Mycobacterium tuberculosis Peptidyl-Prolyl Isomerases: Biochemical, Immunological and Functional Characterization

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By

Saurabh Pandey

(Reg. No. 10IBPH05)

Department of Biochemistry
School of Life Sciences
University of Hyderabad
Hyderabad-500046
INDIA

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University of Hyderabad

(A Central University by an Act of Parliament)

Department of Biochemistry

P.O. Central University, Gachibowli, Hyderabad-500046

DECLARATION

The research work presented in this thesis entitled **"*Mycobacterium tuberculosis* Peptidyl-Prolyl Isomerases: Biochemical, Immunological and Functional Characterization"**, has been carried out by me at Dr. Reddy's Institute of Life Sciences and Department of Biochemistry, University of Hyderabad, Hyderabad, under the guidance of Professor Seyed Ehtesham Hasnain. I hereby declare that this work is original and has not been submitted in part or full for any other degree or diploma of any other university or institution.

Date: **Signature:**

Saurabh Pandey

Name: Saurabh Pandey

Reg. No.: 10IBPH05

University of Hyderabad

(A Central University by an Act of Parliament)
Department of Biochemistry, School of Life Sciences
P.O. Central University, Gachibowli, Hyderabad-500046

CERTIFICATE

This is to certify that **Mr. Saurabh Pandey** has carried out the research work embodied in the present thesis under the supervision and guidance of Professor Seyed Ehtesham Hasnain, for a full period prescribed under the Ph.D. ordinance of this University. We recommend this thesis entitled **"*Mycobacterium tuberculosis* Peptidyl-Prolyl Isomerases: Biochemical, Immunological and Functional Characterization"** for submission for the degree of Doctor of Philosophy of this University. The work is original and has not been submitted in part or full for any other degree or diploma of any other university.

Supervisor

Prof. Seyed Ehtesham Hasnain
Distinguished Research Professor,
Dr. Reddy's Institute of Life Sciences,
University of Hyderabad Campus,
Gachibowli, Hyderabad: 500046

Co-supervisor

Dr. Nasreen Zafar Ehtesham,
Senior Deputy Director,
National Institute of Pathology,
Safdarjung Hospital Campus,
New Delhi-110029

30/12/2015

Head,
Department Biochemistry
University of Hyderabad

Dean,
School of Life Sciences
University of Hyderabad

Dedicated to my Parents

In whose shower of solicitude I rejuvenate

"Imagination is more important than knowledge."

– Albert Einstein

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Publications:

1. Suragani M, Aadinarayana VD, Pinjari AB, Tanneeru K, Guruprasad L, Banerjee S, **Pandey S**, Chaudhuri TK, Ehtesham NZ; Human resistin, a proinflammatory cytokine, shows chaperone-like activity; ***Proc Natl Acad Sci U S A.*** 2013 Dec 17;110(51):20467-72.

2. **Pandey S**, Khubaib M, Tripathi D, Sharma A, Bhuwan M, Chaudhuri TK, Hasnain SE, Ehtesham NZ; Mycobacterial peptidyl-prolyl isomerases show chaperone like activity; in vitro and in vivo;.(Under Review)

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4. **Pandey S**, Tripathi D, Khubaib M, Kumar A, Shaikh J, Ehtesham NZ, Hasnain SE, Mycobacterium tuberculosis peptidyl-prolyl isomerases show immunogenicity, alter cytokine profile and aid in intraphagosomal survival; (manuscript under preparation).

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LIST OF ABBREVIATIONS

%	Percentage
~	Approximately
°C	Degree celsius
βME	Beta mercaptoethanol
µg	Microgram
µl	Microlitre
µM	Micromolar
Ab	Antibody
ADC	Albumin dextrose catalase
Amp	Ampicillin
AP	Alkaline phosphatase
APS	Ammonium persulphate
ATP	Adenosine Triphosphate
BCA	Bicinchoninic Acid
BCG	Bacillus Calmette-Guerin
bp	Base pair
BSA	Bovine Serum Albumin
CD	Complementarity Determinant
CFU	Colony forming Units
CO_2	Carbon dioxide
C-terminal	Carboxy terminal
ddH_2O	Double distilled water
ddNTP	Di-deoxyribose nucleotide triphosphate
DMSO	Dimethyl sulfoxide
DNA/RNA	Deoxyribose/Ribose nucleic acid
dNTP	Deoxyribose nucleotide triphosphate
DTT	Dithiothreitol
EDTA	Ethylene diamine tetracetic acid
EtBr	Ethidium Bromide
FCS/FBS	Fetal calf/bovine serum
GLP	Glycopeptidolipids
gm	Grams

Gm	Gentamicin
h/hrs	Hour/hours
IPTG	Isopropyl-β-D-thio-galactopyranoside
Kan	Kanamycin
kb	Kilo base
kDa	Kilo Dalton
LB	Luria Bertani medium
M	Molarity
m.o.i.	multiplicity of infection
M.tb	*Mycobacterium tuberculosis*
MES	2-(4-morpholino)-ethane sulfonic acid
mg	Milligram
min/mins	Minute/Minutes
ml	Millilitres
mM	Milli Molar
MQ	Milli Q
NBT	Nitro blue tetrazolium chloride
Ni^{2+}-NTA	Nickel-nitrilotriacetic acid
nm	Nanometer
nM	Nanomolar
N-terminal	Amino terminal
OADC	Ovalbumin dextrose catalase
OD	Optical Density
OD_{550}	optical density at 550 nm
OD_{600}	optical density at 600 nm
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCI	Phenol: Chloroform: Isoamylalcohol
PCR	Polymerase Chain Reaction
Pen	Penicillin
pfu	plaque forming units
PMA	Phorbol myristate acetate
PMSF	Phenyl Methyl sulfonyl fluoride
RNase	Ribonuclease

rpm	Revolution per minute
RT	Room Temperature
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis
sec	Seconds
Strep	Streptomycin
TAE	Tris acetate EDTA
TB	Tuberculosis
TE	Tris-EDTA
TEMED	N, N, N′, N′ tetramethyl ethylene diamine
Tris	Tris (hydroxymethyl) amino acid
U	Unit(s)
UV	Ultra Violet
WHO	World Health Organization

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Chapter 1

Introduction

1.1 Tuberculosis: The Disease:

Tuberculosis (TB) is known to humankind for thousands of years. This is evident from its presence in buried mummies and references in oldest available classical texts. Sign of decay that is clearly caused by tuberculosis was found in spinal column of Egyptian mummies dating back to around 2400 BC (Zink *et al*., 2003). The sacred classical text, *Rigveda/Ayurveda* mentions *Mycobacterium tuberculosis* as *Rajyakshma* that goes back to 3500 BCE or more. In the biblical references, the book of Deuteronomy and Leviticus represents tuberculosis as *schachepheth* (Daniel V S & Daniel, T M, 1999). Also, ancient Egyptian and pre-Columbian art depicted the presence of TB.

Historically, tuberculosis is identified with different names as phthisis (Greek, for consumption), Scrofula (swollen lymph nodes in the neck), Tabes mesenterica (TB of the abdomen), Consumption (as consumes people from within), white plague King's evil and Koch's disease. It was Robert Koch who introduced the scientific community with tuberculosis causing bacterium and by his own staining method he presented the bacterial sample in microscopy to them in his famous lecture , "Die Aetiologie der Tuberculose", to the Berlin Physiological Society (Daniel 2006).

Members of tuberculosis complex, a genetically related group, including *M.microti, M.bovis, M.capraae, M.africanum, M.canetti,* etc., along with *M.tb*, cause the disease in humans. Active tuberculosis infection shows symptoms such as blood in sputum, loss of appetite, fever, night sweats, weight loss and loss of energy. The tuberculosis bacilli can infect many organs including gastrointestinal tract, urinary tract, bones, joints etc. Importantly, most of tuberculosis infections are asymptomatic and latent. Ten percent of latent infections progresses to active form of disease eventually.

1.1.1 Transmission:

Infectious aerosol droplets are the most common transmission mode of tuberculosis. When a person with active form of pulmonary TB, cough, sneeze, talk, or spew out; contagious aerosol droplets of 0.5 to 5.0 μm in diameter gets released. A single sneeze of a pulmonary TB patient can release up to approximately 40,000 droplets, each of which can establish the infection in a healthy individual (Nicas *et al.*, 2010).

People with prolonged, frequent or close contact with TB patients are at particularly high risk of getting infected (Ahmed & Hasnain 2011). Untreated TB patients may infect 10–15 (or more) people per year. However, people with latent TB infection do not transmit the disease. The probability of transmission from one individual to another depends on various factors, i.e. number of infectious droplets expelled by the carrier, the duration of exposure, *M tb* strain's virulence capacity, level of immunity of the uninfected person, co-infection with other pathogen, and others. It is thus very important to treat patients with active TB so that they do not remain contagious to others. Another point of concern is early and effective diagnosis that is very crucial especially in remote areas where availability of hospitals and care units are a major hurdle. After two weeks of effective treatment, it has been seen that the patients do not generally remain contagious (Ahmed & Hasnain 2011).

1.1.2 Epidemiology of Tuberculosis:

In 2015, TB prevalence has fallen to 42% lower compared to 1990 level. But Asian countries still have the highest disease burden. India, Indonesia and China are at the frontier in the occurrence of tuberculosis cases, out of global total they carried a disease burden of 23%, 10% and 10%, respectively.

Worldwide tuberculosis incidence rates are estimated to have peaked in 2004 and to have declined at a rate of less than 1% per year since that time. However, the overall disease burden continued to rise as a result of the fast growth of the world population. The estimated number of people fallen sick due to tuberculosis worldwide is 9·6 million in 2014 out of which 37% cases went unreported or undiagnosed. TB related deaths in 2014 was around 1.5 million of which 0.4 million were HIV-positive.

Occurrence of resistance against anti tuberculosis drug is matter of concern. Estimated numbers of MDR-TB are 0.48 million in 2014. A quarter of them were detected and reported. Worldwide prevalence of MDR-TB is estimated as 3.3% of new and 20% of previously treated TB cases in 2014 and this has taken an estimated 190 000 lives in 2014. Extensively drug-resistant TB (XDR-TB) had been reported in more than 100 countries, by 2015. As estimated by WHO, 9.7% of people with MDR-TB have XDR-TB (WHO TB Report 2015).

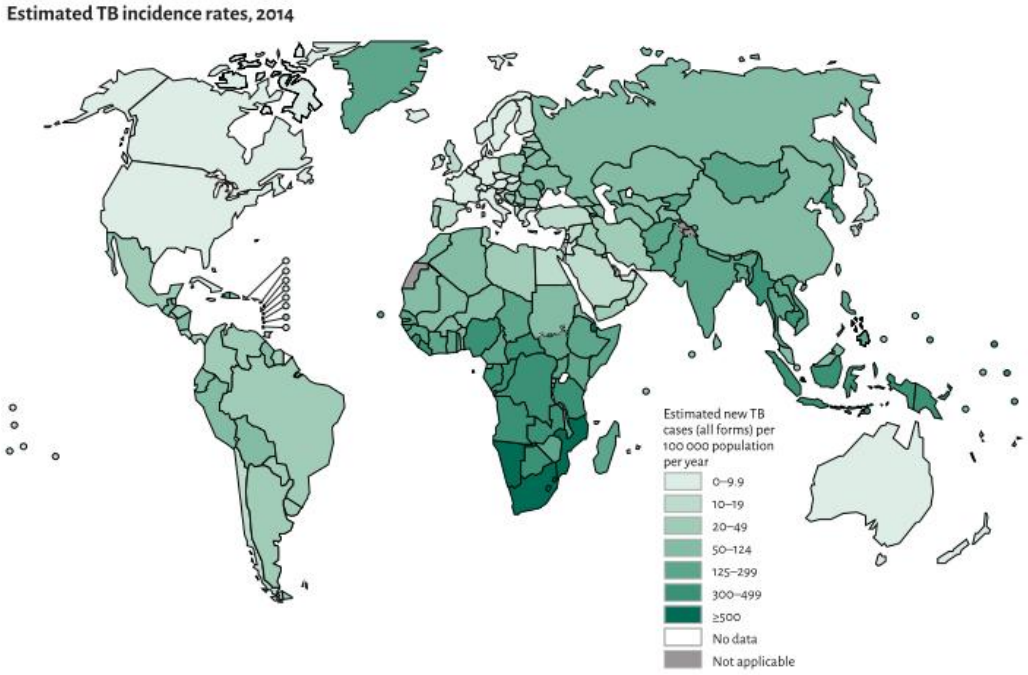

Fig. 1.1.2.a: Coverage of countries showing estimated TB incident rate is shown pictorially. (Global tuberculosis report, 2015).

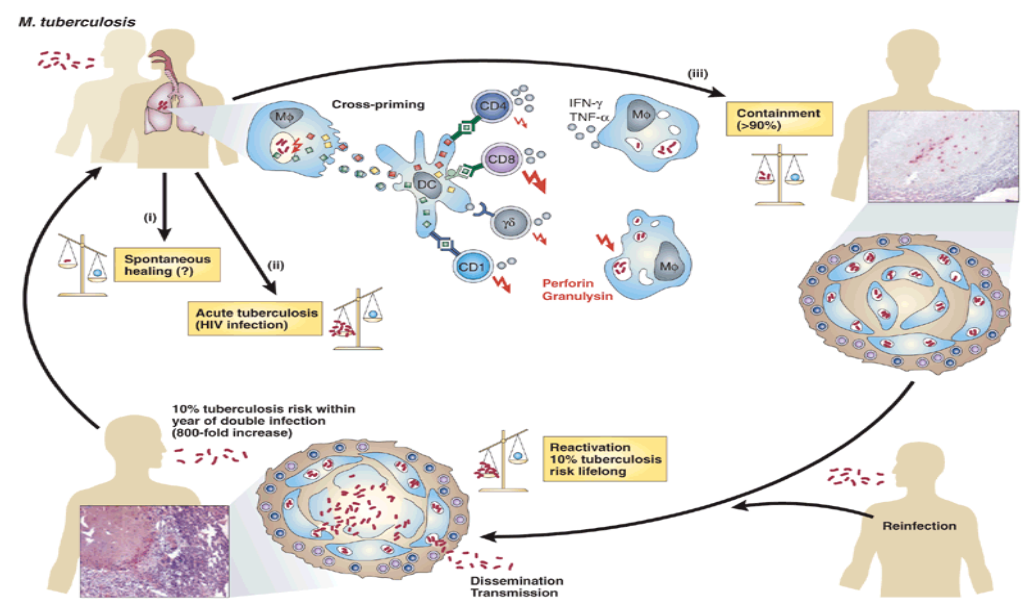

Fig. 1.1.2.b: Outcome of *Mtb* infection and related immune mechanisms. Possible outcomes are. (i) Immediate clearance of *Mtb* by pulmonary immune system. (ii) Infection transforms into tuberculosis. (iii) Infection does not transform into disease because granuloma formation (Kaufmann & McMichael 2005).

1.1.3 Types of tuberculosis depending on the site of infection

1.1.3.1 Pulmonary tuberculosis

Terms used to describe the pulmonary tuberculosis are “consumption” and “phthisis”. These terms are associated with advanced stages of tuberculosis. In case of pulmonary tuberculosis, lungs of the patient were involved mostly and they actively spread the disease through droplet infection. But, about a quarter of patient remain asymptomatic. In rare case scenario, TB erodes pulmonary artery, resulting in massive bleeding. This condition is known as Rasmussen's aneurysm.

It is generally considered that either of better aeration or poor lymph drainage, the upper part of the lung lobes develops TB with higher frequency.

1.1.3.2 Extrapulmonary tuberculosis:

Extrapulmonary tuberculosis (EPTB) refers to TB of organs other than the lungs that include pleura, abdomen, lymph nodes, genitourinary tract, skin, joints and bones, or meninges. In 15–20% of active TB, the disease develops into extrapulmonary form. Young children, immunosuppressed individuals and HIV infected individuals are more prone to extrapulmonary form of TB (Golden *et al*., 2005). Extrapulmonary infection sites generally include the pleura, the central nervous system in tuberculous meningitis, the lymphatic system, urogenital TB, and the bones and joints in Pott's disease. Infection of bones is also known as "osseous TB", a form of osteomyelitis. Not often, tuberculous ulcer bursts as tubercular abscess through skin. A fatal form of TB, Miliary TB, forms massive lymphohematogenous dissemination, thereby tiny lesions disseminates to the whole body. 10% of EPTB cases fall in this form of TB.

1.1.4 Pathogenesis of mycobacteria:

Primarily transmission dynamics initiates with inhalation of droplet infectious agents expelled by infected hosts. The usual aerosol droplets carry 1-400 bacilli to initiate and establish the infection. *M.tb* exploits the natural defenses of the body for its survival and successful colonization. After exposure, bacterium comes in contact with resident macrophages. Alveolar epithelial type II pneumocytes, which are in higher counts too ingest the bacterium in initial stages (Goodman 1996). Dendritic cells are efficient antigen presenting cells, they too participate in the early stages of infection cycle (Tascon *et al*., 2000). This leads to the activation of T cells with specific *M.tb* antigens and for dissemination of *M.tb*. The process of phagocytosis of bacteria is initiated by their association with macrophage mannose and/or complement receptors (Schlesinger 1993). Surfactant protein A, a known glycoprotein at alveolar surfaces can enhance the binding and uptake of *M.tb* by upregulating mannose receptor activity (Gaynor *et al*., 1995). *M.tb* after entry into the macrophages, reside inside the phagosomes. It inhibits the phagosomal maturation cycle by stopping phagosome-lysosomal fusion (Buchmeier & Heffron 1991). By this it avoids the stresses such as pH, ROS, RNS etc. that are natural defenses of host.

The infected macrophages in lung produce chemokines and attract inactivated monocytes, lymphocytes, and neutrophils. Nevertheless, it fails to eradicate the bacteria. Then, the formation of granulomatous lesions composed of giant cells and lymphocytes starts. Formation of granuloma generally clears the infection, but in case of *M.tb* an unusual process happens. *M.tb* bacilli, though, unable to multiply inside granuloma because of its acidic environment and the low oxygen, remain dormant but alive for decades. A person with strong immune status may confine this infection

without progress for the entire lifetime; this is known as latent TB. Compromise in immune surveillance by any reason such as immunosuppressive drugs, HIV infection, malnutrition or aging etc., liquefies the core of granuloma and serves as a rich medium for bacterial revival and replication and reactivate infection eventually.

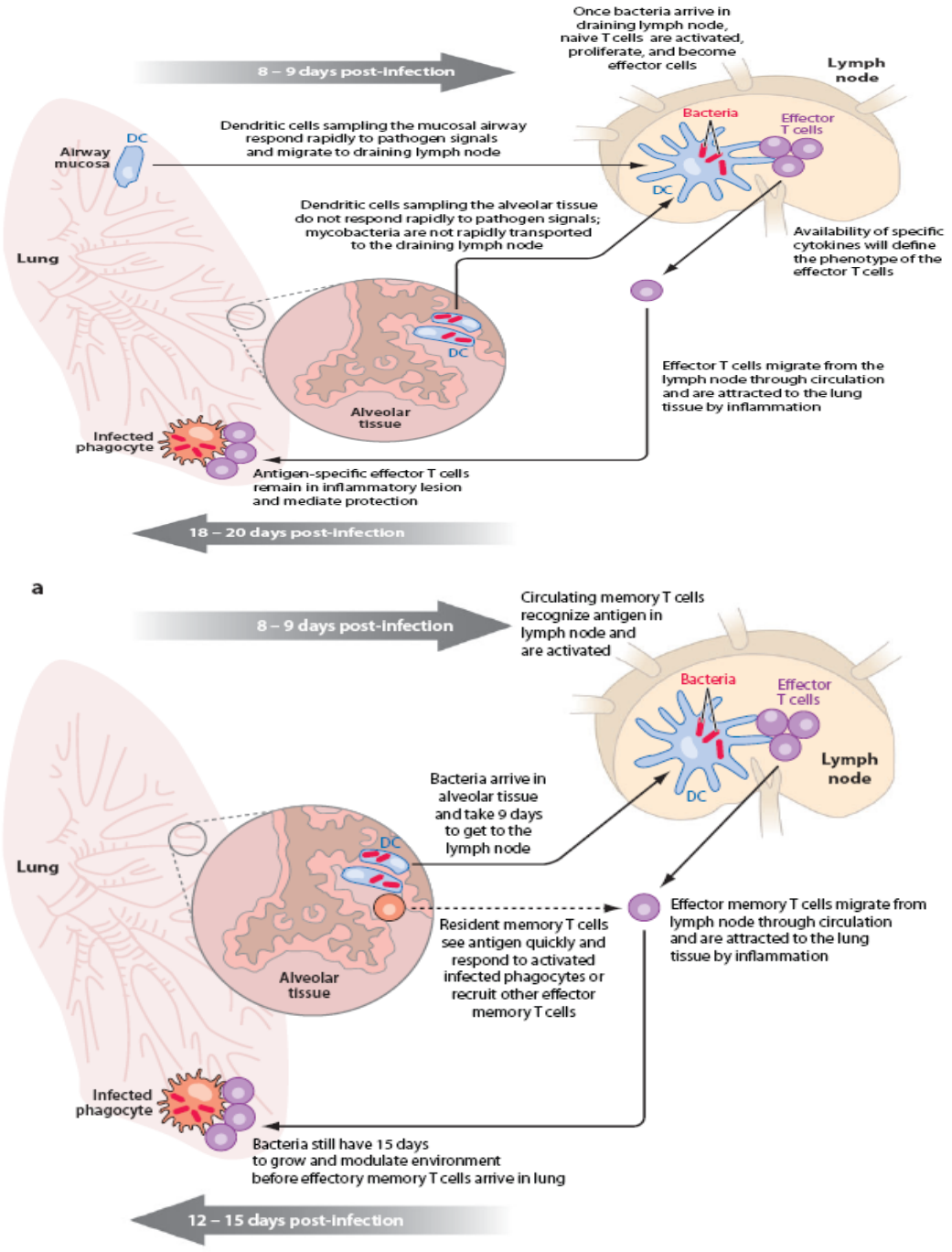

Fig. 1.1.4 Pathogenesis of Mycobacteria (Cooper AM. 2009. Annu. Rev. Immunol. 27:393–422)

1.2 Peptidyl prolyl *cis/trans* isomerases:

1.2.1 Introduction to PPIases:

Peptidyl prolyl cis/tran isomerases (PPIases) were discovered as a proteins with immunomodulatory properties but soon their potential as foldase involved hastening the isomerization of prolyl peptide bond. Later, their pleiotropic role is uncovered. They are ubiquitously expressed and essentially involved in protein processing related works, viz. protein processing for folding of protein, refolding of denatured protein, function in chaperone complexes. Recently, PPIases have been studied in different contexts such as regulatory processes in the cell (Lin *et al*., 2015), cancer pathways (Yao *et al*., 2005; Chen *et al*., 2015), inflammation (Liu *et al*., 2014; Tan *et al*, 2014), virulence, (Bell *et al*., 2006; en *et al*., 2005), stress response (Sykes *et al*., 1993; Mark *et al*., 2001), cell cycle regulation (Are & Heitman, 2005; Lu, Hanes, & Hunter, 1996), chromatin remodeling (Wu *et al*., 2000), transcription factor regulation (Yang, 2001) and RNA-mediated gene expression (Thapar, 2015; Krishnan, 2014) etc. Prolyl isomerases lie in three structurally and sequentially unrelated classes; cyclophilins, FKBPs and Parvullins (K. Kromina *et al*., 2008).

1.2.1 Properties of PPIases and their enzymatic activity:

PPIases are involved in the isomerization of prolyl peptide bond preceding proline. This is very general enzymatic activity whose direct functional impact cannot be scored. Regardless, many attributes of PPIases are listed as follows.

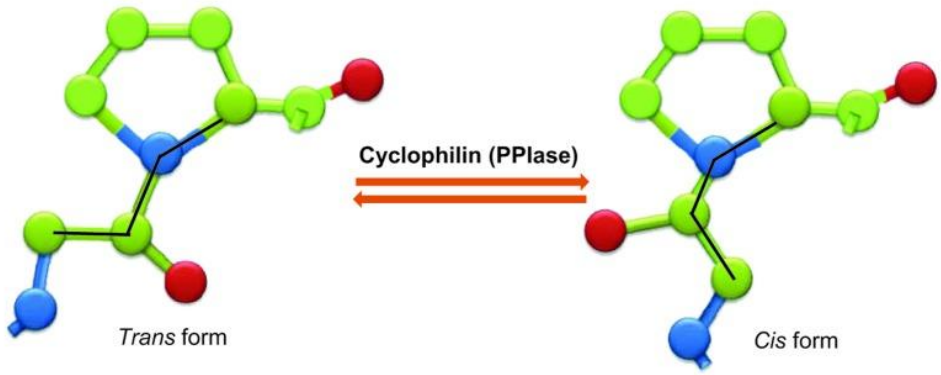

Fig1.2.1 A schematic representation of enzymatic trans/cis isomerization of the peptidyl prolyl bond. (Ref: https://www.scienceopen.com/document/vid/a13180c2-255c-44f2-baba-2b61df6f1129)

1.2.2 Composition of prolyl bond, structure of PPIases, and their folding action:

Proline forms imino moiety in place of amino, this reduces the energetic difference between *cis* and *trans* forms of prolyl peptide bond preceding proline. Lowering of energetic difference leads to the colonization of biological protein space with both *cis* and *trans* forms. Though ribosome synthesizes them into *trans* forms, *cis* form too appear in case of prolyl peptide bands in around 30% cases. The absence of functional enzyme otherwise stalls the protein to some suboptimal structure. Functional PPIases ease this folding bottleneck of prolyl peptide bond but they do not target each of such bonds. There are reports of substrate specificity for PPIase done in assay performed in peptide based substrate system (Harrison & Stein 1990). However, amino acid preceding the proline shows selection bias for enzymatic activity. For example, human FKBP12 for leucine and phenylanaline residue (Fischer *et al.*, 1992); Pin1 of human for phosphotyrosine and phosphoserine (Yaffe, 1997; McNaughton *et al.*, 2010); Macrophage infectivity potentiator (MIP) protein for lysine residues (Fischer *et al.*, 1992). It appears that shapes are essential for enzyme action as they make themselves fit to the active site than sequence specificity. Thus steric hindrance may stop the PPIases to access the site of peptidyl prolyl positions and they remain in *trans* in their native forms (Scholz *et al.*, 1998; Gothel & Marahiel, 1999).

PPIases occur in either with only enzyme domains, e.g. human FKBP12 of 12 KDa, human cypA of 18 KDa and parvulin of *E.coli* of 10 KDa or with additional domains such as TPR domain (Tetratricopeptide repeat) involved in the assembly of multiprotein complex (Schiene-Fischer *et al.*, 2013; D'Andrea & Regan, 2003). PPIases has other protein-protein interaction domains, WD-40, RanBD1, RNA recognition motif, zinc finger domain and calcium binding domain. Availability of these additional domains facilitate protein-protein interactions and allow the PPIase to

be the part of large protein complex to play signaling and chaperone functions along with protein processing (Schiene-Fischer *et al.*, 2013; Blackburn & Walkinshaw, 2011).

Enzymatic domain is significantly conserved in prokaryotic and eukaryotic PPIases. But, among PPIase subfamilies, viz, cyclophilins, FKBPs and parvulins have structural variations (Blackburn & Walkinshaw, 2011). Enzymatic moiety of PPIases are generally consists of 100 to 120 amino acid forming globular structure where β sheets makes the central part of the structure.

1.2.3 Cellular Localization of PPIases:

After enabling the PPIases to interact and form protein complexes, additional sequences and localization signal transport them to different sub-cellular organelles. Eukaryotic PPIases localize at nucleus, cytosol, mitochondria, endoplasmic reticulum, chloroplast and extracellular secretion (Schiene-Fischer *et al.*, 2013; Moro *et al.*, 1995). Bacterial PPIases remain in cytoplasm, periplasm (Hayano *et al.*, 1991), secreted (Kim *et al.*, 2002; Söderberg & Cianciotto, 2008) or membrane anchored (Dartigalongue & Raina, 1998; Trémillon *et al.*, 2012). For example, in the case of yeast PPIases, it has 8 cyclophilins, 4 FKBPs and one parvulin type of PPIases which are localized at different subcellular locations (Wang & Heitman, 2005).

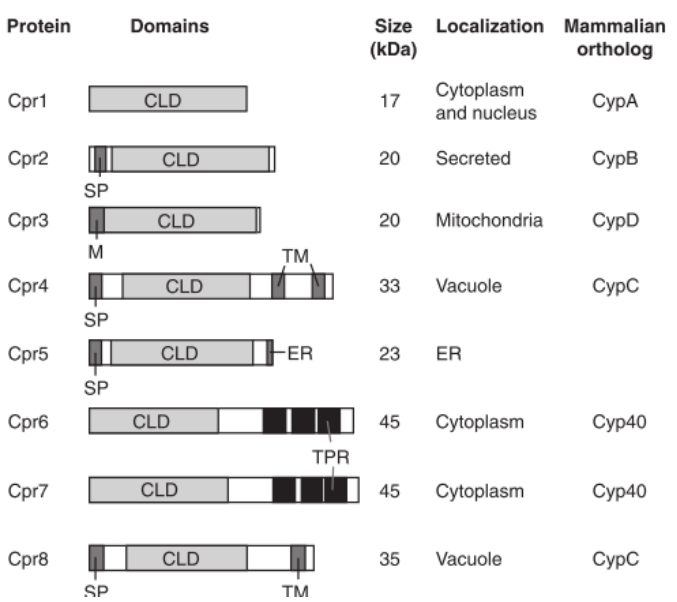

Fig 1.2.3: Yeast PPIases localize at different sub cellular compartments. (Wang & Heitman 2005)

1.2.4 PPIases of Prokaryotes and Eukaryotes:

PPIases are ubiquitously expressed protein families of ancient type expressed from unicellular microbial word to multicellular organisms (Davis *et al.*, 2010; Wang & Heitman, 2005). Eukaryotes expresses large number of PPIases, where as prokaryotes express a few (Bang *et al.*, 2000; Krücken *et al.*, 2009). Biological space is populated with either single domain or multi domain PPIases. The former is primarily the case of prokaryotes unlike large and multi-domain eukaryotic equivalents.

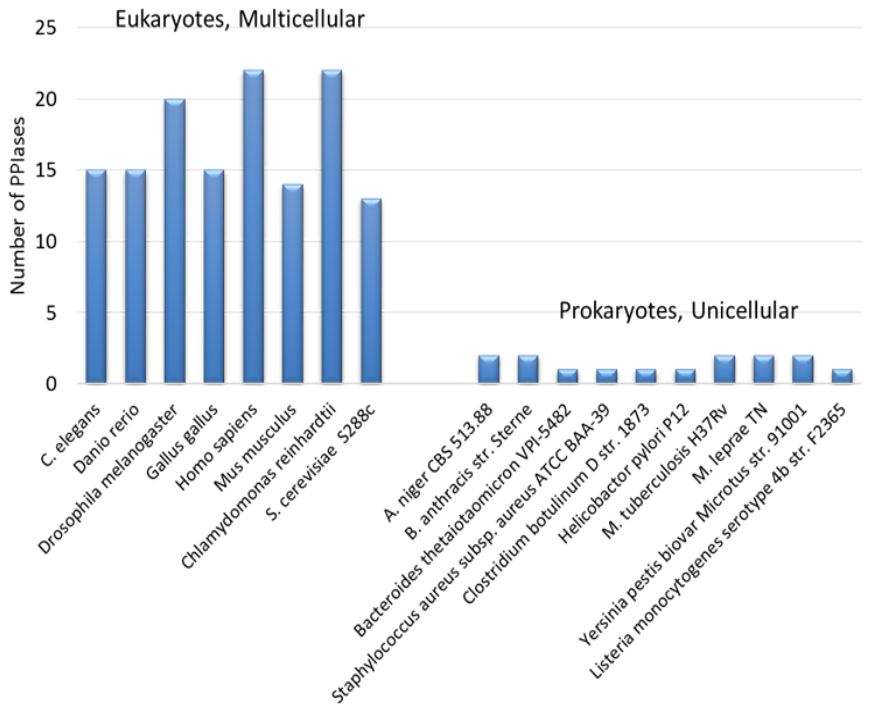

Fig 1.2.4: Number of peptidyl prolyl *cis/trans* isomerases of different organisms is shown in the figure. These are clustered in two groups as prokaryotes with few PPIases and eukaryotes with large number of PPIases.

1.2.5 PPIases in pathogenic processes:

Many pathogens of microbial worlds are discovered that make use of the PPIases for their pathogenesis, some of them even exploit host PPIases too for pathogenesis. A human pathogenic bacteria, *Legionella pneumophila* causing legionellosis, atypical pneumonia, has PPIase site in MIP (Macrophage infectivity potentiator) protein that is involved in infection of host cells (Helbig *et al*., 2003). *Nisseria gonorrhoeae*, has a surface exposed Lipoprotein with PPIase site, Ng-MIP, that aids in survival within macrophages (Leuzzi *et al*., 2005). Similarly, a PPIase fkpa mutant of *Solmonella typhimurium*, containing MIP homologue with PPIase site too helps in intracellular survival (Horne *et al*., 1997). In *Streptococcus pneumonia*, knock out mutant of either of cyclophilin type PPIase SlrA (streptococcal lipoprotein rotamase A) or parvulin type PPIase Ppma significantly looses their colonization efficiency and show increased phagocytosis by macrophages (Hermans *et al*., 2006; Cron *et al*., 2009).

In case of plant pathogens, mutation in FKBP type PPIase of *Xanthomonas campestris* result in significant loss of multiplication and virulence potential within host (Zang *et al*., 2007). A pathogenic fungus of rice, *Magnaporthe grisea* reduces its pathogenic potential and slows down its disease process after mutation in PPIase gene cyp1 (Viaud *et al*., 2002).

In plant world, VirD2 protein *Agrobacterium tumifaciens* interacts with PPIases (CypA) of *Arabidopsis thaliana* in the process of transduction of genetic material, while treatment of cyclosporineA *in vitro* inhibits the transduction (Deng *et al*., 1998). Further, PPIases of mouse (CypA) is essentially required for successful establishment of *Leishmania major*, a protozoan infection (Hoerauf *et al*., 1997) Viruses usually make use of host machinery for their replication and survival. Pathogenicity is also

another feature for which they exploit the host factors. E.g. human immunodeficiency virus capsid protein gag binds to host PPIases CypA and CypB, and it was postulated that it is important for viral infection process, capsid disassembly after infection and protects the capsid proteins from host restriction factors (Luban *et al.*, 1993; Kootstra *et al.*, 2003; Towers *et al.*, 2003). For successful viral replication within the host, host cyclophilins are required, as shown in the case of Hepatitis C virus (Watashi *et al*., 2005), SARS coronavirus (Pfefferle *et al*., 2011) , Japanese encephalitis virus (Kambara *et al*., 2011), rotavirus (He *et al*., 2013) and human cytomegalovirus (Keyes *et al.*, 2012). A tumorigenic virus Hepatitis B (HBV) protein HBx interacts with human Pin1, a parvulin type PPIases, for its stability and successful virus infection in human hepatocyte cell line. Overexpressing HBx and PinI directs the cell line to tumorigenesis (Pang *et al*., 2007).

1.2.6 PPIases in Mycobacterium and their relevance:

M.tb has two PPIases, namely PpiA (Rv0009) and PpiB (Rv2582), situated apart in the genome. PpiA, a 182 amino acid protein known as culture filtrate protein 22 (CFP-22.) is secretary protein with single domain Ppiase containing RGD motif at N terminal site. It is an iron regulated protein, low iron reduces its expression level (Wong *et al.*, 1999). PpiA upregulation in the double knock out mutant of hspR and hrcA (a transcriptional repressor of heat shock proteins), is suggestive of its role as a putative heat shock protein along with a possible virulence factor (Stewart *et al*., 2002). In *M.smegmatis* system, full length PpiA gets secreted but after truncation of N terminal sequence MADCDSVTNSP it is not secreted. Novel secretary sequence present only in pathogenic mycobacterium (Bhaduri *et al*., 2014), though PpiA is not an essential protein for *M.tb* survival as reported by transposon mutagenesis experiment (Sassetti *et al.*, 2003).

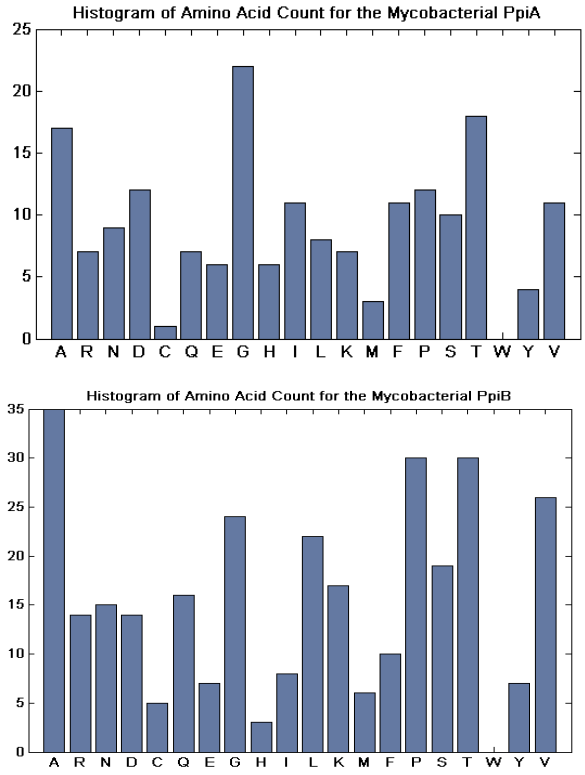

Fig 1.2.6.a: Histogram of Amino acid count of *M.tb* PpiA and PpiB.

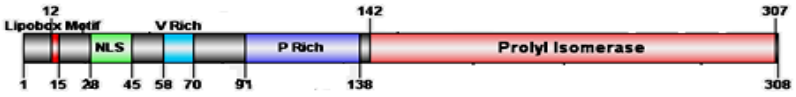

Fig 1.2.6.b: Schematic representation of PpiB showing variety signal sequences. These signals include NLS, lipobox motif, SH3 recognition motif, proline rich region, valine rich region.

PpiB of *M.tb* is a 302 amino acid multi domain protein. Other than cyclophilin like domain (CLD) it has ~140 amino acid sequence at N terminus that makes it unique. This signaling stretch includes nuclear localization signal (NLS), Lipobox motif, SH3 recognition domain, large proline rich region, and valine rich region. Proteome profile reveals that, though it is absent in the secretary proteome, it is present in mannosylated enriched culture filtrate through conA lectine (González-Zamorano *et al*., 2009) and also appears in the membrane fraction (Gu 2003). PpiB is reported to

be essential in the Sassetti's high density transposon mutagenesis experiment (Sassetti *et al*., 2003), expression level PpiB shoots up in the intraphagosomally grown *M.tb* (Mattow *et al*., 2006).

For successful colonization of human host, *M.tb* forms a niche by establishing molecular interaction networks within the host system. *M.tb* has evolved mechanisms to survive in macrophages that represent one of the most stressful environments for bacteria. Successful colonization of the intraphagosomal niche by the pathogen depends on molecular interaction network within the host (Ehrt & Schnappinger, 2009; Russell, 2001). The effector molecules which play crucial role in host pathogen interaction at the molecular level are mostly stress responders, heat shock proteins (HSPs), chaperones and other protein modifying enzymes (Henderson 2010). Pathogen subvert host defenses by quenching reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Ng *et al*., 2004; Cirillo *et al*., 2009), disrupting the membrane repair (Divangahi *et al*., 2009), phagolysosomal fusion (Shukla *et al*., 2014), suppression of autophagy (Huang & Bao, 2014) and by escaping immune challenges (Kugelberg, 2014; Gupta *et al*., 2012) and immune quorum sensing (Tundup *et al*., 2014). Bacterial chaperones play a vital role in protein folding and secretion, thereby indirectly contributing to the virulence and survival of the pathogen inside the host (Stebbins, 2005).

Prolyl isomerases, also known as cyclophilin, are expressed ubiquitously from bacteria to human and as of now 17 cyclophilin proteins in humans, 29 in *Arabidopsis* and 8 in *Saccharomyces* have been reported so far (Wang & Heitman, 2005; Galat, 1993). Isoform diversity, various subcellular localization and differences during evolution are indicative of their functional importance and acquisition of new roles. Moonlighting functions of prolyl isomerases include virulence character (Bell *et al*.,

2006), stress response (Sykes *et al*., 1993; Mark *et al*., 2001), cell cycle regulation (Are & Heitman, 2005; Lu *et al.*, 1996), chromatin remodeling (Wu *et al*. 2000), transcription factor regulation (Yang, 2001), RNA-mediated gene expression (Thapar, 2015; Krishnan, 2014) etc. Infection biology is not only influenced by pathogen encoded PPIases, but host PPIases also play a crucial role in development of the disease. For example, human CypA and Cyp B bind to the capsid protein of HIV and facilitate internalization of the virion particles in CD4 cells. Additionally, human cyclophilin A (PpiA) is recruited with nascent HIV-1 virions as well as incoming HIV-1 capsids where it is involved in isomerization of an exposed proline (Keckesova *et al.*, 2006).

1.3 Hypothesis and Objectives:

Given the fact that the PPIases of various model systems work as chaperone too along with isomerase activity and their role in stress responses and other cellular regulatory activities, It was hypothesized that "Mycobacterial peptidyl-prolyl cis/trans isomerases (PPIases) have additional functions along with prolyl isomerase activity, i.e. moonlighting function"

To validate this hypothesis PpiA (Rv0009) and PpiB (Rv2582) will be characterized biochemically, immunologically, stress response and also assist for other functions. The following constitutes the objectives of my proposed thesis:

- Characterize enzyme activity of rPpiA and rPpiB expressed in *E. coli* (Rational: To prove that PpiB is an active enzyme.)
- Demonstrate that rPpiA and rPpiB function as a molecular chaperone by *in vitro* and *in vivo* studies (Rationale: Many other PPIases show Chaperone potential alongside isomerase action.)
- Investigate immune modulation potential of *M.tb* PPIases (PpiA and PpiB) (Rationale: *M.tb* PPIases available in the host pathogen interaction zone, thus, they may likely have immune modulatory activity.)
- Demonstrate the possible role of *M.tb* PPIases in aiding intraphagosomal survival of the pathogen (Rationale: Chaperones, heat shock proteins and other protein modifiers are potential stress responders, thus may aid in pathogen survival.)

- Demonstrate the possible role of *M.tb* PpiB in biofilm and pellicle formation by the pathogen (Rationale: As PpiB is a putative glycopeptide present on the membrane of bacilli, it may be involved in the biofilm formation)

Chapter 2

Mycobacterium tuberculosis peptidyl-prolyl isomerases also exhibit chaperone like activity *in vitro* and *in vivo*

2.1. Abstract:

Peptidyl-prolyl cis-trans isomerases (PPIases), also known as cyclophilins, are ubiquitously expressed enzymes that assist in protein folding by isomerization of peptide bonds preceding prolyl residues. *Mycobacterium tuberculosis* (*M.tb*) is known to possess two PPIases, PpiA and PpiB. However, as understanding about the biological significance of mycobacterial PPIases with respect to their pleiotropic roles in responding to stress conditions inside the macrophages is restricted. This study describes chaperone-like activity of mycobacterial PPIases. This chapter describes results showing that recombinant rPpiA and rPpiB can bind to non-native proteins *in vitro* and can prevent their aggregation. *E. coli* cells overexpressing PpiA and PpiB of *M.tb*, could survive thermal stress as compared to plasmid vector control. The potential role of mycobacterial PPIases in responding to host generated stresses like hypoxia and oxidative stress was also investigated. HEK293T cells transiently expressing *M.tb* PpiA and PpiB proteins show increased survival as compared to control cells in response to oxidative stress and hypoxic conditions generated after treatment with H_2O_2 and $CoCl_2$. The chaperone-like function of these *M.tuberculosis* cyclophilins may possibly function as a stress responder and consequently contribute to virulence.

2.2. Introduction:

M.tb has two cyclophilins, PpiA and PpiB coded by *ppiA* (Rv0009) and *ppiB* (Rv2582), respectively, which are located apart in the genome. *M.tb* PpiA is known to be structurally and phylogenetically related to eukaryotic cyclophilins. It has been previously reported that it is a secretary protein and interacts with several host proteins such as those involved in iron regulation, immune defense mechanism and signal transduction (Henriksson *et al.*, 2004; Bhaduri *et al*., 2014). Presence of PpiB has been reported in proteomes of membrane fraction (Gu, 2003) and mannosylation enriched culture filtrate (Cole *et al*., 1998), which are indicative of its surface expression. PpiB is reported to be essential for the survival of the pathogen (Sassetti *et al.*, 2003). Functional characterization of the enzymes reflecting their possible role as a stress responder in the pathogen, and thus contributing to its virulence, has not been investigated so far. This chapter presents results that describe the functional characterization of *M.tb* PPIases (PpiA and PpiB) and demonstrate that they display chaperone-like activity. Recombinant *M.tb* PPIases (PpiA and PpiB) expressed in *E.coli* could bind to heat labile MalZ protein *in-vitro* and can prevent its aggregation are also described. *E.coli* transformants expressing *M.tb* PPIases exhibited increased survival under heat shock, as compared to vector control. That these cyclophilins enabled the survival of HEK293T cells under conditions of hypoxic and oxidative stress pointed to the potential role of *M.tb* PPIases to absorb cellular stress *in vivo*.

2.3. Materials and Methods

2.3.1. Materials:

IPTG, imidazole, N-succinyl- Ala-Ala-Pro-Phe-p-nitroanilide, trifluoroethanol, LiCl, α-chemotrypsin, 8-anilino-1-naphthalene-sulfonic acid (ANS), reduced Glutathione, DTT and MTT were obtained from Sigma. All cell culture reagents were obtained from GIBCO. All enzymes were purchased from NEB (USA); ELISA kit from Peprotech and toxicity removal kit from Norgen. All reagents used were analytical grade. The plasmids and strains used in this study are listed in (Table 2.1).

TABLE 2.1. Strains and plasmids used in this study

Plasmids	**Relevant characteristics**	**Source/Reference**
pET28a	Kan^R pET28a (cloning vector), pBR322 origin	Novagen
pET28_ppiA	pET28a containing *ppiA* gene with T7 promoter	This work
pGEX_ppiB	pGEX6p1 containing *ppiB* gene with T7 promoter	This work
pcDNA 3.1 (-)	Mammalian expressing vector, $Neomycin^R$, myc & his tag, CMV promoter	Invitrogen
pcDNA_ppiA	pcDNA3.1(-) containing *ppiA* gene	This work
pcDNA_ppiB	pcDNA3.1(-) containing *ppiB* gene	This work

Strains	**Relevant characteristics**	**Source/Reference**
DH5α	*supE44 ΔlacU(Φ80lacZΔM15) hsdR17 rec*1 *endA*1 *gyrA96 thi*-1 *relA*1	Novagen
BL-21(DE3)	*F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene* 1*ind*1 *sam7 nin5])*	Novagen
Ec_ppiA	BL-21 λ (DE3) containing pET28appiA	This work
Ec_ppiB	BL-21 λ (DE3) containing pGEXppiB	This work

Selection marker resistant to Ampicillin (amp^R) and kanamycin (kan^R).

2.3.2 Cloning of *ppiA and ppiB* in pET28a vector:

The *ppiA* (Rv0009)) and *ppiB* (Rv2582) gene was amplified by PCR using genomic DNA of *M.tb* H_{37}Rv strain as the template for amplification.

2.3.3. Primers:

The sequence specific oligonuceotides used for the cloning as listed in Table 2.2.

Table 2.2: List of primers are as follows:

Serial No.	Oligonucleotide name	Oligonucleotide sequence	Restriction enzyme used
1.	NZE_ppiA_ For	CG**GGATCC**ATGGCAGACTGTGATTCC GTGACTAA	*Bam*H1
2.	NZE_ppiA_Rev	GGG**AAGCTT**TCAGGAGATGGTGA TCGACTCGATC	*Hin*dIII
3.	NZE_ppiB_ For	CG**GGATCC**ATGGGCCACTTGACACCG	*Bam*H1
4.	NZE_ppiB_Rev	GGG**AAGCTT**CTAATCCAGCAGCACCG ACGTG	*Hin*dIII

The highlighted sequence is the restriction sites inserted in the primer sequence.

2.3.4. PCR amplification

The open reading frame corresponding to *ppiA* and *ppiB* were PCR amplified from genomic DNA of *M.tb* H_{37}Rv using forward and a reverse primer set containing *Bam*HI and *Hind*III restriction sites as overhang, respectively. Reaction mix used for PCR amplification of *ppiA* and *ppiB* is given in the table below:

Table 2.3

Serial No.	Reagent	Amount
1.	Template	100ng
2.	Taq Buffer	1X
3.	dNTPs	0.2mM of each dNTP
4.	Primers (Fwd and Rev)	1 µM of each primer
5.	*Taq* DNA polymerase	2.5 units

PCR reaction was set in 0.2 ml flat capped PCR tube using eppendorf thermal cycler.

The PCR reaction conditions are described in Table 2.4 :

Table 2.4:

Step	Temperature	Time	
Initial Denaturation	94°C	5 min	
Denaturation	94°C	30 sec	30 cycles
Annealing	60°C	30 sec	
Extension	72°C	1 min	
Final extension	72°C	10 min	
Hold	4°C	∞	

2.3.5. DNA extraction from agarose gels:

PCR product was fractionated by agarose gel electrophoresis. For extraction of DNA from gel, the protocol described in Sambrook *et al.* 1989 was followed. DNA fragments of right size were excised and eluted from the gel slice using qiagen gel extraction kit as per the manufacturer's instruction. In brief,

1. Gel pieces containing the DNA fragment of interest were chopped into small pieces and transferred to 1.5 ml microfuge tube and gel slices were weighed.
2. For every 100mg of gel slice 100µl of capture buffer was added and then the sample was incubated at 55 ºC till the agarose gets dissolved in the buffer. Tubes were vortexed intermittently. Afterwards, samples were loaded onto the gel extraction spin column and centrifuged at 5,000 rpm for 3 minute.
3. The column was washed by adding 500 µl of wash buffer followed by centrifugation at 5,000 rpm for 3 minute.
4. The column was dried by giving a dry spin at 13,000 rpm for 30 sec.
5. The column was transferred in a fresh microfuge tube and DNA was eluted in 30 µl nuclease free water by centrifugation at 13,000 rpm for 1 minute.

6. Concentration of purified DNA was estimated spectrophotometrically by measuring A_{260}.

2.3.6. Restriction digestion of DNA:

The gel extracted PCR product and pET28a vector were digested with *Bam*HI and *Hind*III enzymes. One unit of enzyme was used per µg of DNA in a final volume of 40 µl with 1X restriction digestion buffer. The reaction mixture was, then, incubated at 37 °C for 3 hours. Subsequent to the digestion, the enzymes were inactivated by incubating the reaction mixture at 60 °C for 20 minutes.

2.3.7. Ligation-Construction of recombinant plasmids:

The digested fragments were cleaned up using the PCR Cleanup kit (Qiagen) as per manufacturer's instructions. The digested *ppiA* and *ppiB* DNA fragment was ligated to *Bam*HI/*Hind*III sites of pET-28a expression vector to obtain pETppiA and pET_ppiB plasmids. Ligation was setup in 10µl reaction volume with vector DNA (10 ng), PCR product (30 ng), 1µl of ligation buffer (10X) and 1 unit of T4 ligase. The ligation reaction mixture was incubated for 3 hrs at 22°C and used to transform *E.coli* DH5α competent cells.

2.3.8. Competent cell preparation:

E. coli DH5α, *E. coli* BL21 (λDE3) and *E. coli* BL21 (λDE3) rosetta competent cells were prepared according to the method described by Cohen (Cohen, Chang *et al.*, 1972). This method yields the transformation efficiency $5x10^6$ to $2x10^7$ transformants per µg of circular plasmid DNA.

1. A single colony of *E. coli* DH5α or *E. coli* BL21 (λDE3) was inoculated into 5 ml LB for overnight growth.

2. 100 ml LB was inoculated with 1% of primary culture and allowed to grow until A_{600} reached 0.4.
3. The culture was chilled on ice, transferred to ice cold 50 ml tubes and centrifuged at 4000 rpm for 10 min at 4°C.
4. The supernatant was decanted; pellet was resuspended in 10 ml of ice cold 0.1 M $CaCl_2$ gently and incubated on ice for 30 minutes with intermittent swirling.
5. Cells were centrifuged at 4000 rpm at 4°C; pellet was resuspended in 4 ml of ice cold 0.1 M $CaCl_2$ containing 20% glycerol.
6. Prepared the aliquots of 200 µl in sterile 1.5 ml vials and checked transformation efficiency and contamination.
7. Aliquots were snap-freezed and stored at -80 ºC.

2.3.9. Transformation into competent cells:

The ligation mixture was transformed in competent *E. coli* DH5α cells according to the established protocol (J. Sambrook *et al.*, 1989) as described briefly below.

1. Aliquot of competent cells was thawed over ice; DNA was added and mixed by tapping. Kept the mixture on ice for 30 minutes.
2. The cells were subjected to heat shock at 42 °C for 90 seconds in a water bath and were immediately transferred to an ice bath for 2 minutes.
3. 1 ml of LB was added to cells and incubated at 37 °C for 1 hour.
4. Afterwards, cells were pelleted at 6000 rpm for 2 minutes and resuspended in 100 µl of LB and plated on pre-made LB-agar plates containing 50 µg/ml of kanamycin. The plates were incubated at 37 °C for overnight.

2.3.10. Screening of transformants:

The transformants were screened for presence of insert by restriction mapping to confirm the presence of *ppiA and ppiB* gene in the putative colonies based on the

amplification of desired size. Briefly, few single colonies of putative clones were inoculated in 3 ml of LB media with kanamycin (50µg/ml) for overnight. Next morning, plasmid DNA was isolated through mini prep, and restriction was set with flanking enzymes along with intermediate enzymes. Digested product was fractionated on agarose gel. Clones releasing the calculated size of the insert were taken as positive clones. Mini-preparations for plasmid DNA and their restriction analysis of putative clones of *ppiA* and *ppiB* in pET-28a were, subsequently, performed. The alkaline lysis method was used for preparations of plasmid DNA (Birnboim & Doly 1979).

2.3.11. Plasmid DNA isolation by Alkaline lysis method:

1. A single colony was inoculated in 3ml of LB medium with kanamycin (50µg/ml) and shaken overnight at 37 °C.
2. Next morning, culture was pelleted in a microfuge tube at 12,000 *g* for 2 minutes.
3. The media was discarded and the pellet was re-suspended in 250 µl of ice-cold solution I by vigorous vortexing and kept on ice for 20 minutes.
4. To the tube, 250 µl of freshly prepared solution II was added. The tube was gently mixed by inversion and incubated at room temperature for 5 minutes.
5. After the incubation, 250 µl of ice-cold solution III was added. The contents of the tube were mixed and incubated on ice for 15 minutes.
6. The mixture was centrifuged at 12,000 rpm for 15 minutes at 4 °C. Supernatant was transferred to a fresh microfuge tube. The DNA was precipitated by the addition of 0.6 volume of isopropanol to this tube.
7. The DNA was precipitated by centrifugation at 13,000 rpm for 15 minutes at room temperature. The pellet was washed with 70 % ethanol, centrifuged at 13000 rpm for 15 min and air-dried.

8. The DNA was obtained by resuspending in 20 µl of nuclease free water.
9. The concentration and purity of the plasmid DNA prepared was analyzed on agarose gel and spectrophotometerically.

2.3.12. Confirmation of putative clones:

Two µg of plasmid DNA was double digested with *Bam*HI and *Hind*III enzymes in 50 µl reaction mix containing compatible 1X buffer. The digestion mixture was incubated at 37 °C for 3 hours and then analyzed on 0.8 % agarose gel, for the presence of insert at the correct position. Clones selected after restriction analysis were then verified by automated dideoxy DNA sequencing to confirm the integrity of cloned gene. Glycerol stocks were prepared for positive clones and stored at -80 °C. The constructs were designated as pETppiA and pETppiB.

2.3.13 Protein expression and purification:

PpiA was over-expressed in *E. coli* cells and purified by affinity chromatography. *ppiB* was not induced in proper soluble form in pET system of poly his tag. Therefore, It was sub cloned in pGEX6p-1 vector using *Bam*HI and *Xho*I restriction site, which gave bigger and soluble tag and increased the chance of purification in native form. Cloning was performed with similar strategy, only PCR being replaced by digestion of pETppiB construct with *Bam*HI and *Xho*I restriction enzymes and *ppiB* was extracted from gel.

2.3.14. Expression of recombinant pETppiA and pGEXppiB:

For high-level expression, recombinant construct pET_ppiA and pGEX_ppiB were transformed into *E. coli* BL21 (λDE3) and BL21 (λDE3) rosetta competent cells, respectively. Cells containing pETppiA and pGEXppiB were grown in LB media containing 50 µg/ml of kanamycin and 100 µg/ml of ampicillin, respectively. When

OD at 600nm reached 0.6, 1ml of culture was taken out as uninduced and. rest of the culture was induced by adding IPTG at concentration of 1 mM for PpiA and 0.1 mM for PpiB. Both uninduced and induced cultures were kept on shaking at 200rpm at 37°C, 3 hours for PpiA and 18°C, 16 hours for PpiB. After induction cells were harvested by centrifugation at 4,000 rpm, at 4°C for 15 minutes. The pellet (of 1 ml induced and uninduced culture) was re-suspended in l00 µl of 1X lysis buffer and centrifuged at 12,000 *g* for 10 minutes at room temperature. The supernatant was loaded onto SDS-PAGE gel for analysis of recombinant proteins.

2.3.15. Purification of rPPiA with Ni^{+2}-NTA agarose under native conditions:

BL21 (λDE3) cells containing pETppiA and BL21 (λDE3) rosetta cells containing pETppiB were grown and induced at the above mentioned conditions. The cells were harvested by centrifugation at 4,000Xg for 15 minutes. Cells were resuspended in lysis buffer and sonicated to break open the cells. The cytosol fraction was separated after centrifugation at 12000 rpm for 30 minutes. 20 µl induced and uninduced culture, corresponding volume of cytosolic fraction and pellet fraction after dissolving the pellet in the lysis buffer, were analyzed by separating on SDS PAGE. Overexpressed proteins were localized in cytosolic fraction. For large scale purification of recombinant rPpiA and rPpiB in *E.coli*, protocol was standardized. Purification protocol Ni-NTA column:

i. A single isolated colony of *E. coli* cells transformed with recombinant proteins, was inoculated into 10mL of sterile LB broth containing 10µL of Kanamycin (50mg/ml), and kept for overnight incubation at 37^0C incubator shaker.

ii. 200mL of sterile LB broth was inoculated with 2% primary culture in a conical flask along with 200µL of kanamycin. The flask was incubated under shaking conditions at 37^0C till A_{600} reaches 0.5.

iii. Culture was induced with 1mM IPTG for 3 hours at 37^oC to express the recombinant protein.

iv. Then cultures were chilled on ice for 20 minutes to prevent further growth of the cells.

v. The culture was pelleted in 50 mL centrifuge tubes at 4000 rpm for 15 minutes. The supernatant was discarded and the pellet was stored at -20^oC.

vi. On the following day, each 50mL pellet was resuspended in 5mL of chilled 1X PBS, pooled and to this was added 1 mM PMSF and 0.3% Sarkosyl.

vii. The sample was then sonicated by keeping at ice to get a clear homogeneous solution and centrifuged at 12000 rpm for 30 minutes at 4^oC.

viii. The pellet was discarded and supernatant was collected in a fresh tube.

ix. Supernatant was added to the Ni-NTA column and flow through was collected in a tube.

x. The column was then washed with 25mL of 12.5mM Imidazole buffer in 1X PBS at 4^oC.

xi. A second wash was followed with 6mL of 25mM Imidazole buffer in 1X PBS.

xii. The elution buffer (250mM imidazole in 1X PBS) was added to the column and elution fractions were collected in 1 ml in labelled tubes.

xiii. Finally, water was passed through the column twice and the beads stored in 30% ethanol at 4^oC to prevent bacterial growth.

xiv. 20µL of the wash and flow through samples were mixed with 5µL of 4X SDS-PAGE gel loading dye.

xv. After elution, 5µL of each elution was mixed with 5µL of the loading dye.

xvi. The protein content in each fraction was estimated by Bradford assay.

xvii. The fractions were analyzed by resolving them on 12 % SDS-PAGE followed by coomassie staining to get the profile of recombinant protein.

2.3.16. Polyacrylamide gel electrophoresis:

Proteins were resolved by SDS-PAGE according to method of Laemmli (Laemmli, 1970). A 12 % resolving and 5 % stacking SDS polyacrylamide gel was utilized for the electrophoretic analysis of proteins. 10 µl of supernatant protein samples were analyzed on gel in a Bio-Rad mini gel apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The gels were stained with Coomassie brilliant blue and destained to remove excess of stain to visualize the resolved proteins.

2.3.17. Purification of rPpiB with Glutathione-agarose resin under native conditions:

The rPpiB was purified in native form as fusion protein with GST (Glutathione-S-Transferase) by the method described by Smith *et al.*(Smith & Johnson, 1988) as detailed below:

i. A single isolated colony of the rPpiB expressing *E. coli* cells was inoculated into 10mL of sterile LB broth containing ampicillin (100mg/mL) and kept at 37^{o}C for overnight incubation under shaking.

ii. 200mL of sterile LB broth was inoculated with 2% of primary culture in a conical flask containing ampicillin solution (50mg/ml) and incubated at 37^{0}C till A_{600} reaches 0.5.

iii. Culture was shifted to 18^{o}C incubator shaker and induced with 0.1 mM IPTG solution for 16 hours to express the rPpiB.

iv. The cultures were then pelleted in 50mL centrifuge tubes by centrifugation at 4000rpm for 15 minutes, the supernatant was discarded and pellet stored at -20^{o}C.

v. On the following day, each 50mL pellet was resuspended in 5mL of 1X PBS and pooled, then 1mM PMSF and 0.3% Sarkosyl was added.

vi. The sample was then sonicated by keeping at ice to get a clear homogeneous solution and centrifuged at 12000 rpm for 30minutes at 4^{o}C.

vii. The pellet was discarded and supernatant was collected in a fresh tube.

viii. The supernatant was collected in a fresh tube and the pellet discarded.

ix. Supernatant was added in the column containing Glutathione-agarose resin and placed in a rotator for 40 minutes at 4^{o}C to allow for proper binding.

x. Afterwards, flow through was collected, 30 ml 1X PBS buffer was used for washing.

xi. Elution fraction was collected at 4^{o}C, by using, 20 mM reduced glutathione in 20 mM Tris buffer pH8.0.

xii. Finally, water was passed through the column and the beads stored in 30% ethanol solution at 4^{o}C.

xviii. 20µL of the wash and flow through samples were mixed with 5µL of 4X SDS-PAGE gel loading dye.

xix. After elution, 5µL of each elution was mixed with 5µL of the loading dye.

xx. The protein content in each fraction was estimated by Bradford assay.

xiii. The fractions were analyzed by resolving them on 12 % SDS-PAGE followed by coomassie staining to get the profile of recombinant protein.

2.3.18. Dialysis of purified protein:

Purified rPpiA and rPpiB were dialyzed against 1X PBS pH-7.4 for buffer exchange and to remove imidazole and reduced glutathione. Dialysis was performed with 0.5 liter 1X PBS at 4^{o}C and buffer was changed 3 times at intervals of 3 hours.

2.3.19. Estimation of Purified rPpiA and rPpiB:

Protein estimation was done using the protein determination dye (BIO RAD) based on the Bradford dye-binding procedure (Bradford 1976). 400 µl of dye was diluted 5 fold by autoclaved MQ water and dispensed in 96 well plate by adding 2 and 5 µl of protein samples of rPpiA and rPpiB in triplicate and mixed well. BSA. Absorbance was measured at 595nm. This was compared with a standard curve of BSA (optical density plotted against varying concentrations of BSA). Amount of recombinant protein was determined by comparing with standard curve.

2.3.20 Enzyme assay of purified recombinant rPPIases:

The ORF encoding *M.tb ppiA* (Rv0009) and *M.tb ppiB* (Rv2582) was PCR amplified from H_{37}Rv genomic DNA by using forward and reverse primers. *ppiA* was cloned in pET28a vector using *Bam*HI and *Hin*dIII restriction sites and *ppiB* in pGEX6p1 vector using *Bam*H1 and *Xho*1 restriction sites. Recombinant proteins were purified using Ni-NTA column for PpiA and glutathione sepharose affinity column for PpiB as described earlier (Banerjee *et al*., 2007). PPIase activity of both, rPpiA and rPpiB was evaluated using a spectrophotometric assay (Fischer *et al.*, 1984). 8mM oligo peptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide substrate solution was prepared in trifluoroethanol containing 0.45 M LiCl in cold. Coupled enzyme α-Chymotrypsin was prepared at a concentration of 60mg/ml in cold solublizing buffer (33 µl of 1 mM HCl and 2mM $CaCl_2$ solution). Reaction mixture included 910µl 0.1 M TrisCl, pH

8.0, 50µl 600µM α-Chymotrypsin and 30µl rPpiA which were incubated for 2 minutes at 15^0C. Subsequent addition of 10µl of peptide solution resulting in a final concentration of 80µM initiated the reaction. The enzyme catalyzed *cis/trans* isomerization of Ala-Pro bond, coupled with cleavage of the *trans* peptide of α-Chymotrypsin was observed as increase in absorbance at 390nm at 15^0C. Measurements were recorded every 0.5 sec till 3 minutes and the final absorbance was measured from each curve. The absorbance at each time point was subtracted from that value.

2.3.21 ANS Fluorescence of rPPIases:

Fluorescence of ANS in the presence of rPpiA and rPpiB was measured by exciting at 390 nm and following the emission between 450 and 550 nm (Suragani *et al*., 2013). 0.5mg/ml of the recombinant proteins, rPpiA and rPpiB, respectively was incubated with 50 µM ANS for 30 min at room temperature, and fluorescence of protein-bound dye was recorded. Fluorescence emission spectrum of ANS alone was used as control. The spectra were corrected with appropriate buffer and protein blanks. The emission and excitation slit widths were set at 10 and 10 nm, respectively.

2.3.22 Aggregation Assay:

Chaperone activity of rPpiases was investigated in terms of its ability to prevent aggregation of MalZ. MalZ loses its native conformation and undergoes aggregation during incubation at 47°C. MalZ and GroEL were purified for the assay as reported earlier (S. Paul & Chaudhuri, 2008; Paul *et al*., 2007). Assay for MalZ aggregation was performed in presence and absence of rPpiA or rPpiB at 47°C. Light scattering was measured by recording the absorbance as described earlier (Goyal *et al*., 2014). The samples used for the assay involved (0.4uM) MalZ alone, rPpiA alone, rPpiA

with lysozyme (negative control) and purified GroEL (positive control) and with increasing molar ratios of rPpiA (10, 20, 40) and rPpiB (5, 10, 20).

2.3.23. Residual activity of denatured *Nde*1:

*Nde*1 (10 U) was incubated at 60°C for 20 min in the absence or presence of rPpiases (rPpiA and rPpiB). BSA (20 μg) was used as a control (Suragani *et al*., 2013; Ellis, 1990). Assessment of the residual enzyme activity was assayed by digesting 150ng of circular pUC18 at 37°C for 1 h. The digestion mixture was electrophoresed on 1% agarose gel, stained with ethidium bromide and visualized under UV light in a Gel doc system (Bio-Rad).

2.3.24 Growth rescue of *E. coli* from thermal shock:

Rescue of *E. coli* cells from thermal shock was performed using the method reported previously (Suragani *et al*., 2013). Fold survival with and without heat shock was calculated and the value was normalized taking the vector control (pET28A and pGEX6p1) as one fold.

2.3.25 Survival Assays under various stress conditions by MTT assay:

Assessment of the hypoxia stress and oxidative stress on the proliferation of HEK293T cells was carried out by MTT assay. Cells were transfected with pcDNA_ppiA, pcDNA_ppiB and vector control. 3 million cells were taken in 35 mm dish in each category. For hypoxia, 5000 cells/well from each category were seeded in a 96-well plate cultured for 12 hours at 37°C in 5% CO_2 in 150ul complete RPMI1640. Dose dependent treatment with $CoCl_2$ (50, 100, 150, 200 μM) was performed for 24 hours (Piret *et al*., 2002). Untransfected cells, only media control and empty wells were used as controls in this experiment. At the end of the treatment,

MTT 25µl (from 5mg/ml in PBS), was added and then incubated for 4 hours. Acidic isopropanol (4%HCl and 0.1% NonidetP-40) was added in each well after removal of the supernatant. After shaking the plate for 10 min, cell viability was assessed by measuring the absorbance at 590 nm with 620 reference filter. Similarly, resistance to oxidative stress was determined by treating the cells expressing PpiA and PpiB, respectively with 10-40µM H_2O_2 in increasing concentrations. All assays were carried out in triplicate.

Results

2.4.1. Cloning of *ppiA* and *ppiB:*

ppiA and ppiB genes was PCR amplified using oligonucleotides that added *Bam*HI and *Hin*dIII restriction sites to the 5' and 3' ends of the gene respectively. Cloning was performed as described in material method and schematically presented below (figure 2.4.1).

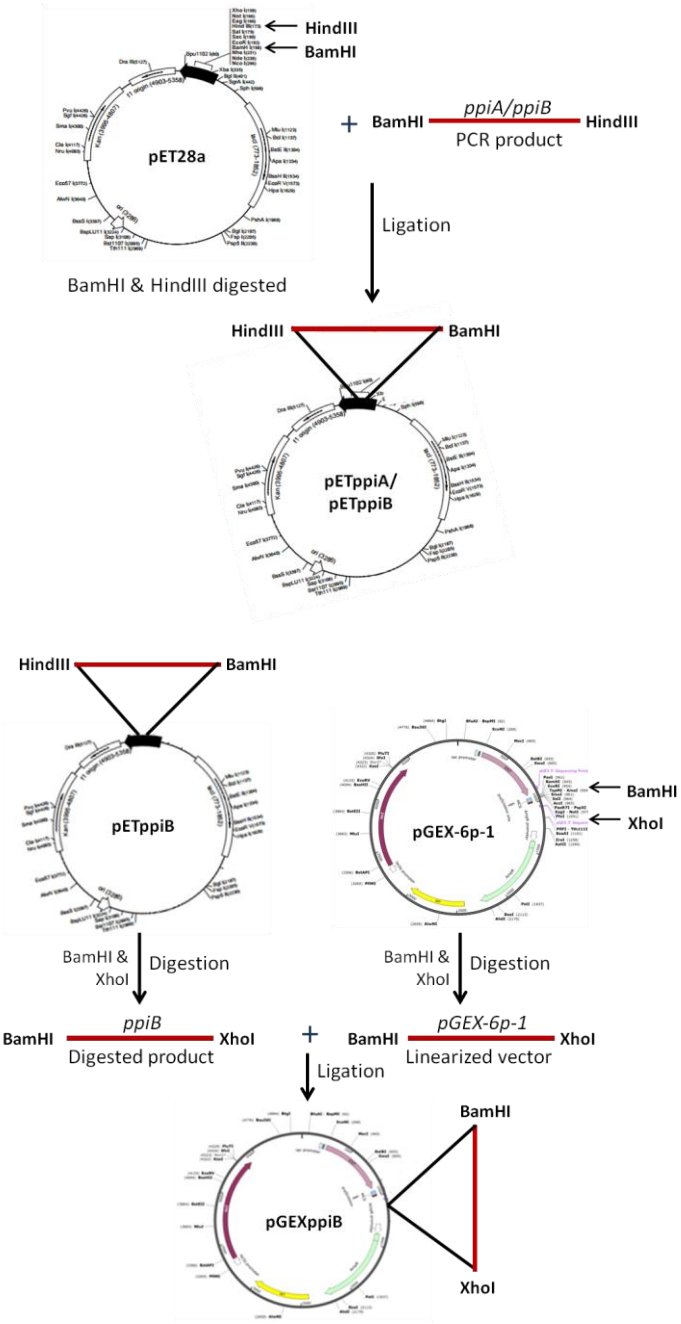

Fig 2.4.1.a: Schematic diagram showing construction of pET_ppiA and pGEX_ppiB.

Purified PCR amplified *ppiA* and *ppiB* and the vector, pET28a were digested with *Bam*HI and *Hind*III restriction enzymes followed by ligation for constructing pETppiA and pETppiB. With the respective digested vector, ligation reaction of the digested PCR product of gene was set up. Ligation mixture was transformed into competent *E. coli* DH5α cells, plated LB-agar plate with appropriate antibiotics. Putative clones were screened by restriction mapping. Release of appropriate size DNA was observed (pET_ppiA, 0.55Kb and pGEX_ppiB, 0.93 Kb).

Restriction mapping of prepared construct was performed that is shown below (Figure 2.4.2.b).

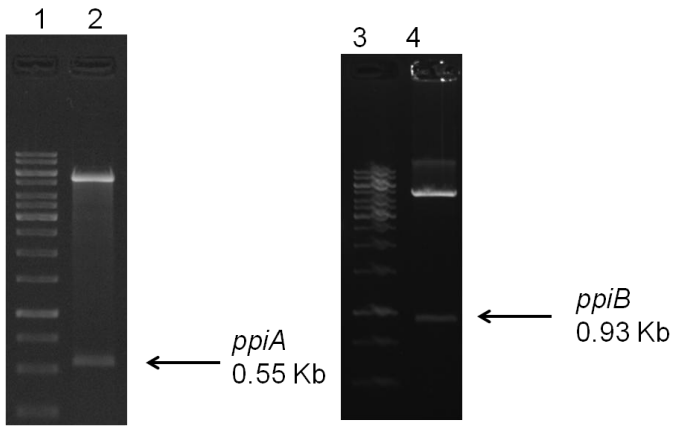

Fig 2.4.1.b: Restriction map of *ppiA* and *ppiB* in pET28a and pGEX-6p-1 digested with *Bam*HI & *Hind*III and *Bam*HIa & *Xho*I, respectively. Fallout of appropriate size was observed in the experiment.

2.4.2. Purification of *ppiA* and *ppiB* in *E. coli* BL21 (λDE3) and BL21 (λDE3) rosetta cells:

E. coli BL21 (λDE3) cells were transformed with pETppiA. Cells were grown till 0.6 OD at 600nm and induced by 1 mM IPTG for 3 hours at 37^{o}C and 0.1 mM IPTG for 16 hours and 18^{o}C. The rppiA was purified from the cell lysate under native condition; employing Ni^{2+}-NTA affinity chromatography. Elution was done with

250mM. rppiB was purified from the lysate under native condition using glutathione-agarose beads. Elution was performed with 20 mM reduced glutathione with 20 mM TrisCl buffer. The fractions of rppiA and rppiB were analyzed on a 12% polyacrylamide gel. The fractions of rppiA and rppiB were pooled, separately and dialyzed with 1X PBS buffer (pH 7.4) with 10 % glycerol. The dialyzed samples were aliquoted and stored at -20°C.

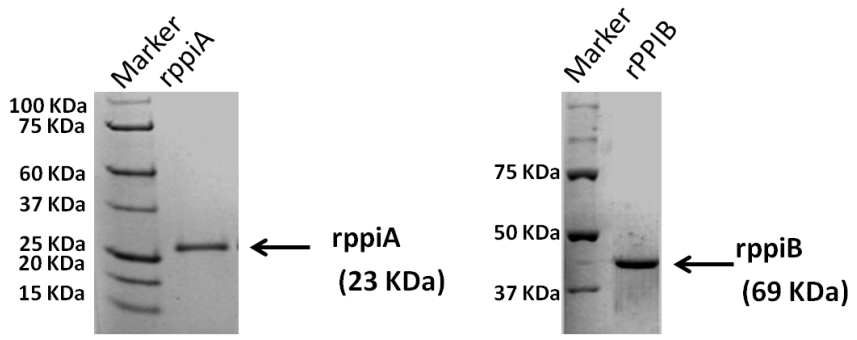

Fig 2.4.2: Purification of rppiA and rppiB: Purified rppiA and rppiB were separated on 12% SDS-PAGE and after staining of protein band was observed at 23KDa and 69KDa, respectively.

2.4.3. Recombinant PPIases are enzymatically active:

His tagged *M.tb* rPpiA and GST tagged rPpiB was purified using Ni-NTA column and glutathione sepharose affinity column, respectively. rPpiA displayed the expected 25kDa molecular size while rPpiB protein band was observed at 69kDA molecular size after SDS PAGE. Enzymatic activity of the purified proteins was measured in a spectrophotometric-coupled assay using the chromogenic peptide N-succinyl-Ala-Ala-Pro-Phe-p-nitro-anilide and α chymotrypsin at 15^{o}C (Figure 2.4.3). Increase in the rate of isomerization activity as compared to control show that the recombinant PPIases are enzymatically active (Henriksson *et al*., 2004).

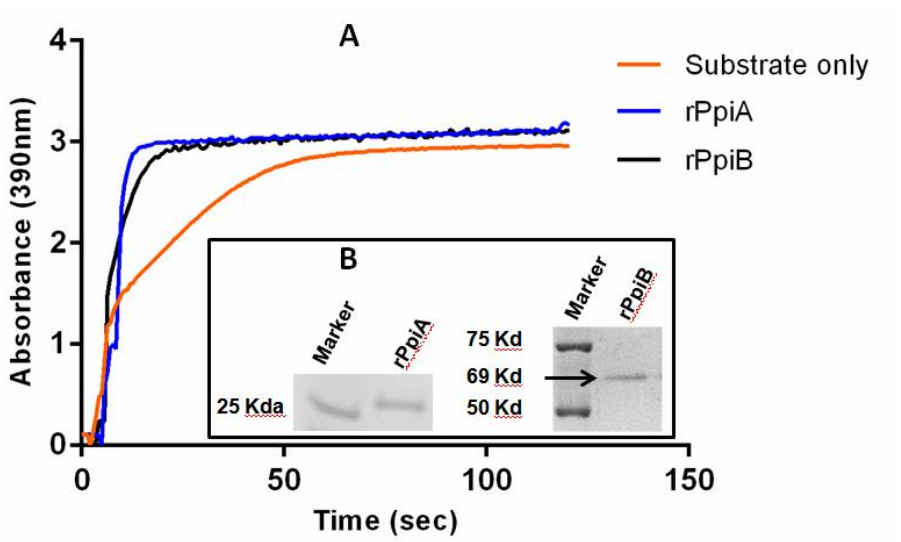

Fig 2.4.3: Purified rPPIases are enzymatically active. A. Isomerization activity of rPpiA and rPpiB at a concentration of 50nM was measured in a coupled assay using the chromogenic peptide N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide and α chymotrypsin compared with the spontaneous background rate of cis-trans isomerization in the absence of the recombinant enzymes. **B.** Molecular weight of purified histidine-tagged rPpiA and GST tagged rPpiB as checked on 10% SDS polyacrylamide gel was around 25kDa and 69kDa, respectively.

2.4.4. *M.tb* PPIases display chaperone like activity as evident by surface hydrophobicity:

ANS has been commonly used as a fluorescent probe to establish surface hydrophobicity in proteins. A blue shift of fluorescence emission maxima and increase of fluorescence intensity is generally attributed to the hydrophobicity of a binding site (H. Yang *et al*., 1999; Gasymov & Glasgow, 2007, Suragani *et al*, 2012).The relative fluorescence intensity and maximum emission wavelength of ANS alone and ANS bound to rPpiA and rPpiB was measured by exciting at 390nm. The maximum emission wavelength of ANS alone was found to be 540nm and a clear blue shift in the emission wavelength was observed in case of ANS bound to rPpiA and rPpiB (Figure. 2.4.4). A significant increase in the fluorescent intensity could be noticed when rPpiA and rPpiB was bound to ANS. These results confirmed surface hydrophobicity in PpiA and PpiB of *M.tb*. which is known to be associated with chaperone like function (Suragani *et al*., 2013). The ANS fluorescence spectra clearly point to the likely function of *M.tb* PPIases as a chaperone.

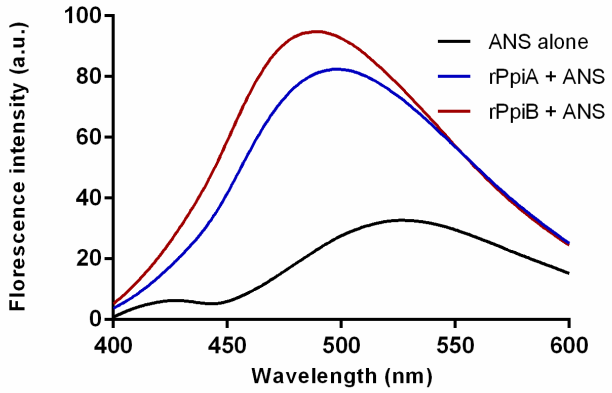

Fig 2.4.4: ANS Florescence spectra reveal surface hydrophobicity in *M.tb* rPPIases. Concentration of ANS, rPpiA and rPpiB used were 20µM, 0.1 mg/ml and 0.1mg/ml, respectively. Blue shift in the position of peak and increase in the intensity of peak was observed upon addition of rPpiA and rPpiB. The ANS emission was scanned in the range of 400 to 600 nm.

To experimentally demonstrate chaperone like activity of *M.tb* rPPIases, their ability to prevent thermal aggregation of a heterologous protein MalZ was assessed. MalZ is known to aggregate under elevated temperature (Paul *et al*., 2007). Light scattering assay to monitor aggregation was carried out by assessing absorbance (500nm) of thermally denatured MalZ in presence and absence of rPpiA and rPpiB. The thermal stability of rPPIases was also monitored and as expected for chaperones, both the rPPIases were highly stable at 47^{o}C, exhibiting negligible aggregation. When rPpiA was co-incubated with MalZ in increasing molar ratio (10, 20, 40), it was able to increasingly prevent aggregation at 47^{o}C (Figure 2.4.5.A). In comparison to rPpiA, rPpiB could inhibit aggregation of MalZ at almost half the concentration (Figure 2.4.5.B). The use of appropriate positive (GroEL) and negative control (lysozyme) confirmed the specificity of the aggregation inhibition activity by the rPPIases. Molecular chaperones are known to exhibit thermal stability and can protect proteins from thermal denaturation and aggregation (Suragani *et al*., 2013) and these result therefore, clearly demonstrate that *M.tb* peptidyl prolyl isomerases can protect proteins from thermal aggregation directly pointing to their chaperone like activity.

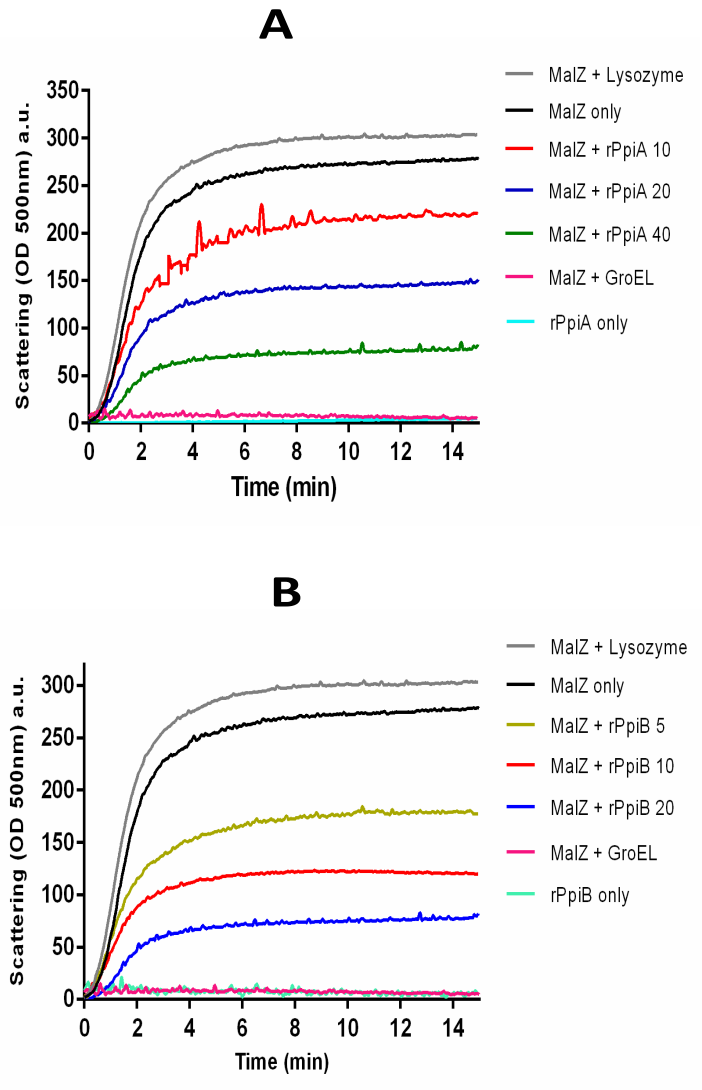

Fig 2.4.5: *M.tb* rPPIases suppresses aggregation of MalZ. The aggregation pattern was monitored by light scattering at O.D. 500 nm with excitation and emission slit width 5 and 2.5 nm, respectively. GroEL was used as a positive control. Lysozyme was used as a negative control. A. Increasing molar ratio of rPpiA (10, 20, 40) was used. B. Increasing molar ratio of rPpiB (5,10, 20) was used.

2.4.6. *M.tb* rPPIases protect *Nde*1 from thermal denaturation and consequent loss of restriction enzyme activity:

Having shown that rPPIases could protect MalZ from thermal aggregation, the ability of rPPIases to protect the enzymatic activity of *Nde*1 restriction enzyme from thermal denaturation was further investigated. Plasmid pET22b has a single restriction enzyme site for the enzyme *Nde*1, thereby generate single DNA band (Figure 2.4.6, lane 2). Upon heat denaturation *Nde*1 loses its ability to linearize pET22b plasmid (Figure 2.4.6, lane3), but when heat denatured in the presence of rPpiA (lane 4) or rPpiB (lane 5) it retained its ability to digest and linearize the pET22B plasmid DNA. BSA when used as a control could not protect *Nde*1 and as a consequence the enzyme activity was lost after heat denaturation (lane 6). These results further demonstrate that *M.tb* rPPIases cannot only protect proteins from thermal aggregation but can also preserve the functional activity of an enzyme under *in vitro* conditions.

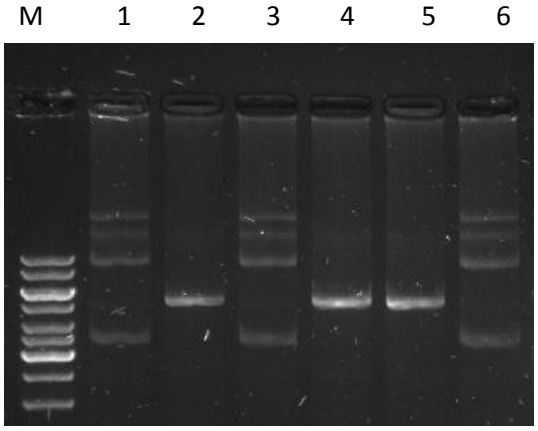

Fig 2.4.6: rPpiA protected *Nde*1 is refractile to thermal denaturation. Enzymatic activity of thermal denatured *Nde*1 in presence and absence of rPpiA, rPpiB and control protein BSA. Lane M, 1-kb molecular size marker; lane1, uncut pET22b; lane2, pET22b digested with native *Nde*1; lane3, with heat denatured *Nde*1; lane4, with rPpiA treated heat denatured *Nde*1; lane5, with rPpiB treated heat denatured *Nde*1; lane6, with BSA treated heat denatured *Nde*1.

2.4.7. rPPIases can rescue *E. coli* from thermal shock:

In order to assay for chaperone like function of rPPIases under physiological conditions, it was investigated if *E. coli* expressing *M.tb* PpiA and PpiB is resistant to thermal shock as compared to the vector control. *E.coli* transformants were assessed for their ability to grow after thermal shock (50^{o}C) as described earlier (Suragani *et al*, 2012). Results indicate that *E. coli* cells transformed with *M.tb ppiA* and *ppiB* showed more than ten folds survival after one hour and about 80 fold survival after 2 hours as compared to the *E.coli* transformed with vector alone (Figure 2.4.7). These results provide conclusive evidence that rPPIases show chaperone like function both under in *in-vitro* conditions and also under physiological conditions.

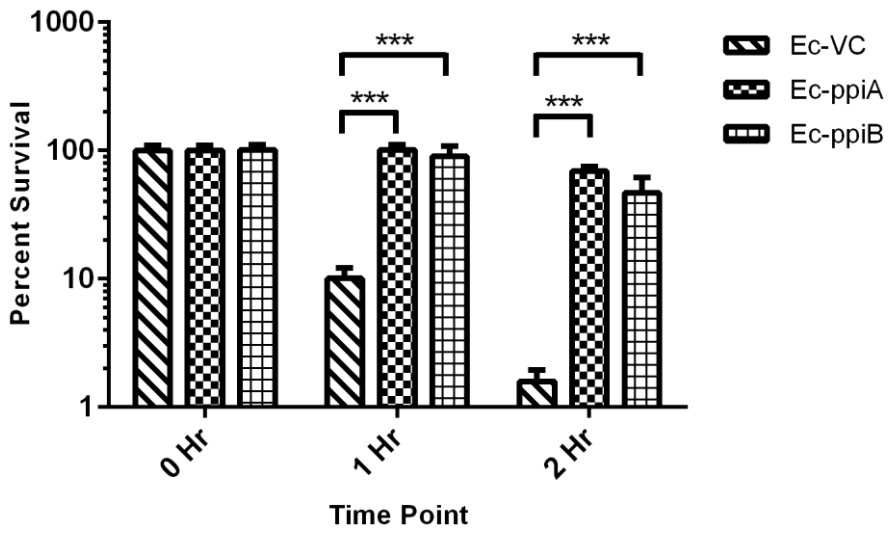

Fig 2.4.7: rPPIases can rescue *E.coli* from thermal shock: *E.coli* expression strain was transformed with pET28a only, pET28a_ppiA and pGEX6p1_ppiB. After heat treatment at 50^{o}C different dilutions of 100ul culture was plated at one hour interval. *E.coli* transformed with *ppiA* and *ppiB* exhibited approximately 10 fold more survival compared with vector control.

2.4.8. *M.tb* PPIases confer protection to HEK cells lines against oxidative stress and hypoxia:

Results presented so far demonstrated the ability of *M.tb* PPIases not only to act as a chaperone, under *in-vitro* conditions and but also under physiological conditions. Given the fact that chaperones help in maintaining cellular homeostasis under various stress conditions (Henderson & Martin 2011) and their presence increases tolerance to heat, toxins, hypoxic shock and increase cell longevity by maintaining proteostasis, Experiments were designed to investigate whether expression of PPIases in HEK cells cultured *in-vitro* could impart resistance against hypoxia and oxidative stress. For this MTT assay was performed to determine viability of HEK cells expressing PpiA and PpiB proteins after oxidative stress and hypoxia. HEK cells transfected with vector alone was used as a control. For oxidative stress cells were treated with different concentration of H_2O_2 (0, 10, 20, 30, 40 µM) whereas for hypoxic stress cells were treated with varying concentrations of $CoCl_2$ (0, 50, 100, 150, 200 µM). A significant increase in viability of cells expressing PpiA and PpiB (Figure 2.4.8.A, 2.4.8.B), as compared to vector control, could be seen. These observations clearly implicate *M.tb* PPIases, in aiding the intracellular survival of the pathogen amid the hostile environment of infected cells pointing to its likely role *in-vivo*.

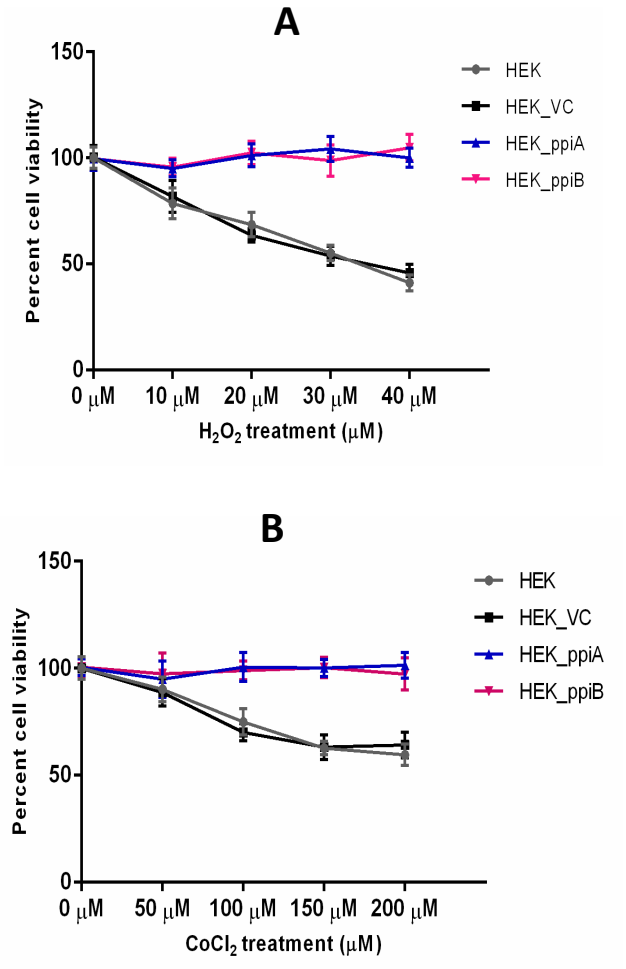

Fig 2.4.8: *M.tb* PPIases impart resistance to HEK293T cells against hypoxic and oxidative stress. MTT assay was performed to score the percent cell viability of HEK293t cells transiently expressing PpiA and PpiB under conditions of oxidative stress (panel **A)** or hypoxia (panel **B**) induced by treatment of cells with varying concentrations of H_2O_2 (0, 10, 20, 30 and 40μM) or $CoCl_2$ (0, 50, 100, 150 and 200μM), respectively. The data are representative of three independent experiments. Error bar represents mean ± S.D.

Discussion:

A multifaceted interplay between the host immune response and pathogen virulence factors govern the outcome of infection caused by *Mycobacterium tuberculosis*. Peptidyl-prolyl isomerase A of *M.tb*, also known as cyclophilin A, is secreted in intraphagosomal niche and is known to be upregulated during infection (Målen*et al*., 2007). Other than ubiquitous expression, prolyl isomerases have large number of isoforms and are localized in different subcellular locations in higher organisms, e.g., in *Saccharomyces*, 8 different prolyl isomerases are targeted to endoplasmic reticulum, mitochondria and nucleus from cytosol (Arevalo-Rodriguez *et al*, 2004). Isoform diversity and various subcellular localization are indicative of their functional importance. Recently, PPIases have been studied in different contexts such as cell regulatory processes (Lin *et al*., 2015), role in different cancer status (Yao *et al*., 2005; Chen *et al*., 2015) and inflammation (Liu *et al*., 2014) and other abnormalities (Tsurubuchi *et al*., 2014 ; Restelli *et al*., 2014).

In addition to their vitality in protein folding, peptidyl prolyl isomerases have also been implicated in many pathological conditions like diabetes, asthma, cancer and microbial infections. *surA* gene coding for PPIase is associated with virulence of pathogenic strains of *Salmonella, E. coli* and *Helicobacter pylori*. Mutation in *surA* gene significantly affects virulence potential of these pathogens (Sydenham *et al*., 2000; Basak *et al*., 2005).

In the present study, it was observed that *M.tb* PPIases exhibit chaperone-like activity. Surface hydrophobicity is considered important during the interaction of molecular chaperones with misfolded proteins (Das & Surewicz, 1995; H. Yang *et al*., 1999). ANS binding analysis of rPPIases showed increase in absorbance and blue shift in the

emission maxima, a reflection of its chaperone-like activity. Unlike Mal Z, *M.tb* rPPIases were refractile to thermal aggregation however; when MalZ was incubated along with rPpiA and rPpiB at 45^{o}C its aggregation was inhibited. About 90% of the enzymatic activity of thermally denatured *Nde*1 was restored when incubated with rPpiA and rPpiB. Molecular chaperones can transiently bind and stabilize an unstable conformation of a protein by preventing its misfolding and aggregation.

With *in-vitro* evidence of rPPIases as a probable chaperone, its chaperone like function *in-vivo* was further investigated It was clearly evident that survival of *E.coli* transformed with *ppiA* and *ppiB* of *M.tb* was quantitatively higher compared to the vector control. Similar examples of proteins that showed chaperone-like activity have been reported earlier (Freeman *et al*., 1996; Ou *et al*., 2001; Lilie *et al*., 1993; Suragani *et al*., 2013)

The ability of *M.tb* to grow under reduced oxygen conditions and resist oxidative stress is directly correlated to its ability to cause disease. Although O_2 is essentially needed for survival of *M.tb*, it can easily adapt itself to the hypoxic micro environment of tissue lesions, sites of active TB and inside microphages (Boon & Dick 2002). In the Wayne model (Wayne 1994), under gradual hypoxic growth environment, bacteria move away from proliferative cycle and tend towards the latent form that is adapted to hypoxia and remain viable for extended period. Turning to hypoxia tolerant status appears to be the key response mechanism for coping stress, and other survival challenges (Gupta *et al*., 2012). In the present work, the role of *M.tb* PPIases in conferring protection to HEK293T cells under hypoxia and oxidative stress was investigated. A significant difference in the survival of HEK cells transiently expressing PpiA and PpiB proteins as compared to the untransformed cells

was evident. There are several other mycobacterial HSPs which could impart survival amid hostile host effector functions such as hypoxia and oxidative stress (Yuan *et al*., 1998).The results presented in this chapter demonstrating chaperone-like function of *Mycobacterium tuberculosis* PPIases have an implication, in terms of possible role in enhancing the stress tolerance of the pathogen which deserves to be investigated.

Chapter 3

M.tb PPIases show immunogenicity, alter cytokine profile and aid in intra phagosomal survival

3.1. Abstract:

Mycobacterium tuberculosis (*M.tb*) has two peptidyl-prolyl isomerases (PPIases) PpiA and PpiB, popularly known as cyclophilin A and cyclophilin B. The role of cyclophilins in processes such as signaling, cell surface recognition, chaperoning and heat shock response has been well documented. In this chapter, evidence was presented that *M.tb* PPIases play role in modulating host immune responses. Sera of TB patients showed high levels of antibody to *M.tb* PPIases as compared to the sera of healthy humans. Treatment of THP-1 cells induced secretion of pro-inflammatory cytokines as a direct function of concentration of rPpiA. Alternatively, treatment with rPpiB inhibited secretion of TNFα and induced secretion of IL-10. Furthermore, heterologous expression of *M.tb* PpiA and PpiB in *Mycobacterium smegmatis* increased its survival in THP-1 cells as compared to vector control. The results in this chapter demonstrated that *M.tb* PPIases are immunogenic proteins that can possibly modulate host immune response and enhance persistence of the pathogen within the host by subverting host cell generated stresses.

3.2. Introduction:

Tuberculosis caused by the intracellular pathogen *Mycobacterium tuberculosis* (*M.tb*), remains a potential threat regardless of strong efforts to reduce its toll on humanity. Intracellular/intraphagosomal survival plays a critical role in the infection cycle of the pathogen, a process which majorly relies on array of virulence factors to colonize and replicate within the host macrophages (Ehrt & Schnappinger 2009). The virulence factors which play a vital role in host pathogen interaction at the molecular level also include stress responders, heat shock proteins (HSPs), foldases and chaperones (Henderson 2010). Studies involving comparative genomic and proteomic approaches using pathogenic, opportunistic and non-pathogenic mycobacteria have identified virulence attributes and intelligent survival strategy adopted by *M.tb* (Hasnain 2014, Rahman *et al*., 2014; Kohli *et al*., 2012; Tundup *et al*.; 2014; Mohareer *et al*., 2011; Saini *et al*., 2012). Immunological characterization of these effector molecules can bridge huge gaps in our understanding of *M.tb* biology and facilitate better therapeutic and diagnostic interventions.

Protein folding in the cell is assisted by molecular chaperones and foldases. The foldases generally include peptidyl-prolyl isomerases and protein disulfide isomerases. In addition to peptidyl prolyl isomerase activity PPIases have been shown to play roles in diverse biological processes such as receptor signaling, apoptosis, stress response, RNA-mediated gene expression (Mark *et al*., 2001; Wu *et al*., 2000; Lu *et al*., 1996). Human cyclophilin A has been reported to possess PPIase independent chaperone like function (Zhang *et al*., 2013). Moreover, in addition to protein folding HSPs, chaperones and stress proteins have long been known to show immune modulatory roles in bacterial infections. For example, Mycobacterial HSP65

induces a strong cellular and humoral immune response (C. J. Raats *et al*. 1994; Friedland *et al*., 2008).

M.tb is known to possess two PPIases (cyclophilins), PpiA and PpiB. *M.tb* PpiA is a part of the secretome and is known to interact with host proteins involved in immune defense mechanism and signal transduction (Henriksson *et al*., 2004; Bhaduri *et al*., 2014), while PpiB has been reported in membrane fraction and mannosylation enriched culture filtrate (Cole *et al*., 1998; Gu, 2003). Immunological characterization of majority of these enzymes owe their possible role in modulating host immune response and aiding intracellular survival of the pathogen have not been investigated till date.

The work presented in this chapter describes the potential role of PPIases in intraphagosomal survival of the pathogen illustrating immunogenic potential of *M.tb* cyclophilins and their involvement in eliciting host immune response and altering the host cytokine profile. *M.tb* PPIases, apart from being a stress protein, possibly alter host immune response and aid in the intracellular survival of the pathogen.

3.3. Materials and Methods

3.3.1. Materials: IPTG, imidazole, α-chemotrypsin, reduced Glutathione,N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide, trifluoroethanol, LiCl, DTT, H_2O_2 and polymixinB were purchased from Sigma. Cell culture reagents were purchased from GIBCO, Thermo Fisher Scientific (USA). All enzymes were purchased from New England Biolabs (USA) and antibodies were from Abcam (UK); and ELISA kit from Peprotech (USA). Mycobacterium growth media, and supplements were purchased from Becton, Dickinson and Company (USA). All reagents used were analytical grade. The strains and plasmids used in this study are listed in (Table 3.3.5.1).

3.3.2. Enzyme assay of purified recombinant PPIases:

The genes encoding *M.tb ppiA* (Rv0009) and *M.tb ppiB* (Rv2582) were PCR amplified from H_{37}Rv genomic DNA by using forward and reverse primers (Table 3.3.5.2). *ppiA* and *ppiB* was cloned in pET28a vector using *Bam*HI and *Hind*III restriction enzymes and *ppiB* was sub cloned in pGEX6p1 vector using *Bam*H1 and *Xho*1 restriction sites. Recombinant proteins were purified using Ni-NTA column for PpiA and glutathione sepharose affinity column for PpiB as described earlier (Banerjee *et al*., 2007). Purified recombinant protein were treated with polymixin B for endotoxin removal as describe (Banerjee *et al*. 2004) PPIase activity of both, rPpiA and rPpiB was evaluated using a spectrophotometric assay (Fischer *et al*., 1984), (Pandey *et al*. under review).

3.3.3. Antigenicity Profiling: Antigenic index of PpiA and PpiB was analyzed *in silico* using protein analysis software (Protean version 4.0, Lasergene Navigator; DNA STAR Inc; Madison, Wis) (Chakhaiyar *et al*., 2004).

3.3.4. Immune assays: Human blood samples were collected and processed as described earlier (Tundup *et al*., 2008). The different samples used in our study were:

confirmed cases of pulmonary TB (category I) (n=43) and healthy volunteers (n=43). Study was approved by institutional Bioethical committee, and informed consent was obtained from all patients. The samples were assayed by enzyme linked immunosorbent assay (ELISA) using microtiter plates (Corning) coated with rPpiA, and rPpiB respectively as described earlier (Banerjee *et al*., 2004).

Differentiation of THP-1 cells by phorbol 12-myristate 13-acetate (PMA) and rPPIases-induced secretion of various cytokines was carried out as described earlier (Silswal *et al*., 2005; Nair *et al*., 2009).

3.3.5. Cloning, expression of *M.tb* PPIases in *M.smegmatis*:

Cloning and expression of *M.tb* PPIases in *M.smegmatis* was carried out as described previously in chapter 2 using pST-2K (*E. coli*-Mycobacterium shuttle vector). The resulting constructs were then electroporated in wild type *M.smegmatis*. The transformed *M.smegmatis* strain was designated as *Ms_ppiA* and *Ms_ppiB*.

3.3.6. Electroporation of mycobacterial cells:

Mycobacterial cells were grown till absorbance 0.4 to 0.6 in 100 ml Middlebrook 7H9 media containing 0.2% glycerol, 0.05% Tween 80 and 10% ADC. Cells were harvested at 6000 X g at 4°C for 15 minutes. Cells were then washed twice with 50 ml of 10% ice cold glycerol and two times with 25 ml ice cold 10% glycerol. Finally the cells were resuspended in 1 ml of 10 % glycerol and 100 µl aliquots were frozen at -70°C. Mycobacterial cells were kept at 0 to 4°C throughout the washings and electroporation procedure.

Purified plasmid DNA (1 µg to 10 µg) was mixed with 100 µl of the electro competent cells and incubated for 5 minutes. The mixture was transferred to a 0.2 cm

gap electroporation Cuvette (Biorad) and a single electrical pulse was delivered through a gene pulser (Biorad) at 2.5 kV, 25 μF, 200 Ω. The cells were immediately transferred to 0.8 ml of Middlebrook 7H9 media chilled in ice. The electroporated cells were kept for revival at 37°C for 4 hours for *M.smegmatis*. Thereafter plated on Middlebrook 7H10 Agar plates containing 0.5% glycerol with 0.05% tween 80 plus 10% OADC containing appropriate antibiotics and incubated at 37°C for 2 to 3 days for *M.smegmatis*.

TABLE 3.3.5.1. Strains and plasmids used in this study

Plasmids	Relevant characteristics	Source/Reference
pET28_ppiA	pET28a containing *ppiA*	This work
pGEX_ppiB	pGEX6p1 containing *ppiB*	This work
pST_ppiA	pST_2K containing *ppiA*	This work
pST_ppiB	pST_2K containing *ppiB*	This work

Strains	Relevant characteristics	Source/Reference
DH5α	*supE44 ΔlacU(Φ80lacZΔM15) hsdR17 rec*1 *endA*1 *gyrA96 thi*-1 *relA*1	Novagen
BL-21(DE3)	*F– ompT gal dcm lon hsdSB(rB- mB-) λ(DE3 [lacI lacUV5-T7 gene* 1	Novagen
Ms_WT	*M.smegmatis* mc2 155	ATCC
Ms_VC	*M.smegmatis* containing pST-2K vector	This work
Ms_ppiA	*M.smegmatis* containing pST-ppiA	This work
Ms_ppiB	*M.smegmatis* containing pST-ppiB	This work

Selection marker resistant to Ampicillin (amp^R) and kanamycin (kan^R).

Table 3.3.5.2 . List of Primers used in the study.

Primers	Sequence	Restriction enzyme site
PpiA_ F	CGGGATCCATGGCAGACTGTGATTCCGTGACTAA	*Bam*H1
PpiA_R	GGGAAGCTTTCAGGAGATGGTGATCGACTCGATC	*Hin*dIII
PpiB_ F	CGGGATCCATGGGCCACTTGACACCG	*Bam*H1
PpiB_R	GGGAAGCTTCTAATCCAGCAGCACCGACGTG	*Hind*III

3.3.6. *In vitro* growth and stress assay:

Log phase cultures of *M.smegmatis* strains (Ms_WT, Ms_VC, Ms_ppiA, Ms_ppiB) were inoculated in Middlebrook 7H9 broth with 10% Oleic Albumin Dextrose Catalase (OADC) in the presence/absence of kanfamycin (25µg/ml). The cultures were grown at 37^{o}C at 200 rpm. Cell density was measured periodically at 600nm (OD_{600}) using spectrophotometer. For hydrogen peroxide stress, log phase cultures (OD_{600} of 0.8–1.0) of *M.smegmatis* strains were diluted 1:100 into Middlebrook 7H9 broth and grown for approximately 12 hrs until the OD_{600} reached 0.4. Re-inoculated cells were then treated with the 7mM concentration of H_2O_2 for period of 3 hrs. At 0, 1, 2 and 3 hrs 100µl samples were serially diluted and plated on Middlebrook 7-H10 broth to determine the viable count (Li *et al*., 2014).

3.3.7: Uptake and intracellular growth of *M.smegmatis* expressing *M.tb PPIases* in human THP-1 macrophages.

THP-1 cell line was cultured and differentiated with PMA in RPMI 1640 medium as reported earlier (Tripathi *et al*., 2015; Chandra *et al*., 2010). The efficiency of uptake by the phagocytic cells was determined by increasing ratio of bacteria per phagocytic cells: the multiplicity of infection (MOI). For each data point, the mean of triplicate wells was used. THP-1 monolayers were infected with washed, exponential-phase bacteria, Ms_WT, Ms_VC, Ms_ppiA and Ms_ppiB strains, for 4 hrs at MOI values of 50. The cells after infection were treated with medium containing 20µg gentamicin ml^{-1} for 30 mins, and washed twice with RPMI medium. The plates were incubated at 37^{0}C after adding fresh complete medium. The cells were dislodged gently at different time points and centrifuged at 2000 r.p.m. for 3 min, washed twice with fresh RPMI1640 medium, and lysed in sterile water. The lysate was then diluted in

Middlebrook7H9 broth and plated on Middlebrook 7H10-OADC agar plates. The plates were incubated at 37^{o}C and colonies were counted after 4 days**.**

3.4. Results

3.4.1. Antigenicity Index:

In silico antigenicity profiling of *M.tb* PpiA and PpiB protein (Figure 3.4.1) using protein analysis software (Protean version 4.0, Lasergene Navigator; DNA STAR Inc; Madison, Wis) displayed major antigenic stretches with a peak value ≥1.7 thereby pointing to a likely role in mounting B cell response (Chakhaiyar *et al*., 2004).

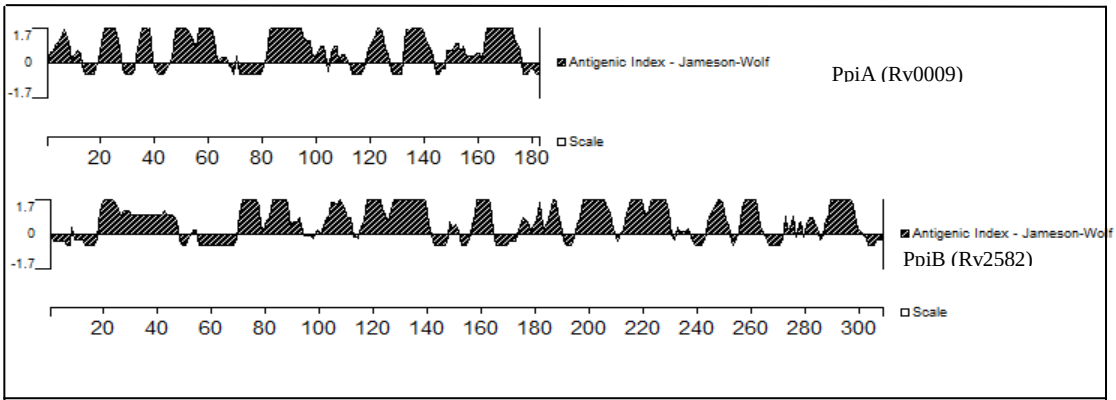

Fig 3.4.1: *M.tb* Ppiases display major antigenic patches: In silico analysis of PpiA and PpiB proteins (182 and 308 amino acids) of *M.tb*, using protein analysis software (Lasergene Navigator; DNA STAR) displayed major antigenic stretches with a peak value ≥1.7.

3.4.2. *Mycobacterium tuberculosis* PPIases elicits B-Cell response:

To investigate if *M.tb* PPIases could indeed elicit humoral immune response, experiments were designed to compare humoral immune response against the *M.tb* PpiA and PpiB in TB patients and healthy human controls. Statistical analysis revealed that TB patient group mounted a significantly higher (P<0.001) immune response against rPpiA and rPpiB compared to healthy group (Figure 3.4.2). These results indicate that *M.tb* PPIases mount B-cell response due to their antigenic nature.

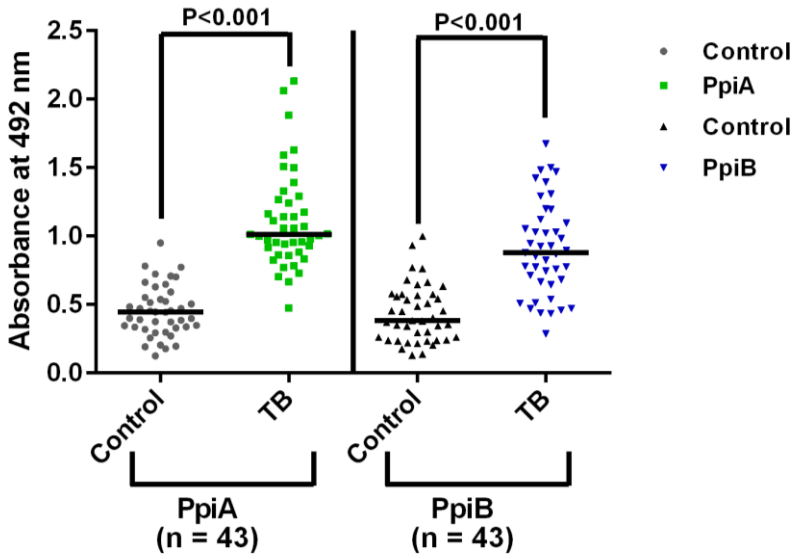

Fig 3.4.2. Mycobacterial Ppiases elicit B cell response in tuberculosis patients. Reactivity to rPpiA and rPpiB were studied in two sets (patients and healthy groups) by ELISA. Statistical analysis of immunoactivity was analyzed, where patient group showed significantly higher (P<0.001) immune response against rPpiases compared to control. The horizontal line indicates the mean of the absorbance values.

3.4.3. rPPIases alter cytokine profile in THP-1 macrophages:

Having shown the ability of *M.tb* PPIases to elicit a B-cell response, further investigations were made to show the ability of these proteins in modulating the expression of various cytokines. Human monocytes differentiated with PMA were treated with increasing concentrations of rPpiA and rPpiB and the soup was collected after 24 hrs to estimate levels of different cytokines. Significant increase in the levels of TNFα, IL-6 (Figure 3.4.3.a) was observed as a direct function of concentration of rPpiA (1, 5, 10µg), however no significant increase in the levels of IL-10 could be noticed. Alternatively, decrease in the level of TNFα, IL-6 and increased level of IL-10 was observed with increasing concentration of rPpiB (1, 5, 10µg) (Figure 3.4.3.b). These results demonstrate that mycobacterial PPIases differentially modulates cytokine secretion in human monocytic-macrophage cell lines and are in agreement with previous reports involving mycobacterial chaperones and heat shock proteins in immune modulation (C. J. I. Raats, *et al*., 1994; Naffin-Olivos *et al*., 2014).

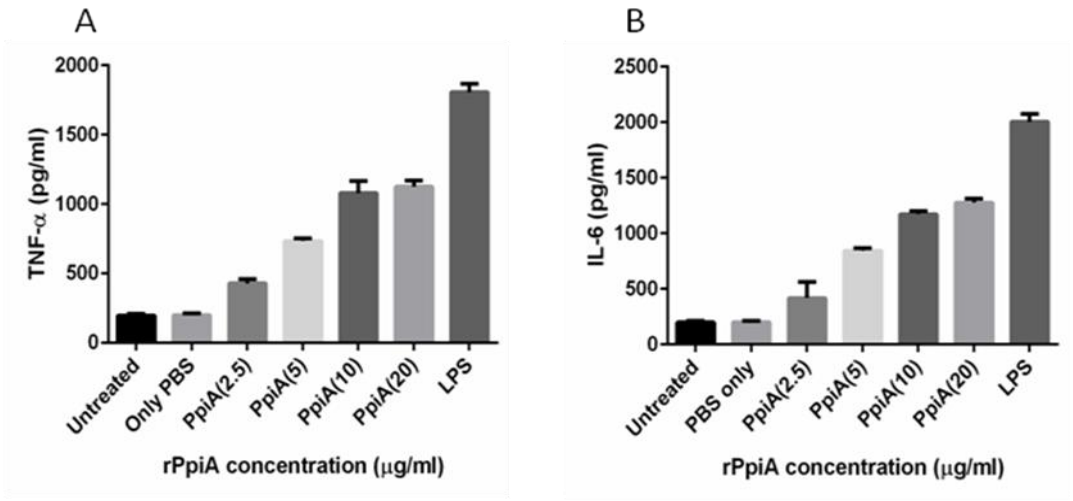

Fig 3.4.3.a: rPpiA stimulates the secretion of proinflammatory cytokines by THP-1 cells. Concentration dependent increase in the release of pro-inflammatory cytokines (A, TNFα; B, IL-6) consequent to treatment by rPpiA (2.5, 10 and 20μg) for 24 hours. Data represent mean ± SDs of three technical replicates. (P value< 0.001).

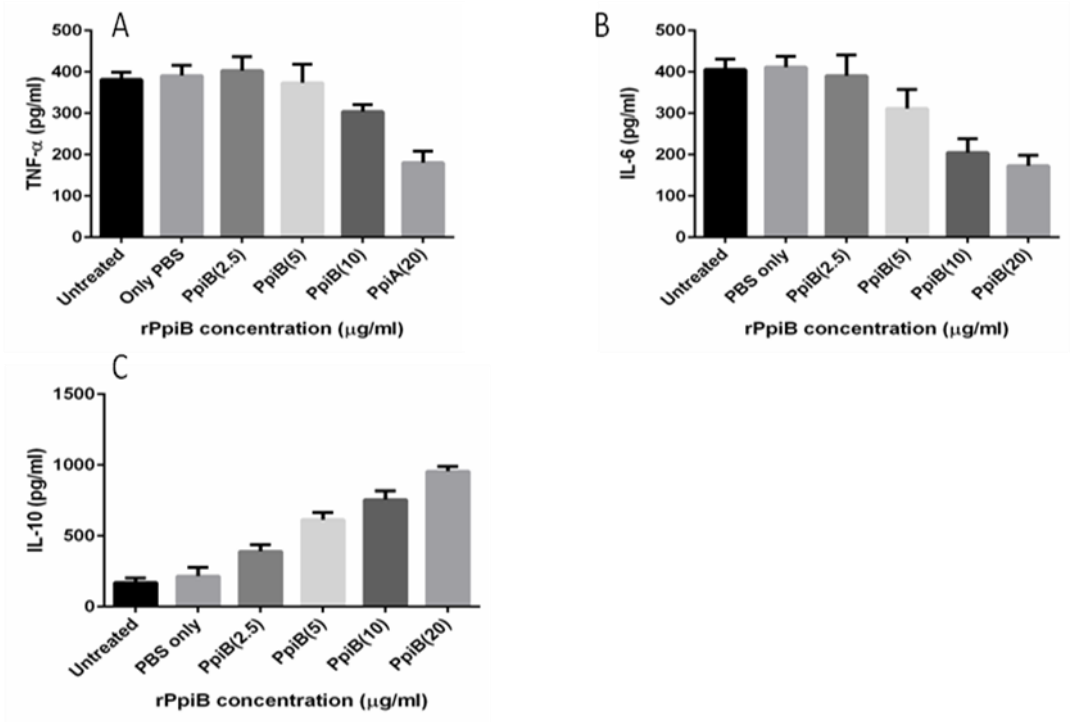

Fig 3.4.3.b: rPpiB decreases secretion of TNF-α, IL-6 and stimulates secretion of IL-10 cytokine by THP-1 cells. Concentration dependent decrease in the release of pro-inflammatory cytokines (A, TNFα; B, IL-6) and concomitant increase in the release of anti inflammatory cytokine (C, IL-10). consequent to treatment by rPpiB (2.5, 5, 10 and 20μg) for 24 hours. Data represent mean ± SD of three technical replicates. (P value < 0.001).

3.4.4.a *M.smegmatis* expressing *M. tb* PPIases show increased survival under condition of hydrogen peroxide stress:

Recombinant M.smegmatis (Ms_VC, Ms_ppiA and Ms_ppiB) and wild type (Ms_WT) were grown in Middlebrook 7H9 broth with 10% OADC and growth profile were monitored by measuring optical density at 600nm. No significant difference was observed in the growth profile of recombinant *M.smegmatis* or wild type *M.smegmatis* (Figure. 3.4.4). These were then subjected to hydrogen peroxide stress, which mimics a similar stress encountered by intracellular mycobacteria. Log phase cultures (OD_{600} of 0.4) of wild type and recombinant *M.smegmatis* were treated with H_2O_2 (7mM) for period of 3 hrs. A significant difference in the survival, as seen from CFU counts on Middlebrook 7H10 plates, of Ms_ppiA and Ms_ppiB as compared to the Ms_WT and Ms_VC cells could be seen (Figure 3.4.4.A). These results clearly indicate that *M.tb* cyclophilins play a critical role in stress adaptation and are in accordance with earlier reports showing their importance in stress adaptation and virulence of intracellular pathogen *Brucella abortus* (Roset *et al*., 2013).

3.4.4.b *M.smegmatis* expressing *M.tb* PPIases show increased survival in human THP-1 cells:

To directly demonstrate the ability of *M.tb* PpiA and *M.tb* PpiB to impact survivability of Mycobacterium in host cells, recombinant *M.smegmatis* strains were assayed for *in vitro* growth in THP-1 cells compared to the *M.smegmatis* transformed with vector alone. As could be seen, *M.smegmatis* transformed with *M.tb* ppiA and ppiB survived in the THP-1 cells longer, up to 72 hrs ($p<0.001$), than the Ms_WT or Ms_VC (Figure 3.4.4.B). An increase of ~1 log was observed after 24 hrs in the control group, after which the colony forming unit (c.f.u.) declined. In contrast,

Ms_ppiA and Ms_ppiB strains continued to replicate and survive up to 72 hrs within the macrophages, establishing the critical role of *M.tb* PPIases for intracellular survival.

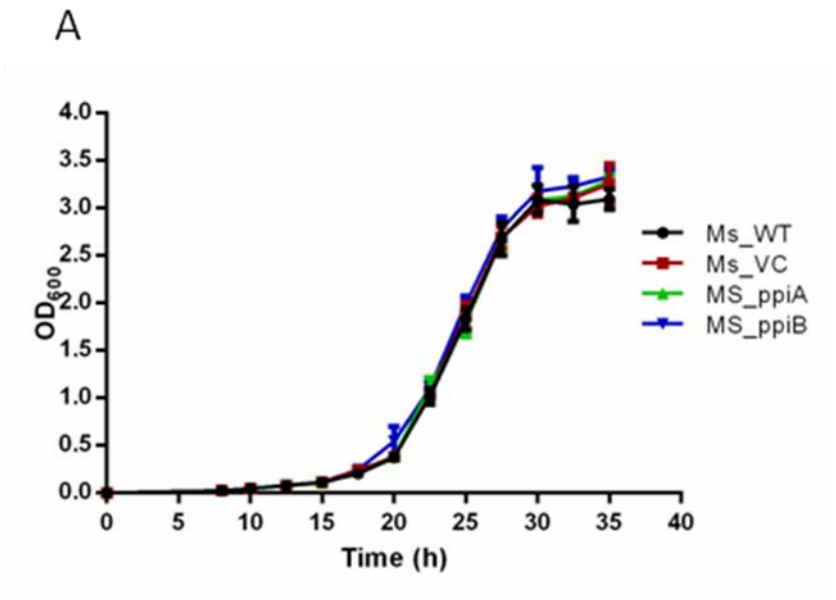

Fig 3.4.4. Growth of the wild type and transformed *M.smegmatis* in 7H9 broth culture. OD_{600} of Ms_WT (wild type), Ms_VC (vector control), Ms_ppiA and Ms_ppiB was plotted. Data represent mean ± SD of values obtained from three independent cultures.

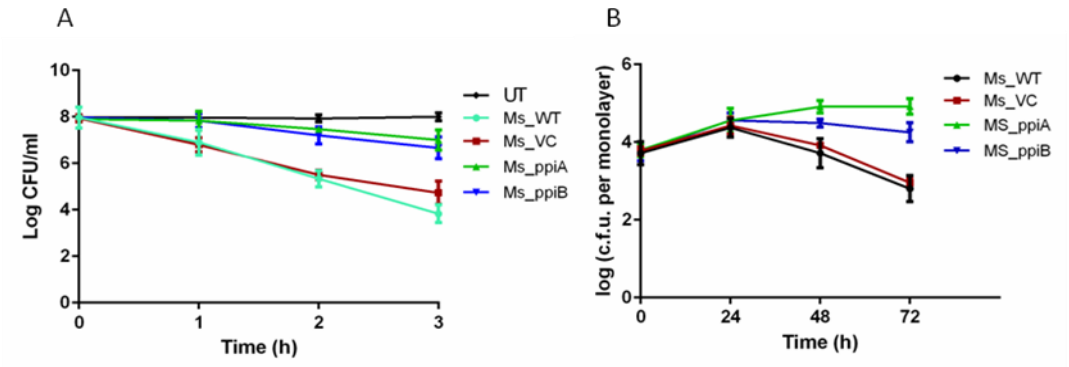

Figure 3.4.4.A. Recombinant *M.smegmatis* expressing *M.tb* PpiA or PpiB under condition of oxidative stress: Secondary culture of wild type *M.smegmatis* mc^2155, vector control *M.smegmatis*, Ms_ppiA, and Ms_ppiB with O.D. 0.4 were treated with 7mM H_2O_2 for 3 hrs and log cfu was calculated at different time points. The data resented are mean ± SEM of triplicate wells and are representative of three individual experiments.

Figure 3.4.4.B. *M.smegmatis* expressing PpiA and PpiB show increased survival in THP-1 cells: THP cells were infected with wild type *M.smegmatis* mc^2155, vector control *M.smegmatis*, Ms_ppiA, and Ms_ppiB and plated after 24, 48 and 72 hrs and log cfu/ monolayer was calculated at different time points. The data presented are mean ± SEM of triplicate wells and are representative of three individual experiments

3.5. Discussion:

Besides their biological function as helpers in protein folding, bacterial molecular chaperones have a distinctive role in virulence and stress tolerance. They are also termed as "moonlighting or multitasking proteins" (Vanghele & Ganea, 2010). Hsp70 is present on bacterial surface and functions as a plasminogen receptor in pathogens like *Mycobacterium tuberculosis, Neisseria meningitides* and *Listeria monocytogenes* (Xolalpa *et al*., 2007*;* Knaust *et al*., 2007). The role of PpiA and PpiB were investigated in immune modulation to aid pathogen survival.

Since B cells can exert influence on T cells, they are considered as important determinants in the outcome of infection with *M. tb*. *M.In silico* analysis of *M.tb* PpiA and PpiB proteins revealed major antigenic stretches in these proteins with a peak value ≥1.7 concomitant with the presence of antibodies to *M.tb* PPIases in sera from TB patients and their absence in sera from healthy individuals indicated that these enzymes are expressed during infection and the native molecules are immunodominant with apparent diagnostic potential. These results are consistent with earlier findings that members of stress proteins and HSPs such as Hsp70 and Hsp10 elicit strong B-cell response (Mehlert & Young, 1989; Young & Garbe, 1991).

During *M.tb* infection cycle, macrophages act as the first line of defense and the pathogen hires a plethora of strategies to counteract the host immune response. Balanced secretion of pro-inflammatory and anti-inflammatory cytokines are necessary for intracellular bacterial survival because cytokines can modulate different host pathways that are responsible for survival of bacteria inside host (Hornef *et al*., 2002; Stuart & Ezekowitz, 2005). Chronic inflammatory pathology is the hallmark of the disease tuberculosis that indicates that overproduction of pro-inflammatory cytokines lies at the heart of the infection. TNF-α is crucial for the formation and

maintenance of granuloma that are main effector sites of antimicrobial activity against the pathogen (Kindler *et al*., 1989). Estimation of proinflammatory cytokines secreted by THP1 cells as a direct function of concentration of rPpiA revealed that the protein is a potent stimulator of TNF-α, IL-6 and thus may play an important role, similar to Hsps, in host defense mechanism (C. J. I. Raats *et al*., 1994; Naffin-Olivos *et al*., 2014). Conversely, assessment of proinflammatory cytokines secreted by THP1 cells on treatment with increasing concentration of rPpiB revealed that the protein is an inhibitor of TNFα, IL-6 and stimulates secretion of IL-10. This result emphasizes on role of *M. tb* PpiB in subverting innate immune response and thus helping in establishment of infection, which is in accordance with previous findings that PpiB is essential for the survival of the pathogen (Sassetti *et al*., 2003).

The kinetics of production along with balance between pro-inflammatory and anti-inflammatory cytokines released by macrophages upon exposure to mycobacterial antigens regulates the T cell responses. Production of pro-inflammatory cytokines is known to contribute to the host response against mycobacteria. These pro-inflammatory cytokines seem to be associated with immunopathological responses in tuberculosis leading to necrosis and cachexy which aid in disease progression. Production of TNF-α has been shown to be directly related to virulence correlating with the intracellular viability (Engele *et al*., 2002; Newman *et al*., 1991) and is also known to promote growth of virulent *M.tb* in monocytes (Byrd, 1997). IL-6 increases the survivability of intracellular pathogen Salmonella inside macrophages by stimulating the fusion between early compartments of endocytic pathway and inhibition of transport of Salmonella to lysosome (Bhattacharya *et al*., 2006). It has been reported that IL-6 increases the survivability of *M.tb* inside host cell by inhibiting the IFN-γ induced autophagy (Dutta *et al*., 2012). Our results show an

interesting phenomenon where *M.tb* PpiA stimulate of pro-inflammatory cytokines thereby playing a vital role in the inflammatory pathology of tuberculosis whereas PpiB through an induction of Th-2 response leads to successful infection (Figure 7). PpiA seems to be expressed late in infection and induces enabling TNF-α and IL-6 (Figure 3.5) response. This pro-inflammatory response induced by high bacterial load is known to induce cell death that is associated with mycobacterial dissemination (Santucci *et al*., 2000, Tundup *et al*., 2014). Conversely PpiB employs the classical approach to aid mycobacterial growth by production of inhibitory cytokine IL-10 (Figure 3.5). It is therefore likely that different strategies employed by the same classes of protein might be due to difference in their expression kinetics with PpiB being expressed early during infection and employing IL-10 to dampen the inflammatory host response. IL-10 secreted by host cell after Mycobacterial infection increases the intracellular bacterial survival by blocking phagosomal maturation (O'Leary *et al*. 2011). It is interesting to note that PpiA and PpiB proteins are not co-operonic but are spaced apart more than 2.8 MB in the *M.tb* genome.

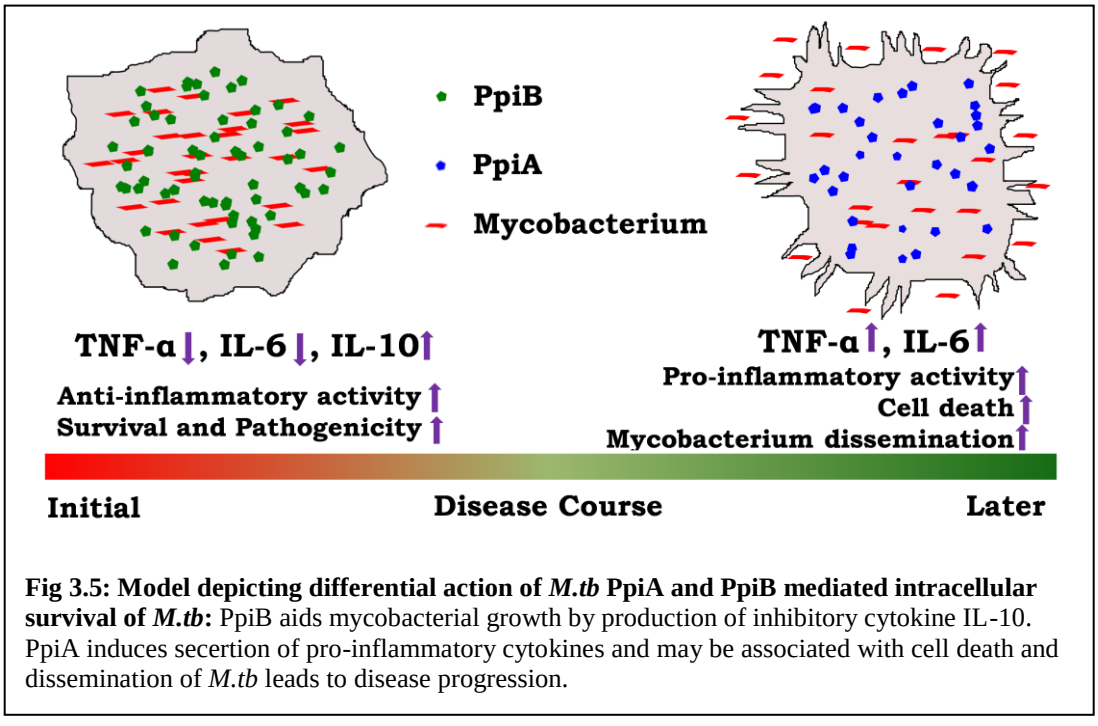

Fig 3.5: Model depicting differential action of *M.tb* PpiA and PpiB mediated intracellular survival of *M.tb*: PpiB aids mycobacterial growth by production of inhibitory cytokine IL-10. PpiA induces secertion of pro-inflammatory cytokines and may be associated with cell death and dissemination of *M.tb* leads to disease progression.

Intracellular infectious agents that have coevolved in long-standing association with the host have acquired mechanisms to persist within the host cell. The persistence of pathogenic mycobacteria within the hostile environment of host macrophages is in part due to the bacterial ability to adapt to the stress conditions encountered. *M. tb* PPIases might play a role in the intracellular survival by subverting the host cell defenses like oxidative stress. Above results implicate that *M. tb* PpiA although help in containment of the active tuberculosis infection by inducing secretion of proinflammatory cytokines, surprisingly it also helps in the intracellular survival of the pathogen by its ability to counter host generated stresses, such as oxidative stress and might play a crucial role in latent tuberculosis infection (Pathakumari *et al*., 2015).

In conclusion, our study shown in this chapter while demonstrating immunomodulatory potential of *M.tb* PPIases also unveils previously unknown function of peptidyl-prolyl isomerases which will aid in understanding infection biology and survival strategies adopted by the pathogen.

Chapter 4

M.tb PpiB promotes biofilm/pellicle formation

4.1. Introduction:

Studies relating to bacterial biofilm and pellicle are gaining considerable importance today mostly due to their medical importance, e.g. their notable property of tolerance to antibiotics and role in disease pathology etc. Example of such biofilm formation in different organisms exists, such as *Vibrio cholerae* (Moorthy & Watnick 2005), *P. aeruginosa* (Whiteley *et al*., 2001), *B. subtilis* (Stanley *et al.*, 2003) and *S. aureus* (Beenken *et al*., 2004) etc. In the simplistic representation, bacterial growth can be classified in two types, plankatonic (free floating) mode of growth and sessile or biofilm mode of growth, forming surface coupled communities. In the biofilm mode of growth, bacteria aggregate physically and form extracellular matrix around them that works as support material. This matrix comprises of both host and bacterial components, such as proteins, DNA and polysaccharides. Biofilm formation is considered as a survival strategy as well as cause of pathogenicity (Costerton *et al.*, 1999; Bjarnsholt & Givskov, 2007) and the overall bacterial biofilm formation is execution of biofilm program, a complex and regulated process (O'Toole *et al*., 2000; Sauer 2003).

In one of the earliest observations about biofilms, Antonie van Leeuwenhoek (1684) observed that animalculs on the scurf of men's teeth shows more resistant to vinegar than animalcules found outside (Leewenhoeck 1684). After the discovery of antibiotics, the slow growing persistent type of infections in patients with clinical symptoms of infection become noticeable as effect of antibiotic was too marginal on them, irrespective of age groups (Costerton *et al*., 1999; Balaban *et al.*, 2004).

Jefferson summarized the biological contexts and driving force for biofilm formation, firstly, as a bacterial defense mechanism; secondly, for colonization of suitable area;

thirdly, to utilize the community cooperation related benefits; and lastly, as natural/default mode of growth in their habitat (Jefferson, 2004). Additionally, biofilm/pellicle formation can also be an adaptation to given nutritional and environmental challenges (Bjarnsholt 2013). The growth pattern of biofilm follows the regulated mode of growth in four steps: *viz*. attachment, sessile growth, maturation of biofilm and dispersal for new colonization (Islam *et al.*, 2012).

There is provisory insight for *M.tb* forming biofilm *in vivo* as part of the persistence program within host and is supposedly cause of long term drug regimen of 6 to 9 months (Hopewell *et al.*, 2006; Hopewell *at al.*, 2014). As a matter of fact, effective killing of the *M.tb* bacilli for first 2 weeks of drug regimen, left the persister population with far lesser drug sensitivity (Jindani *et al.*, 2003; Sirgel *et al*., 2005). The possible explanation, thereby, is formation of immune-defying and drug-tolerant biofilm and this is supported by the linkage between bacterial persistence and biofilm formation (Costerton *et al*., 1999; Roberts & Stewart, 2005).

In minimal liquid culture medium, *M.tb* and *M.smegmatis* form biofilms in the interface of media and air (Darzins & Fahr 1956, Recht *et al.*, 2000). Some more direct evidences indicate that *M.tb* forms biofilm in the lungs of infected animals that shows the clusters of bacilli but it still unclear if these clusters are a genetic growth program for biofilm formation or not (Canetti *et al.*, 1972). Moreover, microcolonies of *M.tb* bacilli found in the rim in the granulomas's acellular part survived even after drug treatment, indicated biofilm like growth pattern in *M.tb* (Lenaerts *et al*., 2007). Also, the discovery of pillin-like protein in *M.tb* expressed *in vivo* with strong binding affinity for eukaryotic extracellular matrix supports the explanation.

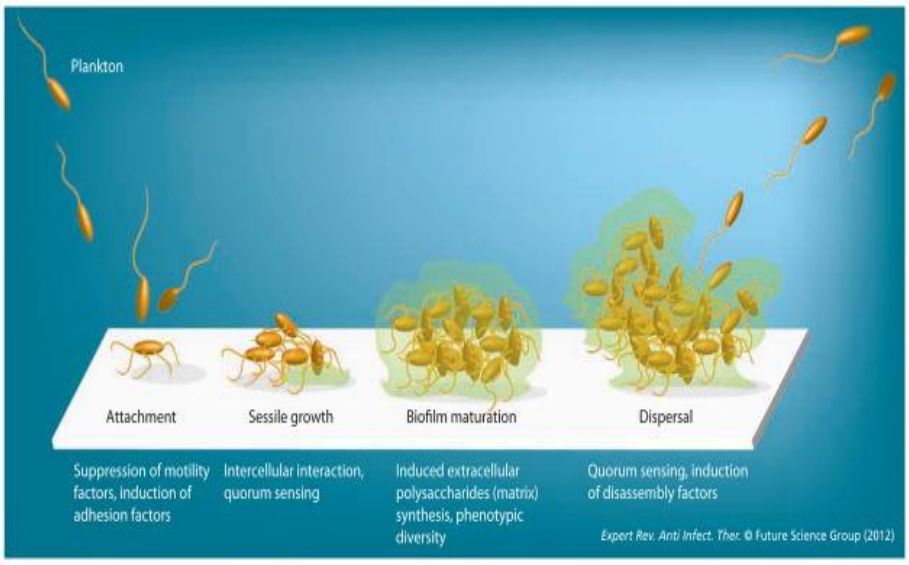

Fig 4.1.1: Representation of steps involved in bacterial biofilm formation. Bacteria adhere to the surface, grow slowly in attachment of the surface and initiate biofilm formation, then secrete components of extracellular matrix and tend towards maturation. At maturation, they disperse to make new colonization. (Islam *et al*., 2012).

Surface glycolpeptidolipids (GPL) are important for biofilm formation and cording in mycobacterium spp. (Bernut *et al.*, 2014) and their absence is inhibitory to biofilm formation (Recht *et al.*, 2001). GPL are available to membrane region and high lipid containing cell wall of mycobacterium (Brennan & Nikaido 1995), that plays the role in antigenicity (Belisle *et al.*, 1993), other than forming support matrix for biofilm and cording (Bernut *et al*., 2014).

Peptidy prolyl cis/trans isomerase B (PpiB) of *M.tb* appears in the con-A lectin concentrate of secretory proteome of *M.tb* (González-Zamorano *et al*., 2009) and, also, present in the membrane fraction of *M.tb* (Xiong *et al.*, 2005; de Souza *et al.*, 2011). Both of these evidences suggest that PpiB is a surface associated glycoprotein, whose

immunological potential is reported first time in current study. Here, an attempt has been made to probe that PpiB has potential to form biofilm. This will strengthen the hypothesis of PpiB's immune-modulatory and virulence associated role.

4.2. Materials and Methods:

4.2.1. Materials:

All analytical reagents were purchased from Sigma (USA). All enzymes were purchased from NEB (UK). Mycobacterium growth media and supplements were purchased from Becton, Dickinson and Company (BD, USA). All reagents used were analytical grade. The strains and plasmids used in this study are listed in (Table 3.1, chapter 3)

4.2.2. Bacterial growth conditions:

M.smegmatis strains were regularly cultured in Middlebrook 7H9 broth supplemented with 10% (v/v) albumin, dextrose and catalase (ADC), 0.2% (v/v) glycerol and 0.05% (v/v) Tween 80 and incubated at 37°C shaking incubator at rotation of 150 rpm. *E. coli* DH5α was grown in Luria-Bertani (LB) medium under appropriate antibiotic resistance. For cloning purpose, *E. coli* DH5α strain from Novagen was used.

The wild type *M.smegmatis* strain (*M.smeg* mc^2155) was used in the study and pST_ppiB, along with vector was electroporated using electroporator (biorad).

4.2.3. Biofilm formation:

The cloning of *M.tb ppiB* gene in *M.smegmatis* has been discussed in Chapter 2. Biofilm formation was studied by growing static cultures of mycobacteria in Middlebrook 7H9 broth in detergent free condition at 37°C. Biofilm formation was assayed qualitatively,

pictorially and quantitatively by using crystal violet staining assay (Recht & Kolter 2001). *M.smeg* cultures, Ms_WT, MS_VC and Ms_ppiB, were grown till stationary phase and absorbance at 600 nm (OD_{600}) was normalized to 1. An aliquote (200 µl) of these cultures was inoculated in Middlebrook 7H9 broth in culture tubes for pellicle and in polystyrene culture plates for biofilm formation for 2 days in static culture. Quantification of biofilm was carried out by carefully decanting medium and staining the biofilm with 1% crystal violet for 45 min. The wells were washed three times with water and air-dried. Afterwards, the dye was dissolved in solubilization buffer (80% ethanol) and absorbance (OD_{550}) was measured.

4.3. Results:

4.3.1. Growth characteristics of *M.smegmatis* strains:

M.smegmatis strains were grown in shaking culture and growth profile was studied by measuring absorbance (OD_{600}). There was no significant difference in the growth pattern of *M.smeg* cultures, Ms_WT, MS_VC and Ms_ppiB in suspension culture (Figure 4.3.1). This shows that in normal condition insertion of vector/construct does not have any inhibitory effect on growth of bacteria.

4.3.2. Biofilm formation study of *M.tb* PpiB:

Biofilm formation of Ms_ppiB showed (Figure 4.3.2a and Figure 4.3.2.b) is in accordance with previous studies that report that surface localized glycopeptidolipids take part in the biofilm/pellicle formation (Bernut *et al.*, 2014). The biofilm/pellicle formation in Ms_ppiB is significantly high compared to vector control in both qualitative and quantitative assays. (Figure 4.3.2a and Figure 4.3.2.b).

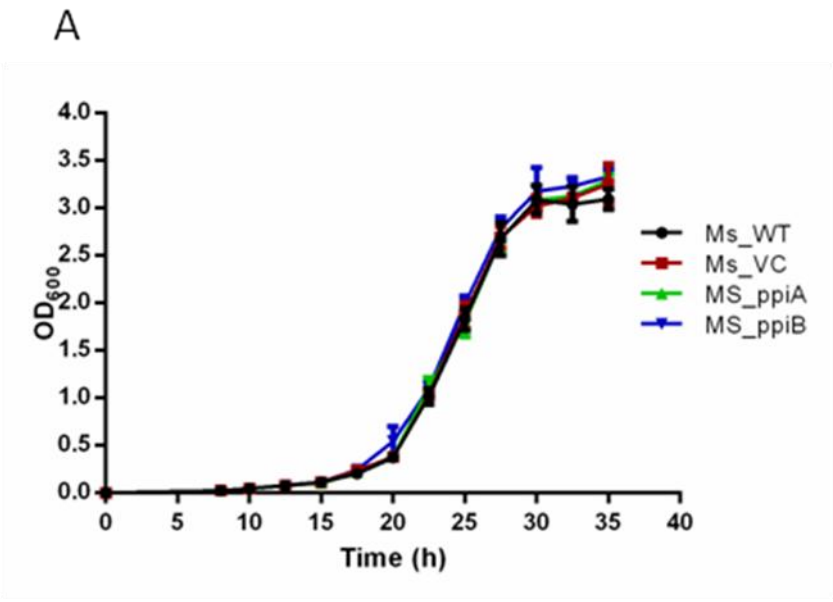

Fig 4.3.1: Growth of the mycobacterial strains in 7H9 broth culture. OD_{600} of Ms_WT (wild type), Ms_VC (vector control), Ms_ppiA and Ms_ppiB was plotted. Data is mean ± SD of values obtained from three independent cultures.

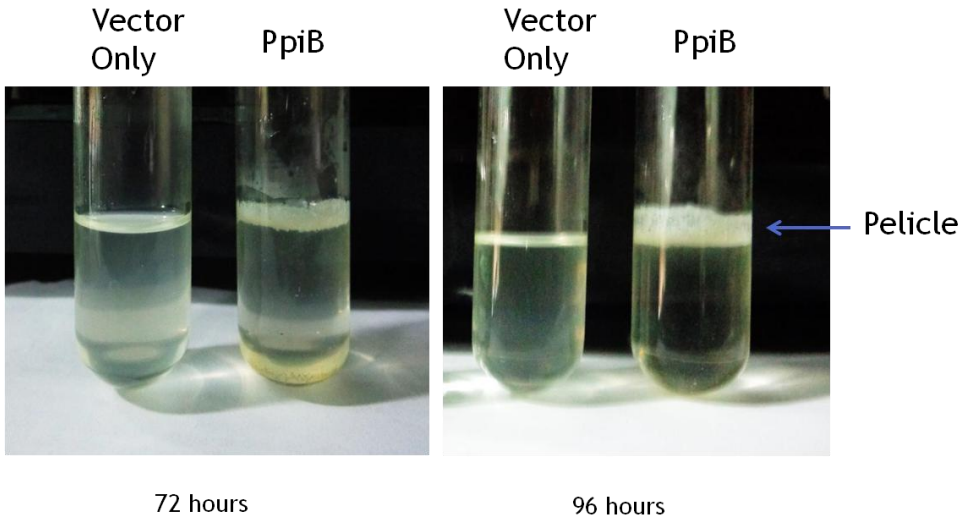

Fig 4.3.2.a: Biofilm and pellicle formation: A. Pellicle formation at the air-liquid interface of the standing 7H9 culture by strains *M.smegmatis* (vector control) and *Ms_ppiB* at different time points.

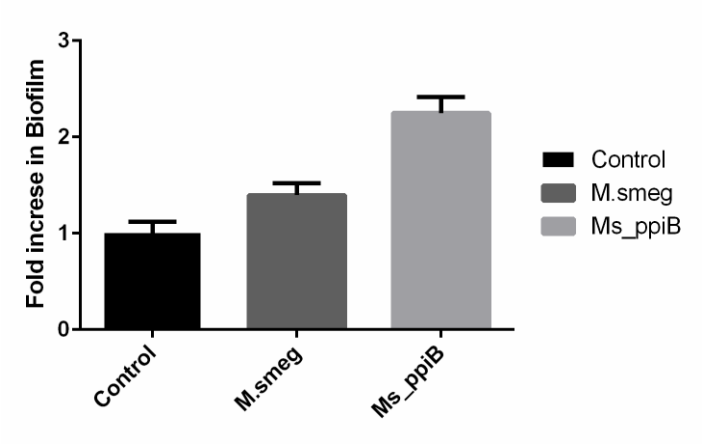

Fig 4.3.2.b: Biofilm formation assay: Biofilm formation assayed using the 1% crystal violet staining assay. The experiments were repeated three times with similar result. Control is medium only and *M.smegmatis* vector control.

Discussion:

Mycobacterial cell wall is lipid rich and numerous glycopeptides are attached to the mycolic acid component of the cell wall. It is an active host-pathogen interaction zone. The results described in the chapter shows the ability of *M.tb* PpiB in biofilm/pellicle formation, whereas host-pathogen interaction is indicative of immune-modulatory role (described in previous chapter). Biofilm formation is also part of stress adaptation as bacteria in the film become highly tolerant to environmental stress. Though planktonic cells contain the genetic stress responders but they easily get overwhelmed by stresses threatening their survival as they do not get time to respond (Etienne *et al*., 2002). *M.tb* biofilm in particular is rich in glycopeptidolipids to a very large extent, rendering the bacterial access difficult to different stresses and thus provide protection. Biofilm formation may help the *M.tb* to attach to the nutrient rich zone, creating matrix for high bacterial density that protects it from antibiotic dosage for months and dense availability signals the expression of quorum associated genes that may change the pathogenic behavior or *M.tb*.

In conclusion, the study reveals that *M.tb* PpiB helps in biofilm formation and thus adds to the virulence potential of the pathogen.

Chapter 5

Summary and Conclusions

5.1. Summary and Conclusions:

The work presented in this thesis entitled '***Mycobacterium tuberculosis* Peptidyl-Prolyl Isomerases: Biochemical, Immunological and Functional Characterization**' was carried out to decipher the moon lighting functions of peptidyl-prolyl isomerases and to reveal their crucial role in intracellular survival of the pathogen.

M.tb has evolved mechanisms to survive in macrophages which are one of the most stressful environments for bacteria. Successful colonization of the intraphagosomal niche by the pathogen depends on molecular interaction network within the host (Ehrt & Schnappinger, 2009; Russell, 2001). The effector molecules which play a crucial role in host pathogen interaction at the molecular level are majorly stress responders, HSPs, chaperones and other protein modifying enzymes (Henderson, 2010). These effector molecules subvert host defenses by quenching ROS and RNS (Ng *et al*., 2004; Cirillo *et al*., 2009), disrupting the membrane repair (Divangahi *et al*., 2009), phagolysosomal fusion (Shukla *et al*., 2014), suppression of autophagy (Huang & Bao, 2014) and by escaping immune challenges (Kugelberg, 2014; Gupta *et al*., 2012). Bacterial chaperones play a vital role in protein folding and secretion, thereby indirectly contributing to the virulence and survival of the pathogen inside the host (Stebbins & Galán, 2003, Stebbins 2005)

PPIase (peptidyl-prolyl *cis-trans* isomerases) are ubiquitously expressed enzymes that (PPIase, EC 5.2.1.8) assist in protein folding by isomerization of Xaa-Pro peptide bonds, which otherwise can act as folding bottleneck and will limit the rate of final folding steps (Schmidpeter *et al*., 2015; Fischer *et al*., 1984; Lang *et al*., 1987; Davis *et al*., 1989; Kiefhaber *et al*., 1990). Prolyl isomerases lie in three structurally and sequentially

unrelated classes, viz. cyclophilins, FKBPs and Parvullins (K. A. Kromina *et al*., 2008). Molecular chaperones play a crucial role, in assisting proper folding of proteins.

It has been previously reported that PpiA of *M.tb* is a secretory protein and interacts with several host proteins such as those involved in iron regulation, immune defense mechanism and signal transduction (Henriksson *et al*., 2004; Bhaduri *et al*., 2014). Since the enzyme is a part of the secretome of the pathogen, it is speculated to play a vital role in survival of *M.tb* in the harsh environment of macrophage. Functional characterization of the enzymes reflecting their possible role in aiding intracellular survival of the pathogen and thus indirectly contributing to its virulence has not been investigated till date.

The work described in this thesis is an attempt to functionally characterize PPIases of *M.tb* (rPpiA and rPpiB), in terms of chaperone activity and virulence attributes. rPPIases was shown to bind to non-native proteins *in vitro* and can prevent their aggregation. *E. coli* cells overexpressing cyclophilins of *M.tb*, could survive thermal stress as compared to control. In addition to this, the potential role of cyclophilins in protecting the pathogen from host generated stresses like hypoxia and oxidative stress was also investigated.

Furthermore, in the results presented in this thesis, using *M.smegmatis* as a model, illustrate the potential role of PPIases in intraphagosomal survival of the pathogen. This study also describes antigenic potential of *M.tb* cyclophilins, its involvement in eliciting host immune response and altering the host cytokine profile. These novel findings suggest that *M.tb* PPIases, apart from being a stress protein, possibly alter host immune response and aid in the intracellular survival of the pathogen. That ppiB expression was

associated into biofilm formation pointed to the likely role of this enzyme in development of drug resistance.

5.2. Salient Findings

The following represents the highlights of the work described in this PhD thesis:

- Cyclophilins of *M.tb* exhibit chaperone like function both in vitro and under physiological conditions.
- rPpiA and rPpiB could protect MalZ from thermal aggregation and could also rescue enzyme activity of heat denatured Nde1.
- *E.coli* cells expressing the cyclophilin proteins of *M.tb* exhibit resistance to thermal stress as compared to control.
- Both the cyclophilins of *M.tb* can modulate host immune response by altering cytokine profile.
- Both the cyclophilins have antigenic patches and induce B-cell response in patients as compared to the healthy individuals.
- *M.smegmatis* strain expressing PpiA and PpiB of *M.tb* exhibit increased survival in THP cell lines as compared to wild type *M.smegmatis*.
- *M.smegmatis* expressing *M.tb ppiB* show increased biofilm formation as compared to the vector control cells.

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Human resistin, a proinflammatory cytokine, shows chaperone-like activity

Madhuri Suragani[a], Varma D. Aadinarayana[a], Aleem Basha Pinjari[a], Karunakar Tanneeru[b], Lalitha Guruprasad[b], Sharmistha Banerjee[c], Saurabh Pandey[a], Tapan K. Chaudhuri[d], and Nasreen Zafar Ehtesham[a,e,1]

[a]Dr. Reddy's Institute of Life Sciences, University of Hyderabad, Hyderabad 500046, India; [b]School of Chemistry, University of Hyderabad, Hyderabad 500046, India; [c]School of Life Sciences, Department of Biochemistry, University of Hyderabad, Hyderabad 500046, India; [d]Kusuma School of Biological Sciences, Indian Institute of Technology, New Delhi 110016, India; and [e]Inflammation Biology and Cell Signaling Laboratory, National Institute of Pathology, New Delhi 110029, India

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Resistin, a cysteine-rich adipocytokine, proposed as a link between obesity and diabetes in mice, was shown as a proinflammatory molecule in humans. We earlier reported that human resistin (hRes), a trimer, was resistant to heat and urea denaturation, existed in an oligomeric polydispersed state, and showed a concentration-dependent conformational change. These properties and an intimate correlation of hRes expression with cellular stress prompted us to investigate hRes as a possible chaperone. Here, we show that recombinant human resistin was able to protect the heat-labile enzymes citrate synthase and Nde1 from thermal aggregation and inactivation and was able to refold and restore their enzymatic activities after heat/guanidinium chloride denaturation. Furthermore, recombinant human resistin could bind misfolded proteins only. Molecular dynamics-based association–dissociation kinetics of hRes subunits pointed to resistin being a molecular chaperone. Bis-ANS, which blocks surface hydrophobicity, abrogated the chaperone activity of hRes, establishing the importance of surface hydrophobicity for chaperone activity. Replacement of Phe49 with Tyr (F49YhRes), a critical residue within the hydrophobic patch of hRes, although it could prevent thermal aggregation of citrate synthase and Nde1, was unable to refold and restore their activities. Treatment of U937 cells with tunicamycin/thapsigargin resulted in reduced hRes secretion and concomitant localization in the endoplasmic reticulum. *Escherichia coli* transformants expressing hRes could be rescued from thermal stress, pointing to hRes's chaperone-like function in vivo. HeLa cells transfected with hRes showed protection from thapsigargin-induced apoptosis. In conclusion, hRes, an inflammatory protein, additionally exhibited chaperone-like properties, suggesting a possible link between inflammation and cellular stress.

protein folding | chaperokine

Resistin, a small cysteine-rich secreted protein, is predominantly produced in human macrophages (1, 2). Resistin levels in human serum could neither be associated with obesity nor linked with insulin resistance (3), pointing to possible other role(s) for this hormone. We, and later others, showed that human resistin (hRes) is a proinflammatory molecule that stimulates the synthesis and secretion of TNF-α and IL-12 from macrophages through an NF-κB–activated pathway (4, 5). hRes mRNA levels are strongly induced by TNF-α and IL-6 in human peripheral blood mononuclear cells (6, 7). Although human and mouse resistin share 64.4 and 59% sequence homology at mRNA and amino acids levels, respectively, they differ considerably in terms of their structural organization (8). We earlier reported, based on extensive biophysical analyses, that recombinant human resistin (rhRes) is a highly stable molecule that exists in oligomeric states as a function of concentration with no major loss in helicity and displays slightly altered tertiary structure with an increase in temperature (9, 10). The variable oligomeric states and poly-dispersity of hRes are features often attributed to chaperones (11, 12). mRNA levels of resistin were earlier found to be down-regulated during endoplasmic reticulum (ER) stress in rodent adipocytes (13).

Cellular stress in any form, including infection, can alter the cellular metabolism, leading to improperly folded, defective, and aggregated proteins within the ER. This induces ER stress, which then triggers unfolded protein response (UPR). Under such conditions, molecular chaperones play a crucial role in assisting proper folding of proteins. The observations that hRes (*i*) is a small molecule of 8–12 kDa; (*ii*) exists in different forms, including high molecular mass oligomers; (*iii*) has a secondary structure that is refractile to increasing temperature; (*iv*) is highly resistant to chemical denaturation; and (*v*) has expression levels that correlate with stress conditions led us to suggest that hRes could act as a molecular chaperone. In this study we show that rhRes binds to nonnative proteins in vitro and protects them from thermal and chemical denaturation while preserving their enzymatic activity. *Escherichia coli* cells, overexpressing hRes, could survive when exposed to higher temperatures. In mammalian cells, an elevated level of hRes was observed upon induction of ER stress by tunicamycin (tn) and thapsigargin (tp). hRes, an otherwise secreted protein, was retained in the cell and localized in the ER upon ER stress. HeLa cells transfected with hRes showed protection from tp-induced apoptosis. These observations prompted us to conclude that hRes, apart from being a proinflammatory molecule, possibly functions as a chaperone under stress conditions.

Results

Homology Modeling of hRes Displayed Surface-Exposed Hydrophobic Patches. The 3D structures of the trimeric and hexameric forms of wild-type hRes, built using MODELER by using mouse resistin as a template (14) (Fig. S1*A*), generated rmsd values of 0.213 and 0.533 Å for trimer and hexamer, respectively. The 3D model of trimer pointed to the presence of surface-exposed

Significance

This is a study of human resistin acting as a molecular chaperone. We show that human resistin, a proinflammatory cytokine secreted by human macrophages, is retained inside the cell during endoplasmic reticulum stress and functions like a chaperone to rescue the cell from apoptosis. The study implicates human resistin as a possible molecular link between cellular stress and inflammation during pathological conditions such as infections.

Author contributions: N.Z.E. conceptualized and designed the research; M.S., V.D.A., A.B.P., and S.P. performed research; L.G. and K.T. carried out computational analyses; N.Z.E. contributed new reagents/analytical tools; M.S., V.D.A., A.B.P., T.K.C., S.B., and N.Z.E. analyzed data; and M.S. and N.Z.E. wrote the paper.

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[1]To whom correspondence should be addressed. E-mail: nzehtesham@gmail.com.

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hydrophobic patches seen typically for chaperones. Four amino acids within the mature polypeptide, namely Leu42, Pro46, and Phe49, and Trp80, were found to be surface-exposed in the homology model. To validate these predictions, one of these amino acids, namely Phe at position 49, was mutated to Tyr by site-directed mutagenesis (Fig. S1*A*), and the impact of this mutation (F49Y) on the surface hydrophobicity was determined using the hydrophobe-selective dye 1-anilinonaphthalene-8-sulfonate (ANS). Although the wild-type rhRes showed ANS binding, as evident from spectroscopic measurement, the mutant F49YrhRes showed negligible binding (Fig. S1*B*). For all subsequent studies the wild-type human resistin (rhRes) and the mutant version (F49YrhRes) were used. The space-filled model of the hexamer is given as Fig. S2.

hRes Trimer Exhibited Molecular Rearrangement. Our earlier studies (9, 10) showed that the molecular mass of hRes varied between 11.3 and 600 kDa in solution. These oligomeric structural features of hRes, reminiscent of chaperone-like proteins, prompted us to carry out molecular simulation studies of hRes and F49YhRes structures in trimer and hexamer forms at 298 and 333 K using GROMACS, which were analyzed using trajectory files obtained during 20-ns simulations. The rmsd plots of trimer of hRes and F49YhRes showed the difference in association and dissociation. hRes trimer started converging after 10 ns of simulations. The rmsd convergence of protein at 298 K reached 0.5 and 0.6 nm for 333 K as observed at the end of simulations (Fig. S3). During the 20-ns molecular dynamic (MD) simulations, the hRes trimer C-terminal β-sandwich head domain rearranges from trimer to dimer–monomer arrangement, thus exposing internal cavity to solvent (Fig. S3, iv). F49YhRes trimer converged after 15 ns of the simulations for both 298 K and 333 K (Fig. S4). At the end of the simulations, the protein at 298 K reached the convergence near 0.5 nm, and at 333 K it converged near 0.6 nm. Compared with rhRes, F49YhRes trimer undergoes less rearrangement and prefers to retain the trimer conformation. However, no dissociation of hexamer hRes was observed during simulations either at 298 K or at 350 K (Fig. S5).

rhRes and F49YrhRes Can Protect Citrate Synthase and Restriction Enzyme Nde1 from Thermal Aggregation. To experimentally demonstrate chaperone-like activity of rhRes, the ability of this adipocytokine to protect heterologous proteins from thermal denaturation was assessed. Light-scattering assay to monitor aggregation was carried out by measuring absorbance (320 nm) of thermally denatured citrate synthase (CS) (15) and Nde1 in the absence or presence of rhRes/F49YrhRes (Fig. 1). These two enzymes were selected based on their thermal sensitivity and availability of convenient assays to monitor activity. The thermal stability of all of the proteins in the experiment, namely rhRes, F49YrhRes, CS, and Nde1, was also checked individually. As expected for molecular chaperones, rhRes and the mutant rF49YrhRes were highly stable at 45 °C for more than 45 min, showing negligible aggregation, where as CS and Nde1 aggregated considerably with time (Fig. 1 *A* and *B*, respectively). We then examined whether rhRes can protect CS and Nde1 from thermal aggregation, a property common to most chaperones (16). Nde1 in the presence of 0.15 μM rhRes was highly stable (Fig. 1*B*) compared with incubation with 0.15 μM lysozyme (negative control). Likewise, rhRes could protect CS against thermal aggregation, and this protection was similar to that seen using a known chaperone, GroEL (Fig. 1*C*). The ability to protect Nde1 and CS from thermal denaturation remained unaltered for the mutant F49YrhRes (Fig. 1 *B* and *C*), consistent with its thermal stability (Fig. 1*A*). These results clearly demonstrate that both rhRes and F49YrhRes can protect proteins from thermal denaturation/aggregation.

Nde1 and CS Protected from Denaturation by rhRes Were Enzymatically Active. Having shown that rhRes can protect Nde1 and CS from thermal denaturation, we investigated if the protected proteins are also functionally active. Enzyme activity of Nde1, after heat denaturation at 60 °C for 20 min, was measured using pUC18. Linearized pUC18 was visualized by electrophoresis on 1% agarose gel. Upon heat denaturation, Nde1 could not cleave pUC18 (Fig. 2*A*, lane 3); however, when *Nde*1 was heat-denatured in the presence of rhRes, much of its DNA cleavage activity was retained as evident from a single linearized pUC18 band after Nde1 digestion (Fig. 2*A*, lane 4). In the presence of either F49YrhRes or control protein BSA, Nde1 activity was lost (Fig. 2*A*, lanes 5 and 6, respectively). It therefore appeared that rhRes not only can prevent physical aggregation of Nde1 upon heating, but also can preserve the functional activity of the protein. The mutant F49YrhRes, although it protected the protein from aggregation, could not prevent loss of enzyme activity upon heat treatment, pointing to the functional importance of the F49 residue of hRes.

The ability of rhRes and F49YrhRes to refold and restore enzyme activity of unfolded protein after denaturation was then assessed. Denaturation with 6 M guanidinium chloride (GdnHCl) resulted in complete loss of CS enzyme activity (Fig. 2*B*). Denatured CS was then diluted in the appropriate buffer to refold in the presence of GroEL (positive control) (17), rhRes, or F49YrhRes. Refolding of CS was assessed in terms of the recovery of its enzyme activity, monitored by the disappearance of acetyl-CoA at 233 nm in a reaction mix containing oxaloacetic acid, acetyl-CoA, and nitrobenzoic acid. CS enzyme activity was plotted as the percentage of the native activity. It could be seen (Fig. 2*B*) that, although GdnHCl-denatured CS showed almost complete loss of enzyme activity, when refolded in the presence

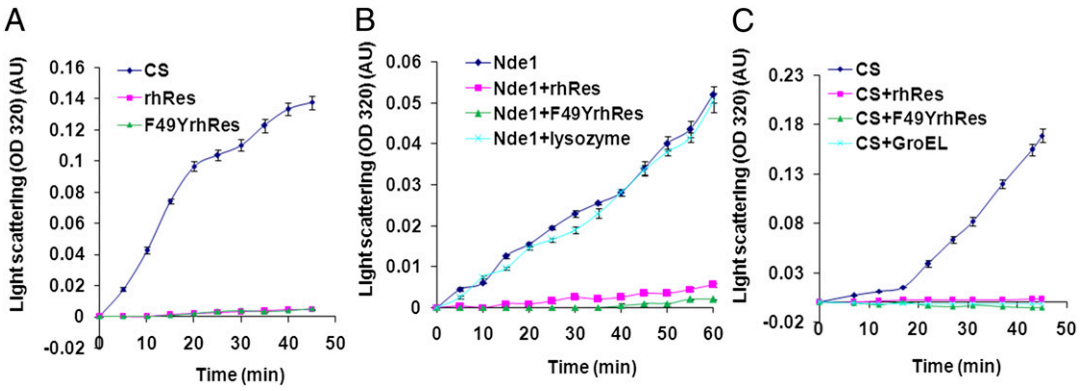

Fig. 1. rhRes and mutant (F49YrhRes) proteins prevent thermal aggregation of proteins. (*A*) Light-scattering assay of thermal aggregation. Absorbance at 320 nm was measured for rhRes, F49YrhRes, and CS at 45 °C for 45 min. (*B*) Resistin prevents thermal aggregation of Nde1 and (*C*) citrate synthase. The kinetics of Nde1 or CS aggregation, alone or in the presence of rhRes, F49YrhRes, or lysozyme, were assayed. Each experiment was carried out in triplicate. The error bars represent SEM.

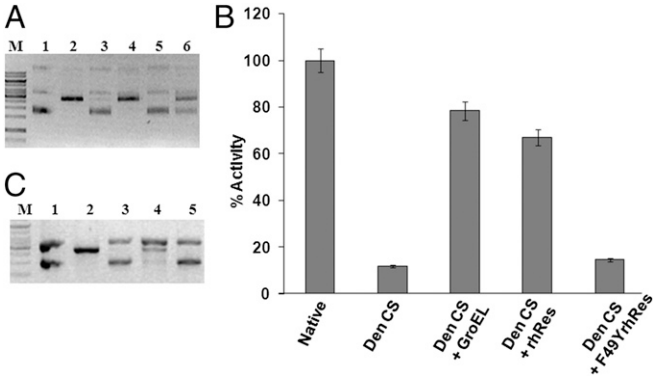

Fig. 2. rhRes-protected Nde1 and CS enzymes are functionally active. (*A*) Residual enzyme activity of thermal-denatured Nde1 in the absence or presence of rhRes, F49YrhRes, and control protein BSA. Lane M, 1-kb molecular size marker; lane 1, uncut pUC18; lane 2, pUC18 digested with native Nde1; lane 3, with heat-denatured Nde1; lane 4, with rhRes-treated heat-denatured Nde1; lane 5, with F49YrhRes-treated heat-denatured Nde1; lane 6, with heat-denatured Nde1 with BSA. The gel is a representative of three independent experiments. (*B*) Residual activity of GdnHCl-denatured CS (Den CS), refolded CS in the presence of chaperone GroEL (Den CS+GroEL+ ATP), refolded CS in the presence of rhRes (Den CS+rhRes), and refolded CS in the presence of F49YrhRes (Den CS+F49YrhRes). Control is the native CS (native). Enzyme activity was assayed by monitoring the disappearance of acetyl-CoA at 233 nm and expressed as the perecentage of activity of native CS (native). Each experiment was carried out in triplicate. The error bars represent SEM. (*C*) Residual Nde1 activity assay after denaturation with bis-ANS photoincorporated hRes. Lane M, 1-kb DNA ladder; lane 1, undigested pUC18; lane 2, pUC18 digested with native Nde1; lane 3, with heat-denatured Nde1; lane 4, with heat-denatured Nde1 in the presence of rhRes; and lane 5, with heat-denatured Nde1 in the presence of bis-ANS–rhRes. The gel is a representative of three independent experiments.

of either rhRes or GroEL, about 75–80% of the activity was regained. CS refolded in the presence of F49YrhRes was found to be functionally inactive (Fig. 2*B*). These results reconfirmed the earlier observation that, although rhRes can restore the activity of the refolded denatured protein, the F49YrhRes mutant cannot do so.

Exposed-Surface Hydrophobicity Is Important for Chaperone Activity. We next evaluated the importance of total surface hydrophobicity, rather than a single hydrophobic amino acid change, on chaperone activity. The dimeric analog of ANS, 4,4′-bis-1-anilinonaphthalene-8-sulfonate (bis-ANS), was photo-incorporated onto the rhRes molecule by UV irradiation and used in our assays. If the hydrophobic patches on the surface of rhRes were important for chaperone-like activity, one would expect that binding of bis-ANS to rhRes (bis-ANS–rhRes) should not protect proteins from functional inactivation at high temperature. Nde1 (10 units) was incubated at 60 °C for 20 min in the absence or presence of either rhRes or bis-ANS–rhRes. The heat-denatured Nde1 was then used to digest pUC18. It is evident that rhRes could protect the functional activity of Nde1 from thermal denaturation (Fig. 2*C*, lane 4), but bis-ANS–rhRes could not do so as evident from the presence of undigested pUC18 (Fig. 2*C*, lane 5). These experiments demonstrate that the hydrophobic residues on the surface of rhRes are important for chaperone-like activity.

rhRes Could Bind Nonnative Proteins Only. To explore if rhRes, like chaperones, differentiates between native and nonnative proteins, we incubated rhRes with native or denatured CS in equimolar concentration, followed by immunoprecipitation with anti-hRes antibodies. The immunoprecipitated complex was then fractionated by SDS/PAGE and stained with Coomassie Blue (Fig. 3). It could be seen that rhRes could complex with denatured CS (Fig. 3*A*, lane 3) whereas native CS could not be coimmunoprecipitated with rhRes (Fig. 3*A*, lane 2). A similar experiment was performed with the randomly selected protein Late Expression Factor 4 (LEF4) (Fig. 3*B*). It once again became evident that rhRes could bind and hence coimmunoprecipitate denatured LEF4 (Fig. 3*B*, lane 3) but not native LEF4 (Fig. 3*B*, lane 2). These observations lead us to conclude that rhRes, like other molecular chaperones, binds only to nonnative proteins.

rhRes Can Rescue *E. coli* Cells from Thermal Shock. Results presented so far provide in vitro demonstration of chaperone-like activity of rhRes. To gain insights into the chaperone activity of rhRes under physiological conditions, we investigated if hRes could rescue *E. coli* growth after prolonged thermal shock. *E. coli* M15 cells were transformed with pQE30 plasmid vector or the recombinant constructs pQE30hRes or pQE30F49YhRes carrying the wild-type hRes gene or F49YhRes mutant, respectively, under the inducible *lac* promoter. The expression of hRes or its mutant was induced by 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 2 h. Uninduced and induced cultures were diluted in a 1:1,000 ratio and grown at either 37 °C or 50 °C for 45 min, and 10 μL of each sample was then spread on agar plates with appropriate antibiotics. It could be seen that *E. coli* cells expressing hRes (Fig. 3*C*, center bar) survived the thermal shock approximately sevenfold more than those transformed with pQE30 basal vector (Fig. 3*C*, left bar) whereas those transformed with pQE30F49YhRes showed marginal (approximately onefold) survival (Fig. 3*C*, right bar). These observations provide evidence that hRes also functions as a molecular chaperone under physiological conditions.

hRes Is Overexpressed During Tunicamycin and Thapsigargin-Induced ER Stress. The human leukemic monocyte lymphoma cell line U937 was used for these studies as they were shown to express resistin in high abundance (18). ER stress was induced in U937 cells either by tn, which blocks the synthesis of *N*-glycans, or tp, which causes Ca^{2+} depletion in ER. The induction of ER stress was confirmed by the observed increase in the levels of Grp78, a well-known ER stress marker (Fig. S6). The levels of hRes were monitored by Western blot with anti-hRes antibody after 24 h of treatment under increasing concentrations of tn/tp (2–15 μg/mL). Levels of resistin were found to increase upon induction of ER stress as a function of tn or tp concentration (Fig. 4 *A* and *B*, respectively). Transcript analyses using quantitative PCR (qPCR) also confirmed the induction of resistin during ER stress by either tn or tp (Fig. 4 *C* and *D*, respectively). Taken together, these results show that ER stress induced resistin expression both at mRNA and protein levels.

Resistin Is Retained in ER upon ER Stress. Having observed that hRes, a secretory protein, is induced during ER stress, we next asked if the secretion of hRes is affected during ER stress. U937 cells were treated with either tn or tp (5 μg/mL), and secretory hRes levels in the supernatant, collected at 6, 12, and 24 h after treatment, were analyzed by ELISA (Adipo Gen). hRes is overexpressed during tunicamycin and thapsigargin induced ER stress; ELISA data showed secretion impairment resulting in an about 40–50% decline in resistin secretion (Fig. 5 *A* and *B*).

To address this issue of differential secretion, we examined the localization of hRes during ER stress by confocal microscopy. Immunocytochemistry was performed on untreated and 5 μg/mL tn- or tp-treated U937 cells for 24 h using antibodies specific to hRes or calreticulin, an ER resident protein. It can be seen that hRes colocalized with calreticulin (Fig. 5*C*, merged panel). Furthermore, the increase in expression of hRes after 24 h of treatment in terms of increased Cy3 intensity is also evident. It is therefore apparent that, under conditions of ER stress, although resistin continues overall to be induced, the bulk of it remains within ER. These results demonstrate that tn/tp-induced stress caused a reduction in the levels of secretory resistin despite increased expression levels and that the bulk of the intracellular hRes was localized to the ER.

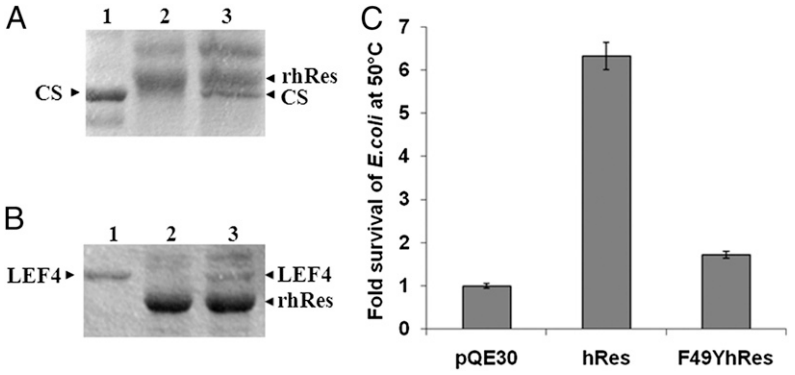

Fig. 3. rhRes binds only to nonnative proteins, and hRes can rescue *E. coli* cells from thermal shock. (*A*) Coimmunoprecipitation of rhRes with native and denatured CS. The different lanes are the following: native CS (lane 1); coimmunoprecipitation of rhRes after incubating with native CS (lane 2); or with GdnHCl-denatured CS (lane 3). (*B*) Coimmunoprecipitation of rhRes with native and denatured LEF4. Native LEF4 (lane 1); coimmunoprecipitation of rhRes after incubating with native LEF4 (lane 2); or with GdnHCl-denatured LEF4 (lane 3). Arrowheads indicate relative position of the proteins. The gel pictures are representative of three independent experiments. (*C*) *E. coli* M15 cells were transformed with plasmid pQE30, pQE30hRes, or pQE30F49YhRes. Note that after heat treatment for 45 min at 50 °C, *E. coli* M15 cells transformed with hRes showed a more than sevenfold survival compared with pQE30 vector control, whereas those transformed with mutant F49YhRes showed significantly reduced survival compared with wild-type hRes. Each experiment was carried out in triplicate. The error bars represent SEM.

Resistin Can Rescue Mammalian Cells from ER Stress-Induced Apoptosis. HeLa cells (human cervical adenocarcinoma cells) were used for studying the impact of transiently expressed hRes in rescuing cells from ER stress-induced apoptosis. HeLa cells were transfected with either empty vector pCDNA or pCDNAhRes carrying hRes gene (schematically represented in Fig. S7), and expression of resistin was confirmed by RT-PCR (Fig. S8). No endogenous expression of resistin at the transcript level could be registered in HeLa cells (Fig. S8, lanes UT and T-EV). Apoptosis was induced in these cells using 15 μg of tp for 24 h, and the percentage of cells undergoing apoptosis was scored using Annexin V staining per the manufacturer's guidelines (Invitrogen). It could be seen that expression of hRes gene in HeLa cells could successfully rescue these cells from thapsigargin-induced apoptosis (Fig. 6). These results demonstrate that resistin aids cells in restoring ER homeostasis, thereby protecting them from apoptosis.

Summarizing, hRes, earlier known to be a proinflammatory cytokine, exhibited chaperone-like activity and can provide protections to *E. coli* from thermal stress and to mammalian HeLa cells from ER stress-induced apoptosis. In line with its role as a possible chaperone under stress conditions, this secretory protein was retained in ER during stress, suggesting a possible link between inflammation and cellular stress.

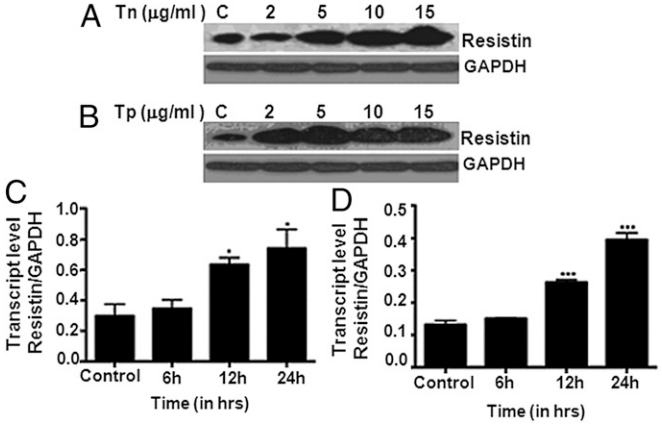

Fig. 4. Resistin is up-regulated during tunicamycin or thapsigargin treatment. U937 cells were treated with increasing concentrations (0, 2, 5, 10, and 15 μg/mL) of tn (*A*) or tp (*B*) for 24 h, and resistin expression levels were determined by Western blot. Western blot figures are representative of three independent experiments. Note the up-regulation of resistin protein expression with increasing concentration of tn or tp. In another experiment, U937 cells were treated with 5 μg/mL of tn (*C*) or tp (*D*) for 0, 6, 12, and 24 h, and resistin transcript levels were quantified using qPCR. Note the increase in resistin mRNA levels over time during tn/tp-induced ER stress. Statistical analysis was performed using one-way ANOVA and post-Dunnett's multiple comparison test. ***$P < 0.0001$, *$P < 0.01$. Each experiment was carried out in triplicate.

Discussion

hRes, a small cysteine-rich secretory protein produced in macrophages, although initially proposed to be a link between diabetes and insulin resistance, is emerging as an important player in inflammation (4, 5). Our previous studies showed that rhRes is an extremely stable molecule whose structure was insignificantly altered as a function of temperature (9). Extending our earlier studies, we now report that hRes has chaperone-like activity that can protect proteins from denaturation and retain their activity.

The molecular model of hRes, constructed using a mouse 3D model as a template, shows an interesting funnel-shaped structure, with an orifice at one end, for probable interaction with the unfolded proteins. Based on this homology model, four sites on hRes, namely Leu42, Pro46, Phe49, and Trp80, were found to be exposed on the surface. These residues may play a role in imparting surface hydrophobicity to the molecule. Surface hydrophobicity is critical for chaperones as these are considered instrumental in interactions with unfolded or misfolded proteins (19), and replacement of any of these hydrophobic residues with hydrophilic residues should result in significant structural alterations, which would then disrupt interaction with misfolded proteins, as reported for some heat-shock proteins (20). We observed that the same replacement of Phe-49 by Tyr drastically reduced the ability of hRes to interact with misfolded proteins, implicating its importance in wild-type rhRes. Increased molecular rearrangements accompanied by higher affinity for unfolded proteins is considered a paradigm of chaperone action (21). Comparison of mutant F49YhRes with wild-type hRes, in terms of chaperone activity, gave results consistent with these predictions. Furthermore, ANS-binding analysis of F49YrhRes showed a decrease in absorbance compared with rhRes, a reflection of the loss of surface hydrophobicity due to F49Y mutation.

The computational simulation data served as the basis for experiments to demonstrate chaperone-like function of the hRes protein by using heat- and the alkali-labile enzyme CS and the restriction enzyme Nde1. Unlike CS and Nde1, rhRes was refractile to heat-induced aggregation, pointing to its thermal stability. However, when both heat-labile CS and Nde1 proteins were individually incubated at 45 °C for 45 min in the presence of rhRes not only could aggregation be prevented, but also almost 80% of activities could be restored. The mutant F49YrhRes could not assist in restoring enzyme activities, although it could provide protection from thermal aggregations. It is likely that structural alteration and the declined molecular reassociation due to F49Y change, although rendering the mutant protein refractile to thermal aggregation, caused subtle change(s) in structure that prevented the ability of F49YrhRes to act as a chaperone. F49YrhRes was able to make a complex with denatured CS, similar to wild-type rhRes, when assayed by coimmunoprecipitation.

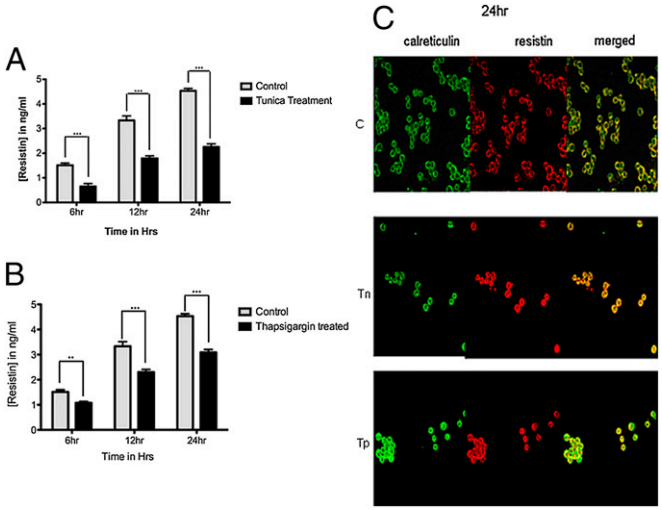

Fig. 5. Levels of secreted resistin were reduced under ER stress. U937 cells were treated with 5 μg/mL of tn (*A*) or of tp (*B*) for 6, 12, and 24 h. ELISA was performed with the supernatants of the treated cells. Note the reduced secretion of resistin in tn/tp-treated cells compared with untreated controls. Statistical analysis was performed by two-way ANOVA and post-Bonferroni test (***$P < 0.0001$, **$P < 0.001$). Each experiment was carried out in triplicate. (*C*) Colocalization of resistin and calreticulin in ER. Untreated U937 cells and those treated with 5 μg/mL of tunicamycin (Tn) or thapsigargin (Tp) for 24 h were processed for confocal microscopy using respective fluorescence-labeled antibodies. ER marker (calreticulin) is shown in green color and resistin in red. Note the merging of the green and red signals in the merged column indicating colocalization of resistin in the ER. Figures are representative of two independent experiments.

Bis-ANS was used to mask the hydrophobic patch on the surface of wild-type hRes. The activity of Nde1, when incubated with bis-ANS–treated hRes, could not be protected from thermal denaturation, indicating the importance of the hydrophobic patch in the chaperone property of hRes. ATP was not required for preventing the aggregation of CS due to high temperature, suggesting that hRes is an ATP-independent chaperone. Similar examples of proteins that work like a chaperone under cellular stress conditions in the absence of ATP hydrolysis have been reported; these include Clusterin (22), which is a secretory mammalian chaperone, and Spy (23), an *E. coli* periplasmic protein that showed chaperone-like activity in vitro. Measurement of ATPase activity of rhRes revealed 0.193 mM (K_m) and 0.128 nM/μg/min (V_{max}), which were lower measurements than those of GroEL, DnaK, HSP100, and HSP104. The same measurements of ATPase activity for F49YhRes were 0.362 mM (K_m) and 0.428 nM/μg/min (V_{max}).

In vivo evidence on the role of hRes as a possible chaperone came from *E. coli* growth rescue after heat treatment. It was apparent that survival of *E. coli* transformed with pQE30hRes was quantitatively higher compared with cells transformed with pQE30F49YhRes and pQE30 vector alone. These data confirm that overexpression of hRes can rescue growth of *E. coli* after heat shock. hRes expression could also successfully rescue HeLa cells from thapsigargin-induced apoptosis.

Cellular responses to stress can progress to apoptosis or adaptive responses (24). Stress induces different responses depending upon its nature, duration, and severity (25). The adaptive response involves the induction of both constitutively expressed or induced conserved heat-shock proteins and chaperones. Celluar stress is often manifested by the accumulation of unfolded or misfolded proteins in the ER, leading to UPR in eukaryotes (26). With in vitro evidence of hRes as a probable chaperone, we further tested its role during induced ER stress by checking the expression levels and localization of hRes during tn- and tp-induced ER stress in U937 cells. Levels of hRes protein were observed to be substantially elevated under both these treatments. It therefore appears that, like many other chaperones, hRes was also overexpressed during ER stress. Interestingly, hRes also colocalized with calreticulin in a time-dependent manner, indicating that it remained in the ER during stress induced by tn and tp treatment. This is in agreement with literature reports that chaperones are restricted to the ER upon stress to assist in the refolding of misfolded proteins (27).

The logical question, then, is that, if hRes is secreted out, how can it possibly function as a chaperone inside the cell? We therefore looked at the levels of secreted hRes under normal and stress conditions. Levels of secreted hRes during ER stress were significantly less compared with nonstress conditions. However, intracellular levels of hRes under conditions of ER stress were significantly higher. This supports the scenario that, upon ER stress, a significant fraction of hRes was retained inside the cell, within the ER, to aid in protein folding. As a corollary, under non-ER stress conditions, the secretory hRes then functions as a proinflammatory molecule.

In two prospective studies of patients with sepsis (28), resistin was observed to be a marker of severity of disease and was a possible mediator of prolonged inflammatory state seen in critically ill patients. hRes was suggested as a biomarker for disease staging and treatment end-point determination in tuberculosis (29). Some human disorders, such as Alzheimer's disease, Parkinson's disease, cystic fibrosis, and neuronal damage by ischemia, are related to ER stress, which leads to intraluminal accumulation of unfolded proteins (30). The presence of an ER stress response element in the resistin gene additionally points to its role in stress-signaling pathways. The unfolded protein responses are central to inflammatory disorders, thereby establishing a fine link between protein folding and inflammation (31).

Cellular stress and strong inflammatory response are thus increasingly being linked to a number of human disorders (31). Our observations on chaperone-like features of hRes constitute a line of evidence in support of this function of hRes. The hypothetical model in Fig. S9 explains the dual nature of human resistin. Human resistin is predominantly secreted from macrophages as a proinflammatory cytokine upon stimulations such as those caused by infection. Upon stress, resistin is retained inside the ER of stressed cells where it functions like a chaperone,

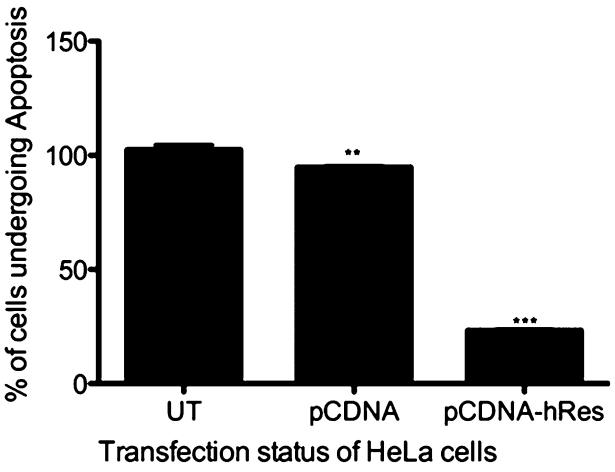

Fig. 6. Resistin expression causes an inhibition of apoptosis of HeLa cells. HeLa cells were transiently transfected with pCDNA plasmid vector, recombinant pCDNAhRes plasmid construct, or mock transfected (UT) HeLa cells. Apoptosis was induced by treating cells with thapsigargin for 24 h, and the percentage of apoptosed cells was scored by Annexin V staining using FACS. The percentage of cells undergoing apoptosis in untransfected condition was normalized to 100 and results were plotted. Statistical analysis was performed by two-way ANOVA and post-Bonferroni test (***$P < 0.0001$, **$P < 0.001$). Each experiment was carried out in triplicate.

helping in refolding of misfolded proteins, thereby rescuing the cells from stress-induced apoptosis. hRes therefore may have a role in modulating UPR during ER stress under physiological conditions in addition to its role as a regulator of inflammation (4, 32). The role of hRes as a chaperone-like molecule is strongly indicative of this chemokine acting as a connecting link between stress response and inflammation.

Materials and Methods

Also see *SI Materials and Methods* for more information.

Cell Lines and Cell Culture. U937 and HeLa cells (procured from National Centre for Cell Science, Pune, India) were cultured in RPMI-1640 media supplemented with 2 mM L-glutamine, 100 U of penicillin/mL, 100 μg of streptomycin/mL, and 10% FBS and maintained at 37 °C with 5% (vol/vol) CO_2.

MD Simulations. MD simulations of hRes and F49YhRes structures in trimer and hexamer forms at 298 K and 333 K for 20 ns were carried out using GROMOS96 53a6 force fields in GROMACS-4.0.7. The graphs were generated using "Grace."

Purification of Recombinant Wild-Type and Mutant hRes Protein. *E. coli* M15 cells were transformed with plasmids pQE30hRes (Fig. S7) and pQE30-F49YhRes. Recombinant proteins (rhRes and F49YrhRes) were purified by affinity chromatography as described (9), and the purity was checked by FPLC and mass spectrometry. Protein had no detectable contaminants.

Aggregation Assays. One micromolar of each protein, namely of rhRes, F49YrhRes, CS, and Nde1 (5 U), was incubated at 45 °C for 45 min, and the aggregation was monitored by light scattering at 320 nm using a Perkin-Elmer spectrofluorometer as per protocol described earlier (15).

Coimmunoprecipitation Assays. Five microliters of polyclonal antisera against hRes was used to coimmunoprecipitate native and denatured CS enzyme and LEF4 (33) protein.

Growth Rescue of *E. coli*. The IPTG-induced and uninduced *E. coli* M15 cells, transformed with either pQE30 vector alone or pQE30hRes or pQE30F49YhRes and incubated at either 37 °C or 50 °C for 45 min, were checked for cfu to assess fold differences upon survival.

Tp-Induced Apoptosis of HeLa Cells. Resistin was cloned with its secretory signal in pCDNA (mammalian expression vector) (pCDNAhRes) (Fig. S7*B*) and transfected in HeLa cells using Lipofectamine 2000 (Invitrogen) after 80% confluency in serum and antibiotic-free DMEM media. Empty pCDNA was used as control. After 24 h, expression of resistin was confirmed by RT-PCR. Apoptosis was induced by 15 μg of tp for 24 h. The percentage of apoptosed cells were scored by FACS (BD) after Annexin V (Invitrogen) staining. Statistical analysis was performed using one-way ANOVA and post analysis was carried out using Dunnet's multiple comparison test.

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