also controlled by other effectors that include reactive oxygen species, which induce the disassembly of the Fe-S cluster of IRP-1 (Brown *et al.*, 1998), and nitric oxide and hypoxia, which affect both IRPs (Rouault *et al.*, 1997).

Alternatively iron homeostasis regulated through other pathways, principally through HIF-1α. TfR expression is up-regulated by the HIF-1 (Bianchi *et al.*, 1999), which is typically activated under hypoxic conditions but can also be turned on by a number of non-hypoxic stimuli, including inflammatory signals such as NO and LPS (Dery *et al.*, 2005). Hypoxia in conjunction with NF-kB regulates many physiologically important conditions and has been

shown to be involved in iron homeostasis and redox regulation of cells. In this study our results of gel shift assays showed that CT infection leads to dampening of NF-kB and HIF-1α activation pathway and down-regulation in iron acquisition protein TfR. Further increased expression of PHD-1 was observed in CT infected cells in hypoxia mimetic condition generated by the use of DFO. CT infected cells always pose to continuous flux of inflammatory stimuli and responses are modulated in favour of chlamydia. Transcription factor NF-kB, the master regulator of inflammatory pathway and is major player in iron homeostasis due to its presence upstream to TfR and FHC gene (Torti *et al.*, 2002). The molecular mechanisms of TfR transcriptional regulation under inflammatory conditions are complex and involve at least two pathways. The induction of TfR expression is regulated by a signaling pathway that successively involves NF-kB activation, HIF-1α induction, and TfR transcription. NF-kB activation, which is presumably induced through the TLR4-dependent signaling cascade and possibly involves a transient and early increase in the low molecular weight iron pool leading to a prompt increase in HIF-1α activity (Tacchini *et al.*, 2008). In cytoplasm NF-kB is anchored with Ikk and on activation IkBα get phosphorylated followed by polyubiquitination and rapid ubiquitin-dependent degradation by the 26S proteasome (Chen *et al.*, 2006). Thus released NF-kB transcription factors then translocate to the nucleus, where they coordinate the transcriptional activation of several hundred target genes, directly involved in innate immunity and inflammation (Hoffmann *et al.*, 2006). However, status of NF-kB in CT infected cells falls in the favour of deactivation by various means like degradation and deubiquitination (Mackern-Oberti *et al.*, 2006).Many animal and plant pathogenic bacteria and viruses interfere with the host ubiquitin-proteasome system to subvert the host signalling system as part of their infection strategy (Rosebrock *et al.*, 2007).

Among these signaling systems, bacterial effector proteins have been shown to inhibit the NF-kB pathway by subverting the host ubiquitin machinery (Neish, 2004). In Chlamydiae *Chla*Dub1 suppresses NF-kB activation induced by several pro-inflammatory stimuli, including cytokines, the LPS receptor TLR4, and signal-transducing components of NF-kB activation pathways (Le Negrate *et al.*, 2008). Further our study is in consistent with Lad *et al*. wherein the authors have reported that *Chlamydia* infection does not induce NF-kB activation and appears to protect IkBα from TNF-α induced degradation (Lad *et al.*, 2007a). In that report, a molecular mechanism was proposed in which p65/RelA is specifically cleaved by the bacterial protease CT441, thereby preventing activation of the NF-kB pathway (Lad *et al.*, 2007b). NF-kB has been shown to be activated by hypoxia in a number of studies (Bonello *et al.*, 2007, Schmidt *et al.*, 2007). Cyclooxygenase 2, TNF-α, IL-6 and macrophage inflammatory protein 2 are among the target genes identified for hypoxia-induced NFkB, and these underline the factor's importance in inflammatory signaling (Viemann *et al.*, 2007). Upregulation of HIF by bacterial and viral compounds in cells of the immune system prepares the cells for migration to the hypoxic environment of inflamed and injured tissues. Furthermore, HIF-1α is essential for myeloid cell mediated inflammation (Haddad, 2002). Myeloid cells lacking HIF-1α had a lower glycolytic capacity, resulting in impairments in myeloid cell aggregation, motility, invasiveness and bacterial killing (Nanduri *et al.*, 2007). HIF therefore appears to have an important role in the coordination of cellular responses under conditions of inflammation (Oliver *et al.*, 2009). During chlamydial infection, HIF-1α was actively degraded by CPAF, a chlamydial protease secreted into the cytoplasm of the cells within 48 to 72 h after infection (Rupp *et al.*, 2007). It is known that in normoxia, chlamydiae exhibit anti-apoptotic activities to stabilize the infected host cell

during mid to late stages in the developmental cycle (Miyairi *et al.*, 2007). Our study is consistent with earlier finding and additionally provides the clue that CT blocks activation of HIF-1α in presence of iron chelator/hypoxia mimetic DFO. Hypoxia is a feature of sites of chronic inflammation, for example in the RA synovium, in atherosclerotic plaques, in sites of bacterial infection and at growing tumours (Murdoch *et al.*, 2005). This occurs when the cellular demand for oxygen, in order to meet the metabolic needs of the tissue to produce ATP, exceeds the supply. Inflammatory reactions to bacterial infections usually reduce tissue oxygenation and induce cellular responses to hypoxia in diseased organs (Koury, 2005). Iron is major player in innate immune responses and essential for the living. Iron in free form catalyses generation of reactive oxygen species and directly cause the damage to cellular component. Iron acquisition is one of the evolutionary most conserved innate immune defence of host from pathogens, however in case of intracellular pathogens this mechanism is controlled by many other pathway along IRP-IRE system. Cross-talk of NF-kB and HIF-1α is responsible the higher transcription of TfR mRNA (Tacchini *et al.*, 2008). This study have shown the deactivation of NF-kB and HIF-1α that is indicating other region of lower level of TfR other than attenuation of binding activity IRP to IRE. We further reported higher expression of PHD-1 in CT infected cells under hypoxia mimetic condition thereby indicating towards involvement in degradation of HIF. Thus these finding suggest that chlamydia device the mechanism to divert the iron acquisition by lowering the TfR expression level by deactivating NF-kB and HIF-1α. Thereby reduce the availability of intracellular iron and its catalytic potential to generate toxic free radicals. Further these finding is augmented by the results showing lower level intracellular free iron as shown by calcein staining. These results indicate that the level of intracellular free iron (Labile Iron

Pool) is maintained at a critically functional state in chlamydia-infected cells. In a recent study, Nupur et al. have shown that *Leishmania donovanii* uses labile iron pool (LIP) as a dynamic source of iron inside the cells (Das *et al.*, 2009). These observations further extend the possible involvement of LIP as a source of iron for intracellular development of CT.

Hence, this study showed that chlamydial infection has a modulatory effect on the activity of IRPs which leads to decreased TfR and increased FHC expressions thus limiting the intracellular availability of catalytic-free iron and finally reactive oxygen species. Additionally this study provides the insight to deactivation of alternate pathway which consists of NF-kB and HIF-1α for transcription of TfR mRNA. Furthermore, chlamydial infection may accelerate the recycling of TfR and transferrin to the surface, thus recharge the required iron in LIP by faster shuttling. Transiting iron in LIP may be sequestered in FHC as stored available iron for *CT* in HeLa infected cells. Thus, the current findings suggest attenuation in the binding activity of IRP-1/IRE may be one of the mechanisms involved in modulation of iron homeostasis in *CT*-infected cells (Fig 5.8).

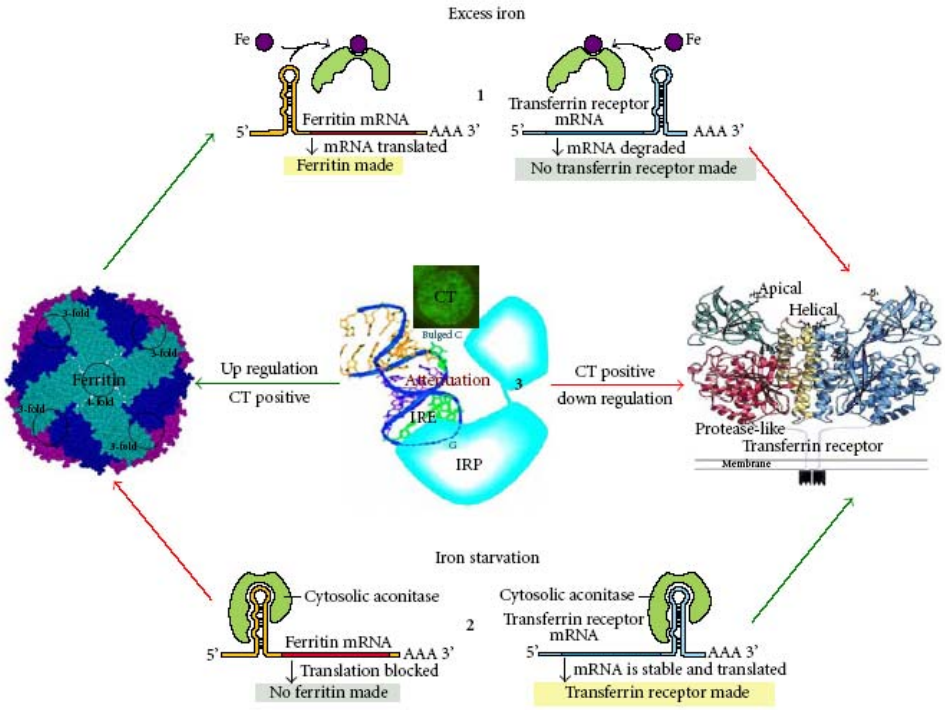


Fig 5.8: Translational initiation of ferritin and TfR mRNA degradation are regulated by binding of IRP to IRE in response to availability of iron. (1) In the excess iron, IRP forms an open conformation and is not able to bind IRE, subsequently translation of ferritin gets initiated and degradation of exposed TfR mRNA takes place. (2) In iron starved cells, IRP attains a closed conformation and binds to IRE present on 5*'* UTR of ferritin mRNA thus interferes with translation initiation and simultaneously stabilize TfR mRNA by binding 3*'* UTR. (3) Binding affinity of IRP-IRE is attenuated in presence of chlamydia leading to lower expression of TfR and higher expression of FHC.

Chapter 6

Chlamydia trachomatis: Iron and cytokine regulation

Science, in the very act of solving problems, creates more of them.
Abraham Flexner

Chapter 6

Chlamydia: Iron and cytokine regulation

Introduction

Chlamydia trachomatis is the most common sexually transmitted pathogen which causes severe sequelae in women including pelvic inflammatory disease, ectopic pregnancy and infertility (Byrne *et al.*, 2004, Vats *et al.*, 2004, Singh *et al.*, 2003). Most of the pathological consequences associated with chlamydial infection are conferred to host immunological reaction to pathogens. Endosymbiont chlamydiae were evolved with host immune resistance and developed the strategy for survival. Evolution conferred *CT* ability to infect and multiply within a broad range of eukaryotic cells, including macrophages, smooth muscle, epithelial, and endothelial cells (Hackstadt *et al.*, 1991). Chlamydial infection of epithelial cells at mucosal surface induces secretion of proinflammatory factors such as interleukin (IL)-1α, IL-6, IL-8, IL-11, GRO-α, and granulocyte-macrophage colony-stimulating factor (Dessus-Babus *et al.*, 2000, Rasmussen *et al.*, 1997). It can lead to an acute inflammatory response characterized by neutrophil infiltration to the primary sites of infection, followed by a subepithelial accumulation of mononuclear leukocytes during the chronic phase of infection (Patton *et al.*, 1989). These cellular responses promote cellular proliferation and tissue damage of affected organs (Wylie *et al.*, 1997). Most of the invasive bacterial pathogens often induce rapid but transient responses (Ulevitch, 1999). In contrast *CT* infection induces delayed proinflammatory responses, especially IL-8 production in epithelial cells and is dependent on bacterial replication (Rasmussen *et al.*, 1997). In addition TNF-α and IL-1β have been reported to be the most powerful inducers of IL-8 in a multitude

of cell lines and a major contributor in clearance process for intracellular pathogens (Rupp *et al.*, 2003).

Several studies using various pathogenic infection models have shown that IL-10 plays an important role in balancing the protective and pathological immune responses during intracellular parasitic infection (Gazzinelli *et al.*, 1996, Hunter *et al.*, 1997, Linke *et al.*, 1996). Chlamydia associated immunopathological changes are the product of pro or anti inflammatory responses and as a result of infection. Chlamydial infection induces production of many pro- and anti- inflammatory cytokines, in which IL-10 is the key determinant of disease pathology (Yang *et al.*, 1996, Yang *et al.*, 1998). Earlier data suggests that IL-10 inhibits host clearance of chlamydial infection and that IL-10 plays a negative regulatory role in the immune responses to chlamydial infection (Morrison *et al.*, 2000). It is reported that IL-10 inhibits antigen presentation via suppression of major histocompatibility complex (MHC) classes I and II expression (Mach *et al.*, 1996).The immune response to microbial infection is determined by complex interactions between cells, cytokines milieu and micro-environment (Abbas *et al.*, 1996, Fresno *et al.*, 1997, Mosmann *et al.*, 1989, Scott *et al.*, 1991).

Cellular iron availability alters the proliferation and activation of immune cells, and is able to modulate immune effector pathways and cytokine activities (Dagvadorj *et al.*, 2009b). Moreover, iron is directly involved in cytotoxic immune defense mechanisms, where iron is needed to catalyze the formation of the hydroxyl radical ($OH^{\bullet}$) via Fenton reaction (Gray *et al.*, 2002). During microbial infection, competition for iron between the host and microorganisms is therefore inevitable. Given the potential physiological importance of iron in immune function, iron chelators have been implicated to modulate certain inflammatory

mediators and regulate inflammatory processes (Marx *et al.*, 2002, Choi *et al.*, 2004). Bacterial iron chelator (desferal, deferoxamine mesylate) triggers inflammatory signals, including the production of CXC chemokine IL-8 in human intestinal epithelial cells (IECs) by activating ERK1/2 and p38 kinase pathways (Choi *et al.*, 2004). Chlamydia enters into persistence stage in presence of iron-chelating drug (Desferal), thereby showing its dependence on iron for completion of developmental cycle (Raulston, 1997). Persistence can also be induced by antibiosis and tryptophan starvation induced by penicillin G and IFN-γ respectively (Ramsey *et al.*, 2001, Pantoja *et al.*, 2001). Earlier model of *C. pneumoniae* persistence showed that after IFN-γ and penicillin treatment chlamydia-induced IL-8 expression was inhibited, while it stayed up regulated in iron-depletion (Peters *et al.*, 2005). It has long been accepted that iron is key determinant of infection state of chlamydia and is centrally placed at the crossroad of immune responses and infection. Cellular iron availability alters the proliferation and activation of immune cells, and is able to modulate immune effector pathways and cytokine activities (Bahia-Oliveira *et al.*, 2009, Dagvadorj *et al.*, 2009a, Vardhan *et al.*, 2009). Moreover, iron is directly involved in cyto-toxic immune defense mechanisms, where iron is needed to catalyze the formation of the hydroxyl radical (OH^{o}) via Fenton reaction (Gray *et al.*, 2002). Ubiquitously distributed ferritin is an iron-storage protein that plays a key role in cellular iron homeostasis and regulated by availability of iron (Rouault *et al.*, 1997). Some low molecular weight substances and cytokines also affect transcriptional and/or posttranscriptional ferritin synthesis (Harrison *et al.*, 1996). Our study describes (Chapter 5) the upregulation of ferritin heavy chain (FHC) resulting from attenuated binding activity of iron regulatory protein and iron responsive element in CT

infected HeLa cells. Further earlier studies have established FHC as a potent immunosuppressive protein, which induces IL-10 production (Gray *et al.*, 2001).

It is reported that patients receiving higher doses of IL-10 developed anemia and presented with a dose-dependent increase in ferritin and soluble TfR levels, an indicator of iron restriction to erythroid progenitor cells (Gray *et al.*, 2002). Hyper-ferritinemia results from direct stimulation of ferritin translation by IL-10 in activated monocytic cells, most likely by cytokine-mediated reduction of the binding affinity of translational repressor, iron-regulatory proteins, to the 5'-untranslated region of ferritin mRNA (Weiss *et al.*, 1995).

In order to understand chlamydia pathogenesis, it is essential to define the activation pathways in which iron chelation controls IL-8 induction in chlamydial infection. Although many studies have shown induction of anti and pro- inflammatory cytokines in chlamydial infection; however the effect of these cytokines with intracellular redox regulator iron and its homeostasis is still in infancy and need to be studied.

Thus, this study is aimed:

(I) To Study the involvement of intracellular iron in chlamydia induced production of proinflammatory cytokine IL-8 from HeLa 229 cells and autocrine effect of TNF- α and IL-1β in production of IL-8 from *CT* infected cells under iron deprived condition and

(II) To understand the association IL-10 and host iron homeostasis.

Experimental Methods

Materials

All the bio-chemicals and cell culture media were purchased from Sigma-Aldrich (Saint Louis, USA) and antibodies from Santa Cruz (LA, USA) unless otherwise mentioned. Deferoxamine mesylate (DFO)-bacterial iron chelator; Mimosine- a plant based iron chelator; Ferric ammonium citrate (FAC)- iron source; Glucose oxidase (GO)- oxidant; DETA-NANOate -nitric oxide producer; Pyrrolidin dithiocarbamate (PDTC)- nitric oxide inhibitor; Ebselen- anti-oxidant.

Propagation of chlamydiae

CT serovar D was propagated in HeLa 229 cell monolayers and purified on discontinuous gradients of Renograffin (Squibb, Montreal, Canada) as described previously (Schachter *et al.*, 1994). Purified elementary bodies (EBs) were resuspended in isotonic sucrose-phosphate-glutamate (SPG) buffer and stored at -80°C. The infectivity of purified EBs was titrated by counting chlamydial inclusion forming units (IFUs) on the monolayer of HeLa 229 cells grown in a 96-well plate.

Infection

Sub-confluent human HeLa 229 cervix epitheloid cells (CCL2) were grown in 12-well culture plates and infected with ~2 multiplicities of infection (moi) of EBs in SPG. The plates were then rocked for 2 h at 37°C after which the extracellular bacteria were removed by washing and subsequently cells were cultured in Delbecco's Minimum Essential Media (Sigma, Saint Luis, USA) containing 10% Fetal bovine Serum (PAA Laboratories GmbH, Pasching, Austria), 10 μg/ml gentamicin (Sigma, Saint Luis, USA), 1 μg/ml Amphotericin B (Sigma, Saint Luis, USA) and incubated at 35 ^{o} C in 5% CO_2 environment. After 2 hours post

infection (hpi), cells were treated with DFO (50μm) or Mimosine (1mM) or Ferric ammonium citrate (0.5 mM) or DETA-NANOate or Ebselen or PDTC or Glucose oxidase along with their respective controls. At 6, 12, 24, 30, 48, 72 and 96 hpi, supernatants were collected after centrifugation at 12000g and stored at -80°C until cytokine assay.

Cytokine assays using ELISA

Levels of secreted cytokines (IL-8, TNF-α, IL-1β, and IL-10) in culture supernatants were determined using ELISA kit (Pierce Biotechnology Inc, Rockford, USA and eBiosciences, San Diego, USA)) having detection limit of 2pg/ml, 4pg/ml and 8pg/ml respectively. All the assays were performed in triplicate according to manufacturer's instructions.

IRE-GFP construct, transfection and flow-cytometric quatitation:

Iron response element containing plasmid was kind gift from Michael Kibler. The construct was a modified pd2EGFP-N1 vector containing ferritin IRE, in place of indigenous CMV promoter, ahead of NLS-GFP cassette. In abundance of iron, cytoplasmic IRPs lose their binding activity to IRE, thus no translation blockage occurs and expression of GFP is registered as green fluorescence in FL-1 detectors (Macchi *et al.*, 2003).

Fig 6.1. Schematic representation of the construct: The ferritin promoter containing the IRE element drives the expression of the NLS-GFP.

HeLa 229 cells were grown in 6 well tissue culture plates with cell density of 5×10^5 cells/well in Earl's modified eagle's medium (EMEM) containing 10% fetal calf serum (FCS). On reaching the sub-confluence, cells monolayer were washed twice with hank's

balanced salt solution (HBSS) and transfected with 1µg construct using of 3µl of HD fugene tranfection reagents as per manufacturer's instructions. EMEM with 5% FCS was added to these cells and subsequently placed in incubator at 37^{o}C in 10% CO_2 for 24 hours, prior to infection with CT.

Transfected cells were washed with HBSS and infected with chlamydial EBs at multiplicity of infection (MoI) of 2. For homogenous infection tissue culture plates were placed on a rocker for 2 hour at 35ºC after addition of serum free media containing EBs. Media containing unbound EBs were aspirated and supplemented with complete Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum (FCS). Infected HeLa 229 cells were incubated at 37^{o}C with 5% CO_2 in a humid environment. Thereafter at 18 hours post infection (hpi) media was aspirated and replaced with fresh media containing 50µm deferoxamine (DFO) and 1mM Ferric ammonium citrate (FAC) in respective wells with their controls. Media was aspirated for ELISA and cells were analysed for green fluorescence using flow cytometer. For negating auto- fluorescence same pool of untranfected cells were used and appropriate setting was used for further acquisition and analysis.

Immunoblotting of proteins:

CT-infected HeLa cells were washed with phosphate buffered saline (PBS) and subsequently treated with lysis buffer (0.5% Nonidet P-40, 150mM NaCl, 0.1% sodium dodecyl sulfate (SDS), 50mM NaF, 1mM Na_3VO_4, and 1 mM phenyl methyl sulfonyl fluoride containing the complete protease-inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were determined by the Bradford protein assay (BioRad Laboratories) with BSA as standard. Extracted proteins (40 µg) were electrophoresed on 8–12% SDS

polyacrylamide gels and transferred to polyvinyl difluoride membranes (BioRad Laboratories); the membranes were then reversibly stained with Ponceau S (Sigma Aldrich) to confirm complete transfer. Membranes were blocked with 5% nonfat dry milk in PBS-Tween-20 and incubated with rabbit anti-IgGs against TfR-1, ferritin heavy chain (FHC), phosphorylated-Smad (p-Smad) and further incubated with the goat anti-rabbit IgG conjugated with horseradish peroxidase. Subsequently, they were developed using 3,3-diaminodbenzidine as the detection agent and analyzed using the Image J software (NIH, Bethesda, USA).

Statistical analysis

A statistical analysis was performed using GraphPad Prism software (version 5.0). Differences were tested for statistical significance by 2-way repeated measurements analysis of variance (ANOVA) followed by Bonferroni post-test. Every experiment was done twice in triplicate.

Results

Elevated level of exogenous IL-8 in CT infected HeLa 229 cells:

Level of IL-8 was significantly increased ($P < 0.001$) in culture supernatant of *CT* infected cells in comparison to mock infected cells. Further significant increase in IL-8 level was observed in *CT* infected cells on addition of Iron chelator deferoxamine (DFO). In time dependent study, significantly elevated level of IL-8 secretion was detected at 12 hpi, ($P < 0.001$) in *CT* infected cells comparison to control (mock infected Hela cells). Further in control cells two crests were seen at 24 and 72 hpi through at 30 hpi representing a biphasic curve, whereas in *CT* infected cells secreted IL-8 level represented a monophasic curve which showed consistently significantly increased levels from 12 hpi till 72 hpi ($P < 0.001$) [Fig.

6.2]. In contrary significant decline in level of secreted IL-8 was observed in mock and CT on supplementation of Iron (FAC) in comparison to CT control and DFO treated mock (♣).

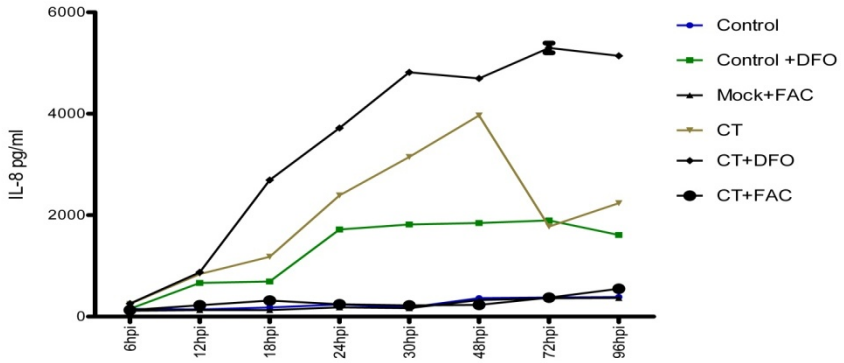


Fig 6.2: *CT* infected HeLa 229 cells showing significantly ($P < 0.001$) increased levels of IL-8 as detected by ELISA in comparison to mock infected cells.

Secretion of IL-8 is independent of exogenous IL-1β and TNF-α in chlamydia infected cells:

Temporal expression of IL-1β and TNF-α was assessed to ascertain the involvement of these cytokine in IL-8 induction. TNF-α concentration as determined by ELISA in *CT* infected culture supernatant was significantly (P<0.001) increased at early time points (6, 12 and 18 hpi) and late time points (72 and 96 hpi), whereas there was decrease at mid time points (24, 30 and 48 hpi) in comparison to mock infected (Fig 6.3A). Levels of IL-1β in supernatants of *CT* infected culture were significantly decreased (P<0.005) at early (6, 12 and 18 hpi) and late (72 and 96 hpi) time points in comparison to mock infected culture, whereas at mid time point no significant (P<0.001) change was observed (Fig 6.3B).However, at the mid phase of *CT* growth, level of secreted TNF-α declined, whereas IL-1β level did not show any deviation from basal level [Fig. 6.3A and 6.2B] and secretions of TNF-α, IL-1β and IL-8 did not show synergy at different time points. Further addition of DFO lead to decline,

conversely addition of FAC lead to increase in TNF-α and IL-1β level in both CT and Mock condition (♣).

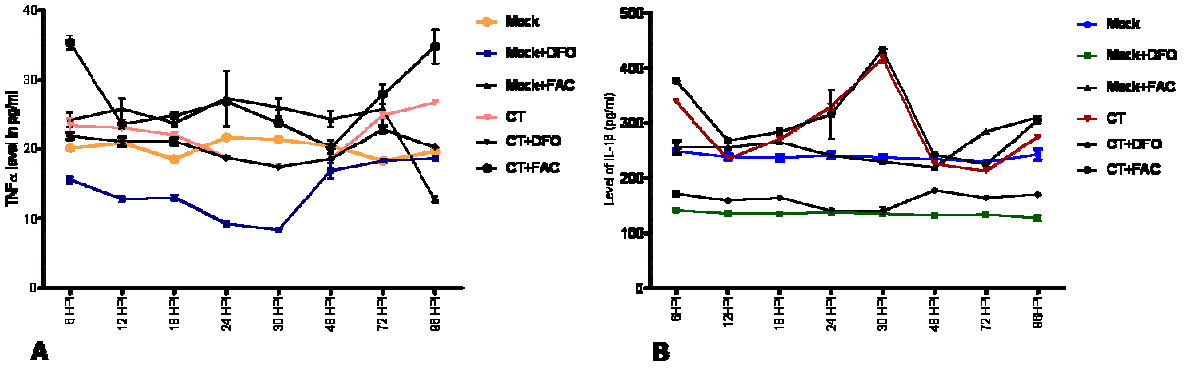


Fig 6.3: (A) TNF-α concentration as determined by ELISA in culture supernatant (B) Levels of IL-1β in supernatants

IL-8 expression with iron chelator DFO and Mimosine in *CT* infected HeLa cells:

Gradual increase was detected in levels of IL-8 in culture supernatants; highest at 30 hpi thereafter subsequent decrease was observed from 48 hpi onwards in *CT* infected cells treated with Mimosine. Mimosine, a plant based iron chelator showed earlier induction of IL-8 at 6 hpi in *CT* infected HeLa cells whereas in presence of DFO there was delayed (12 hpi) induction and was consistent at all time intervals(Fig 6.4).

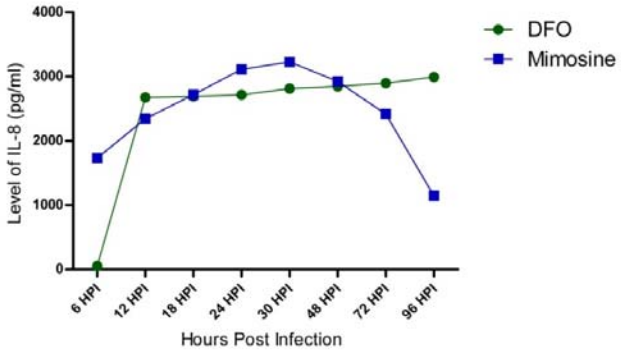


Fig 6.4: IL-8 concentration as determined by ELISA in culture supernatant after addition of DFO and Mimosine.

Chlamydia induced IL-8 production is mediated by reactive nitrogen species in iron restricted condition

IL-8 production was significantly increased (P<0.001) in *CT* infected HeLa cells after stimulation with DETA-NANOate nitric oxide (Fig 6.5). Co-stimulation of CT infected cells with DFO and NO lead to further significant increase in induction of IL-8 (P<0.001, Fig6.4). However there was non-significant changes observed in FAC treated mock and CT infected cells (♣). In addition Glucose oxidase treatment induced IL-8 production in both the conditions *CT* infected as well as Mock (P<0.005, Fig 6.5). Nitric oxide also showed prominent induction of IL-8 and DFO had further additive effect on IL-8 secretion in *CT* infected cells in comparison to mock (P<0.001, Fig 6.5). Moreover, significantly higher inhibition (P<0.05, Fig 6.5) of IL-8 induction was observed after addition of nitric oxide specific inhibitor PDTC than Ebselen.

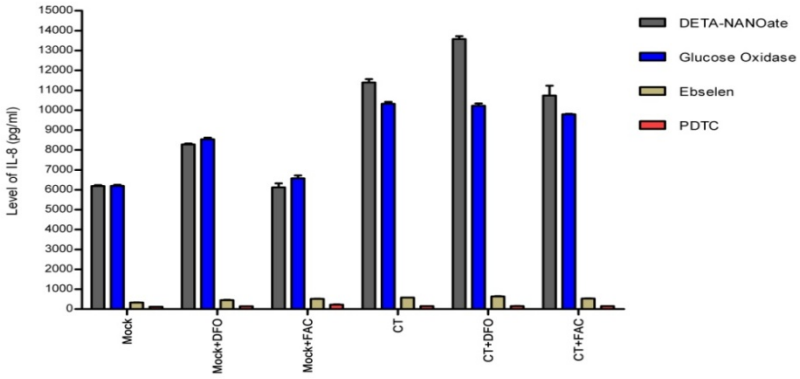


Fig 6.5: IL-8 induction was significantly (P<0.001) increased in *CT* infected HeLa cells after stimulation with DETA-NANOate and there was synergic effect with DFO. Glucose oxidase treatment induced IL-8 production at same degree in both the *CT* infected and Mock

conditions (P<0.005). Higher inhibition (P<0.05) of IL-8 induction was observed after addition of nitric oxide specific inhibitor PDTC than Ebselen.

Cytokine and regulation of iron homeostasis in CT infected cells

Level of IL-10 in supernatant:

CT infected cells showed significant (P<0.001) increase in anti-inflammatory IL-10 level in comparison mock infected cells. On addition of ferric ammonium citrate (FAC) significant increase was in secreted IL-10 was observed in both mock and CT infected cells in comparison to untreated mock and CT infected cells respectively. Moreover change was more significant in mock (p<0.001) than CT (p<0.01) infected cells. When mock infected cells were treated with iron chelator DFO, significant decrease in IL-10 level observed in comparison untreated mock. In contrast CT infected cells showed lesser degree (p<0.01) of decrease in IL-10 level in comparison to untreated CT when treated with DFO (Fig 6.6).

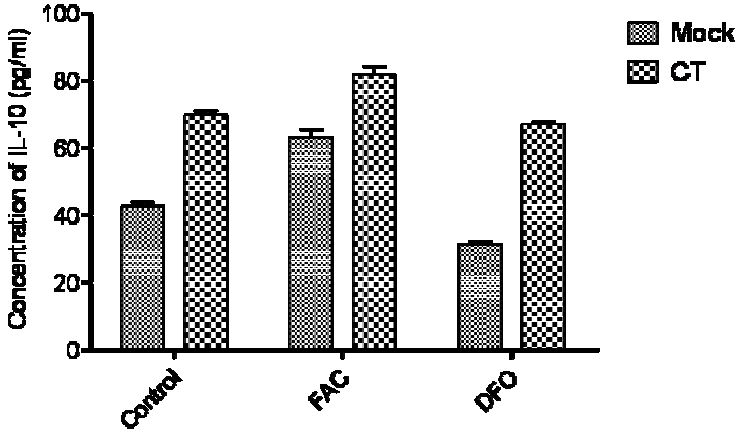


Fig 6.6: Supernatant of treated and untreated cells were analysed at 24 hpi using ELISA. Level of IL-10 is responsive to iron supplementation (FAC) and iron depletion (DFO) in mock infected, not in CT infected cells.

Concerted secretion of IL-10 and IRE-IRP regulated GFP expression:

Level of IL-10 in Mock and CT infected cells was compared with mean fluorescence intensity (MFI) of GFP as observed in flow cytometer in FL-1 channel. Level of IL-10 increased on addition of iron supplement FAC and decreased on addition of iron chelator DFO in mock infected cells in comparison to untreated mock (Control). Similarly iron regulated GFP expression (MFI) was increased in case of iron supplementation with FAC and decrease was evidenced in case of iron chelation with DFO in mock infected cell in comparison to mock. However in CT infected cells there was significant increase in level of IL-10 in comparison to mock control. Increased level was remains consistently increased irrespective of iron supplementation with FAC and iron depletion with DFO in CT infected cells. When these results compared with GFP expression (MFI) of CT infected cells, similar trend of consistently elevated MFI was observed, irrespective of treatment with FAC and DFO (Fig 6.7).

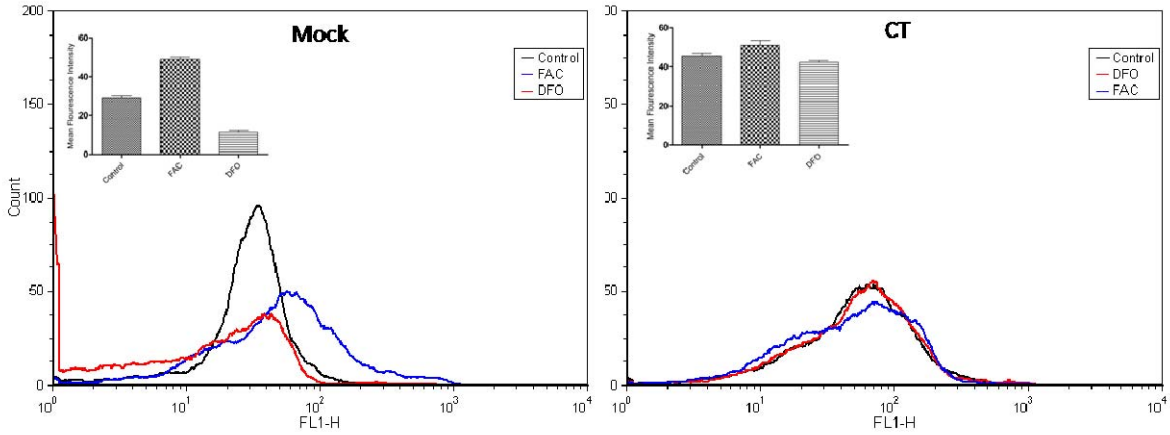


Fig 6.7: Effect of CT infection on IRE regulated GFP expression: Histogram showing GFP fluorescence as analysed in flowcytometric quantitation of mean fluorescence intensity.

Ferritin and Transferrin Receptor expression:

In CT infected cells level of ferritin was increased significantly whereas non significant decrease was observed in transferrin receptor level in comparison to control (Fig 6.8). Expression of TfR increased in control on addition of DFO; whereas there was no change observed in CT infected cells in comparison to CT control (untreated) (Fig 6.8). Further CT infected cells were treated with FAC resulted in lesser increase of FHC expression, however greater increase was observed in mock infected cell. However FHC level remained at the basal level (mock) in CT-infected cells after addition of DFO. Expression of transferrin receptor remains constant in CT infected cells, however decrease was observed in control on addition of FAC. These results was in corroborated with findings appeared in flow cytometric analysis, indicating iron homeostasis in CT infected cells favoring storage of free iron (Fig 6.7). Moreover higher temporal expression of anti-inflammatory IL-10 in CT infected cells, which was undeterred by iron chelation and supplementation, indicating involvement in regulation of iron homeostasis (Fig 6.6).

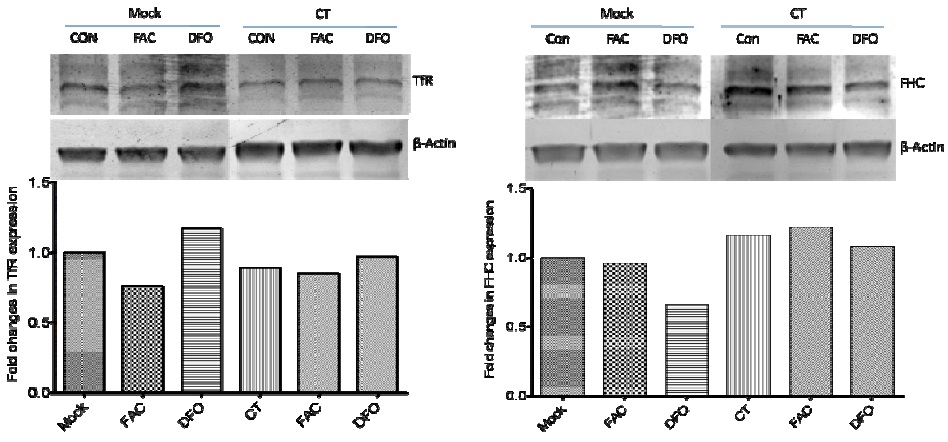
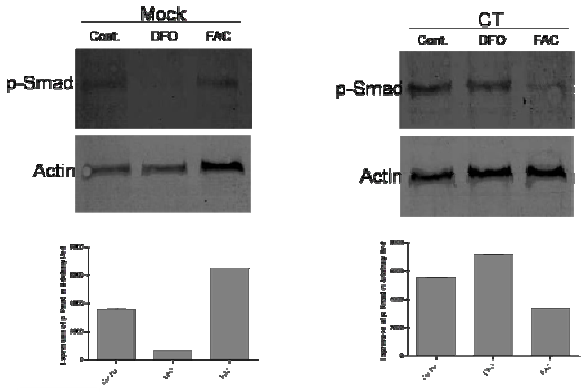


Fig: 6.8 Effect of CT infection on TfR and FHC expression in HeLa cells: Western blot analysis using anti-TfR and anti-FHC antibodies, with beta-actin as the loading control.

Level of p-Smad 2/3:

More than two fold increase in p-Smad level was observed in CT infected cells in comparison to mock. On addition of DFO, decreased level of p-Smad observed in Mock, however it remained higher CT. On addition of FAC in CT infected cell, level of p-Smad decreased significantly in comparison to untreated CT. In contrast, increase in p-Smad level was observed in FAC treated mock cells in comparison to untreated mock (Fig 6.9)



F**ig 6.9:** On addition of DFO, up-regulation in p-Smad was observed in CT and mock showed down-regulation.

4. Discussion

Chlamydial infections, such as sexually transmitted or respiratory diseases occur frequently however, course of these illnesses tends to be mild. Hence, they often pass unrecognized and untreated. These predominantly mild acute infections are considered as possible starting points of other chronic diseases, such as tubal inflammation with infertility, or reactive arthritis for *CT* (Pal *et al.*, 1998, Mardh, 2004). Disease outcome of Chlamydial infection is predominantly governed by mediators of inflammation like IL-8 and balancing anti-inflammatory IL-10.

Here we have studied *CT* infection and its association with iron and modulation of immune responses. The first part of the study reveals the regulatory effect of iron on expression of chemokine IL-8 and second part is about IL-10 secretion and regulation of iron distribution in chlamydia infected cells.

Elevated level of IL-8 is dependent on chlamydial growth (Rasmussen *et al.*, 1997) and it is reported that in case of DFO induced persistence, level of IL-8 remains higher (Peters *et al.*, 2005). This raises the question; Are chlamydia directly involved in IL-8 induction or intracellular level of iron contributes to some extent?

In our study it was observed that in *CT* infected HeLa 229 cells, level of secretory IL-8 increased significantly ($P < 0.001$) at 12 hpi till 72 hpi in comparison to mock infected cells showing thereby the probable involvement of early phase of chlamydial differentiation and metabolic activities responsible for induction and secretion of IL-8. This is in accordance with earlier study wherein it has been shown that *CT* and *C. psittaci* up regulate mRNA expression and secretion of the proinflammatory cytokines like IL-8, GROα, GM-CSF, and IL-6. Further IL-8 production in HeLa 229 cells infected with *CT* was significantly delayed (from 24 hpi), unlike most invasive microorganisms that promote rapid but transient inflammatory response (Rasmussen *et al.*, 1997). Elevated level of IL-8 might help epithelial cells at the mucosal surface to communicate with professional immune cells in response to pathogenic insult**.** Alternatively chlamydia switches delayed expression of IL-8 to transmigrate with the help of neutrophil and macrophage, as they come to site of infection.

Further it was observed that IL-8 induction is independent of TNF-α and IL-1β in *CT* infected HeLa 229 cells. Earlier studies had also suggested that TNF-α and IL-1β act as co-stimulatory signal for IL-8 induction in NF-kB dependent pathway (Chaly *et al.*, 2000).

Chlamydiae contain a tail serine protease (Tsp) that selectively cleaves the p65/RelA subunit of NF-kB to potentially interfere with host inflammatory response (Lad *et al.*, 2007). In a recent study lower level of TNF receptor 1 was observed on cell surface of chlamydia infected cells, inhibiting TNF mediated signaling (Paland *et al.*, 2008) thereby enticing the possibility of TNF-α and IL1β independent expression of IL-8 in *CT* infected HeLa 229 cells. We also demonstrated chlamydial infection act synergistically with DFO to activate IL-8 secretion. Intracellular level of available iron has been shown to modulate inflammatory mediators and to regulate inflammatory process in several cell types. Two important signaling pathway represented by p38 and ERK1/2 are required for DFO-induced IL-8 secretion (Weiss *et al.*, 1992, Choi *et al.*, 2004). IL-8 induced by *CT* has been shown to be dependent on ERK and independent of p38 and Jun N-terminal MAPK (Buchholz *et al.*, 2007) showing thereby that chlamydial infection and DFO follows the same course for IL-8 activation. However, further study of post-transcriptional mechanisms for DFO induced IL-8 involving p38 or ERK is warranted.

In our study DFO-induced IL-8 was increased by DETA-NANOate and H_2O_2, although extent of stimulation is less than that of NO in HeLa cells infected with *CT*. However, in iron supplemented condition decline in IL-8 level was observed. It has been reported that NO is the initial factor in the signaling cascade that mediates IL-8 gene transcriptional activation by DFO. NF-κB is also an important transcriptional factor for IL-8 production in cancer cells. PDTC, one of the most effective inhibitors of NF-κB, inhibits the NO-dependent induction of the IL-8 gene (Turpaev *et al.*, 2003). It may be elucidating that NO plays important role in IL-8 induction in *CT* infected cells treated with DFO.

In *CT* infected cells, Mimosine treatment showed gradual increase and subsequent decrease in IL-8 expression. Conversely, delayed induction and consistent expression of IL-8 expression was observed with DFO. Further this hypothesis gets more impetus by decrease in labile iron in CT infected cells as shown in calcein quenching experiment. Thus synergy was observed only with DFO having bacterial origin, indicating that level of intracellular iron play an important role in induction of proinflammatory responses. Further it may indicate towards involvement of iron quenching mechanism of chlamydial origin. Results of this study elucidated the involvement of intracellular iron in activation of IL-8 in chlamydia infected HeLa229 cells.

Balance of inflammatory reaction is becoming key determinant in disease pathology. Iron plays vital role in key processes of host and pathogen and catalyst of innate immune system. Free iron ion catalyses many reactions which generate free radicals of oxygen that is toxic to pathogen as well as for the host if not controlled. Chlamydiae have shown ability to modulate host pathways in favour of own survival. Here, in this study we showed that chlamydia induced increase in IL-10 secretion is associated with FHC expression in HeLa cells.

Significantly higher levels of IL-10 were detected by ELISA in chlamydia infected cells as compared to control cells. In earlier studies IL-10 has been shown to be related to the suppression of immune reactions (Bahia-Oliveira *et al.*, 2009, Dagvadorj *et al.*, 2009a). The function of IL-10 production induced by pathogen exposure may have an opponent effect on host-pathogen interaction. On one hand, IL-10 may lead to dysfunctional immune protection, thus providing an opportunity for immune evasion by the microbes (McGuirk *et al.*, 2002, Ocana-Morgner *et al.*, 2003, Jeong *et al.*, 2009). On the other hand, IL-10 production may be beneficial to the host, given the fact that IL-10 is essential in regulating immune responses

and thus preventing an excessive inflammatory response that may be detrimental (Akbari *et al.*, 2001, Higgins *et al.*, 2003).The fact that certain pathogens/microbial products can induce IL-10 producing cells that show an immuno-regulatory function has been reported in several studies (Pulendran *et al.*, 2001, Romagnoli *et al.*, 2004, Srivastava *et al.*, 2008, Han *et al.*, 2006). IL-10 effectively suppresses production of pro-inflammatory cytokines , generation of reactive oxygen intermediate and expression of surface MHC class II and co-stimulatory molecules such as B7 (Shibata *et al.*, 1998). Unique immuno-stimulatory function of IL-10 is broadly dependent on the microenvironment and cytokine milieu (Shibata *et al.*, 1998, Herrero *et al.*, 2003).

Iron metabolism and immune responses are closely interconnected. This is due to divergent regulatory effects of the metal on immune cell proliferation and on the effectiveness of cellular immune effector pathways (Ludwiczek *et al.*, 2003, Tilg *et al.*, 2002, Weiss, 2005). In this study we did comparative analysis of temporal level of IL-10 and Ferritin-IRE regulated GFP expression as MFI. The analysis showed the direct association of temporal level of IL-10 and expression of GFP (MFI) as FL-1 shift was seen in flow histogram. In presence of FAC and DFO, mock infected cells showed significant increase and decrease in the IL-10 level, similarly increased and decreased expression of GFP (MFI) as observed in flow cytometric analysis in comparison to untreated mock infected cells respectively. Moreover, irrespective of iron supplementation and depletion CT infected cells showed consistently increased IL-10 level and MFI in comparison to untreated mock infected cells. Hence these results show the reciprocal regulation of IL-10 and FHC. It is further seen in this study that higher MFI indicative of higher expression of ferritin heavy chain and slight decrease in transferrin receptor expression as detected in immunobloting. Cytokines

influence the posttranscriptional control of iron homeostasis by modulating the binding affinity of iron regulatory proteins (IRP-1 and IRP-2) to specific RNA stem loop structures, termed iron responsive elements (IREs), which are found within the 5' untranslated region of ferritin mRNA and within the 3' untranslated region of transferrin receptor (TfR) mRNA, the surface protein involved in the uptake of transferrin bound iron (Hentze *et al.*, 2004, Pantopoulos *et al.*, 1995, Kell, 2009). It is reported that patients receiving higher doses of IL-10 developed anemia and presented with a dose-dependent increase of ferritin and soluble transferrin receptor levels, an indicator of iron restriction to erythroid progenitor cells (Weiss, 2009) . These studies indicate the involvement of anti-inflammatory (IL-10) cytokine in modulation of iron homeostasis. However, consistently increased level of IL-10 and its independence from iron supplementation and depletion in CT infected cells needs further investigation. This study suggests that CT might induce level of IL-10 to modulate iron homeostasis for higher expression of ferritin, thereby restrict availability of free iron for catalytic reaction resulting in production of harmful reactive oxygen species (data not shown). These data further showed the ability of chlamydia to modulate interwoven host regulatory pathways for own existence.

We further examined the p-Smad (2/3), major link between cytokines and iron (Fiocchi, 2001, Ruiz *et al.*, 2005, Anderson *et al.*, 2008). On western blot analysis, higher level of p-Smad was detected in DFO treated and untreated CT infected cells in comparison to DFO treated and untreated control cells. However after addition of FAC such a significant difference was not observed in CT infected cells in comparison to their control. Further, the increased level of p-Smad in DFO treated CT infected cell may have protective role.

However these finding needs further study to establish direct relation of p-Smad, balancing act and iron homeostasis.

Thus, in the present study we have shown the essential role of anti and pro- inflammatory cytokines signal in iron homeostasis in CT infected cells. Extrapolation of our data may provide the clue that iron is also involved in regulation of the cellular defense against chlamydial infections. Conversely it will be possible to modulate iron homeostasis thereby immune responses leading to clearance of chronic chlamydial infection.

Chapter 7
Conclusion and Future Scope of Work

Life preys upon life. This is biology's most fundamental fact.
Martin H. Fischer

Chapter 7

Conclusion and future scope of work

In the present study chlamydial-host interaction was assessed in HeLa229 cells under iron depletion and repletion conditions. In CT infected cells, cellular viability was critically balanced at 25-50μM concentration of DFO, however mock infected cells loss their integrity at lower concentration (25μM) as inferred from OD value of MTT assay. In the presence of excess iron supplementation with FAC (1mM), cellular viability was compromised in both CT and mock infected cells at varying degree, however CT infected cells showed greater sustenance. Thus these iron stress conditions were selected for the entire study including-establishment, regulation and immuno-pathogenesis of chlamydial infection.

First part of the study revealed the association of iron, redox status of cell and apoptosis in CT and mock infected cells. Flow cytometric analysis showed decline in surface expression of iron acquisition protein TfR, concomitant with dampened intracellular ROS in CT infected cells. Further upon addition of DFO and FAC, mock infected cells showed increased and decreased TfR surface expression respectively. In contrary to CT infected HeLa cells showed lesser degree of change in TfR expression on addition of iron chelator (DFO) and supplementation (FAC). Mitochondrial membrane potential was higher in CT infected cells than mock infected cells on apoptotic induction by H_2O_2, DFO and excess FAC. This MMP stabilizing effect may be due to decline in ROS in CT infected cells. Downstream component of apoptosis was analysed to understand modulatory effect of CT under iron stress conditions. In CT infected HeLa cells apparent increase in ratio of Bcl/Bax, retention of cytochrome 'C' and no activation of caspase 3, 9 and 8 was observed, which is indicative of anti-apoptotic properties of CT. Further increase in anti-oxidant Trx and MMP stabilizing

protein PAPR-γ was favouring reduced oxidative stress and retention of MMP in CT infected cells. Thereby results suggest that critical concentration (25μM) of DFO have augmented the anti-apoptotic effect of CT in HeLa cells. This effect in part mediated through decrease in catalytic free iron by down-regulating TfR (transport) and up-regulating FHC (storage), resulting in decrease of ROS generation in CT infected cells. Declined ROS level provide conducive environment for establishment of infection and increases cellular sustenance for completion of chlamydial developmental cycle.

Second part of study revealed the modulatory effect of CT on host iron regulatory mechanism. To explore regulation of iron acquisition and storage in CT infected cells cytoplasmic extract was incubated with radio-labeled IRE transcript. CT infected HeLa cells showed attenuated binding activity of IRP-IRE in comparison to mock infected cells as detected in gel shift assay. The attenuated binding (IRP-IRE) followed by apparent decrease in TfR and increase in FHC expression in CT infected cells. Additionally calcein staining of free divalent iron showed decrease in LIP in CT infected cells. The shift in iron homeostasis might result in change in distribution of free iron from cytoplasm to bound iron in ferritin (storage). The attenuating effect is consistent in CT infected cells irrespective of iron depletion and supplementation; however on addition of chloramphenicol this effect was revesed. These results suggested the involvement of CT in attenuated binding (IRP-IRE) activity. However TfR expression was not in full accordance with regulatory effect conferred to IRP-IRE interaction, moreover it is alternatively regulated through NF-kB and HIF-1α. Further it was revealed that decreased expression of TfR was attributed to loss of Nf-kB and HIF-1α activation.

Third part of study showed the immunodulatory effect conferred to chlamydia was in part mediated through iron and protein (FHC) associated with iron. Therefore the effect of LIP on in vitro expression of IL-8 and effect of iron homeostasis on anti-inflammatory IL-10 was studied. In CT infected cells delayed (12hpi onward) and higher expression of IL-8 was observed, which is consistent with earlier study. Additionally our study able to show that level of IL-8 was dependent on availability of free iron in converse order in CT infected HeLa cells. Further time kinetics of IL-8 production was not in accordance with temporal IL-1β and TNF-α production, thus denying the involvement both the later cytokine in regulation of earlier in CT infected HeLa cells. Also it was demonstrated that CT act synergistically with iron chelator DFO to activate IL-8 secretion. Further investigation reveals that iron dependent regulation of IL-8 is mediated through nitric oxide not by ROS in CT infected cells. These results showed that iron plays important role in regulation of IL-8 and thereby immuno-pathogenesis of chlamydial diseases. The study provided the new facets to regulation of IL-8 expression, showing delayed expression (12 Hpi) which is hallmark of chlamydial infection. Further it was investigated that whether level of anti -inflammatory cytokine (IL-10) and the iron regulatory system is associated or not? In CT infected HeLa cells there was evident increase in IL-10 levels, which was consistent with higher expression of IRE-regulated GFP expression (representing FHC expression). These results were supported by higher expression of FHC as observed in immunoblot. Thus it may indicate that expression of FHC is associated with IL-10 production in CT infected cells. Moreover CT infected cells showed higher level of p-Smad, which might be indicating toward signalling cascade involved in balancing act played by pro and anti inflammatory cytokines. This study

provided the possible association between regulation of iron and mediators of immune responses in CT infected cells.

Future Scope of work

The ultimate objective for our *in vitro* studies on iron stress and CT was to advance our understanding of the human disease process. These findings will further extend to understanding interaction of CT with the host immune system. The regulatory network between iron and immune function has attracted great interest, especially in association with underlying inflammatory processes which are found in diseases with chronic immune stimulation, such as in chronic infections, cancer or autoimmune disorders. The control over iron homeostasis under these circumstances is one of the most important determinants of the fate of an infectious or autoimmune disease. The close interaction between iron and immunity is underscored by observations that certain immunological proteins alter cellular iron metabolism, changes in immune function thus affect iron homeostasis and vice versa. These immune responses can be controlled by changing the availability of iron in infected cells. Apoptosis is the key determinant in establishment of infection and centrally controlled by redox status of the host cells. Redox status of cells is reciprocally connected to iron metabolism. Chlamydia takes the control over apoptosis through redox status of cell. Extrapolation of these *in vitro* results opens up many point of investigation to control or eliminate the chlamydial infection in *in vivo*.

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Appendix

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.

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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.

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.

2X SDS-PAGE sample buffer

The composition of sample buffer is as follows

Tris-Cl (pH6.8) 100mM
DTT 200mM
SDS 4%
Bromophenol blue 0.2%
Glycerol 20%
b-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 'C'

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

Publications and Brief Bio-Data

Publications:

1. **Vardhan H**, Bhengraj AR, Jha R and Mittal A. *Chlamydia trachomatis* alters Iron-regulatory protein-1 binding capacity and modulates cellular iron homeostasis in HeLa 229 cells. **J Biomed Biotechnol**. Epub 2009 Aug 16.

2. **Vardhan H**, Dutta R, Vats V, Gupta R, Jha R**,** Jha HC, Srivastava P, Bhengraj AR and Mittal A. Persistently elevated level of IL-8 in *Chlamydia trachomatis* infected HeLa 229 cells is dependent on intracellular available iron. 2009. **Mediators of Inflammation** Epub 2009 May 26**.**

3. **Vardhan H**, Bhengraj AR, Jha R, Mittal A. Higher expression of ferritin protects *Chlamydia trachomatis* infected HeLa 229 cells from reactive oxygen species mediated cell death. **Biochemistry and Cell Biology**, 2010 (In press).

4. Jha H C, **Vardhan H**, Gupta R, Verma R, Prasad J and Mittal A. Higher incidence of persistent chronic infection of *Chlamydia pneumoniae* among CAD patients in India is a cause of concern. **BMC Infectious Diseases** 2007, 7:48.

5. Jha R**, Vardhan H**, 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. **Gynecol Obstet Invest.** 2009 Jul 29; 68(3):160-166.

6. Gupta R, Srivastava P**, Vardhan H,** 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

7. Gupta R, **Vardhan H**, Srivastava P, Salhan S, 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. Reprod Biol Endocrinol. 2009 Aug 22;7(1):84. [Epub ahead of print].

8. Bhengraj AR, **Vardhan H**, Srivastava P, Salhan S, Mittal A. Decreased susceptibility towards azithromycin and doxycycline in clinical isolates of *Chlamydia trachomatis* obtained from recurrently infected female patients in India. **Chemotherapy**, April 2010.

Submitted for publication

9. **Vardhan H**, Gupta R, Jha R, Bhengraj AR, Mittal A. Ferritin heavy chain mediated-iron homeostasis regulates expression of IL-10 in *Chlamydia trachomatis* infected HeLa Cells.

10. **Vardhan H**, Bhengraj AR, Jha R, Mittal A. Thioredoxin-1 plays protective role in *Chlamydia trachomatis* infection.

11. **Vardhan H**, Bhengraj AR, Jha R, Srivastava P, Mittal A. Deactivation of NF-kB and HIF-1α leads to repression in transferrin receptor mediated iron intake in *Chlamydia trachomatis* infected HeLa 229 cells.

Harsh Vardhan

There are grounds for cautious optimism that we may now be near the end of search for the ultimate laws of nature.

Stephen W. Hawking

Personal Information

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Institute of Pathology (ICMR), Safdarjung Hospital Campus, Delhi, India
Phone: +91-9968419670 ; +91-9717757674
Email: harshiop@gmail.com, harsh_bio@rediffmail.com

Current Position (August 2004 - Present):

Graduate (Ph.D.) student at the Microbiology and Tissue Culture Division, Institute of Pathology (ICMR), New Delhi, India.

Ph D Project: "*Role of Iron in pathogenesis of chlamydia trachomatis infection in vitro*".

Education:

B. Sc (Hons)	September 1999
Vinoba Bhave University, Hazaribagh, India	59.5%
M. Sc (Microbiology)	June 2003
Jiwaji University, Gwalior, India	76.5%
Ph D	
Institute of Pathology/BITS, Pilani, New Delhi, India	Thesis Submitted

Honors:

- Selected in All India Level Exam (Centralized) held by **CSIR-UGC-NET (2003).**

  http://csirhrdg.res.in/jrfsrfra2.htm

- Selected in All India Level Exam (Centralized) held by Indian Council of Medical Research **(ICMR), 2003**. http://www.icmr.nic.in/jrf.htm

- Qualified in **Graduate Aptitude Test in Engineering (GATE)**, conducted Indian Institute of Technology, Govt. of India (2003).

  http://www.gate.iitb.ac.in/gate2010_website/

- Selected in All India Level Exam (Centralized) held by Indian Council of Medical Research **(ICMR) as Junior Research Fellow** (2004).

http://www.icmr.nic.in/jrf.htm

- Selected in All India Level Exam (Centralized) held by **CSIR-UGC-NET (2004).**

  http://csirhrdg.res.in/jrfsrfra2.htm

- Junior Research Fellowship from Indian Council of Medical Research, Govt. of India (from **August 2004 – August 2006**). http://csirhrdg.res.in/jrfsrfra2.htm

- Senior Research Fellowship from Indian Council of Medical Research Govt. of India (from **September 2006 – ongoing**). http://www.icmr.nic.in/jrf.htm

- Complete fee waiver to attend **3D microscopy of live cells course** at University of British Columbia, Canada (**June 2009**). http://www.3dcourse.ubc.ca/2010/public.php?page=alumni&year=2009#tableTop

Research Experience:

During my PhD and Dissertation I have gained experience in the following techniques:

- Trained in 3D and live cell imaging techniques on various microscopic systems.
- Friendly with Image processing software's like Image J, Imaris and Velocity.
- Protein chemistry, SDS-PAGE, Immuno- blotting and 2D- PAGE.
- DNA, RNA and Plasmid Isolations; PCR, RT-PCR.
- Transformation of competent cell with different construct.
- Animal Handling: Viral infection to mouse pups and adult animals (M.Sc. dissertation).
- Cell Biology: Culture of different Cell lines, transfection with constructs and siRNAs.
- DNA/RNA-Protein-protein interaction ; EMSA and Immuno-précipitation.
- Immunocytostaining, Immunohistoschemistry.
- Flow Cytometry: Surface and Intracellular staining, Reactive Oxygen Species (ROS) using DCFH-DA probe , intracellular Calcium measurement using Fluo4-AM probe ,Mitochondrial membrane permeability (MMP) using JC-1 and GFP analysis for transcriptional activation; Analysis of results by Cell Quest Software. Cell cycle analysis using PE by FlowJo software.

Research Interest:

- Iron and redox signaling in disease pathogenesis.
- Infection and Iron homeostasis.
- Inter and intracellular interactions.
- Pathogens transmigration and Inflammation.

Conference/ Presentation:

1. **Vardhan H,** Mittal A. *Chlamydia trachomatis* modulates iron homeostasis of infected HeLa cells, National conference on Emerging trends in life sciences research Frontiers in biological sciences, BITS, Pilani, 5-7th March 2009. (**Oral presentation**)

2. **Vardhan H**, Aruna Mittal. Modulation of Iron homeostasis in chlamydia infected Cells, International conference on Mechanism of microbial pathogenesis, IISc, Bangalore, 10-12th March 2009.

3. **Vardhan H,** Mittal A. Persistently elevated level of IL-8 in *Chlamydia trachomatis* is iron responsive and mediated through nitric oxide. IFRR, King George Medical College, Lucknow, 19-21th March 2009.(**Oral presentation**)

4. Jha R, Jha HC, **Vardhan H**, Gupta R, Srivastava P, Bhengraj AR and Mittal A. Three-dimensional structure of *Chlamydia trachomatis* heat shock protein 60 reveals cross presentation of epitopes in infected women causing infertility. ICSB 2007, Chinese University of Hong Kong, **Hong Kong**, 19-22nd Nov. 2007.
5. Gupta R, Srivastava P, Bhengraj A R, Jha R, Jha H C, **Vardhan H**, Salhan S and Mittal A. *Chlamydia trachomatis* heat shock protein 60-specific antibody and cell-mediated responses can predict tubal factor infertility in infected women. Sixth meeting of the europian society of chlamydia research, **Aarhus, Denmark**, July 1-4th 2008.

Courses/ workshop/ Symposia

- Successfully survive the course on **3D microscopy of live cells** at University of British Columbia, Vancouver, Canada. 13-25th June 2009.
- Participate in **3D image processing workshop** at University of British Columbia, Vancouver, Canada. 27-29th June 2009.
- Advances in **Protein Sciences Research**: From Sample Preparation to Protein Analysis, This seminar will showcase latest technologies in protein sciences research including DIGE, advances in ECL and SPR technology. Organized by GE Healthcare Life Sciences New Delhi, 21st May 2007.
- 21st Annual Conference, Indian Association of Pathologists & microbiologists. 16th April 2006, Institute of Pathology (ICMR), New Delhi.
- Attended lecture on “Secondary structure of RNA and 5’ regulatory elements” delivered by Paul Fox at Jawaharlal Nehru University, New Delhi, March 2006.
- Attended “**2-D – Clinic**” information and troubleshooting meet with Dr. Reiner Westermeir, organized by GE Healthcare Life Sciences on 23-24th January 2006, India Habitat Center, New Delhi.
- Attended a talk on “New Avenues to Health” by Noble Laureate David Baltimore at Teen Murti Auditorium, New Delhi, on 14th Jan. 2008.

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) **Membe**r- 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. Bhengraj AR, Dar SA, Talwar GP, **Mittal A**. Potential of a novel polyherbal formulation BASANT for prevention of *Chlamydia trachomatis* infection. **International Journal Antimicrobial Agents**. 2008;32:84-88 .
2. Jha HC, **Mittal A**. Coronary artery disease patient's first degree relatives may be at a higher risk for atherosclerosis. **Int. Jour of Cardiology**.2008, doi:10,1016/j.ij card 2008. 03 .031.
3. Agarwal,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. **Clin. Micobiol. Infec**. **2008**,15:50-59.
4. Dutta,R.Jha,R.Salhan,S.and **Mittal A**.: Chlamydia specific heat shock protein 60 antibodies can serve as prognostic marker for chronic Chlamydia infection. **Infection**. 2008; 36;374-378 .
5. 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**.**
6. Srivastava P, Jha R, Salhan S, **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

7. Srivastava P, Gupta R, Jha HC, Bhengraj AR, Jha R, Salhan S, **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
8. 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. **Jour. of Cardiology.** 2008; 52: 140-45**.**
9. Aggarwal,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.
10. Aggarwal 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
11. 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. **Inter. Jour. Microbiology.** 2008, 5(2):1-8.
12. Srivastava, P. Jha,H.C. Salhan,S and **Mittal,A.** Azithomycin treatment modulates cytokine production in Chlamydia trachomatis infected women**. Basic and Clinical Pharmacology & Toxicology.**2009:104, 478-482.
13. Aggarwal,T. Vats,V., Salhan,S and **Mittal,A.** Determination of chlamydial infectious load and immune parameters in asymptomatic, symptomatic and Infertile women.**FEMS,**2009 ,55:250-257
14. Jha,H., Prasad,J and **Mittal A.** Why first degree relatives of coronary artery disease patient's have Chlamydia pneumoniae infection? **Int Jour Cardiology.**2009 doi:10.1016/j.ijcard.2008.12.062.
15. 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.
16. Gupta R, Salhan S and **Mittal A.** Seroprevalance of antibodies against C.trachomatis inclusion protein B & C in infected women. **Jour of Infection in developing countries.**2009,3:3.
17. Jha R**,** Vardhan H, 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. **Gynecol Obstet Invest.** 2009 Jul 29; 68(3):160-166.
18. Gupta R, Srivastava P**,** Vardhan H**,** 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
19. Gupta R, Vardhan H, Srivastava P, Salhan S, **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. Reprod Biol Endocrinol. 2009 Aug 22;7(1):84.
20. 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 .

21. 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.**Jour.Biomed.Biotech,**2009 doi:10.1155/2009/342032**.**
22. Sachdeva,P. Patel, A. Sachdev, D, Ali, **Mittal, A**. and Saluja, D. Evaluation of indigenous PCR assay for detection of Chlamydia trachomatis using Direct fluorescent assay and Roche amplicor detection kit. **Jour.Med.Microbiol.**2009,58:867-873**.**
23. Bhengraj AR, Goyel,A. Talwar GP, **Mittal A**. Assesment of antichlamydial effects of a novel polyherbal tablet "BASANT". Sexually transmitted infection.**2009(Accepted for publication)**
24. 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)
25. Jha,H.Srivastava,P Prasad,J. and **Mittal,A:** Coronary artery disease: Biomarkers in first degree relatives. **Atherosclerosis Supplements.** 2009;10(issue2)
26. Jha,H.Prasad,J and **Mittal A.:** Sequencing of Chlamydia pneumoniae in Coronary artery disease patients attending tertiary hospital in New Delhi, India. **American Jour of Inf. Control.2009(Accepted for publication)**
27. Jha,H. Prasad,J. and **Mittal,A.** The plasma circulatory markers in male and female coronary artery disease patients. **Heart and Lung. 2009(Accepted for publication)**.
28. 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 Microbiol Immunol Scan .2009** (**Accepted for publication**)