Functional Characterization of Zmp1 (Rv0198c), a Zinc metalloprotease of *Mycobacterium tuberculosis*

Thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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CERTIFICATE

This is to certify that this thesis entitled **"Functional Characterization of Zmp1 (Rv0198c), a Zinc metalloprotease of *Mycobacterium tuberculosis"*** submitted to the University of Hyderabad by Mani Harika Vemula, for the degree of Doctor of Philosophy, is based on the studies carried out by her under my supervision. I declare to the best of my knowledge that this work has not been submitted earlier for the award of degree or diploma from any other University or Institution.

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I, Mani Harika Vemula, hereby declare that the work presented in my thesis is entirely original and was carried out by me in the Department of Biochemistry, University of Hyderabad, under the supervision of **Dr. Sharmistha Banerjee.** I further declare that this work has not been submitted earlier, in part or in full, for the award of degree or diploma to this University or from any other University or Institution.

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ABBREVIATIONS

µg	**micro gram**
µm	**micro meter**
µM	**micro Molar**
µL	**micro litre**
AIDS	**Acquired Immune deficiency syndrome**
AIM2	**absent in melanoma 2**
ANOVA	**Analysis of variance**
APC	**Antigen presenting cell**
ATP	**Adenosine Triphosphate**
BAL	**Bronchoalveolar lavage**
BCG	**Bacillus Calmette-Guerin**
BSA	**Bovine serum albumin**
CCL	**chemokine (C-C motif) ligand**
CD	**Circular Dichroism**
CD	**Clusters of Differentiation**
CFU	**Colony forming unit**
CFP10	**10 kDa culture filtrate antigen**
CHO	**Chinese Hamster Ovary**
CTL	**Cytotoxic-T-lymphocyte**
CXCL	**chemokine (C-X-C motif) ligand**
DCs	**Dendritic cells**
DC-SIGN	**Dendritic cell-specific intercellular adhesion molecule-3-**
DMEM	**Dulbecco's Modified Eagle Medium**
DMSO	**Dimethyl Sulphoxide**
DNA	**Deoxyribonucleic acid**
dNTP	**Deoxynucleotide triphosphate**
dpf	**day post fertilization**
dsDNA	**Double stranded DNA**
DTT	**Dithothreitol**
E. coli	***Escherichia coli***
EDTA	**Ethylene diaminetetraacetic acid**
ELISA	**Enzyme-linked immunosorbent assay**
EPTB	**Extra-Pulmonary tuberculosis**
ESAT6	**6 kDa early secretory antigenic target**
ESX	**Type VII secretion systems**
FACS	**Fluorescence-activated cell sorting**
FBS	**Fetal bovine serum**
FP	**Forward Primer**
HEK	**Human embryonic kidney cells**
HIV	**Human Immunodeficiency virus**
HRP	**Horse radish peroxidase**
IL	**Interleukin**
IFN-γ	**Interferon gamma**
kDa	**Kilo Dalton**
M. bovis	***Mycobacterium bovis***
M. smegmatis	***Mycobacterium smegmatis***

M. leprae	***Mycobacterium leprae***
M. marinum	***Mycobacterium marinum***
M. tb	***Mycobacterium tuberculosis***
MCP-1	**Monocyte chemoattractant protein1**
MHC	**Major Histocompatibility complex**
MIP	**Macrophage inflammatory protein**
mL	**Milli Litre**
mM	**Milli Molar**
MMP	**Matrix metalloproteases**
MOI	**Multiplicity of infection**
TBC	**Mycobacterium tuberculosis complex**
MΦ	**Macrophage**
NaCl	**Sodium Chloride**
NFκB	**Nuclear factor kappa-light chain enhancer of activated B cells**
NK	**Natural Killer cell**
NLRP3	**NACHT, LRR and PYD domains-containing protein 3**
nm	**nano metre**
nM	**Nano Molar**
NO	**Nitric oxide**
NRAMP1	**Natural resistance-associated macrophage protein 1**
OADC	**Oleic acid Albumin Dextrose Catalase**
^{o}C	**Degree Celsius**
OD	**Optical density**
PAMP	**Pathogen associated molecular pattern**
PCR	**Polymerase chain reaction**
PDB	**Protein databank**
PMA	**Phorbolmyristate acetate**
PMSF	**Phenyl methane sulfonyl fluoride**
PNK	**Poly nucleotide kinase**
PPD	**Purified protein derivative**
PRR	**Pattern recognition receptor**
PTB	**Pulmonary tuberculosis**
RANTES	**Regulated on activation, normal T cell expressed and secreted**
Rip	**Regulated intramembrane proteolysis protein**
RNA	**Ribonucleic acid**
RNI	**Reactive Nitrogen Intermediates**
RNS	**Reactive Nitrogen Species**
ROI	**Reactive oxygen Intermediates**
ROS	**Reactive oxygen species**
RP	**Reverse Primer**
RPM	**Revolutions per minute**
RPMI	**Roswell Park memorial institute**
RT	**Room temperature**
SD	**Standard deviation**
SDS-PAGE	**Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis**
TB	**Tuberculosis**
TEMED	**N,N,N',N'-tetramethyl ethylene diamine**
TFA	**Trifluoro acetic acid**

TGF	**Tumor growth factor**
Th1	**T-helper cell 1**
Th2	**T-helper cell 2**
THP-1	**Human monocytic cell line**
TLR	**Toll like receptor**
TNF	**Tumor necrosis factor**
TNFR	**Tumor necrosis factor-receptor**
Treg	**Regulatory T cells**
Tris	**Tris (hydroxymethyl) amino methane**
WHO	**World health organization**
ZN	**Ziehl-Neelsen staining**

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Chapter 1
Introduction

Introduction

1.1 Tuberculosis: preview

Tuberculosis (TB) is a highly contagious, chronic, airborne infection caused by bacilli belonging to genus mycobacteria, primarily, *Mycobacterium tuberculosis* (*M. tb*) (Tuberculosis:FactSheet 2000). *M. tb* mainly attacks lungs (called pulmonary TB), however may also target a plethora of other tissues in human host, including bone, kidney, lymph nodes etc (called extra-pulmonary TB) (Smith 2003). The clinical symptoms of Pulmonary TB (PTB) includes recurring fever, night sweats, persistent cough, weight loss, fatigue, chest pain and blood in sputum, subjected to severity of the disease, while Extra-pulmonary TB (EPTB) are more difficult to diagnose owing to highly diffused symptoms but are certainly associated with unexplained fever, cough and weight loss (Sharma and Mohan 2004). Most infections, however, remain asymptomatic and are referred to as latent TB. TB is completely curable, however, despite remarkable progress in social awareness, better diagnosis, availability of drugs and effective TB care through direct observed therapy (DOTS), TB continues to pose a major health challenge worldwide, especially as a co-epidemic with HIV. In India, approximately 2 million TB cases registered annually, equivalent to about one-fifth of the global cases and tops the list of TB afflicted countries. With World Health Organization (WHO) estimating 40% of Indian population infected with TB causing 3 lakh deaths annually, TB is one of the major health concerns of India (2014). Though there is a decline in the prevalence, incidence and mortality of TB cases in comparison to 1990 (Figure 1), India still exists among the 22 high-burden countries of TB (Globaltuberculosis 2015).

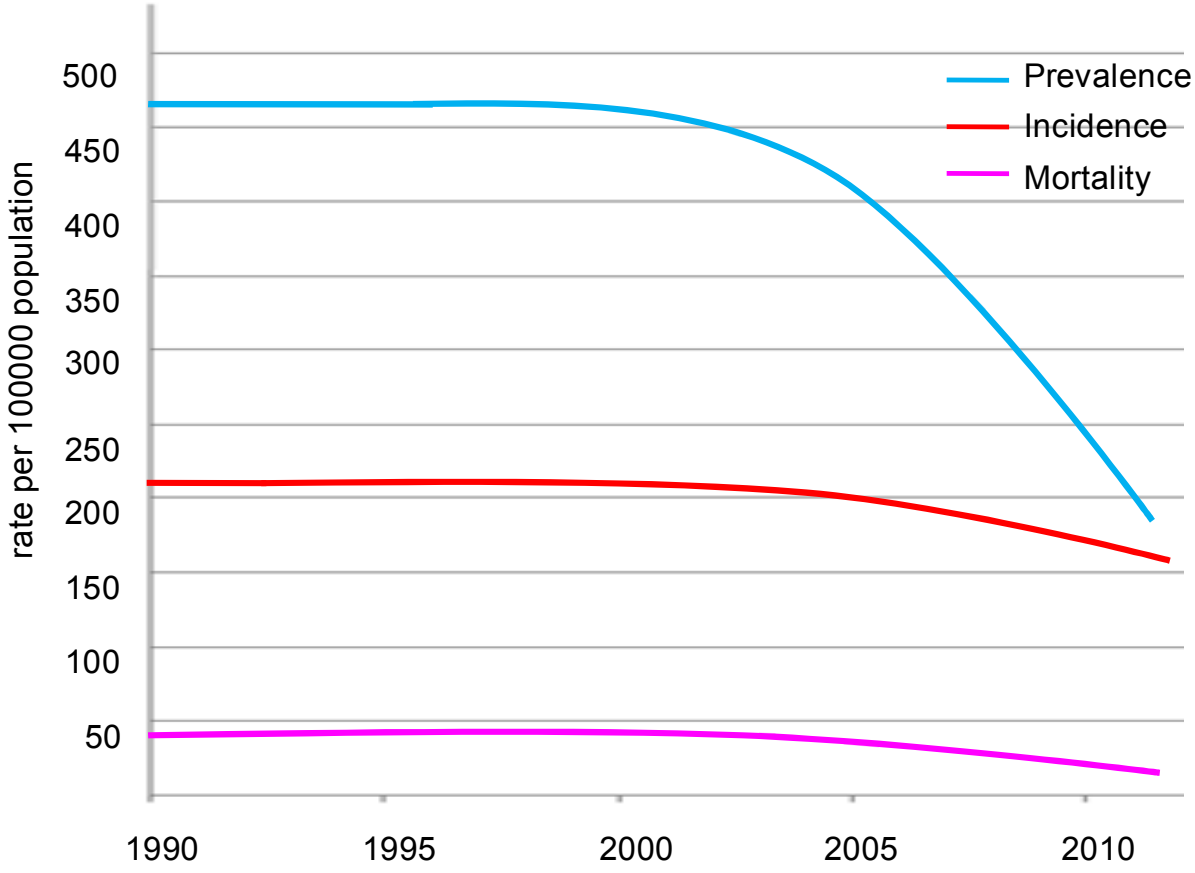

Figure 1: Representing estimated TB Mortality, Incidence and Prevalence rates 1990-2015, in India (adapted from (Global Tuberculosis Report 2015)).

1.2 *Mycobacterium tuberculosis* (*M. tb*): Historical perspective

M. tb is an ancient cohabiter of mankind. Modern molecular tools have enabled palaeomicrobiologists to detect the presence of tubercle bacilli dating back to nine thousand years old Neolithic settlement in Eastern Mediterranean (Hershkovitz, Donoghue et al. 2008). Not even in the nineteenth century could people envisage that TB is communicable, which required decades of work with seminal contribution from Jean-Antoine Villemin, a French army doctor. The breakthrough discovery of the etiological microorganism responsible for this deadly disease came from Dr. Robert Koch, a German physician and microbiologist, in the year 1882. He also formulated a glycerin extract of pure *M. tb* culture, which he called 'tuberculin' and claimed to cure TB. In recent times, tuberculin is replaced with purified protein derivative (PPD) of *M. tb* culture filtrate and is used routinely in detection of TB cases by a test called Mantoux

test (Snider 1982; Booth 2001; Nayak and Acharjya 2012). Thirteen years, from 1908 to 1921, rigorous efforts by French bacteriologists Albert Calmette and Camille Guerin resulted into development of a vaccine which was named after them and commonly known as Bacillus Calmette Guerin (BCG) vaccine. This strain has been potent in curbing TB in childhood cases efficiently but had varying results in case of adults (Luca and Mihaescu 2013; Hawn, Day et al. 2014). Though the efficacy of this vaccine is questionable, BCG is still administered in many countries, including India (Fine 1995). With the commencement of the era of antibiotics and the discovery of the antimycobacterial drugs such as streptomycin, isoniazid, the rifampicins, and pyrazinamide, periodically from 1944 to 1980, tremendously helped TB disease management and control (Kanabus 2016). Today, TB is curable with a six months regular course yet it accounts for 2-3 million deaths per year, which surely is unacceptable. Emergence of drug resistance, HIV epidemic, inability to detect asymptomatic yet infective cases and decreasing efficiency of the vaccine in adults possibly are some of the major reasons for this resurgence (Globaltuberculosis 2015). This necessitates novel drugs, new strategies for target identification & vaccine identification along with new biomarkers for monitoring disease progression and detecting latent TB.

1.2.1 The pathogen

Mycobacterium species are non-sporulating, immotile, and gram positive acid fast bacilli with rod shaped structures of 1-4 μm in length and 0.3-0.6 μm in width. They belong to the order *Actinomycetales* under the family *Mycobacteriaceae*. These bacteria have unique mycolic acids in their cell envelope which are crucial for the structure and function of the cell wall. The properties of the cell wall such as acid-fastness with high hydrophobic nature, resistance towards pH variations, antibiotics which confer resistance towards host are characteristics specific to the *Mycobacterium* genus (Sakamoto 2012). *Mycobacterium tuberculosis* is a member of *Mycobacterium tuberculosis* complex (TBC) which consists of 6 other obligate pathogenic species such as *M. africanum*, *M. bovis*, *M. canetti*, *M. caprae*, *M. microti* and *M. pinnipedii*. Though all these species have the ability to cause TB, they differ distinctly in their phenotypic characteristics and host range but genetically seem to be closely related (Tuberculosis:FactSheet 2000). *M. bovis* species causing TB in cattle differs from *M. tb* in 0.05% of its total genome. M. *tb* is a slow growing pathogenic bacterium with a replication time of 12-24 hrs with unique

immunomodulatory properties. This slow growth is probably attributed to its limited nutrient intake by the extremely impermeable cell wall and the rate of RNA synthesis (Harshey and Ramakrishnan 1977; Daffé and Draper 1997). Under infection conditions mycobacteria mostly shifts its metabolism by using lipid as its carbon source to survive in the host. *M. tb* is a facultative intracellular parasitic bacterium with ability to multiply inside the host phagocytic cells such as monocytes and macrophages whereas other mycobacterial species are mostly environmental saprophytic bacteria (Forrellad, Klepp et al. 2013).

1.3 Infection and disease

1.3.1 Recognition of *M. tb* by the cells of innate immune system

M. tb enters human body through aerosolic route when droplets carrying infective bacteria are inhaled. The inhaled bacteria are carried to lower airways and then to alveoli where these are phagocytosed by alveolar macrophages and sub mucosal dendritic cells. In 70% of the cases, infection gets cleared by the host, with the remaining 30% causing active or latent disease. Infection by *M. tb* strain leads to active disease with symptoms in only 10-20% of the cases whereas in the remaining cases (80-90%), host immune response helps in the arrest of bacteria, which are either cleared or contained inside the granuloma. In cases when disease is established, *M. tb* succeeds in evading host immune mechanisms by degrading the microbicidal mechanisms of the host immune cells such as arresting phagolysosomal fusion, modulating antigen presentation by MHC class I, class II and CDI molecules, inhibiting the release of NO, RNI and ROS and, if required, remain in dormant state known as Latent Tuberculosis Infection (LTBI) (Chan, Tanaka et al. 1995; Smith 2003; Barth, Remick et al. 2013). The non-replicating bacilli revives and cause active TB (5-10%) by perturbing host immune mechanisms upon any secondary infection such as HIV infection (Walker, Meintjes et al. 2013) (Figure 2).

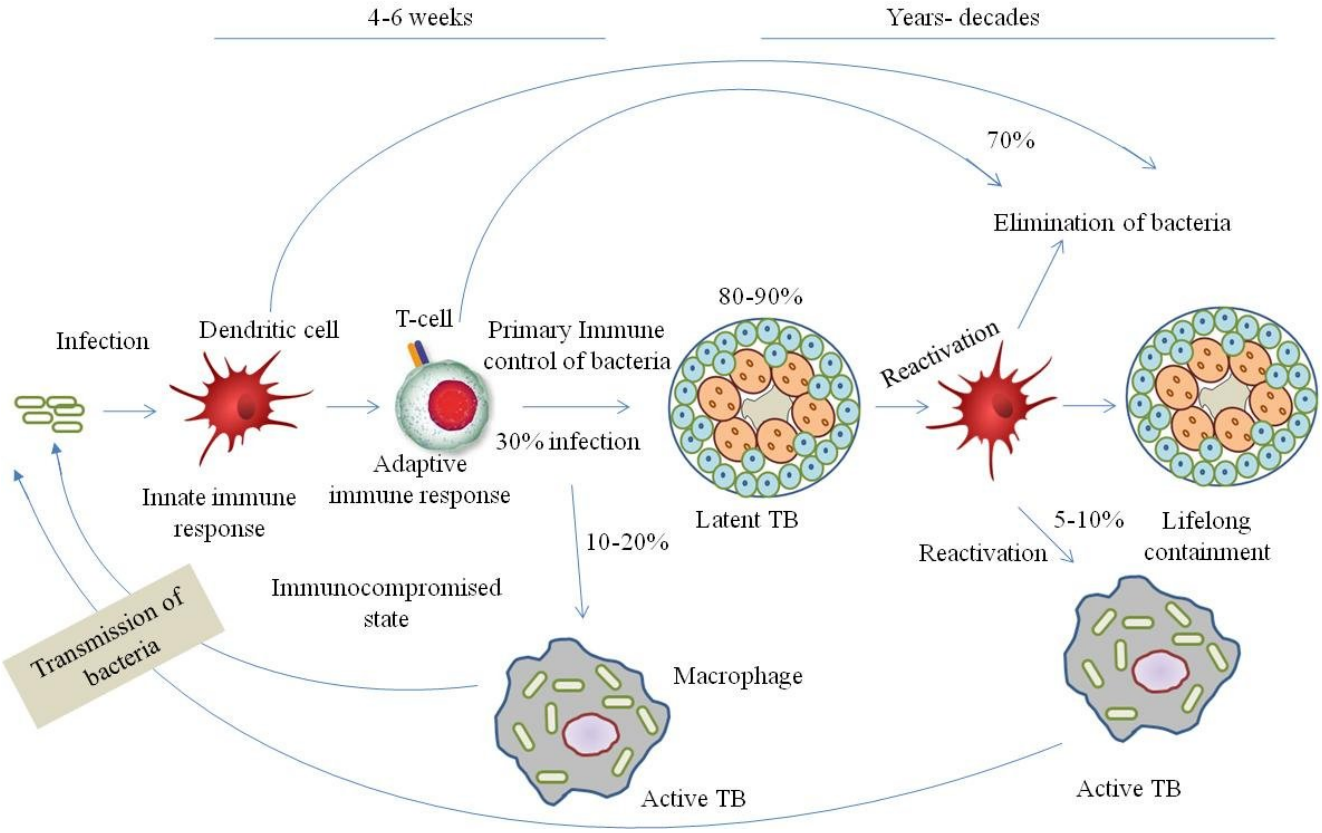

Figure 2: Infection cycle of *Mycobacterium tuberculosis*. On exposure to *Mycobacterium*, based on the immunity, there would be elimination of bacteria (70%) or an active primary TB infection (10-20%). Another outcome which leads to slow progression of disease is the latent infection with the containment of bacteria in the granuloma (80-90%). Reactivation (5-10%) of latent infection by any other secondary infection leads to active disease (adapted from (Young, Stark et al. 2008)).

Granulomas are formed by the lining of immune cells, macrophages and activated T lymphocytes which separate the growing necrotic tissue which can limit the increase and spread of the infection. Most of the Mycobacteria are killed in these caseating granulomas, further arresting the disease progression (Ramakrishnan 2012; Guirado and Schlesinger 2013).

As mentioned earlier, *M. tb* infects the host by phagocytosis of bacteria by antigen presenting cells in the lung such as alveolar macrophages. Pathogen-associated molecular patterns (PAMPs) are recognized by Pathogen recognition receptors (PRRs) of the host which initiate the host immune response. Mycobacterial cellular components and cell surface ligands which are exposed onto the surface are recognized by host receptors such as Nod like receptors (NLRs), C-type

lectins and Toll like receptors (TLRs). These mycobacterial ligands include lipomannan and mannose-capped lipoarabinomannan (ManLAM), 27 kDa, 19 kDa, LprA, and LprG lipoproteins, mammalian cell entry (Mce) proteins and 38 kDa glycolipoprotein (Noss, Pai et al. 2001; Kang, Azad et al. 2005; Jung, Yang et al. 2006; Ahmad 2011). Other strong receptors are complement receptors, cholesterol receptors, surfactant protein A receptors and scavenger receptors that recognize mycobacterial cell wall and cell components (El-Etr and Cirillo 2001). The interaction of *M. tb* with TLRs is the initial event in the course of infection. TLR2 and TLR4 and their polymorphisms specifically modulate the innate immune response towards mycobacterial lipopeptides and sensitivity to pathogens. Interaction of *M. tb* ligands with TLRs stimulate NFκB and release battery of cytokines such as TNFα (Tumor Necrosis Factor alpha), IL-12, chemokines involved in pro-inflammatory responses and nitric oxide release by myeloid differentiation primary response protein 88 (MyD88)-dependent or MyD88-independent pathways. CD207, DC-SIGN, and Dectin-1 which are membrane bound PRRs of C-type lectins transmit inflammatory signals whereas NLRs and cytosolic PRRs regulate pathogen identification by the host (Jo 2008; Ahmad 2011; Kim, Sohn et al. 2012).

Neutrophils, that are yet another cell of innate wing, not only function by randomly engulfing, but also by secreting anti-bacterial proteins, like cathelicidins etc. The infected neutrophils undergo apoptosis, thereby activating macrophage further. Professional phagocytic cells like macrophages also generate stress to intracellular mycobacteria by either sequestering essential metal ions like iron, zinc or copper or pumping excess of these ions to poison intracellular microorganisms (Stafford, Bokil et al. 2013). Natural Killer cells reach the site of bacterial infection and in the process of destroying infected host cells secrete interferon gamma (IFN-γ), which further activates macrophages making them aggressively attack intracellular mycobacteria by oxygen-dependent and independent pathways. IFN-γ also induce macrophages to secrete a battery of pro-inflammatory cytokines like IL-12, IL-15 and IL-18, which activate CD8+T-cells, thus linking the innate to the adaptive immune system.

Conversely, the *M. tb* has evolved with strategies that can dampen the innate immune responses for its survival inside the host. TLR-induced signaling pathway needs to be modulated by the host to avoid over damage to the host-tissues. This is performed by a group of tyrosine kinase receptors Tyro3/Axl/Mer (TAM) by a negative feedback mechanism (Rothlin, Ghosh et al. 2007). The 19 kDa lipoprotein of *M. tb* has taken advantage to exploit this mechanism by using

TLR2 receptors and regulate the host immunity and presentation of antigen in the cell. This interaction for over a period of time suppresses the expression of MHC class II molecules and further dampens the processing of antigen by macrophages (Fulton, Reba et al. 2004; Pai, Pennini et al. 2004). So, part of the infected macrophages have lowered antigen presenting function and are unable to present the *M. tb* antigens leading to decreased activation of effector cells and further providing an environment suitable for mycobacteria to develop niches and therefore its survival. Another mechanism of evasion is by ManLAM which can inhibit mannose-receptor dependent IL-12 production and also limits phagolysosome fusion. This inhibits macrophage response towards *M. tb* and promotes the infection (Kang, Azad et al. 2005). Pathogenic strains of *M. tb* have evolved with abilities to inhibit NFκb mediated macrophage activation using antigens like ESAT-6, ManLam and CFP-10 (Natarajan, Kundu et al. 2011). ManLam is known to down regulate the levels of IL-12 and ESAT-6 inhibits MyD88-IRAK4 interaction further affecting the TLR signaling towards NFκb. *M. tb* infected macrophages non-specifically kill the infected cells and CFP-10, another antigen of *M. tb* decreases this ability of macrophages and thereby their NO and ROS production (Gupta, Kaul et al. 2012). ESX5, a part of ESX, the protein transport system of *M. tb*, was found to modulate macrophages by curbing inflammasome activation (Bottai, Di Luca et al. 2012). These survival mechanisms of *M. tb* help its escape from phagolysosomes. Although innate immune responses play role in protecting from TB infection, adaptive immune responses are more effective to clear the infection.

1.3.2 Humoral response

Mycobacteria have developed new evasion strategies using the adaptive immune responses. They impede the antigen presentation and augment the secretion of IL-10 shifting the balance towards Th2 response thereby inhibiting the secretion of IFN-γ (Gupta, Kaul et al. 2012). Also, they may attract the Treg cells to diminish the pro-inflammatory response. Studies with TB infected rabbit model demonstrated that mycobacteria hinder the macrophage activation and allow them to develop an infection inside the pulmonary tissue (Subbian, Tsenova et al. 2011). During latency, mycobacteria consist of a specific set of genes called *rpf* genes coding for Rpf proteins to activate bacteria from dormant state. They also possess DosR regulon which codes for anti-dormancy genes to regulate bacterial growth when required (Leistikow, Morton et al. 2010).

Patients with active TB disease along with latent infection have no defects in innate or acquired immunity whereas they show an increased number of lymphocytes with profuse cytokine environment. This is a result of the immunocompromised state in the case of TB infection (Handzel, Barak et al. 2007). Adaptive immune markers such as CD4+ T cells along with CD8+ cells, Th17 and B-cells are involved in antigen presentation via Class I MHC molecules provoking cytotoxic response (Gupta, Kaul et al. 2012; Philips and Ernst 2012). Immuno-dominant mycobacterial antigens such as ESAT-6, CFP-10, Rv2031c, Rv2654c and Rv1038c act independent of the stage of infection and release various pro-inflammatory cytokines such as IL-1β, IL-6, IL-21 and IL-12p40 (Torrado and Cooper 2010; Arlehamn, Sidney et al. 2012). γδ T cells secrete IL-17 under acute TB infection which induces IL-12 thereby forming a self inducing inflammatory loop which is regulated by TGFβ. TGFβ further inhibits the surfeit responses from Treg cells which allows the replication of *M. tb* in the macrophages by shifting the balance from Th1 type (Marin, París et al. 2010). Once the T-cells are activated, inflammatory cells release chemokines which help T-cells to undergo clonal expansion to reach the site of infection. Macrophage gets activated at the infection site by the release of IFN-γ which stimulates IL-18 production by activating the microbicidal machinery leading to dominant Th1 protective response (Al-Muhsen and Casanova 2008; Handzel 2013). However, in Multi Drug Resistant (MDR) strains alteration towards the Th2 response is observed due to discrete polymorphisms in few genes such as NRAMP1 whereas in children it is because of Mendelian heritability defects for the shift towards Th2. Mutational defects in these mechanisms make host more vulnerable to TB disease. So it distinctly shows that host response towards TB is preferably a Th1 kind of response except in the cases of Multi Drug Resistant TB (MDR-TB) and child TB where IL-4 levels are inflated leading to a Th2 type response (Alcaïs, Fieschi et al. 2005; Tan, Xie et al. 2012).

Previous reports have shown that the protective immunity from the serum in *M. tb* infection does not have much role to access the mycobacterial components (Johnson, Cooper et al. 1997). Recent studies have shown that monoclonal antibodies can be more potent and disproved the ineffective role of humoral immunity (Bosio, Gardner et al. 2000). The mycobacterial antigens induce B-cell response which can be a major breakthrough to identify diagnostic candidates. In this regard, several antigens have been identified using mice studies such as 71, 65, 38, 23 kDa proteins and so on, but all does not seem to be probable targets. These immuno-dominant

antigens identified can be cloned into recombinant vectors and checked for the humoral response in infected patients. Further the specific mechanism of action of B-cells in TB disease in humans needs to be resolved to encounter the pathogen (WHO 1986), as these ancient microbes easily get adapted to the counter attacks of the host. Therefore, the combat between the bacteria and various cellular components of the immune system stimulates intricate responses from the host and pathogen playing a vital role in their survival (Handzel 2013).

1.4 Cytokines and Chemokines during *M. tb* infection

M. tb infection induces a large subset of cytokines where few are known to play role in control of infection and others to associate with pathogen damage. The immunological response is mostly towards Th1 type in the infected patients inducing IL-12 and production of IFN-γ (Cooper, Roberts et al. 1995).

TNFα: TNFα is stimulated by multiple ways and is known have to several functions. In response to *M. tb*, it induces macrophages and neutrophils in both autocrine and paracrine fashion to stimulate apoptosis and release of Reactive Oxygen Intermediates (ROI) and Reactive Nitrogen Intermediates (RNI) species which destroys the phagocytosed pathogen (Gan, He et al. 2005). TNFα is critical in granuloma formation by recruitment of leukocytes and stimulation of chemokines. It enhances the expression of adhesion molecules affecting the formation of granulomas (Roach, Bean et al. 2002). Mutations in TNF and TNFR (TNF receptor) genes are more succumbed to TB infection. TNFα deficient mice were defective in granuloma formation leading to acute TB infection. TNFα neutralizing drugs have increased the recurrence of latent TB cases. After TNFα neutralization, expression of *nos2 (nitric oxide synthase 2)* was reduced; granulomas were disorganized with less activated macrophages and diffused cellular infiltration was observed. Studies have shown that TNFα neutralized mice had severe pathology in lungs with meager increase in the bacterial load implicating its contribution towards restricting the pathologic response for the TB infection (Guirado and Schlesinger 2013). Experiments with recombinant BCG overexpressing TNFα resulted in destructive inflammation. Shifting the balance of TNFα towards pathogen increases lung pathology and necrosis of the infected tissues.

IL-12: IL-12 is an important cytokine in controlling *M. tb* infection. Exogenous administration of IL-12 in BALB/c mice had improved survival under infection whereas IL-12-/- mice were more susceptible. In humans, defective genes of IL-12p40 or IL-12 receptor decreased production of IFN-γ from T-cells with increasing susceptibility towards infection. Reports have also shown that in chronic infections, bacterial numbers can be lowered in mice by using IL-12 DNA implying its role in the design of TB vaccine (Ottenhoff, Kumararatne et al. 1998; Raja 2004).

IFN-γ: IFN-γ is a crucial cytokine in control of *M. tuberculosis* infection and activates macrophages secreting ROS and RNS. It is majorly produced by CD4 and CD8 T cells, Natural killer cells, antigen presenting cells and limited amounts by B-cells (Martinez, Helming et al. 2009). Mutations in IFN-γ or IFN-γ receptor genes or downstream signaling pathways makes an individual more susceptible to chronic and non-tuberculous mycobacterial infections (Dorman and Holland 2000). IFN-γ knockout mice are the extremely susceptible mouse strains for TB infection as they have impaired macrophage activation with decreased levels of iNOS (inducible nitric oxide synthase). But still inos-/- mice studies have shown an increased survival rate in comparison with IFN-γ knockout mice indicating an inos independent mechanism for IFN-γ. When IFN-γ-/- mice were infected with BCG, there is an increased incidence of bacterial load and large disorganized granulomas were formed. Patients treated with exogenous IFN-γ had clinical improvement in their condition whereas patients with low levels of IFN-γ in conditions such as AIDS were more prone to *M. tb* infection (Law, Jagirdar et al. 1996). Thus, IFN-γ contributes for the control of *M. tb* infection and studies also suggest additional factors associated for its protective role against infection (Sakamoto 2012).

Chemokines: Chemokines are small proteins that can bind to heparin which comprise a large family of peptides that are structurally related to cytokines. Their main function is to modulate cell trafficking and are secreted in response to pro-inflammatory cytokine signals for selectively recruiting monocytes, neutrophils and lymphocytes (Callewaere, Banisadr et al. 2007). They are also known to involve in pathogenesis of various diseases such as HIV, auto-immune diseases, pulmonary diseases etc., Based on their function they have been classified as homeostatic and inflammatory chemokines (Deshmane, Kremlev et al. 2009). Homeostatic chemokines include

CCL14, CCL19, CCL20, CCL21, CCL25, CCL27, CXCL12 and CXCL13 which are constitutively expressed and are responsible for basal leukocyte migration inducing adaptive immune responses. Inflammatory chemokines are CXCL-8, CCL2, CCL3, CCL4 mostly involved in pathogenesis by attracting cells to the site of infection. Expression of chemokines and chemokine receptors is known to be associated with the formation and maintenance of the granulomas in chronic TB infections (Rhoades, Cooper et al. 1995). In this regard, we discussed below the chemokines involved in the pathogenesis of TB.

Interleukin 8 (IL-8) is a an important chemokine produced by macrophages, epithelial cells, airway smooth muscle cells, fibroblasts and in lower amounts by keratinocytes and lymphocytes. IL-8 induces a cascade of reactions essential for migration of cells and their phagocytosis by increasing intracellular Ca2+ levels, exocytosis by release of histamines and oxidative burst by the release of ROS. In the host-pathogen interaction, IL-8 is released which employs neutrophils, T-cells and granulocytes near the site of infection. Studies have shown that increased levels of IL-8 in BAL fluid of the patients and their mRNA levels have been more in comparison to normal subjects (Zhang, Broser et al. 1995). Mostly IL-8 is stimulated by the *M. tb* pathogen directly or by the lipoarabinomannans (LAMs) but deacylated LAMs are unable to stimulate this chemokine in the macrophages. It is also observed in pleural TB patients in their pleural fluids (Ceyhan, Ozgun et al. 1996; Law, Jagirdar et al. 1996).

Other chemokines involved in host response towards infection of TB are MCP-1, MCP-3, MCP-5, RANTES, MIP-1α, and MIP-1β. *In vitro* and *in vivo* studies have shown that mycobacterial infection increased production of various chemokines such as RANTES, Macrophage inflammatory protein 1-α (MIP-α), MIP2 (Macrophage inflammatory protein 2), MCP-1 (Monocyte chemoattractant protein), MCP-3, MCP-5 and IP10 (IFN-gamma-inducible protein 10 or CXCL10) (Rhoades, Cooper et al. 1995). TNFα has the ability to upregulate the expression of MIP1-α, MIP1-β, MIP2, MCP-1, cytokine-induced neutrophil chemo attractant (CINC) and RANTES (Regulated on activation, normal T cell expressed and secreted) thereby effecting the recruitment of various cell types at certain sites of infection (Lane, Markovitz et al. 1999). Release of MCP-1 and MIP1-β was observed in human alveolar macrophages when infected with mycobacterium. MCP-1 over expressing mice were more susceptible to *M. tb* infection than MCP-/- deficient mice. Chemokine receptor CCR2, receptor for MCP-1, 3 and 5 chemokines, deficient mice showed extreme susceptibility to infection and were defective in macrophage

recruitment (Lu, Rutledge et al. 1998). Till date the chemokine pathways are not explored and the other effector molecules involved in these mechanisms remain unknown. Their role in the disease severity suggests their use as biomarkers and so expansion of this study in terms of Tb disease may help in diagnostic therapies (Slight and Khader 2013).

1.5 Mycobacterial genome

Mycobacterial genome sequencing and annotation have been done by the institute for genomic research and Sanger-Center-Pasteur institute consortium (Fleischmann, Alland et al. 2002). *M. tb* H37Rv genome consists of 4.4 million base pairs coding for about 4000 genes. About 6% of the total genome has been annotated for enzymes involved in lipid metabolism functions conferring its ability to survive inside the pathogens and use fatty acids as their major carbon source (Cole, Brosch et al. 1998). 4% of the genes comprise the unrelated PE and PPE families which are acidic-glycine rich proteins (Brosch, Gordon et al. 2000). They contain proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) sequences in the conserved N-terminal region. Similar abundance of this class is observed in other members of *M. tb* complex (TBC) such as *M. leprae* and *M. marinum* causing virulence and also in non-pathogenic strains like *M. smegmatis*. PE family consists of 104 genes in *M. tb* which has been further classified into 3 classes with 29 proteins having PE domain alone, 8 proteins having an unrelated C-terminal sequence along with the PE domain and 67 proteins possess C-terminal extensions with repeats of PGRS (polymorphic GC-rich repetitive sequences) with Glycine-glycine-Alanine or Glycine-glycine-Aspargine amino acids. These putative families of proteins are uncharacterized but probably may play crucial role due to their presence in the cell wall and envelope regions of *M. tb*. The antigenicity plots of these proteins show that they are involved in antigenic variation during infection (Banu, Honore et al. 2002). Apart from these groups, *M. tb* has 13 sigma factors contributing to the transcriptional specificity for RNA polymerases, 22 other regulatory factors including 13 two component systems which are involved in transduction of environmental signals. Also approximately 125 annotated genes corresponding to 3 % of the total genome are involved in transport functions which are equivalent to half of those genes coded by *Streptomyces* species, which is a soil-dwelling bacterium. Probable evidence comes from gene duplication and divergent evolution allowing bacteria to rapidly change to the radical environmental conditions (Bentley, Chater et al. 2002).

1.6 Mycobacterial Virulence factors

M. tb carries an array of factors that contribute for the virulence either directly or indirectly by interacting with host environment. Pathogenesis of *M. tb* involves acidification of phagosomes and their maturation processes which are inhibited by a secretory protein PtpA (tyrosine phosphatase) of *M. tb*. It binds to the H subunit of vacuolar proton ATPase machinery which helps in the acidification of phagosomes (Wong, Bach et al. 2011). *M. tb* over expresses Rv3361c-Rv3365c operon and secretes Rv3364c which is involved in inhibition of caspase-1 dependent apoptosis. It binds to cathepsin G and inhibits its activity on the membrane thereby inhibiting pyroptosis (Danelishvili, Everman et al. 2011).

Secretory proteins of *M. tb* are found to play major role in pathogenesis and are potential virulent factors. These extracellular factors are much exposed to the host receptors than the internal molecules of mycobacterium in both *in vitro* and *in vivo* studies and being studied for their immuno-dominant antigenic properties. Similar to gram negative bacteria which uses complex nano machines like type I-VI secretory systems for host cell death, mycobacteria uses type VII secretion systems coded by esx operon (Smith 2003). Mechanisms involved in pathogen survival inside the host include mainly the ESX-1 secretion system which controls the NLRP3 inflammasome activation and AIM2 inflammasome activation and cell to cell spread of infection (Briken, Ahlbrand et al. 2013). Various reports using ESX-1 secretory systems of *M. tb* and *M. marinum* have shown that they are important for virulence. In addition, studies have shown that there is no/low range of spread of infection into new host cells in non-virulent strains in comparison with the virulent strains advocating that the possible mechanism for host colonization with *M. tb* could be apoptosis (Feltcher, Sullivan et al. 2010). *M. tb* has exploited the TLR mechanisms for its benefit by using ~19 kDa secreted lipoprotein of *M. tuberculosis* which acts as an agonist of the TLR2 thereby modulates the innate immunity and antigen presenting cell function (Noss, Pai et al. 2001). Recently, factors like *Mycobacterium tuberculosis* porin A (MtpA), is observed to be secreted into the macrophage cytosol causing necrosis (Handzel 2013) and secretory proteins CFP-10 and ESAT6 are known to induce apoptosis by down regulating ROS production. ESAT-6 is also known to cause translocation of *M. tb* into the cytoplasm from phagolysosomes by causing lysis of the cells by pore formation thereby helping in cellular invasion and dissemination of *M. tb* (Kinhikar, Verma et al. 2010).

Earlier reports found a mycobacterial protein, Rv3903c (channel protein with necrosis-inducing toxin, CpnT) with ability to induce cytotoxicity. It possesses an N-terminal channel domain and a secretory toxic C-terminal domain. Mechanism of action of CpnT is by secreting the toxic domain into the extracellular milieu by forming a channel in the outer membrane suggesting it is essential for the escape of mycobacteria from macrophage and thereby help in the spread of infection (Danilchanka, Sun et al. 2014). Several enzymes such as KatG, SodA, SodC and NoxR3 play an essential role in the host susceptibility towards pathogen (Sakamoto 2012).

1.7 Metalloproteases

Physical barriers to pathogen invasion are the first line of defense exercised by the host. Quite often, tissue barriers, like mucous, extracellular glycoprotein matrices, keratin, collagen, laminin etc are targeted by bacterial proteases secreted in the milieu, for infiltration. An interesting group of secreted proteins is zinc-metalloproteases. These have been documented to contribute to the virulence of pathogenic bacteria by a variety of mechanisms. Several of these are established exotoxins and virulence factors. Extracellular zinc-containing metalloproteases constitute one such class of proteases that are omnipresent in both gram-positive and gram-negative bacterial pathogens. These zinc-containing proteases have HEXXH motif in their primary protein structure, suggesting strong evolutionary conservation across genera (Jongeneel, Bouvier et al. 1989). Several extracellular zinc-containing metalloproteases from pathogenic bacteria function as toxins and have been closely associated with their virulence and invasive properties. Some of the well-characterized are those from *Clostridium botulinum* (botulinum neurotoxins), *Clostridium tetani* (Tetenospasmin), *Bacillus anthracis* (anthrax toxin lethal factor), *Legionella pneumophila* (neutral zinc metalloendopeptidase); *Vibrio cholera* (PrtV protein) etc. Toxins of these nature causing necrotic or hemorrhagic tissue damage, are highly immunogenic and can be heat labile or highly stable to inactivation (Hase and Finkelstein 1993). Most of the known microbial toxic proteases are Zinc dependent metalloproteases like metalloproteases produced by *Vibrio vulnificus* causes septicemia (Miyoshi, Oh et al. 1993) by inducing inflammatory mediators like histamine and bradykinin and two other metalloproteases produced by *Pseudomonas aeruginosa* digest wide variety of host proteins such as structural components of the basement membrane and proteins involved in coagulation or complement action (Wretlind and Pavlovskis 1983).

These metalloproteases have zinc as their integral component playing role in various aspects of metabolism. Structural studies such as x-ray crystallographic analysis of these zinc proteins have shown that the catalytic zinc atom is coordinated to three amino acid residues along with an active water molecule where as structural zinc atoms are coordinated to four cysteine residues. Addition of this metal in to the protein induces conformational changes and further allows the regulation of their enzymatic activity (Vallee and Auld 1990). Zinc activates the water molecule and acts as a nucleophile to attack the carbonyl group of the peptide. Later a base in the active site turns water molecule into a nucleophile by removing a proton to act on the peptide bond (Figure 3B). The bacterial metalloproteases such as thermolysin from *Bacillus thermoproteolyticus* (Holmes and Matthews 1982), neutral proteases from *Bacillus cereus* (Stark, Pauptit et al. 1992) and elastase from *Pseudomonas aeruginosa* (Thayer, Flaherty et al. 1991) have been serving for comparison with sequences of metalloproteases for which x-ray crystallographic studies are yet to be done. The first consensus sequence for the metalloproteases family members was based on homology with human fibroblast collagenases and the 11 amino acids flanking the zinc binding site of Serratia proteases, which shares strong homology with thermolysin at this site (McKerrow 1987). Later on HEXXH motif was found to be the primary sequence motif in many zinc metalloproteases which are extra cellular in nature identified from the pathogenic organisms have been known to play role in virulence. Though these are found in all kind of species, proteases of organisms which are pathogenic are of industrial importance and being pursued further (Hase and Finkelstein 1993).

Metalloproteinases belonging to closely related zinc dependent proteases are highly regulated by gene expression and enzymatic activation and inhibition. Metalloproteinases are known to degrade extracellular matrix thereby affecting several physiological processes such as cell proliferation, migration, and cell–cell interactions. MMPs (Matrix metalloproteases) are initially expressed as holoenzymes which are further activated by cleavage. Many MMPs such as MMP-1 are known to play essential role in wound healing and neovascularization processes directly or by stimulating other effector cells. MMP1 by degrading dermal collagen type-I and MMP-2, expressed near acute wounds in association with laminin-332 helps in the migration of keratinocytes (Caley, Martins et al. 2015). MMP-7 and 9 play role in wound healing by recruitment of neutrophils and also in cell signaling. Apart from degrading cellular matrices, recent studies have shown that MMPs also have non-matrix substrates such as several cytokines,

chemokines and other growth factors indicating role of MMPs in various physiological processes of the host (Butler and Overall 2009). Besides these functions, these proteases occasionally act as toxic factors to the host especially in pathogenic micro-organisms helping their survival. Apart from these, there are other proteases which play indirect role in pathogenicity. Metalloprotease from *V. cholerae serovar O1* type is known to be responsible for the epidemic cholera by digesting small intestinal mucosa and thereby increasing the attachment of bacteria to the intestine (Finkelstein 1996). The consensus HEXXH motif with 2 histidine residues as first and second ligands were also found in clostridial neurotoxins, *Bacteroides fragilis* enterotoxin and *Bacillus anthracis* lethal factor indicating all these proteins to be zinc metalloproteases but have substrate specific proteolytic activity (Miyoshi and Shinoda 2000).

1.7.1 Zinc Metalloproteases of *M. tb*

Mycobacterial zinc metalloproteases are also known to play role in pathogenesis but still the mechanisms are unclear. The deciphered genome of *M. tb* and experimental validation have so far identified Rv0198c (zmp1), Rv2869c (rip), Rv0563 (htpX), Rv2467 (pepN), Rv3610c (ftsH), Rv1977 and Rv2625c (Stewart, Wernisch et al. 2002; Griffin, Gawronski et al. 2011; Kelkar, Kumar et al. 2011; Mazandu and Mulder 2012; Schneider, Sklar et al. 2014) zinc-metalloproteases. Rv2467c an amino peptidase specifically cleaves leucine, arginine or lysine rich peptide bonds in the substrates and therefore, a causative of virulence. Rv3610 (ftsH), a membrane bound ATP-dependent zinc metallopeptidase proteolytically regulates membrane specific and cytoplasmic proteins and is a stress-response protein allowing pathogen to surpass ROI stress helping its survival inside the host. Rv2869 (Rip), a secretory metalloprotease regulating the intramembrane proteolysis is involved in the proteolytic degradation of anti-sigma substrates like RsdA controlling the SigD axis of mycobacteria and thereby helping mycobacterial virulence. Rv0198c, a secretory zinc metalloprotease hydrolyzes neuropeptides and plays role in phagolysosome arrest and inflammasome activation. Other metalloproteases like Rv2625 is a probable conserved protein involved in DosR regulon, Rv1977 possess chaperonin function and Rv0563 hydrolyzing specific peptides needs to be explored. Although all these proteases are not well-characterized, direct or indirect evidences indicate their role in virulence and survival of pathogen inside the host. This study involved characterization of one of

the secretory zinc metalloproteases of *M. tb*, which has been implicated in regulating pathogen survival through suppressing inflammasome activation.

1.7.2 Rv0198c, Zinc metalloprotease1

Zmp1 is a putative mycobacterial zinc metalloprotease. It possess a HEIGH motif from 493-497 residues with two histidines at 493 and 497 positions along with the glutamic acid coordinated to Zinc atom (Figure 3A). Phylogeny of Zmp1 showed that it is an M13 family of Zn2+ metalloprotease involved in macrophage functions (Turner, Isaac et al. 2001; Wahl, Goetsch et al. 2005). Using DNASTAR, a phylogenic tree was made and found Zmp1 of *M. tb* and *M. bovis* (Mb0204c) to be highly similar (Figure 3C).

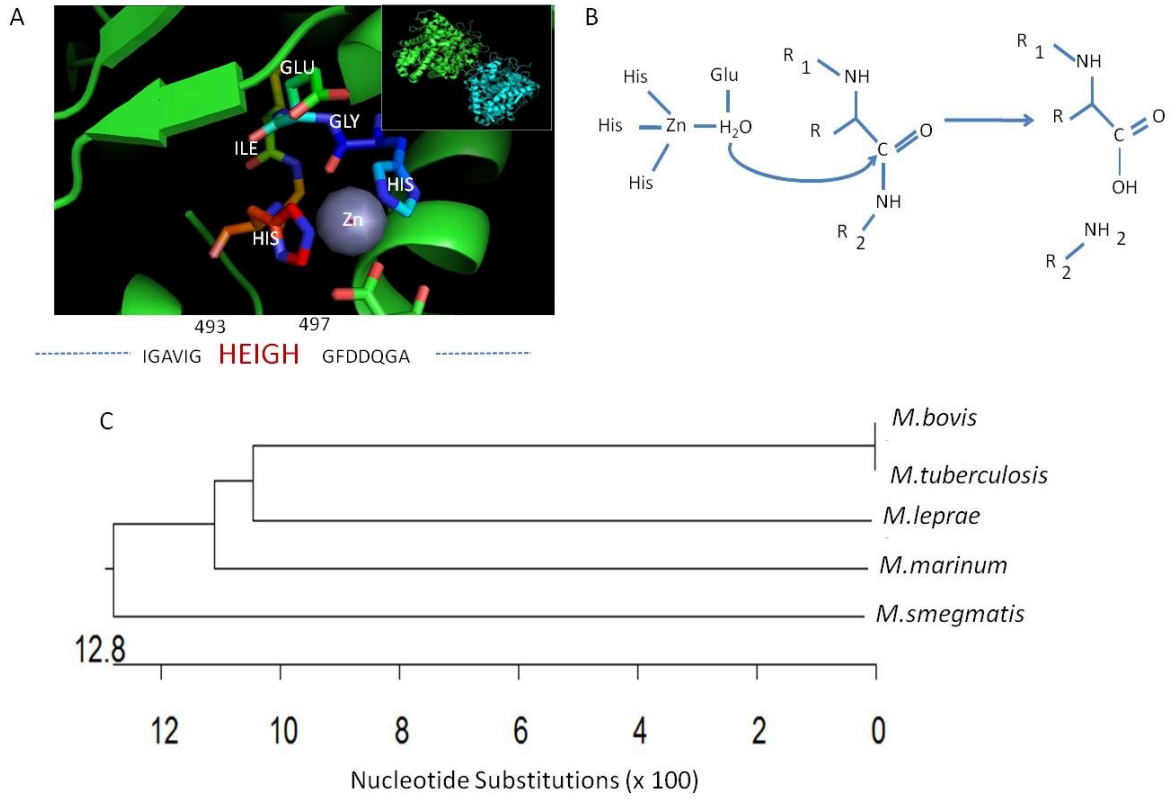

Figure 3: *M. tb* Zmp1 structure and Phylogeny. (A) Representative image of Zmp1 crystal structure highlighting the HEIGH catalytic motif coordinated with Zinc (inset Zmp1 structure) GLY-Glycine, GLU-Glutamic acid, ILE-Isoleucine, HIS-Histidine residues (adapted from (Ferraris, Sbardella et al. 2011)). (B) Reaction mechanism of Zinc metalloproteases. (C) Phylogenic tree of *M. tb* Zmp1 with Zmps of other Mycobacteria's aligned using ClustalW (MegAlign 5.00, DNASTAR Inc). *M. tuberculosis - Mycobacterium tuberculosis, M. bovis -*

Mycobacterium bovis, M. leprae - Mycobacterium leprae, M. marinum - Mycobacterium marinum, M. smegmatis - Mycobacterium smegmatis.

Reports suggest that Rv0198c (*zmp1)* gene impedes stimulation of caspase-1 and curbs IL-1β processing *in vivo*. By various measures of immune reactivity, it was shown that Zmp1deletion augmented the immunogenicity of BCG both *in vitro* and *in vivo*. (Turner, Isaac et al. 2001; Johansen, Fettelschoss et al. 2011) In contrary, (Muttucumaru, Smith et al. 2011) *zmp1* gene knock out studies using mice suggested that it is essential for hypervirulence of mycobacteria. This report discords which leads to impaired inflammasome activation and phagolysosome arrest, which are two important pathways of pathogen defense in host. Studies supported that by obstructing zmp1 gene, there is a reactivation of caspase-1 and other downstream processes helping in the clearance of pathogen (Master, Rampini et al. 2008). It has also been proposed that *M. tb* zinc metalloproteases (Fratti, Chua et al. 2002; Vergne, Chua et al. 2003) help in the survival of mycobacteria inside the cell. *M. tb* gene Rv0198c (*zmp1*) is essential in preventing caspase-1-dependent stimulation of IL-1β. Using an *M. bovis* BCG mutant lacking the *zmp1* gene, it has been assessed that the possible influence of Zmp1 on presentation of mycobacterial antigens by professional antigen presenting cells and the induction of CD4- and CD8- positive T-cell responses to mycobacterial antigens with the previous reports which implied the same to attenuation of virulence. Nonetheless, these two studies propose an important role for Zmp1 for pathogenicity with mechanism of action unexplored.

1.8 Rationale

With some metalloproteases playing a distinct role in bacterial pathogenesis and emerging evidence of Zmp1 as a possible virulence factor, I further wanted to explore its potential role in the physiology of *M. tb* and being a secretory protein, its impact on the immune status of the host.

With this two major objectives were designed:

1. Immunological characterization of secreted *M. tb* Zinc Metalloprotease 1 (Rv0198c) to identify Tuberculosis-specific Humoral Immune Response.
2. Biochemical and physiological characterization of Zmp1: possible role in dissemination.

Chapter 2

Immunological characterization of secreted *M. tb* Zinc metalloprotease 1 (Rv0198c) to identify Tuberculosis-specific Humoral Immune Response

(Vemula, et al., Frontiers in Microbiology 2016)

2.1 INTRODUCTION

Mycobacterium tuberculosis (*M. tb*), the tuberculosis (TB) causing bacilli are facultative intracellular parasite, residing primarily in phagocytic cells like alveolar macrophages and monocytes, but may also colonize other cells, such as alveolar epithelial cells, bones, meninges, peritoneal linings of the intestines, etc (Golden and Vikram 2005). As discussed in chapter 1, Traditionally, like all intracellular pathogens, protective immunity to *M. tb* has been credited to the cell mediated immunity (CMI) with CD4+ T cells playing a crucial role in granuloma formation while antibody mediated immunity (AMI) is considered non-protective (van Crevel, Ottenhoff et al. 2002). The argument that *M. tb* is strictly intracellular, however, is debatable as *M. tb*, during some point of its infectious cycle, has also been observed in extracellular spaces (Grosset 2003), where in principle they can be vulnerable to antibody action. There are a number of reports where despite being intracellular pathogen, antibodies have been shown to modulate the immune response in favour of the host against pathogens (Casadevall 2003). Studies with antibodies, monoclonal or otherwise, have now demonstrated passive protection for several microbes, such as *Candida albicans* (Han and Cutler 1995), *Listeria monocytogenes* (Edelson, Cossart et al. 1999), *Leishmania Mexicana* (Anderson, David et al. 1983), *M. tb* (Teitelbaum, Glatman-Freedman et al. 1998; Zhao, Shi et al. 2011) etc, though experiments with immune serum have provided inconsistent results. Surface-exposed or secreted proteins of *M. tb* are customarily the targets of immune responses in the infected host. Several *M. tb* proteins, including culture filtrate proteins have been evaluated for their immunogenic properties, such as CFP10, ESAT-6, Ag85B, ICDs etc (Banerjee, Nandyala et al. 2004; Sinha, Kosalai et al. 2005; Malen, Softeland et al. 2008; Floss, Mockey et al. 2010). Similarly, a considerable expanse of data propose that defense against intracellular and extracellular pathogens are not stringently restricted to either Th1 (promoting CMI) or Th2 (promoting AMI) responses. Citing a few examples, humoral immunity have been shown to be protective against intracellular pathogens like Plasmodium or Mycobacteria, while protective immunity against extracellular parasitic flatworm *Schistosoma* was due to CMI triggered by Th1 response (Abebe and Bjune 2009; Greenhouse, Ho et al. 2011; Wen, He et al. 2011; Dups, Pepper et al. 2014). Recently, Modified Vaccinia Ankara 85A (MVA85A) failed to clear the phase 2b trial, where *M. tb* major secreted antigen complex 85A (Ag85A) that induces a strong Th1 immune response in BCG-primed host

was used (Tameris, Hatherill et al. 2013). Therefore, though CMI may remain the mainstream immune response, the role of AMI in conferring protection against intracellular pathogens, including *M. tb*, cannot be dismissed.

Serological studies advocate that *M. tb* infection, beside CMI, also evokes a strong humoral response in patients against a variety of mycobacterial antigens (Steingart, Dendukuri et al. 2009). Corroborating these observations are other studies where *M. bovis* BCG vaccination led to generation of mycobacterial antigen specific IgG and IgM (Beyazova, Rota et al. 1995; Brown, Cruz et al. 2003; de Valliere, Abate et al. 2005). Some anti-M.tb antibodies enhanced both innate and CMI responses during mycobacterial infection (de Valliere, Abate et al. 2005). Antibodies, through a range of mechanisms, including simple opsonization to complicated FcR activation can regulate the fate of intracellular pathogens. Some vaccine trials have included induction of antibody-mediated immunity to transduce protection against fungal diseases (Vecchiarelli, Pericolini et al. 2012). A recent study has evaluated the feasibility of using humoral immunity in vaccine development against *M. tb* by comparing immunoglobulin titers (IgG and IgA) with a variety of clinical and immunological parameters (Niki, Suzukawa et al. 2015). While these studies strongly support the inclusion of evoking AMI alongside CMI in TB vaccine development program, there is a need for systematic investigation of mycobacterial antigens for a strong and specific humoral response that can be employed against TB.

Several proteins like *M. tb* GlcB (malate synthase), MPT51 (FbpC1) and HSPX (alpha crystalline) have also been evaluated for humoral response in clinically asymptomatic Health-care workers with latent infections, suggesting the possibility of these responses to be protective (Reis, Rabahi et al. 2009). In this study, we have evaluated the humoral response to *M. tb* H37Rv zinc metalloprotease-1, Zmp1 (Rv0198c), a protein present in the culture filtrate (de Souza, Leversen et al. 2011)Extracellular zinc-containing metalloproteases are ubiquitously present, quite a few of them from pathogenic bacteria function as exotoxins, such as, Clostridial neurotoxins, Anthrax toxins, Botulinum neurotoxin, *Bacillus sp.* thermolysin etc (Hase and Finkelstein 1993; Miyoshi and Shinoda 2000). These zinc-metalloproteases are also known to elicit a strong and specific humoral response, for which the inactivated toxin (toxoid) function as vaccine candidate (Hase and Finkelstein 1993; Miyoshi and Shinoda 2000). The annotated zinc-metalloproteases from *M. tb* H37Rv are, namely, Rv0198c (zmp1), Rv0563 (htpX), Rv2467 (pepN), Rv2869c (rip) and Rv3610c (ftsH), Rv1977 (Stewart, Wernisch et al. 2002;

Griffin, Gawronski et al. 2011; Kelkar, Kumar et al. 2011; Mazandu and Mulder 2012; Schneider, Sklar et al. 2014). Mutant studies of Zmp1 in *M. tb* strain H37Rv and *M. bovis* BCG suggested that it is essential for the intracellular survival of the bacteria and possibly impairs inflammasome activation and phagosome maturation (Master, Rampini et al. 2008; Johansen, Fettelschoss et al. 2011). We further checked the antigenic index of Zmp1 by Protean software (Protean 5.00, DNASTAR Inc.) and found to be highly antigenic (Figure 4).

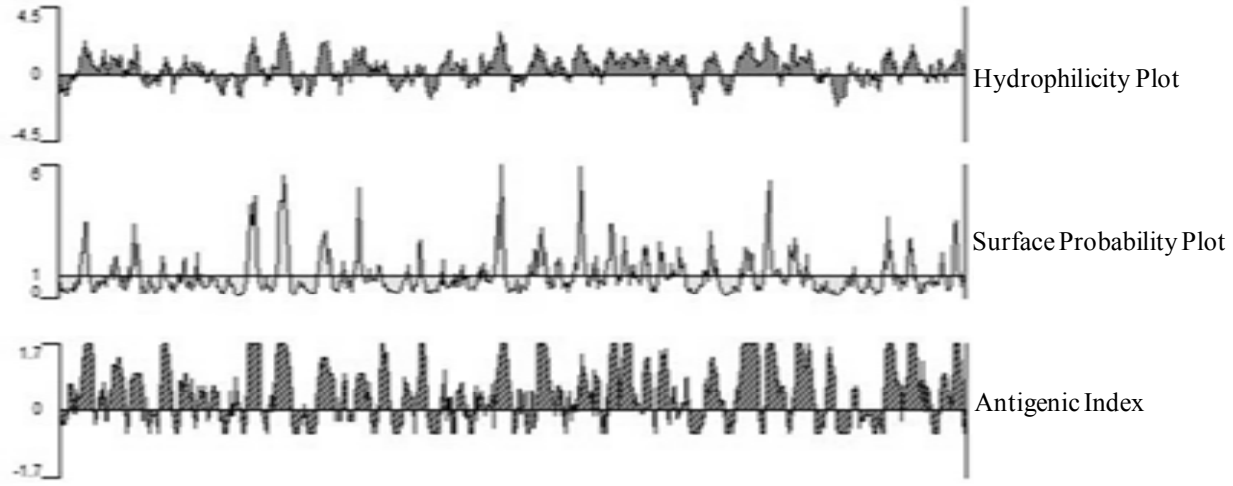

Figure 4: Rv0198c (Zmp1) protein is antigenic. Antigenicity prediction of Zmp1 using PROTEAN software. Kyte-Doolittle plot for hydrophilicity; Emini plot for Surface probability and Jameson-Wolf analyses for Antigenic index.

With these cues suggesting Zmp1 as an immunomodulator and upon recording high antigenic index by Jameson-Wolf plot using Protean software, we hypothesized that Zmp1 could be a strong immunostimulant and provoke an effective humoral response during infection. Presence of anti-Zmp1 antibodies in TB patient sera confirmed that it is indeed expressed during infection. We established that Zmp1 is secreted by H37Rv under granuloma-like in vitro growth conditions and is capable of orienting the immune response towards Th2 cytokine microenvironment. Further, we compared the humoral response to Zmp1 in various TB patient categories, including smear-negative Extra-pulmonary TB cases. Presence of high titers of anti-Zmp1 antibodies in smear-negative Extra-pulmonary Tuberculosis (EPTB) cases similar to PTB patients that ranged from low to high bacillary load indicated the potential of Zmp1 to evoke an effective humoral response independent of mycobacterial load. The study points to the potential of further

exploration of mycobacterial proteins, such as Zmp1, as antigen candidates for prime-boost vaccination strategies or as markers for disease progression.

2.2 MATERIAL AND METHODS

Strains, Constructs and plasmids used: *E. Coli* DH5α strain was used for cloning and *E. Coli* BL21 DE3 strain was used for expression studies. pET 28a (+) vector was used for cloning of *zmp1*. Primers used for cloning are listed below in Table 1.

Table 1: Primers used for cloning *M. tb zmp1* gene.

Primer	Sequence	Enzyme
Forward primer	5' ataGGATCCgtgacacttgccatcccctcgg 3'	BamHI
Reverse primer	5' agtCTCGAGgtcttagcctagttccagat 3'	XhoI

2.2.1 Cloning, Expression and Purification of recombinant Zmp1 (rZmp1) protein:

Rv0198c (*zmp1*) gene was amplified from the genomic DNA of H37Rv using specific primers. The amplicon was cloned into BamHI and XhoI sites of pET28a vector. The positive clones were confirmed by sequencing. pET28a (+) plasmid carrying *zmp1* (Rv0198c) gene was transformed into E. coli BL21-DE3 cells (Vemula, Ganji et al. 2016). A single bacterial colony carrying plasmid was inoculated into LB medium containing kanamycin (34 µg/mL) and was incubated overnight at 37°C with vigorous shaking (180 rpm). The next day, secondary culture was set up in LB media with 2 % inoculum of overnight grown primary culture and incubated at 37°C with vigorous shaking (180 rpm). When OD at 600nm reached to 0.6, the culture was induced with 0.5mM β-D-Isopropyl thiogalactoside (IPTG) kept for 4hr at 37ºC. pET28a-*zmp1* construct was expressed in BL-21 DE3 cells and N-terminal Histidine tagged recombinant Zmp1 (rZmp1) was purified under native conditions (50mM Tris-Cl buffer, 300mM NaCl, pH-8.0) by affinity chromatography using cobalt based resin. The purified protein was dialyzed against dialysis buffer (50 mM Tris-Cl buffer pH-8.0, 100mM NaCl, 4% glycerol, 1mM PMSF) at 4°C. Dialyzed protein was treated with polymyxin-B agarose beads to remove endotoxins. Heat-inactivation of rZmp1 was performed by heating the purified rZmp1 at 100^{o}C for 10 min and later snap freezed.

2.2.2 In vitro mycobacterial growth conditions:

The mycobacterial strain used was *Mycobacterium tuberculosis* H37Rv. The growth of mycobacteria was performed as described earlier (Ganji, Dhali et al. 2015). The mycobacteria were plated on 7H10 agar media supplemented with 10% Oleic acid, Albumin, Dextrose and Catalase (OADC) and incubated at 37^{o}C. The colonies were picked into the 7H9 broth media supplemented with 10% OADC and incubated at 37^{o}C at 180 rpm until the OD_{600} nm reached 0.5 to 0.6. The culture was checked for any contamination using Ziehl-Neelsen (ZN) staining procedure. The culture was then centrifuged at 3700 rpm for 7 min. The culture pellet was washed with Phosphate Buffered saline (PBS) pH 7. The pellet was then resuspended in Sauton's media under granuloma-like stress conditions, such as acidic pH 5.5, oxidative stress (10 mM H_2O_2) (Voskuil, Bartek et al. 2011), Iron deprivation and Nutrient starvation for 36 hr. For Nutrient stress the culture was resuspended in PBS. For Iron deprivation, the glassware and the media were made Iron-free as described earlier (Hall and Ratledge 1982).

2.2.3 Sample Collection:

A total of 295 subjects in the age group of 15-60 yrs were recruited at Mahavir Hospital and Research Centre (MHRC), Hyderabad and University of Hyderabad (UH) after taking prior ethical committee approvals (ECR/450/Inst/AP 2013 and UH/IEC/2014/36) and written consents from the subjects. 2-5 mL of blood was collected in vacutainers with EDTA and later sera were separated for the experiments. The study population was divided into four categories, namely, Clinically Healthy donors (n=62) (Annexure I), TB patients (n=121) (Annexure II and Annexure III), household contacts (n=89) (Annexure IV and Annexure V) and Non-specific infection controls (n=23) (Annexure VIII). Clinically healthy donors had no symptoms of any disease at the time of sera collection and were tested for TB-Interferon-Gamma Release Assays (TB-IGRA) using QuantiFERON®-TB Gold (QFT®) ELISA kit (Reference# 0594-0201) and the results were analysed using QuantiFERON-TB Gold Analysis software (Version 2.62) as per the manufacturer's instructions (Supplementary Table 1). For TB patients, the sputum microscopy for AFB was performed as per Revised National Tuberculosis Control Programme (RNTCP), government of India, guidelines with confirmed diagnosis of sputum, culture and chest X-ray in patients (http://www.tbcindia.nic.in/rntcp.html). Tuberculin skin test (TST) was

performed in all the subjects. TB patients were further categorized into Pulmonary TB (PTB) (n=66) and Extra-Pulmonary TB (EPTB) (n=55) cases. EPTB patients were defined atleast with one culture-positive specimen from an extra-pulmonary site, or histological or radiological, or strong clinical evidence consistent with active extra-pulmonary TB. Household contacts of the respective patients were those who resided in house of the TB patient during 3 months period for atleast 7 consecutive days prior to the diagnosis of tuberculosis. Mostly they were siblings and spouses. The household contacts, though, were clinically asymptomatic but many of them were Mantoux positive (Out of 89 household contacts, 60 subjects were Mantoux positive with diameter of Induration >15 mm). Non-specific infection controls comprised of patients with random infections other than TB such as viral, bacterial and parasitic infections. Pregnant women, terminally ill patients, immunocompromised patients, patients undergoing any chemotherapy or with chronic illness were not included in the study.

2.2.4 ELISA:

The PBMCs were isolated from blood collected from healthy donors using Ficoll gradient. They were either left untreated or exogenously treated with 50 nM and 100 nM of purified, endotoxin-free rZmp1 or 0.5 µg/mL of LPS in RPMI media supplemented with 10% FBS and kept at 37^{o}C for 24 hr. For measurement of cytokine levels we have used BD OptEIA ELISA sets and performed as per manufacturer's instructions. For measurement of anti-Zmp1 antibody titers, 100 ng of purified rZmp1 in 100 µL of coating buffer was coated per well at 4°C overnight. The plates were then washed 3 times with PBST (PBS containing 0.05% Tween-20) before blocking with 120µL of blocking solution (3% BSA in PBS) at 37°C for 1 hour. After blocking, plates were washed thrice with PBST. 1:100 times diluted (100 µL/well) sera samples were added and incubated at 37°C for 1hour followed by 5 washes with PBST. Anti-Human IgG conjugated with HRP (Sigma) was used as the secondary antibody in 1:10000 dilution (100 µL/well) and incubated at 37°C for 1hour. After 7 washes with 1X PBST, 100 µL/well of chromogenic substrate (Tetramethylbenzidine) was added and kept for incubation at 37°C for 30 minutes. The reactions were stopped using 100 µL of 2N H_2SO_4. The absorbance was measured at 450 nm and 570 nm in multi-well plate reader (Biotek).

2.2.5 Sensitivity and Specificity calculations:

The specificity and sensitivity of rZmp1 towards identifying EPTB was calculated using the following formula: Sensitivity = True Positives/ (True positives + False negatives)**;** Specificity = True Negatives/ (True Negatives + False Positives). A cut-off value of Absorbance 0.95 at 450 nm was used for calculations to achieve 100% specificity and avoid false positive samples. Values 0.95 and above in EPTB category were considered true positives and those in Healthy or Contacts categories were considered false positives. Similarly, values below 0.95 at Abs_{450} in EPTB category were considered false negatives and those in Healthy or Contacts categories were considered true negatives (Parikh, Mathai et al. 2008).

2.2.6 Graphs and Statistical analyses:

Statistical analyses were carried out using SigmaPlot software version 11.0.0.77 (Systat Software, Inc., USA). For cytokine data, One-way ANOVA was performed with Holm–Sidak multiple pair-wise comparison method and the threshold for significance was set at $p<0.05$. The error bars represent the ± standard deviation (SD) from the mean of at least three independent experiments. For statistical analyses of anti-Zmp1 antibody titers measured from the blood samples, One-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method. The threshold for significance was set at $p<0.05$. They were represented as box plots using SigmaPlot. Within the plots, the upper quartile of the box represents the 75^{th} percentile and the lower quartile for the 25^{th} percentile. The line inside the box represents the median. The whiskers arising from either side of the upper half and the lower half of the box correspond to 1.5 times the interquartile range (IQR) (Benjamin, Banerjee et al. 2013). Any datum to the further extreme of the whiskers is termed as outlier.

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2.3 RESULTS

2.3.1 Cloning, Expression and Purification of recombinant Zmp1 (rZmp1) protein: Mycobacterial *zmp1* was cloned into pET28a(+) vector under and later expressed in BL21 DE3 strain of *E. coli* (Figure 5A, 5B). The culture was induced at 18^0C using 0.5mM IPTG concentration. Cell pellets were resuspended in lysis buffer and sonicated. The recombinant Zmp1 (rZmp1) protein was purified using the talon resin. Further purified protein was subjected to dialysis and polymyxin treatment for removal of endotoxins. Purified rZmp1 was checked on SDS-PAGE and used (Figure 5C).

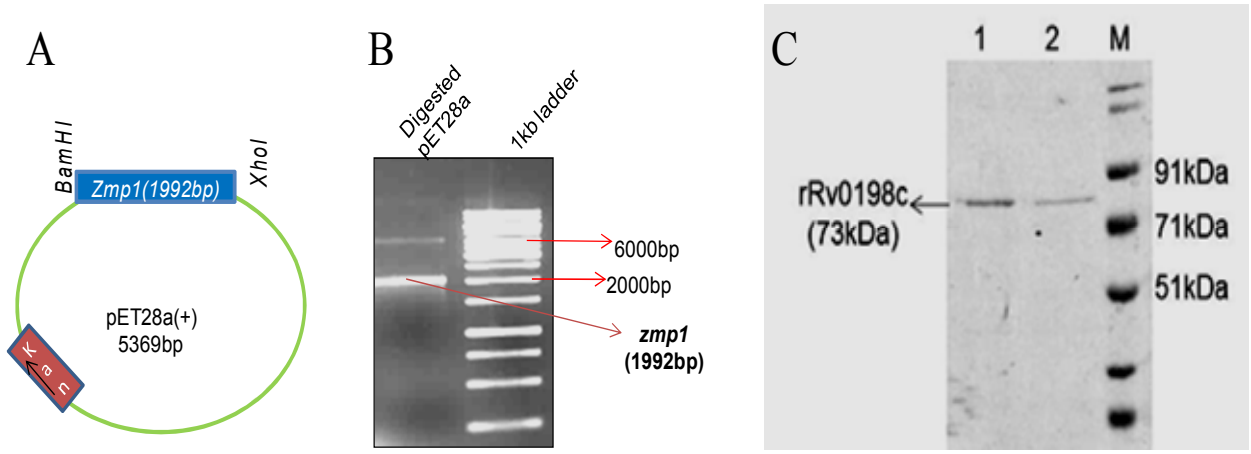

Figure 5: Cloning and purification of rZmp1 protein (A) pET28a (+) vector map with *zmp1* construct (B) Agarose gel showing clone confirmation of *zmp1* gene by XhoI and BamHI digestion (C) SDS-PAGE with purified recombinant Zmp1 protein purified using Cobalt-based affinity chromatography, M: marker

Purified recombinant Zmp1 was subjected to polymyxin B treatment for the removal of endotoxins. The Endotoxin units of rZmp1 were found to be 0.374461±0.105479 from standard curve (Figure 6) which is approximately equivalent to 37.44 pg of E. coli LPS per microgram of recombinant protein (Dawson 1988). As reported earlier, THP-1 macrophages were least sensitive cell type and require a minimum of 2ng of LPS to stimulate them. The levels of endotoxin in rZmp1 were in the permissible range, so was used for further experiments (Schwarz, Schmittner et al. 2014).

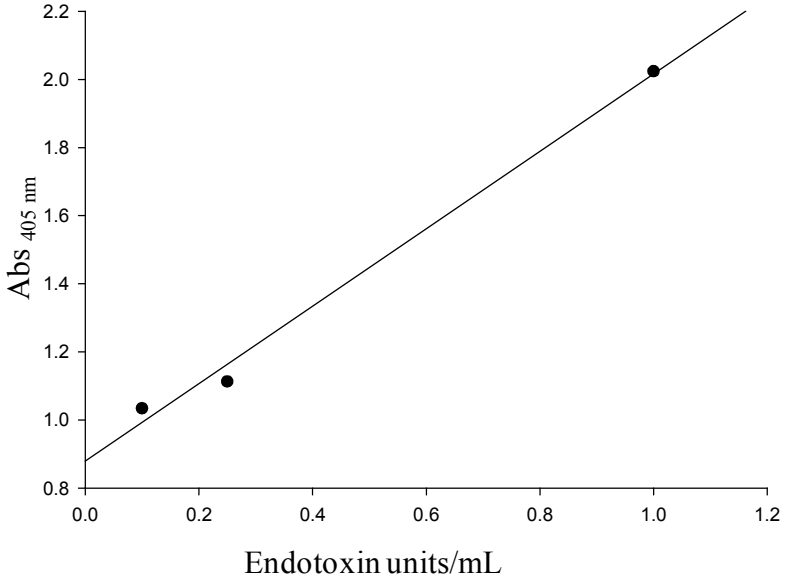

Figure 6: Standard curve for the quantitation of endotoxin in a chromogenic assay.

2.3.2 Zmp1 protein is expressed during infection and is secreted under granuloma like in vitro growth conditions by *M. tb* H37Rv:

The functionally active, endotoxin free, purified recombinant Zmp1 (rZmp1) was used as antigen to capture anti-Zmp1 antibodies in the sera samples of TB patients (results presented and discussed later). The presence of anti-Zmp1 antibodies in *M. tb* infected patients confirmed that Zmp1 was indeed expressed by *M. tb* during infection. Zmp1 was identified as one of the culture filtrate proteins of in vitro grown H37Rv (de Souza, Leversen et al. 2011). We extended the study to check if the same holds true for granuloma-like conditions. To study the same, H37Rv was grown under different stress conditions known to simulate acellular caseous environment of TB granulomas, that is, acidic pH 5.5, H_2O_2 induced oxidative stress, nutrient deprivation and iron deficiency (Stallings and Glickman 2010). Bacteria were grown to mid-log phase and then subjected to various stresses for 36 hrs. The mycobacterial cells were then harvested and the culture supernatants separated. Culture supernatants were then precipitated using 10% TCA and the precipitate was used to detect presence of Zmp1 protein using Western blotting with in-house

generated anti-Zmp1 antibody (Figure 7A upper panel). CFP10 and GroEL1, which were probed with their respective antibodies, were used as positive and negative controls respectively (Figure 7A middle and lower panel). CFP10 is a known mycobacterial secretory protein (Malen, Berven et al. 2007; Malen, Softeland et al. 2008) and hence was used as a positive control for culture supernatant preparations while GroEL1 is an intrabacterial, membrane associated protein which is not secreted out (de Souza, Leversen et al. 2011; Malen, De Souza et al. 2011) and is used as negative control. Absence of GroEL1 in the culture supernatants indicated absence of cell lysis products in the culture supernatant (Figure 7A lower panel). The presence of band corresponding to Zmp1 in Western blots suggested secretion of the protein under all the tested stress conditions (Figure 7A upper panel). The assay was a qualitative check to confirm the secretion of Zmp1, though it is possible that the levels of secreted Zmp1 in culture supernatants may vary with stress conditions. Figure 7B represents the Western blots with the whole bacterial lysates as control experiment. This suggested that Zmp1 is indeed a secreted protein, possibly secreted within granuloma of infected host.

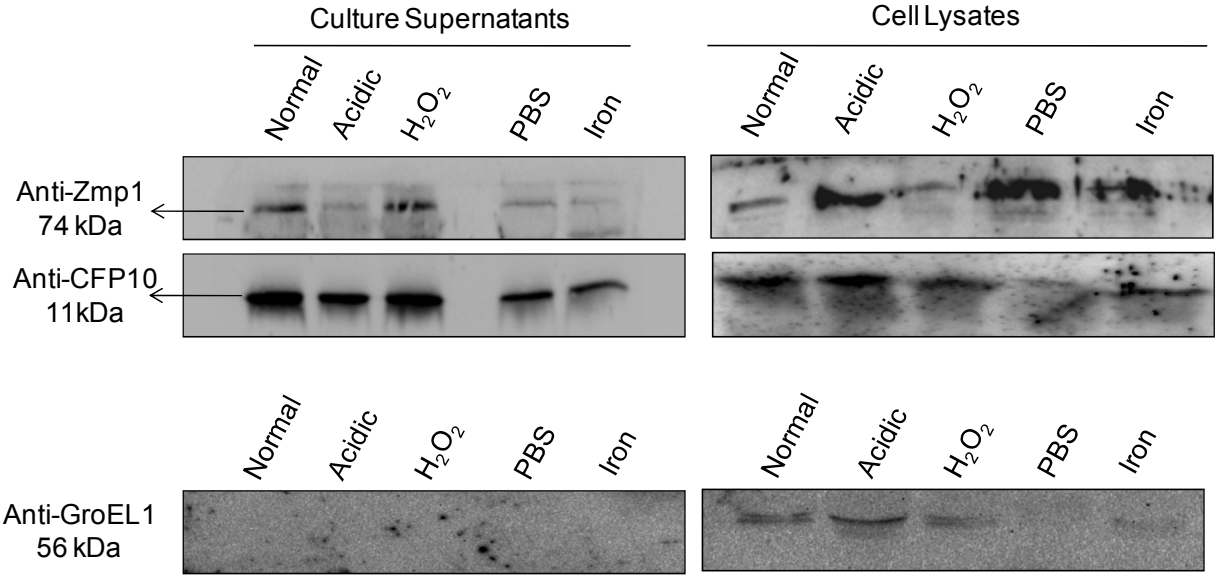

Figure 7: Zmp1 is expressed and secreted by *in vitro* grown *Mycobacterium tuberculosis* H37Rv under granuloma-like conditions. (A) The culture supernatants of *in vitro* grown *M. tuberculosis* H37Rv under normal conditions and under various stress conditions, such as, acidic pH5.5, H2O2 induced oxidative stress, nutrient starvation and iron depletion, were precipitated by 10% trichloroacetic acid and the resultant precipitate was subjected to Western blot using Mouse anti-Zmp1 antibody (1:1000 dilution; Upper panel). Rabbit anti-CFP10 antibody (1:1000 dilution) against CFP10, a culture filtrate protein 10, was used as a positive control (Middle

Panel) and Mouse anti-GroEL1 antibody (1:1000 dilution) against GroEL1, a cytoplasmic chaperone was used as a negative control (Lower Panel) to evaluate for cell lysis products in supernatants. The absence of band corresponding to GroEL1 in the supernatant fractions suggests the purity of culture filtrate preparations. (B) Figure represents the Western blots with the whole bacterial lysates as control experiment

2.3.3 rZmp1 stimulated PBMCs to release Th2 class of cytokines

We next evaluated the immunostimulatory potential of Zmp1 in terms of release of Th1/Th2 cytokines from exogenously treated PBMCs derived from healthy volunteers to elucidate the association of Zmp1with CMI or AMI. To do the same, functionally active, endotoxin free rZmp1 was used for stimulatory assays.

PBMCs were treated with rZmp1 at 50nM and 100nM for 24 hrs. LPS, a known strong immunostimulant of PBMCs, was used as a positive control (Jansky, Reymanova et al. 2003). The culture supernatants were then collected to assay for the levels of a minimal battery of cytokines. TNFα and IL-1β are the cytokines of innate response that stimulate the acute phase reaction and represent initial stimulation of immune cells. High titers of TNF-α (Untreated: 91.98±27.67 pg/mL; rZmp1 100 nM: 408.71±52.96 pg/mL) and IL-1β (Untreated: 205.62±65.63 pg/mL; rZmp1 100 nM: 634.34±51.72 pg/mL) (Figure 8A and 8B) upon exogenous treatment of PBMCs with rZmp1 established that rZmp1is indeed a strong immunostimulant, comparable with LPS (TNF-α: 255.89±107.67 pg/mL; IL-1β: 485.46±111.66 pg/mL) (Figure 8A and 8B). We next measured the levels of pro-inflammatory cytokines IFN-γ and IL-12p70 and regulatory cytokines IL-4 and IL-10. It was observed that upon treatment of PBMCs with rZmp1 protein, there is an increased secretion of regulatory cytokines, IL-10 (Untreated: 562.41±244.02 pg/mL; rZmp1 100 nM: 1218.77±270.60 pg/mL) and IL-4 (Untreated: 168.91±60.62 pg/mL; rZmp1 100 nM: 292.31±49.13 pg/mL) (Figure 8C and 8D) and while no change was observed in the levels of IL-12p70 (Untreated: 46.32±2.15 pg/mL; rZmp1 100 nM: 45.23±8.31 pg/mL) and IFN-γ (Untreated: 81.84±31.44 pg/mL; rZmp1 100 nM: 52.49±19.43 pg/mL) (Figure 8E and 8F). When the ratio of IFN-γ to IL-10 was considered, rZmp1 treatment showed lower IFN-γ: IL-10

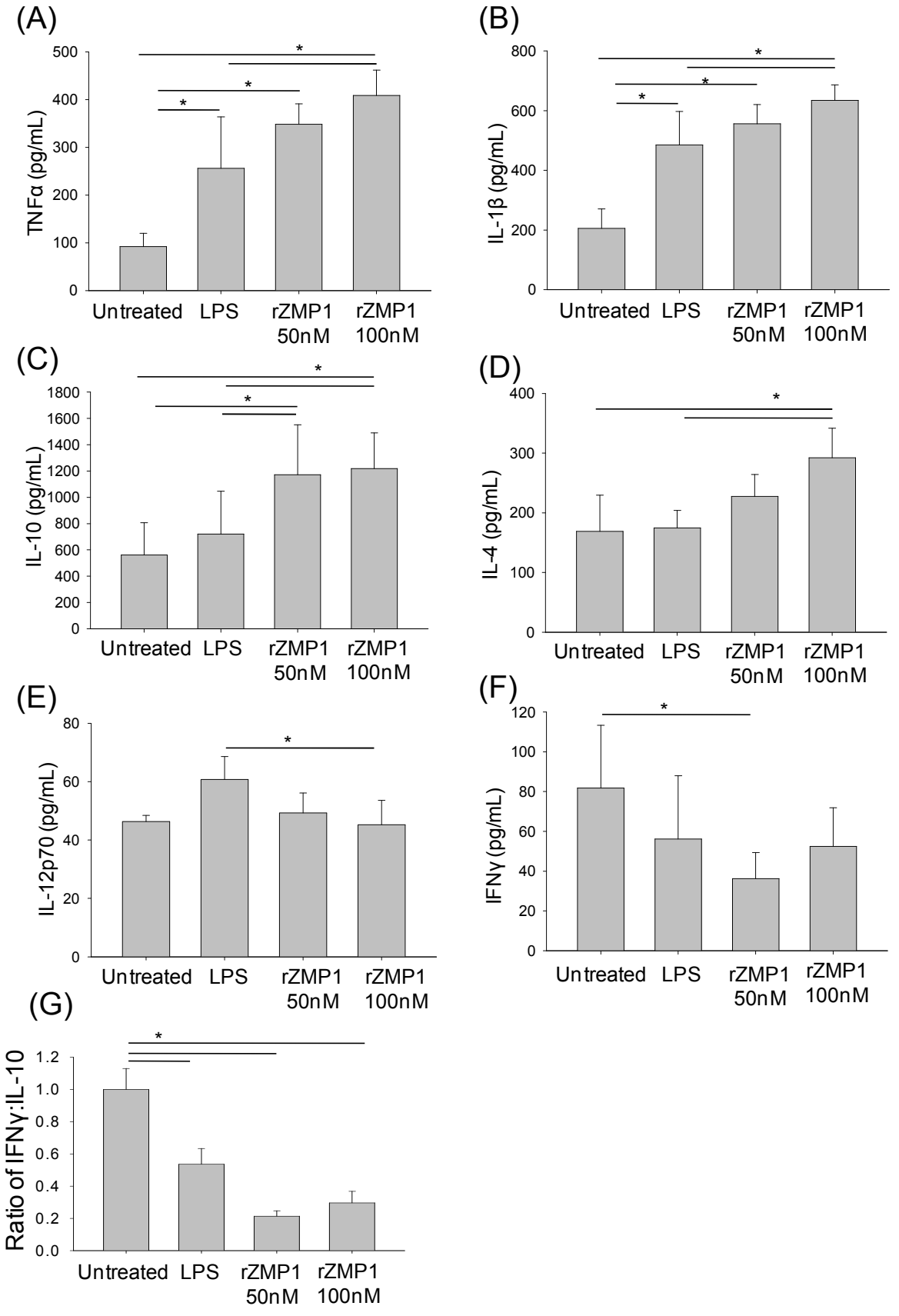
(A)
TNFα (pg/mL)
0
100
200
300
400
500
*
*
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(B)
IL-1β (pg/mL)
0
200
400
600
800
*
*
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(C)
IL-10 (pg/mL)
0
200
400
600
800
1000
1200
1400
1600
1800
*
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(D)
IL-4 (pg/mL)
0
100
200
300
400
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(E)
IL-12p70 (pg/mL)
0
20
40
60
80
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(F)
IFNγ (pg/mL)
0
20
40
60
80
100
120
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM
(G)
Ratio of IFNγ:IL-10
0.0
0.2
0.4
0.6
0.8
1.0
1.2
*
Untreated
LPS
rZMP1 50nM
rZMP1 100nM

Figure 8: Treatment of PBMCs with recombinant Zmp1 protein inclines the immune status to Th2 response. Cytokine profiles of the PBMCs either untreated or treated with LPS, 50 nM rZmp1 and 100 nM rZmp1 was estimated. (A) TNFα, (B) IL-1β, (C) IL-10, (D) IL-4, (E) IL-12p70, (F) IFN-γ titers were measured using capture ELISA. (G) Ratio of IFN-γ: IL-10. All the experiments were performed more than three times. Statistical analyses were done using one-way ANOVA with Holm-Sidak multiple pair-wise comparison method. Error bars represent ±SD (standard deviation). ∗ Represents $p < 0.05$.

ratio of 0.30±0.07 as compared to untreated (1±0.13) or LPS (0.54±0.10) treated (Figure 8G). This indicated that rZmp1 promoted a Th2 immune response. Summing up, these experiments indicated that the secreted *M. tb* Zmp1 should incline the immune system towards Th2 response, promoting humoral immunity.

With the above experiments indicating that Zmp1 induces Th2 response, we next assessed if this is manifested in the form of anti-Zmp1 antibody production in TB patients. To verify the same, we measured anti-Zmp1 antibodies in the sera samples of TB patients using rZmp1 as the bait antigen in ELISA based assays. Prior to that we checked for the cross-reactivity of rZmp1 with other anti-TB antibodies, and performed Western blotting for the rZmp1 using anti-ESAT6 antibody. ESAT6 was not detected by Zmp1 antibody (Figure 9A) and rZmp1 was not detected by the anti-ESAT6 antibody in the Western blot (Figure 9B) and thus confirms the specificity of rZmp1 and fidelity of rZmp1-based ELISA method to determine the anti-Zmp1 antibody titers in the sera samples

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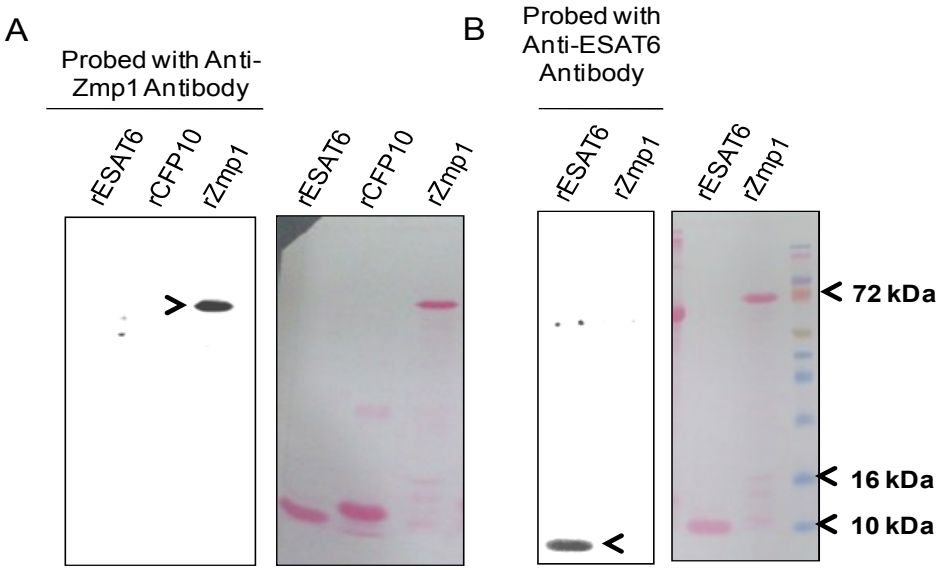

Figure 9: Cross-reactivity check of anti-Zmp1 antibody and rZmp1. (A) Recombinant Mycobacterial antigens, ESAT6 (5 µg), CFP10 (5 µg), and Zmp1 (5 µg) were loaded on to 15% SDS-PAGE followed by Western blot with Mouse anti-Zmp1 antibody. The corresponding Ponceau S stained blot showing transferred protein is provided. (B) Recombinant Mycobacterial antigens, ESAT6 (5 µg) and Zmp1 (5 µg) were loaded on to 15% SDS-PAGE followed by Western blot with rabbit anti-ESAT6 antibody (1:1000 dilution). The corresponding Ponceau S stained blot showing transferred protein is provided.

2.3.4 Zmp1 elicited a strong B-cell response which was specific for Tuberculosis (TB) infection

Humoral response of the host against mycobacterial secretory protein, Zmp1 was scored in a study population comprising 295 subjects. This included four groups, TB patients, Healthy controls, House hold contacts of TB patients and volunteers with non-specific infections. Purified rZmp1 was used as bait antigen in the indirect ELISA to score for the anti-Zmp1 antibodies in the serum samples of healthy (n=62), TB patients (n=121), house hold contacts (n=89) and non-specific infection control samples (n=23) (Figure 10). We observed that there was a significant increase ($p<0.001$) in the absorbance at 450nm corresponding to the anti-Zmp1 antibody titers in TB patient sera as compared to the healthy or house hold contacts or non-specific infection controls (Figure 10). The median values for the Healthy was 0.702 (IQR: 0.506-0.893), TB patients was 1.264 (IQR: 0.961-1.982), household contacts was 0.606 (IQR: 0.486-0.78) and non-specific infection control was 0.539 (IQR: 0.394-0.708) showing distinct difference of TB patients from other groups. The non-reactivity of rZmp1 to sera samples of non-specific infection controls clearly indicated that the rZmp1 did not cross-react with the antibodies generated due to other infections in human host. In addition, negligible absorbance observed in sera of healthy donors and Household contacts strongly points to the specificity of rZmp1 to TB infection. This clearly indicated that Zmp1 could elicit a strong humoral response that was specific to TB patients and could clearly distinguish TB patient category from all other categories, including asymptomatic household contacts.

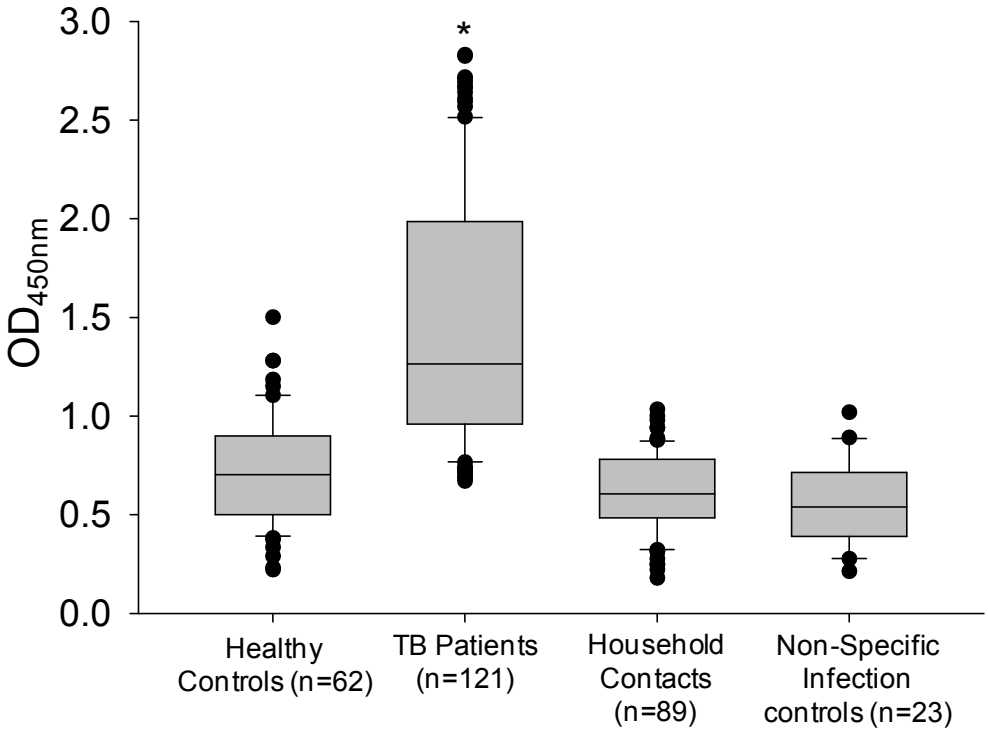

Figure 10: rZmp1 ELISA was specific for tuberculosis (TB) infection. Box plots representing titers of anti-Zmp1 antibody titers as indicated by values at Absorbance 450 nm in Healthy, TB, Household or patient contacts and Non-specific infection control. ∗The differences in Abs450 values for TB vs. Healthy; TB vs. Household contacts and TB vs. non-specific infection controls were highly significant and a p-value of <0.001 was observed in all the three cases.

2.3.5 Zmp1 elicits strong B-cell response independent of mycobacterial load in TB patients

Having seen that rZmp1 stimulated release of Th2 cytokines over Th1 in PBMCs and accordingly Zmp1 elicited a strong B-cell response that could be detected in terms of high anti-Zmp1 IgG titers in TB patients, we next checked if these titers differed between PTB (n=66) and EPTB (n=55) cases. This was important as all EPTB patients were sputum smear-negative but recorded presence of mycobacteria by Acid-fast bacilli (AFB) staining and caseous necrosis in their biopsy samples, suggesting localized mycobacterial load. We observed that the median values for both PTB and EPTB cases were approximately similar, viz; for PTB it was 1.257 (IQR: 0.956-1.8) and for EPTB, it was 1.421 (IQR: 0.979-2.259) (Figure 11). We then compared the anti-Zmp1 titers in PTB patients with various gradients of mycobacterial load. Table 2 lists the representative ELISA readings of PTB patients ranging from a high bacilli load (3+) to smear

negative, synonymous with extremely low bacilli titers, clearly showing humoral response to Zmp1 was independent of *M. tb* load. This observation was significant, as it suggested that Zmp1 could elicit a strong humoral response even in paucibacillary TB cases and can detect EPTB cases significantly.

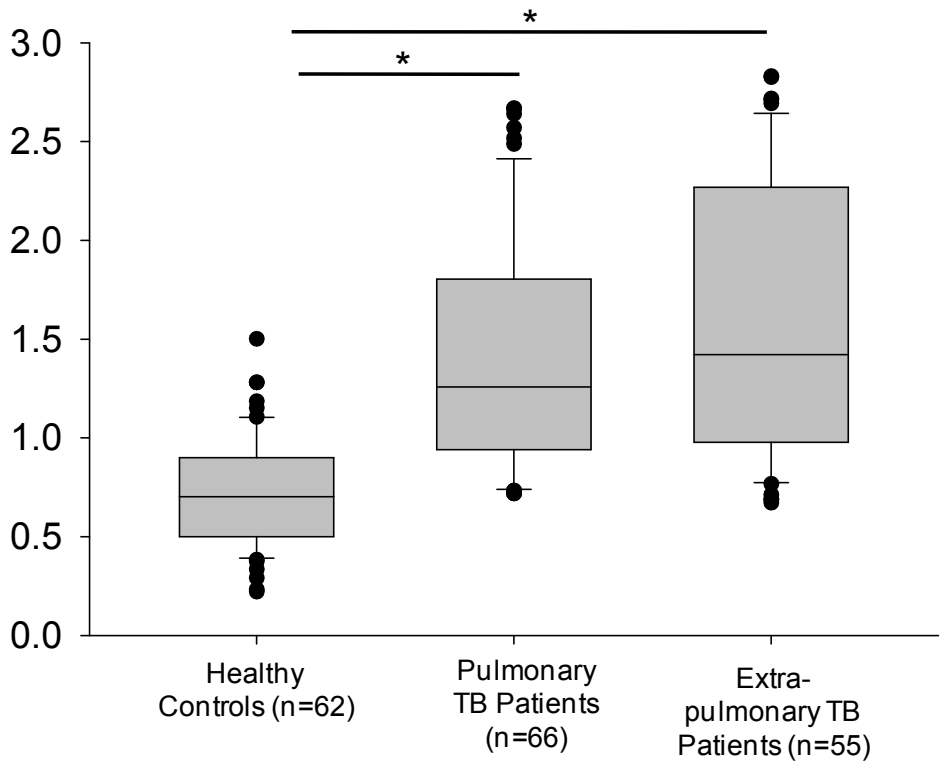

Figure 11: The box plots representing the anti-Zmp1 antibody titers in Healthy controls, Pulmonary TB patients and Extra-pulmonary TB patients. Statistical analyses were done using one-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method. ∗ Represents $p < 0.05$.

Table 2. Representative table to show that pulmonary TB (PTB) cases with varying loads of bacilli load in sputum does not show variation in their Abs450 nm when detected by rZmp1 ELISA test.

Sample No.	AFB (Bacilli load)*	Abs_{450}
PTB# 1	P I -VE	1.260667
PTB# 2	P I -VE	1.297333
PTB# 3	P I -VE	1.407333
PTB# 4	P I -VE	1.716
PTB# 5	P I -VE	1.936333
PTB# 6	P I -VE	2.3335
PTB# 7	P I 1+	1.127333
PTB# 8	P I 1+	1.178333
PTB# 9	P I 1+	1.725667
PTB# 10	P I 1+	1.978333
PTB# 11	P I 1+	2.317333
PTB# 12	P I 2+	1.249667
PTB# 13	P I 2+	1.319667
PTB# 14	P I 2+	1.773
PTB# 15	P I 2+	1.800333
PTB# 16	P I 2+	2.487
PTB# 17	P I 3+	1.107
PTB# 18	P I 3+	1.274333
PTB# 19	P I 3+	1.564667
PTB# 20	P I 3+	2.67

*PI 1+ indicates more than 100 acid fast bacilli (AFB) in sputum, PI 2+ indicates more than 200 acid fast bacilli in sputum, PI 3+ indicates more than 300 acid fast bacilli in sputum, PI -VE indicates no acid fast bacilli in sputum.

2.3.6 Humoral response to Zmp1 was detected only in active TB cases and not in their household contacts

Some of the reports have indicated antibodies against specific *M. tb* antigens in the sera of clinically healthy, latently infected Health-care workers (Reis, Rabahi et al. 2009). With the anti-Zmp1 antibodies detected in active EPTB and even in active paucibacillary PTB cases (Table 2), we wanted to check if anti-Zmp1 titers were also detectable in the respective household contacts. These household contacts had stayed with active patients for at the least 7 consecutive days during the 3 months prior to the diagnosis of TB. They were expected to be exposed to *M. tb* though it is reported that EPTB patients, specifically those with tissue TB, are unlikely to transmit the bacilli. Most of these clinically asymptomatic contacts were tested positive for Mantoux's test (Out of 89 household contacts, 60 subjects were Mantoux positive with diameter of Induration >15 mm) and hence may represent possible cases of latent TB. To evaluate that, ELISA readings of EPTB (n=55) and their respective household contacts (n=55) and PTB (n=66) and their household contacts (n=34) were plotted (Figure 12A and 12B). It was observed that compared to EPTB patient contacts (Median: 0.663; IQR: 0.524-0.808) or PTB patient contacts (Median: 0.570; IQR: 0.387-0.630), titers of anti-Zmp1 antibody were distinctly high in EPTB patients (Median: 1.421; IQR: 0.979-2.259; $p<0.001$) or PTB patients (Median: 1.257; IQR: 0.956-1.8; $p<0.001$), respectively (Figure 12A and 12B), suggesting that Zmp1 humoral response is restricted to active infection cases as against asymptomatic household contacts including Mantoux positive cases under the category which may be possible latent subjects.

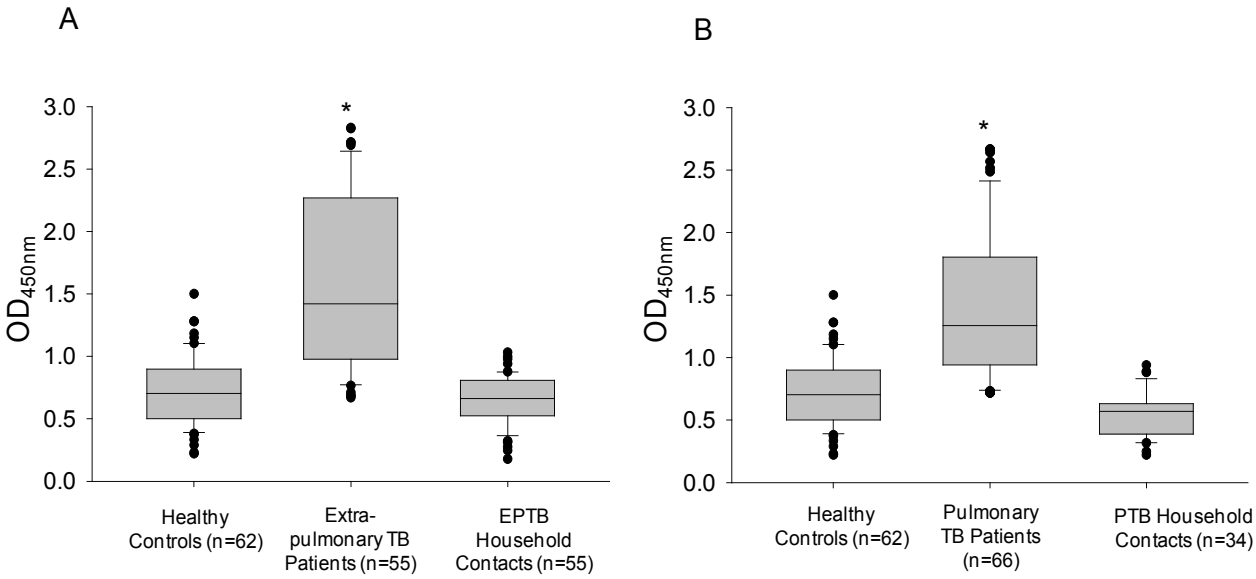

Figure 12: rZmp1 ELISA detected all active TB cases with high specificity and sensitivity. (A) Box plots representing titers of anti-Zmp1 antibody titers as indicated by values at Absorbance 450 nm in Healthy, extra-pulmonary TB (EPTB), EPTB patient contacts (Household contacts of EPTB patients). (B) Box plots representing titers of anti-Zmp1 antibody titers as indicated by values at Absorbance 450 nm in Healthy, pulmonary TB (PTB), PTB patient contacts (Household contacts of PTB patients). ∗The differences in Abs450 values for PTB vs. Healthy; PTB vs. PTB Household contacts; EPTB vs. Healthy; EPTB vs. EPTB Household contacts were highly significant and a p-value of <0.001 was observed in all the cases. Statistical analyses were done using one-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method.

Since rZmp1 ELISA showed the potential to detect paucibacillary cases, we calculated the specificity and sensitivity of rZmp1 ELISA to detect EPTB cases in our conditions.

Table 3: Sensitivity and Specificity of rZmp1 ELISA for EPTB with respect to different control population.

A. With respect to healthy controls		
rZmp1 ELISA test	Positive	Negative
Test +ve	44	9
Test –ve	11	53
Total	55	62
	Sensitivity	Specificity
	44/55 (80%)	53/62 (85.48%)
B. With respect to EPTB Patient contacts		
rZmp1 ELISA test	Positive	Negative
Test +ve	44	2
Test –ve	11	53
Total	55	55
	Sensitivity	Specificity
	44/55 (80%)	53/55 (96.36%)

Table 3: Sensitivity and Specificity of rZmp1 ELISA for EPTB with respect to different control population. A) With respect to healthy controls; B) with respect to EPTB patients contacts (Household contacts of EPTB patients).

Keeping a stringent cut-off value of 0.95 at $Abs_{450,}$ the specificity and sensitivity were calculated for the detection of EPTB with respect to all control populations (refer 2.2.5). With respect to Healthy controls, the specificity and sensitivity were 85.48% and 80% respectively (Table 3A). The same with respect to asymptomatic patient contacts were 96.36% and 80% (Table 3B).

2.3.7 Strong response to Zmp1 was observed in paucibacillary pleural EPTB cases

Since rZmp1 ELISA showed the potential to detect EPTB cases, we further grouped paucibacillary pleural TB cases from our study and checked. ELISA readings between Pleural TB cases (n=20) (Annexure VI) and their respective contacts (n=20) (Annexure VII) were plotted. It was observed that pleural TB patients had high titers of anti-Zmp1 antibody (mean value 1.573) that significantly distinguished them from pleural TB contact populations with mean value 0.79 ($p<0.001$) and Healthy controls (0.702) (Figure 13). This study needs to be further extrapolated by taking large number of datasets.

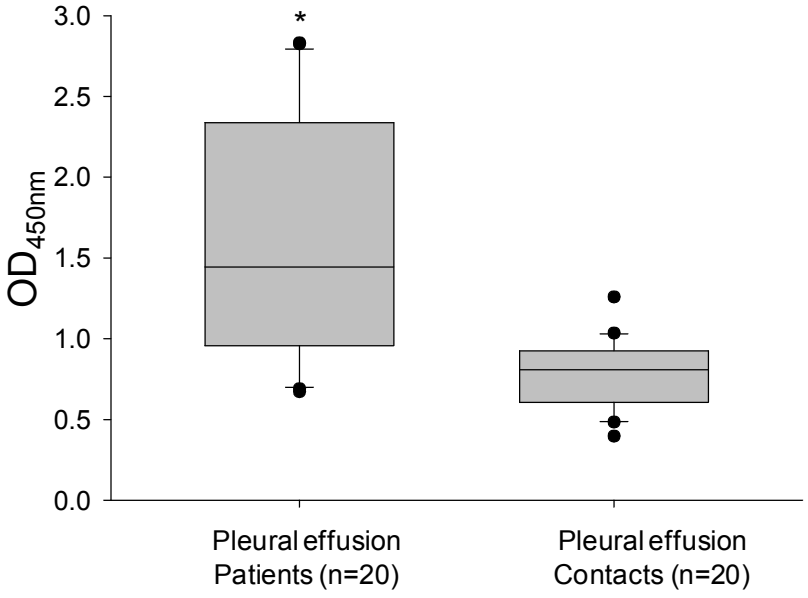

Figure 13: rZmp1 ELISA detected Pleural TB cases. Box plots representing titers of anti-Zmp1 antibody titers as indicated by values at Absorbance 450 nm in pleural TB cases and Pleural TB patient contacts (Household contacts of pleural TB patients) *The differences in Abs_{450} values for pleural effusion vs. Pleural TB Household contacts were highly significant and a p-value of <0.001 was observed.

2.4 DISCUSSION

Till recently, TB vaccine program was driven by the belief that protective immunity against *M. tb* infection is chiefly because of CMI generated by Th1 microenvironment. The majority of candidate vaccines were focused on improving CMI either by engineering the present BCG vaccine or using mycobacterial antigens that elicited Th1 responses as boosters after BCG priming. Interestingly, the vaccine strategies that use the whole cell (such as *M. indicus pranii*) has reached phase III of clinical trials (Weiner and Kaufmann 2014; Tye, Lew et al. 2015). The success of this may be owing to a balanced stimulation of both CMI and AMI wings rather than trying to bias the same towards CMI. Additionally, with monoclonal antibodies conferring passive immunity against several intracellular pathogens, including *M. tb*, humoral response to TB is being explored with a new enthusiasm.

In this study, we deliberated on the immunomodulatory function of a secreted *M. tb* protein Zinc metalloprotease-1. *M. tb* Zmp1 is a well characterized protein that alters phagosome maturation and is considered essential for intracellular survival of *M. tb*. With the X-ray structure available, this protein has been screened for small molecule inhibitors to evaluate it as a potential drug target (Ferraris, Sbardella et al. 2011; Mori, Moraca et al. 2014). Zmp1 works optimally under slightly acidic conditions with neuropeptides as possible substrates (Petrera, Amstutz et al. 2012). It was also shown to be involved in inflammasome activation (Master, Rampini et al. 2008), suggesting that it definitely has immunomodulatory function besides its role in regulating phagosome maturation. Protein sequence analyses by Jameson-Wolf plot using Protean software, which recorded high antigenicity indices based on surface probability and hydrophobicity of its amino-acid sequence of Zmp1 further supported the notion that apart from an active enzyme, it can also be a B-cell stimulant (Figure 4).

We confirmed the secretory nature of Zmp1 in granuloma-like in vitro growth conditions and concluded that Zmp1 could indeed be released out in the extracellular milieu when *M. tb* is growing in acidic, nutrient deprived and oxidatively stressed acellular environment at the centre of TB granulomas (Figure 7). In addition to that, we also observed that it is a strong immune-stimulant and could stimulate mono-nuclear cells to release high titers of TNF-α and IL-1β, the shock inducing cytokines that also start the innate mechanisms towards acute phase reaction.

However, subsequently, it was not the pro-inflammatory, but Th2 cytokines that were predominantly released upon rZmp1 stimulation of PBMCs (Figure 8). Corroborating the Th2 response, in a cohort of about 121 TB patients, high titers of anti-Zmp1 antibodies could be recorded (Figure 10). This study revealed yet another facet of *M. tb* Zmp1 as a highly immunogenic mycobacterial antigen that could elicit strong and specific humoral response in TB patients.

There are many reports analyzing the levels of IgG antibody titers against *M. tb* antigens, secretory or otherwise, in different clinical stages (Wu, Yang et al. 2010; Baumann, Kaempfer et al. 2014; Baumann, Kaempfer et al. 2015). The studies also claim that these antibodies are present during active disease, but reduce upon decrease in bacterial load with treatment (Singh, Dong et al. 2005). That would mean antibody titers are bacterial load dependent and a low bacterial infection may not sufficiently trigger a good humoral response. To check the same, we compared the anti-Zmp1 antibody titers in patients with high or low bacillary loads. To our surprise, anti-Zmp1 titers were equally high in paucibacillary cases as in patient with sputum bacilli score of 3+ (Figure 11; Table 2). This suggested that a very low dose of *M. tb* Zmp1 could induce very strong and specific B-cell response, a property that can be suitably explored for prime–boost vaccination strategy. In total, with the evidence that Zmp1 is secreted by active *M. tb* in granuloma like growth conditions and stimulated PBMCs to release Th2 class of cytokines, one may hypothesize that very low concentration of this protein when released in the granulomas of infected host, stimulate the neighboring mono-nuclear cells to eventually generate a Th2 immune response that supports B-cell specific immunity towards Zmp1 resulting in high titers of anti-Zmp1 antibodies in patients. However, unlike reported humoral response to GlcB (malate synthase), MPT51 (FbpC1) and HSPX (alpha crystalline) in latent TB cases (Reis, Rabahi et al. 2009), anti-Zmp1 antibodies could not be detected significantly in healthy household contacts who were asymptomatic, Mantoux's positive and possibly represent latently infected population in this study.

Likewise, we observed that rZmp1 ELISA was highly specific for TB cases, both PTB and EPTB (Figure 11). The group comprising of non-specific infections were used to rule out if, Zmp1 cross-reacts with antibodies generated against other bacterial or viral proteins. This group had patients with random infections other than TB such as viral, bacterial and parasitic infections. Non-reactivity of rZmp1 with antibodies in the sera of non-specific infection controls

as indicated by low-absorbance values (Figure 9) points to the specificity of rZmp1 to distinctly differentiate TB cases from all other categories. This suggests the potential of rZmp1 ELISA as a disease marker which can be further explored. Identification EPTB disease remains challenging for reasons like diffused symptoms, low *M. tb* load at the site of infection and difficulties in obtaining clinical specimens from deep-seated organs (Lawn and Zumla 2012). Dependence on cultures for EPTB frequently leads to substantial delays, compromising patient care and spread of infection to others. Though serological tests are less acceptable in the field of TB detection, the problem possibly lies in the selection of an antigen that shows a good serological response even when infection load is less. In comparison with microscopy and cultures, ELISA based serological tests offer several advantages such as in terms of time, infrastructure and ease of sample collection in the form of peripheral blood.

In this study, we revealed hitherto unknown immunogenic property of *M. tb* Zmp1. Zmp1 is a strong *M. tb* specific immune-stimulant, the properties of which can be further explored both as a potential vaccine candidate or a disease marker. The possibility of taking the study on antigenicity of Zmp1 to the next level by using larger cohort, blinded samples and multi-centric study is very stimulating from both scientific point of view and translational research.

Chapter 3

Biochemical and physiological characterization of Zmp1: role in dissemination

3.1 INTRODUCTION

As mentioned in the chapter 1, the TB causing bacilli, *Mycobacterium tuberculosis* enter the host through aerosol route and are engulfed by alveolar macrophages, polymorphonuclear neutrophils and type 2 pneumocytes by phagocytosis (Smith 2003). In most of the cases, the infection does not result in disease as the bacillus has evolved with nimble strategies to live in balance with the immune response, thus remaining latent for decades (Babalola 2015). A hallmark of immune reaction to TB bacilli is the formation of granuloma which helps in the containment of the infection (Guirado and Schlesinger 2013). The granuloma is an aggregation of various cells triggered in response to the cytokine environment. It consists of various cells of immune system such as macrophages, dendritic cells, and lymphocytes, (Ordway, Harton et al. 2006). Some of the infected cells undergo necrosis and create an acellular central zone where TB bacilli persist within granuloma. This necrotic zone eventually disintegrates in certain immunocompromised hosts, triggered by mechanism still unknown, causing reactivation (Silva Miranda, Breiman et al. 2012; Guirado and Schlesinger 2013).

A significant aspect of the pathogenesis of virulent mycobacteria, like *M. tb*, is the ability to modulate cell death pathways where apoptotic cell death is considered bactericidal and necrotic cell death possibly assists bacterial dissemination and transmission (Chen, Divangahi et al. 2008; Gan, Lee et al. 2008; Divangahi, Chen et al. 2009; Behar, Divangahi et al. 2010). In this regard, several secretory proteins of *M. tb* have been implicated in either initial establishment of lung infections or extrapulmonary dissemination. To exemplify, ESAT-6 limits macrophage responses by inhibiting signaling from Toll like receptor-2 (TLR-2) and causes phagosomal membrane lysis, thus helping establishment of infection, while HbhA, a glycoprotein, found on *M. tb* surface and also in culture filtrates, is not required for initial infection, but has possible role in dissemination to extrapulmonary regions (Pethe, Alonso et al. 2001; de Jonge, Pehau-Arnaudet et al. 2007; Pathak, Basu et al. 2007). Yet other proteins, unique to mycobacteria genus, like PE25/PPE41 protein complex, has been shown to induce necrosis in macrophages and speculated to have role in dissemination and disease reactivation (Tundup, Mohareer et al. 2014).

As discussed in chapter 1.7, several secretory proteins belonging to zinc metalloprotease family play role in the virulence of pathogenic bacteria by various mechanisms. Many proteases such as *V. cholerae O1* serotype are established exotoxins and virulence factors known to damage tissues

and help in dissemination (Finkelstein and Hanne 1982; Hase and Finkelstein 1993). So far, we have evidences of three zinc-metalloproteases from *M. tb* in the culture filtrate, namely, Rv2869 (Rip), Rv2467 (pepN) and Rv0198c (Zmp1). Rv2869 (Rip), a secretory metalloprotease has been shown to regulate intramembrane proteolysis and proteolytic degradation of anti-sigma substrates like RsdA controlling the SigD axis of mycobacteria and thereby playing a role in mycobacterial virulence (Makinoshima and Glickman 2005; Sklar, Makinoshima et al. 2010). Rv0198c (Zmp1), supported by deletion mutant studies, was implicated in suppression of inflammasome activation by inhibiting caspase-1 activity and phagosome maturation, leading to decreased pathogen clearance suggesting a key role of Zmp1 during M. tb pathogenicity (Master, Rampini et al. 2008; Johansen, Fettelschoss et al. 2011). Interestingly, the purified recombinant Zmp1 protein was earlier shown to cleave synthetically generated neuropeptides (Petrera, Amstutz et al. 2012). Zmp1 of *M. tb* possess a HEIGH motif which is predominant feature of a zinc metalloproteases. Length of Zmp1 is 1992 basepairs consisting of 663 aminoacid residues corresponding to 74kDa. With *M. tb* Zmp1 reported as virulent factor holding the properties of immunomodulation, high immunogenicity and proteolysis of synthetic neuropeptides, we further extended the study on the possible role of Zmp1 in pathogenesis of *M. tb*.

3.2 MATERIAL AND METHODS

The cell lines used were CHO cell line and THP-1 Monocyte leukemia cell line (Cat#TIB-202, ATCC). Bacterial strain used was *Mycobacterium tuberculosis*, H37Rv and *Mycobacterium marinum.* Cell culture media and fetal bovine serum were purchased from Himedia, India. Pierce™ LAL Chromogenic Endotoxin Quantitation Kit from Thermoscientific, India, Casein was obtained from Sigma-Aldrich, USA. BD OptEIA^{TM} capture ELISA sets and eBioscience Human cytokine ELISA Ready-Set-Go kits were used. MTT, DMSO and other chemicals were purchased from HiMedia, India. All plastic ware and glassware was obtained from Tarsons, India; Eppendorf, Germany and Corning, USA. pTEC27 plasmid was obtained as a gift from Dr. Lalita Ramakrishnan and Dr. Deepak Saini for electroporation into *M. marinum.*

3.2.1 Maintenance of cell lines:

The THP-1 cell lines were maintained in RPMI 1640 media supplemented with 10% FBS (South American origin, Gibco) and incubated at 37^{o}C with 5% CO_2. The media was changed when the confluency of the cells reached 90%. The CHO cell lines were maintained in RPMI media supplemented with 10% FBS and incubated at 37^{o}C with 5% CO_2. The media was changed when the cells reached 90% confluency, the cells were trypsinized with the Trypsin-EDTA (Sigma) and washed with phosphate buffered saline pH 7.4 (PBS) (Ganji, Dhali et al. 2016).

3.2.2 Growth and Maintenance of mycobacterial culture:

Mycobacterium marinum transformed with pTEC27 harboring Td-Tomato (TdM. marinum) was plated on 7H10 agar media (Hi-media, India) supplemented with 10% Oleic acid, Albumin, Dextrose and Catalase (OADC, Hi-Media, India) in presence of Hygromycin (50 µg/mL). The plate was incubated at 30^{o}C until colonies appeared. The colonies were picked into the 7H9 broth supplemented with 10% OADC, Hygromycin (50 µg/mL) and the broth culture was incubated at 30^{o}C at 180 rpm until the $OD_{600\ nm}$ reached 0.8 to 1. The culture was checked for any contamination and single suspension of bacteria was obtained as described earlier (Ganji, Dhali et al. 2016).

3.2.3 Cloning, Expression and Purification of Zmp1 (rZmp1) protein: Refer 2.2.1

3.2.4 Caseinase activity:

Hydrolysis of casein by rZmp1 was performed at 37^{o}C for 1hr in Tris-Cl buffer (50mM Tris-Cl pH-8.0 and 100mM NaCl). rZmp1 (5µg) and casein (8µg) were added to the reaction mix at respective concentrations. The reaction was stopped by adding Laemmli's buffer to the reaction mix followed by fractionation on SDS-PAGE. Casein alone and rZmp1 alone were used as controls.

3.2.5 Mucinase activity:

50ul of 4x buffer (100mm Tris-Cl pH-8.0, 10mM KCl, and 10mM $MgCl_2$) was added to each well and mucin (10mg/ml) was taken. rZmp1 (20µg) was added to the reaction mix at respective concentrations and the reaction mix was made up to 200ul/well using MilliQ. Shake well and Incubate at 37^{0}C for 1hour.100ul/well of protamine sulphate (1mg/ml) was added and incubated for 5min. absorbance was read at 492nm. Trypsin was used as positive control.

3.2.6 MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay:

About 0.2 × 10^{5} cells (THP-1 and CHO cell lines) per well were seeded in 200 µL media into a 96 well plate. Purified rZmp1 protein was added exogenously at varying concentrations ranging from 5 nM to 2 µM and incubated for 24 hr at 37^{o}C with 5% CO_2. Later 20 µl of 5 mg/mL MTT solution was added to each well, followed by incubation at 37^{o}C with 5% CO_2 for 4 hr. 150 µL of spent media was removed and the formazan crystals were dissolved in 150µl of DMSO. Absorbance at 540nm was read using multi-well plate reader.

3.2.7 Toxicity assay in Zebra fish:

Zebra fish breeding pairs were set up and embryos were collected. Embryos were placed in a 96 well plate (approximately 6 per well) filled with E3 media. Zebra fish embryos were taken and incubated with varying concentrations of rZmp1 in E3 media for 24 -72 hours. Images were taken at different time points using inverted microscope and checked for the survival.

3.2.8 Exogenous treatments with rZmp1:

THP-1 monocytes were stimulated with 10 pg/mL of PMA and differentiated macrophages were treated with and without nigericin (5µM) for 30 mins. Cells were washed twice with PBS and

later treated with rZmp1 protein at 50 and 100 nM concentrations for 24 hours. The culture supernatants were taken and performed ELISA.

3.2.9 Gap closure assay:

CHO cells were allowed to reach >90% confluence in 35 mm cell culture dish. For the assay, prepared 10ml of base media with 50 nM of rZmp1 protein and filter sterilized. Meanwhile, a line was drawn with a marker on the bottom of the dish. Using a sterile tip, three separate wounds were scratched through the cells moving perpendicular to the line drawn before. Cells were rinsed gently with PBS and replaced with 1.5 mL of media either containing rZmp1 protein or only buffer (control set). Pictures were taken using phase contrast at 10× magnification at 16, 24 and 48 hr. The same was repeated with the control set without rZmp1. The gap closure was measured in both control set and rZmp1 containing set using ImageJ software (Liang, Park et al. 2007).

3.2.10 Boyden chamber assay:

24-well tissue culture plate containing cell culture inserts with polycarbonate membrane of 8µm pore size were taken. Added 200ul of media with 0.4×10^5 cells without rZmp1 to upper chamber of cell insert and 750ul of media without cells containing only 50 nM of endotoxin-free rZmp1 protein was added to lower chamber. The plate was then placed at 37^{o}C with 5% CO_2 for 24 hours. For Staining: Cells were fixed in Paraformaldehyde (3.7% in PBS) for 3-5 mins. Later, cells were rinsed with 1X PBS and diluted Giemsa Stain (1:20 with deionized water) was added and incubated for 15 mins at RT. Cells were washed with water to remove excess stain using deionized water, air dried and used for obtaining representative images. For counting: The cells in the upper and lower chamber were collected separately by centrifuging at 200-300g for 5 min. The cell number was then determined by Trypan blue exclusion assay using hemocytometer.

3.2.11 Capture ELISA Protocol:

Capture ELISA for cytokine measurements was performed as per the manufacturer's instructions. Briefly, 50 µL of diluted capture antibody was added to the wells of ELISA/RIA compatible 96-well plate for overnight at 4^{o}C. Followed by three PBS-T (PBS containing 0.05% Tween-20) and one PBS washes. The coated plate was blocked using 3% bovine serum albumin

in PBS at 37^{o}C for 1 hr. After blocking, washes were performed as mentioned earlier. 50 µL of sample was added and incubated at 37^{o}C for 2 hr which was followed by five PBS-T and one PBS washes. Then 50 µL of diluted detection antibody along with streptavidin-HRP conjugate was added and incubated at 37^{o}C for 1 hr, followed by 7 × PBS-T and one PBS washes.3,3',5,5'-Tetramethylbenzidine (TMB) was used as substrate according to manufacturer's directions. Dispensed 100 µl into each well in dark and incubated at RT (5-30 min) for color development. The reaction was stopped using 100 µL of 2N H_2SO_4. Read the optical density (OD) for each well with a microplate reader set to 450 nm.

3.2.12 Lactate Dehydrogenase (LDH) assay:

The lactate dehydrogenase activity was determined by measuring the decrease in the absorbance at 340 nm resulting from the oxidation of NADH. The reaction mix consisted of 0.66 mM NADH and 3 mM Sodium pyruvate in 0.2M Tris-HCl, pH 7.3. To 100 µL of reaction mix 20 µL of culture supernatant of THP-1 cells treated with or without rZmp1 was added and monitored the change in NADH levels at 340nm.

3.2.13 Propidium Iodide staining for flow cytometry;

THP-1 cells were treated with or without rZmp1 (500 nM and 1 µM) for 24 hr. After incubation, the media was removed, added prewarmed RPMI complete media containing 40 µg/mL of propidium iodide (PI) for 15 min at room temperature (RT) in dark. After staining, the cells were washed twice in PBS and then fixed with 3% paraformaldehyde at RT for 20 min. Then the cells were washed twice with PBS followed by RNase (50 µg/mL) treatment at 37^{o}C for 15 min. The cells were then washed with PBS and resuspended in PBS for flow cytometry analyses.

3.2.14 TdM. marinum dissemination assay in Zebrafish:

Fluorescently labeled TdM. marinum was generated by electroporation of *M. marinum* with pTEC27 (1 µg) carrying tdTomato at 2500 Volts for 5 milliseconds in a pre-chilled 2mm cuvette. After electroporation, the cells were allowed to recover in antibiotic-free media at 37^{o}C for 3-4 hr. After recovery the transformants were selected on 7H10 agar media containing 50 µg/mL of hygromycin at 37^{o}C for 2-3 weeks. The fluorescently labeled *M. marinum* (TdM. marinum) colonies obtained were picked into 7H9 broth media with antibiotic and used for Zebrafish

studies. Fluorescently labeled TdM. marinum was injected at 200 cfu (colony forming unit) with and without rZmp1 (25 nM to 100 nM) into yolk sac of 1 dpf (day post fertilization) zebrafish. Images were taken at the 0 day of injection and on the 5th day after injection of TdM. marinum using fluorescent microscopy. Fish were scanned for the presence of TdM. marinum in the whole organism and images were taken accordingly. Fluorescence intensity of TdM. marinum presence in the fish was quantified using ImageJ software. Total fluorescence was calculated by taking count of all the regions in a whole fish (Benard, van der Sar et al. 2012). Dissemination of TdM. marinum towards tail region was calculated by two parameters and plotted: 1) Relative percentage of fish with TdM. marinum disseminated to tail region = (Number of fish with TdM. marinum disseminated to tail region / total number of fish injected with TdM. marinum in the category) × 100; 2) Dissemination to tail region = (Fluorescence intensity in tail region / total fluorescence intensity in whole fish) × 100. Survival of Zebrafish for the rZmp1was evaluated by injecting the fish with rZmp1 (ranging from 25 nM to 100 nM) alone without any TdM. marinum and monitored the fish over the course of 5 days.

3.2.15 Graphs and Statistical analyses:

SigmaPlot software version 11.0.0.77 (Systat Software, Inc., USA) was used for different statistical analyses. For cytokine data and cell migration data, One-way ANOVA was performed with Holm–Sidak multiple pair-wise comparison method and the threshold for significance was set at $p<0.05$. The error bars represent the ± standard deviation (SD) from the mean of at least three independent experiments. For statistical analyses of mycobacterial dissemination data, One-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method for comparing more than two groups or Students t test with Mann-Whitney Rank sum test was performed for comparing two groups. The threshold for significance was set at $p<0.05$They were represented as box plots using SigmaPlot. Within the plots, the upper quartile of the box represents the 75th percentile and the lower quartile for the 25th percentile. The line inside the box represents the median. The whiskers arising from either side of the upper half and the lower half of the box corresponds to 1.5 times the interquartile range (IQR) (Benjamin, Banerjee et al. 2013; Vemula, Ganji et al. 2016). Any datum to the further extreme of the whiskers is termed as outlier.

3.3 RESULTS

As discussed in chapter 2.3.1, the endotoxin free Purified recombinant Zmp1 was used for further experiments.

3.3.1 Confirmation of the protease activity of recombinant Zmp1:

As earlier reports indicate rZmp1 to be a protease, we checked the proteolytic activity using a common substrate like casein. rZmp1 was checked for protease activity using casein as the substrate. The endotoxin-free rZmp1 protein was checked for functional activity using casein as a substrate (Rowland, Ruckert et al. 1997; Coffey, van den Burg et al. 2000; Grandgenett, Otsu et al. 2007). Proteolytic degradation of casein can be assessed on SDS-PAGE by monitoring for cleavage products at lower molecular weights or apparent shift in the casein band to lower molecular weight after proteolytic digestion. As anticipated hydrolysis of casein by rZmp1, two bands corresponding to apparent molecular weights of 27 kDa and 20 kDa were observed (Figure 14A). Band corresponding to 20kDa is the cleaved product of casein which was observed when casein was incubated with rZmp1 suggesting that the purified protein was functionally active and that protein was used for further studies (Vemula, Ganji et al. 2016). Heat-inactivated rZmp1 (HI) was later checked for caseinase activity (Figure 14B) and was observed that after heating rZmp1 at 100^{o}C, the protein lost its original confirmation and activity, therefore was used in further experiments as an inactive protein.

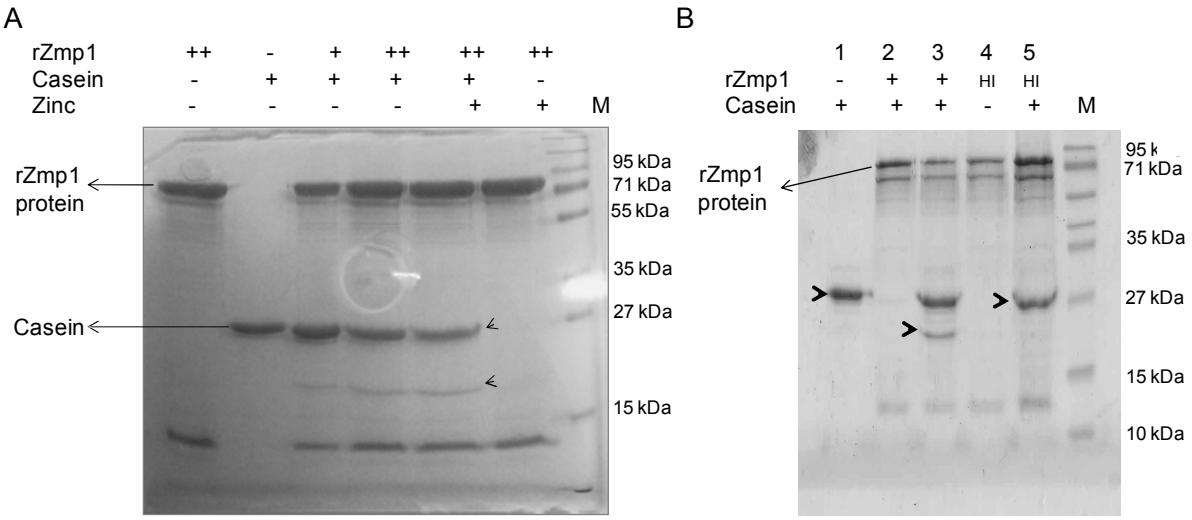

Figure 14: SDS-PAGE depicting the functional activity of purified rZmp1 using casein as substrate. A) Endotoxin-free rZmp1 was incubated with casein in presence or absence of Zinc ions. Casein was hydrolyzed by rZmp1 yielding two bands (marked by arrowheads) corresponding to apparent molecular weights of 27 kDa and 20 kDa. B) Endotoxin-freerZmp1protein or heat-inactivated rZmp1 (+ represents ~ 4.8 µg and HI represents heat inactivated rZmp1) was incubated with casein (+ represents 10 µg) at 37^{o}C in 100 mM Tris pH-8.0. Small black arrowheads show the Casein (lane 2) and cleaved product of Casein (lanes 3 and 4).

The proteolytic activity of Zmp1 was further characterized using mucin as substrate. Earlier reports on prokaryotic zinc metalloproteases suggested their ability to degrade mucin (Luo, Kumar et al. 2014; Valeri, Rossi Paccani et al. 2015). rZmp1 was assayed for colorimetric based mucinase activity, where cleaved mucin show decrease in the absorbance maxima at 492nm (Figure 15). The decrease in absorbance at 492nm was used to calculate the % mucinase activity (refer methods). rZmp1 could degrade mucin as indicated by decrease in the absorbance at 492nm. Trypsin was used as a positive control. The percent mucinase activity by normalizing with standard mucin for rZmp1 was 38.5859±1.43 and trypsin was 54.65±2.308 (Figure 15A). Lipopolysaccharide (LPS) was also used to check the mucinase activity and found the percent mucinase activity to be 5.2920±0.0084853 in comparison with rZmp1 (38.8686±0.0070711) (Figure 15B). From these results, we concluded that *M. tb* Zmp1 could degrade mucin

From these results we could infer that rZmp1 was an active protease. This protein was further used for characterization.

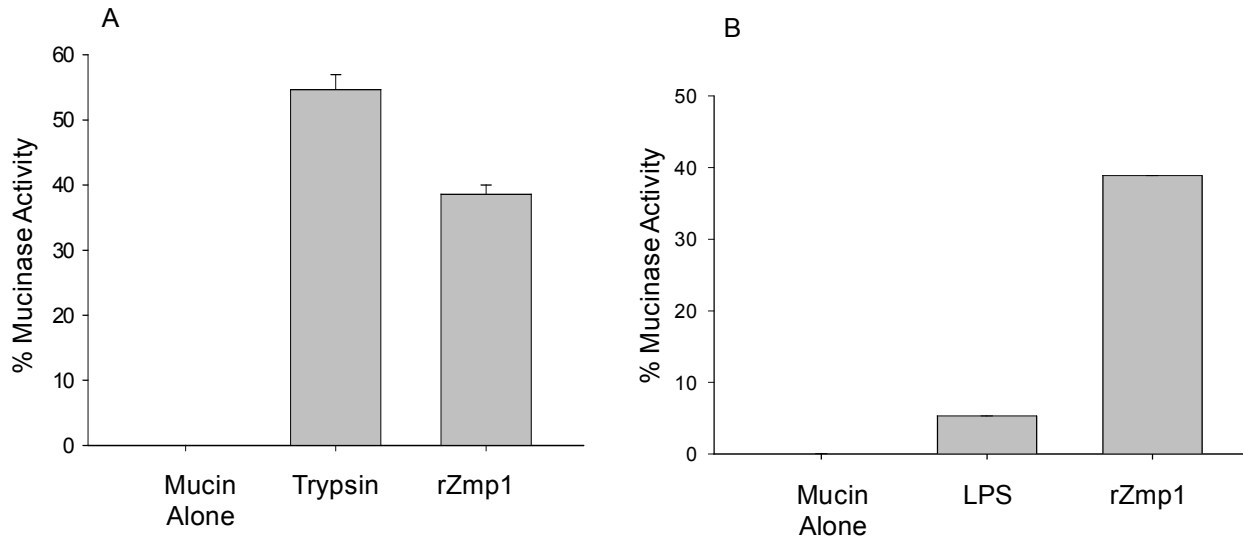

Figure 15: rZmp1 possess mucinase activity. (A) Bar graph represents the percentage mucinase activity with mucin alone as blank and trypsin as positive control (B) Bar graph represents the percentage mucinase activity with LPS and rZmp1. All the experiments were performed more than three times. The error bar represents standard deviation from mean

3.3.2 Higher concentrations of exogenous rZmp1 treatment caused necrotic cell death:

Secreted zinc metalloproteases across pathogenic bacteria are known to act as toxins causing a range of pathological effects by virtue of their proteolytic activities, quite often causing necrotic tissue damage (Hase and Finkelstein 1993). To begin with, we checked for the toxicity of the recombinant Zmp1 (rZmp1) on human monocyte leukemia cell line THP-1 and CHO cell lines (Figure 16A and 16B). THP-1 monocytes or CHO cells were treated with varying concentrations of endotoxin-free rZmp1 and assayed for cell death using MTT assay. It was observed that though rZmp1 protein was not toxic at lower concentrations of 50 nM but was toxic at high concentration starting from 500 nM at 24 hr (Figure 16A and 16B).

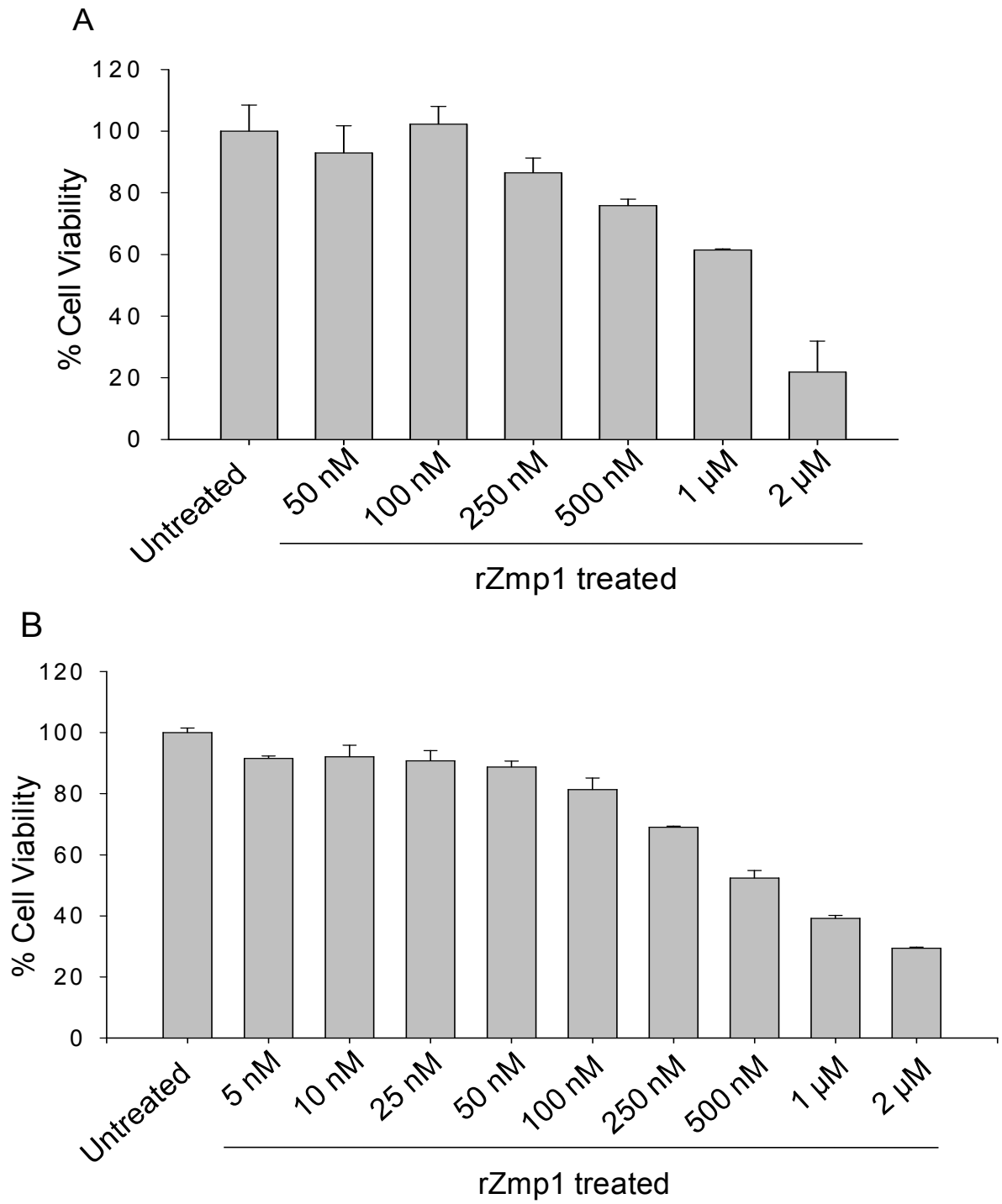

Figure 16: MTT assay showing toxicity of rZmp1 in THP-1 and CHO cell lines. (A) Bar graph represents the percentage THP-1 cell viability upon exogenous treatment with varying concentrations of endotoxin-free rZmp1.(B)Bar graph represents the percentage CHO cell viability upon exogenous treatment with varying concentrations of endotoxin-free rZmp1 cytotoxicity of exogenous treatment of endotoxin-free rZmp1 on CHO cells as determined using

MTT assay. All the experiments were performed more than three times. The error bar represents standard deviation from mean

The IC_{50} calculated for rZmp1 cytotoxicity on THP-1cells based on the MTT assay. It was found that the IC_{50}value was 550 nM (Figure 17).

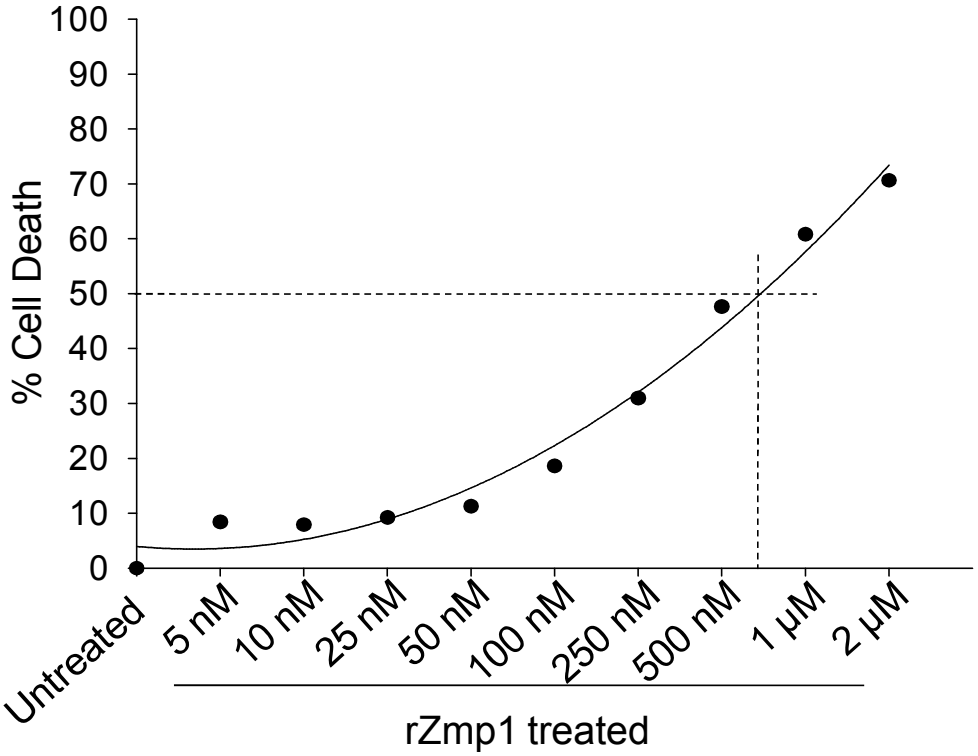

Figure 17: rZmp1 at higher concentrations leads to cell death with IC_{50} of ~550 nM. Non Linear Regression curve represents the percentage THP-1 cell death upon exogenous treatment with varying concentrations of rZmp1. The dotted lines depict the concentration of rZmp1 at which there was 50% cell death (IC_{50}).

We further observed that the toxicity at higher concentrations was due to necrotic mode of cell death as determined by flow cytometry using Propidium Iodide (PI) staining of the rZmp1 treated cells and lactate dehydrogenase (LDH) assay of the supernatants of the rZmp1 treated cells (Figure 18A, 18B and 18C). It was observed that rZmp1 treated cells at both 500 nM and 1 µM concentrations showed increased PI stained population suggesting cell death due to necrotic damage (Figure 18A and 18B). The 500 nM and 1 µM rZmp1 treated cells showed 66.6% and 75.51% PI positive population, respectively (Figure 18A and 18B). Upon LDH assay of the

culture supernatants of the rZmp1 treated and untreated THP-1 cells, it was observed that culture supernatants of rZmp1 (both 500 nM and 1µM) treated cells showed increased LDH activity (500nM rZmp1: 0.548±0.03 Abs340nm; 1µM rZmp1: 0.558±0.02 Abs340nm) as compared to untreated controls (0.388±0.02 Abs340nm) suggesting the mode of cell death to be necrotic in nature (Figure 18C).

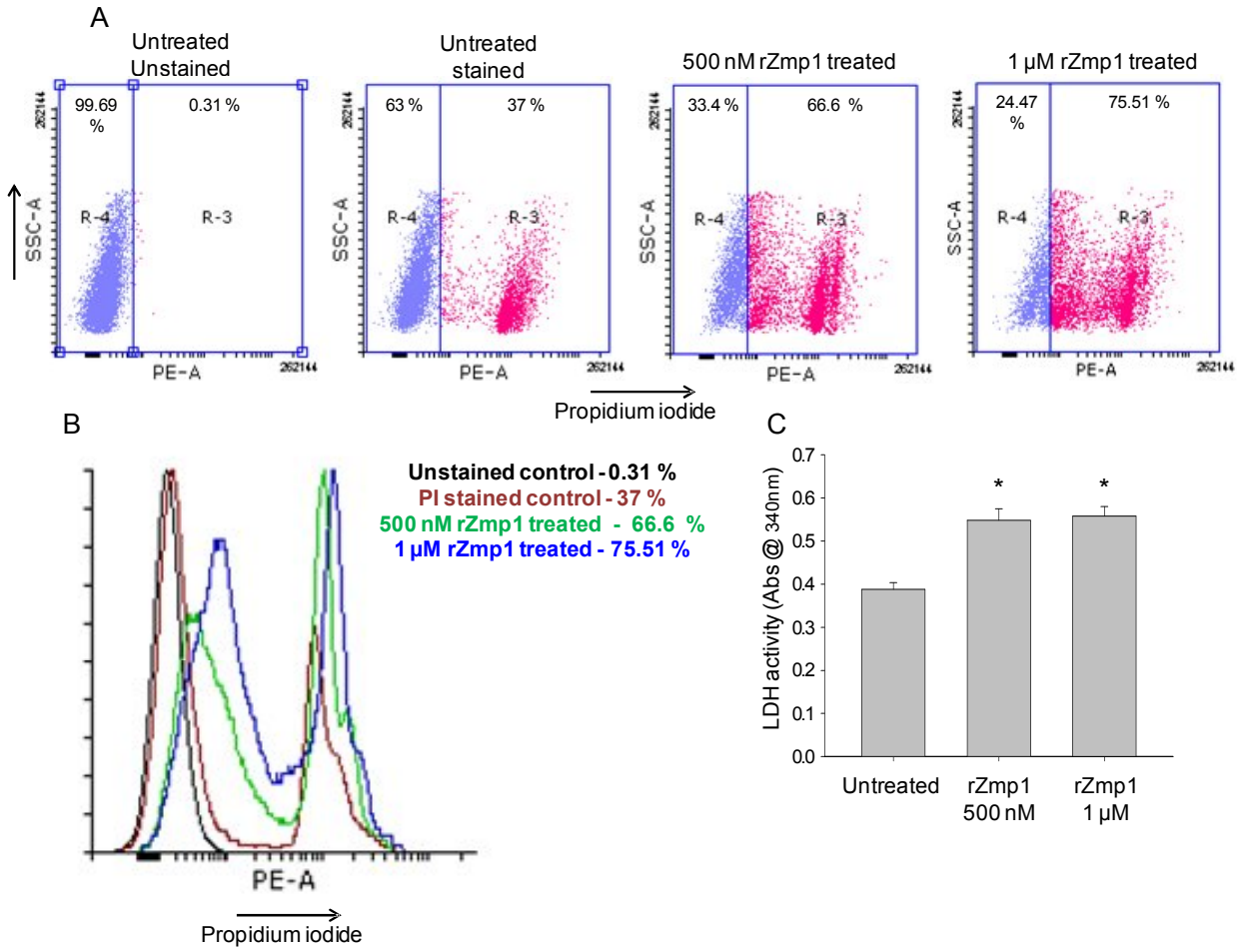

Figure 18: Exogenous treatment of THP-1 with rZmp1 induces necrotic cell death. (A) The dot plot shows the propidium iodide staining for the conditions, Untreated unstained, Untreated stained, 500 nM rZmp1 treated and 1 µM rZmp1 treated cells. The THP-1 cells were either only buffer treated or rZmp1 treated (500 nM and 1 µM) for 24 hr. Propidium iodide staining followed by flow cytometry was used to evaluate for necrosis of THP-1 cells. (B) The histogram plot depicts the extent of PI staining upon rZmp1 treatment. (C) Bar graph represents the Lactate dehydrogenase activity (Absorbance at 340nm) of the culture supernatants of THP-1 cells either buffer treated or rZmp1 treated.

We further checked the levels of cytokines, TNFα, IL-6 and IL-1β, which are implicated in necrosis (Kaczmarek, Vandenabeele et al. 2013; Moriwaki, Bertin et al. 2015), in the culture

supernatants of the THP-1 cells treated with rZmp1 (500 nM and 1 µM) (Figure 19A, 19B and 19C). The levels of cytokines as measured by ELISA were TNFα (Untreated: 54.82±14.75 pg/mL; 1 µM rZmp1 treated: 239.46±35.31 pg/mL; $p<0.05$) (Figure 19A), IL-6 (Untreated: 91.93±15.92 pg/mL; 1 µM rZmp1 treated: 1318.47±74.23 pg/mL; $p<0.05$) (Figure 19B) and IL-1β (Untreated: 328.19±168.89 pg/mL; 1 µM rZmp1 treated: 2665.31±1366.98 pg/mL; $p<0.05$) (Figure 19C), respectively. As anticipated there was significant increase in the secretions of these cytokines by THP-1 into the culture supernatants upon rZmp1 exposure as compared to untreated cells further pointing that exogenous treatment of rZmp1 at higher concentrations induced necrosis. Many secreted bacterial zinc-metalloproteases across various species have been reported to be cytotoxic in nature and help in the bacterial pathogenesis (Hase and Finkelstein 1993). In general, necrosis of the *M. tb* infected cells has been associated with the dissemination of bacilli and further infection of fresh cells. Careful evaluations with complementary experiments are further required to understand the exact mechanism of necrotic death caused by rZmp1.

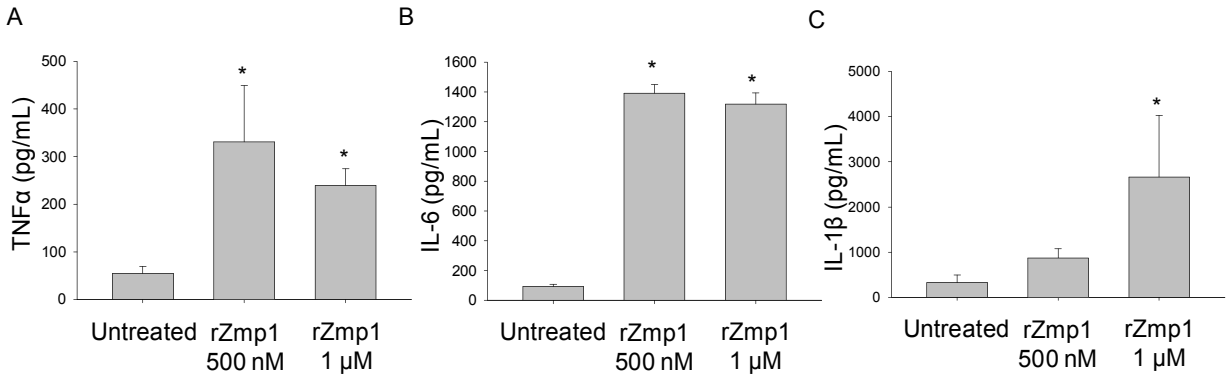

Figure 19: Induction of necrotic cytokines by rZmp1. Bar graph represents the levels of cytokines such as, (D) TNFα, (E) IL-6 and (F) IL-1β determined in the culture supernatants of rZmp1 treated cells, using capture ELISA method. All the experiments were performed atleast three times. The error bars represent standard deviation from mean. * represents statistical significance with $p<0.05$.

From the previous results indicating rZmp1 to help necrosis, we performed Toxicity assay using Zebra fish embryos. Groups of 12 zebra fish embryos were exposed at doses from 1µM to 0.001µM of rZmp1. Zebra fish embryos were placed in a 96 well plate (approximately 6 per well) with 200ul E3 media. Zebra fish embryos were incubated with 50nm rZmp1 in E3 media

for 24 -72 hours. The minimum lethal dose is 0.05µM having mortality less than 10% after 72hrs exposure (Figure 20A). Images were taken at 72 hrs and found that tail region was found to become opaque with dentations on rZmp1 treatment (Figure 20B).

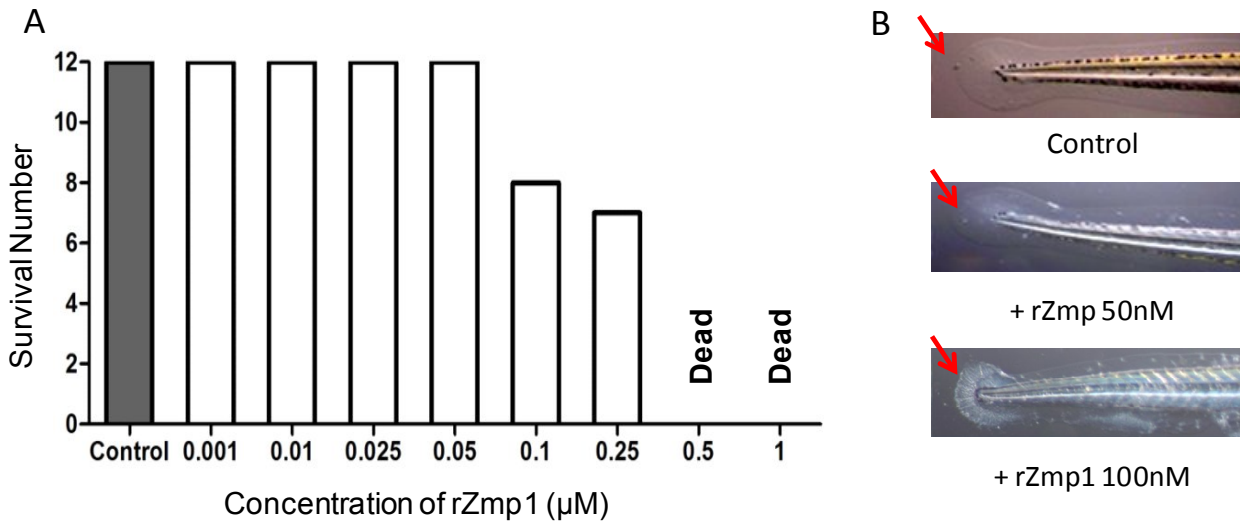

Figure 20: Toxicity effect of rZmp1 on Zebrafish embryos (A) Bar graph shows survival rate of Zebrafish when treated with varying concentration of rZmp1 after 72 hrs. (B) images showing different morphology of the tail region upon treatment with rZmp1 at 50 and 100nM concentrations.

3.3.3 Role of Zmp1 in inflammasome pathway:

From the previous reports using knockout studies it has been shown that Zmp1 inhibits inflammasome activation with decrease in IL-1β levels (Master, Rampini et al. 2008). To further check the molecular players involved in this process, we performed exogenous treatments with rZmp1 on THP-1 macrophages. We further observed if rZmp1 treatment of THP-1 cells resulted in decline of the pro-inflammatory cytokine IL-1β thereby helping mycobacterial survival. The cytokine levels in the culture supernatants of THP-1 cells untreated or rZmp1 treated were, Untreated: 303.5570±53.3230 pg/mL; 50nM rZmp1: 697.0480±132.4060pg/mL; 100 nM rZmp1: 783.2830±140.6190 pg/mL; ($p<0.001$). Nigericin was used as a positive control as an inducer of inflammasomes (Mariathasan, Weiss et al. 2006; Pelegrin and Surprenant 2007) and cells were treated with rZmp1 in the presence of nigericin. Addition of rZmp1 to nigericin treated cells

further augmented the levels of IL-1β. The cytokine levels in the culture supernatants of THP-1 cells with nigericin or nigericin + rZmp1 treated were, nigericin treated: 872.1390±116.1080 pg/mL; 50nM rZmp1: 1061.3990±112.0970 pg/mL; 100 nM rZmp1: 1066.7580±117.6990 pg/mL; ($p<0.001$) (figure 21). Cells upon incubation with rZmp1 at 50 nM and 100 nM showed increased levels of IL-1β compared to the untreated cells. This was in contrary to the earlier studies using Zmp1 mutant, suggesting that exogenous treatment and mutant may not function by same mechanism. Most likely, there is a cross-talk between other mycobacterial protein and Zmp1, which is reflected in the activity of the mutant. So, there must be possibly a different molecular mechanism with other players involved in to decrease IL-1β in case of *M. tb* infection which needs to be explored.

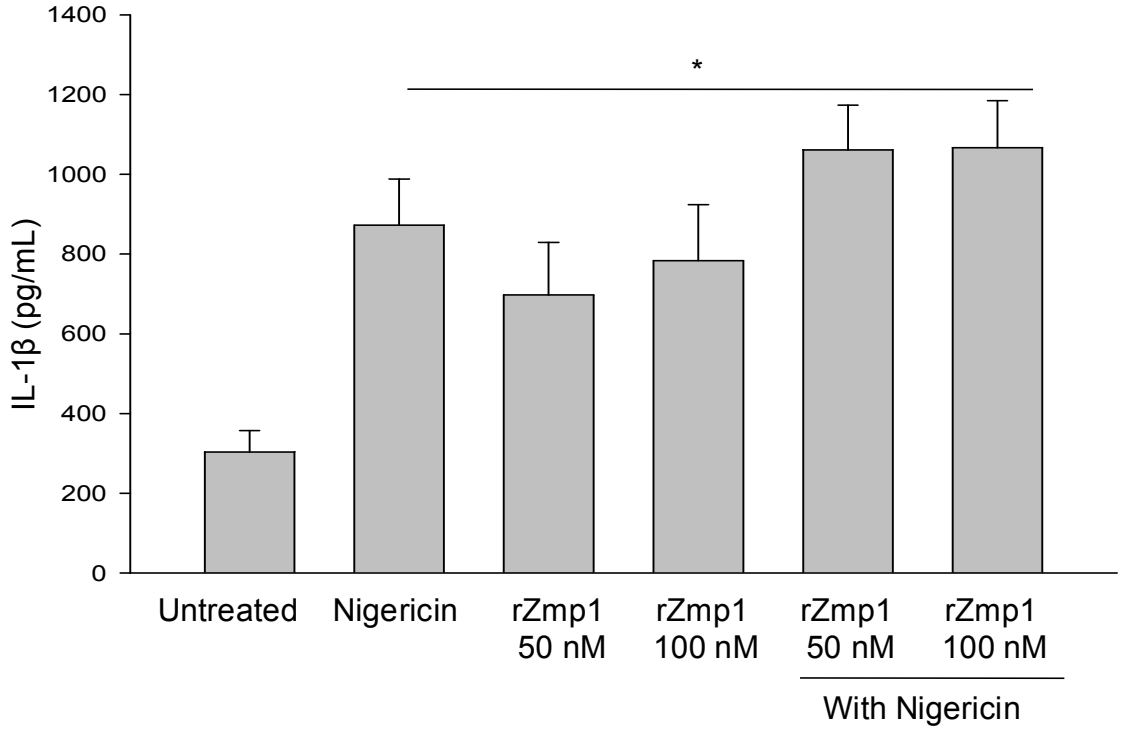

Figure 21: Role of Zmp1 in inducing inflammasomes. Bar graph represents the levels of IL-1β determined in the culture supernatants of rZmp1 treated cells, using capture ELISA method. All the experiments were performed atleast three times. The error bars represent standard deviation from mean. * represents statistical significance with $p<0.001$.

3.3.4 rZmp1 promoted cell migration in THP-1 cells and induced secretion of chemotactic chemokines:

Necrotic cell death of the *M. tb* infected cells has been linked to mycobacterial dissemination (Molloy, Laochumroonvorapong et al. 1994; Lee, Repasy et al. 2011; Butler, Brodin et al. 2012; Roca and Ramakrishnan 2013). With high concentration of rZmp1 causing necrotic cell death, and increase in the inflammatory cytokine environment such as TNFα, which help in the spread of infection, we proceeded to assess for the ability of rZmp1 to promote cell migration. For these assays, a sub-toxic level of rZmp1 concentration was used, so that excessive cell deaths can be avoided. The preliminary experiments were performed on CHO cells using the gap closure assays, which were further supported by Boyden chamber assays using THP-1 cells (Figure 22 and 23). Gap closure assays were performed using untreated or CHO cells treated with 50nM rZmp1 (Figure 22A). Images were taken at different time points (0, 16, 24 and 48 hr) and gap width was calculated and plotted (Figure 22B). Exogenous treatment of rZmp1 resulted in increased gap closure as compared to the untreated cells by 2.05±0.47 folds at 48 hr (Figure 22B).

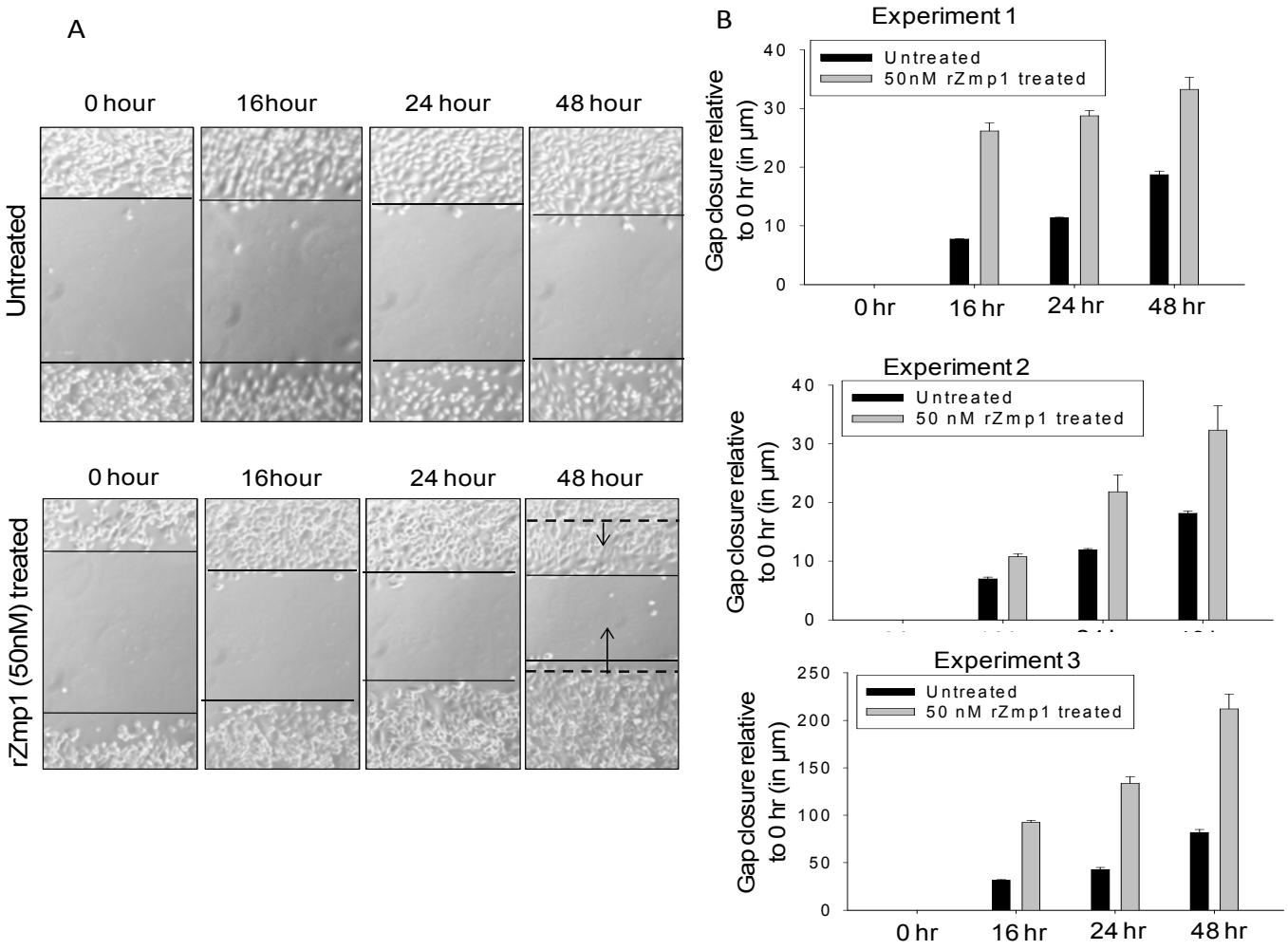

Figure 22: rZmp1 treatment helps in CHO cell migration as observed by gap closure assay. (A) Representative microscopic image showing CHO cell migration as function of time upon 50 nM rZmp1 exogenous treatment. Monolayer of CHO cells were grown in 35mm cell culture dish. A scratch was introduced using a sharp tip and the cells were either only buffer treated (Upper panel) or 50 nM rZmp1 treated (lower panel). The extent of gap closure was monitored microscopically at regular intervals (0, 16, 24 and 48 hr). (B) Bar graphs represent the distance of gap closure in µm (micrometers) relative to 0 hr calculated using ImageJ software at regular intervals from three independent experiments. The error bars represent the standard deviation from mean.

The ability of rZmp1 to promote cell migration was further confirmed by Boyden chamber assays using THP-1 monocyte cell line. In this assay, we preloaded the cells in the upper chamber with complete media and the lower chamber with media containing 50 nM and 100 nM endotoxin-free rZmp1, expecting that if rZmp1 indeed influenced migration of cells, the cells

would move from the upper chamber to the lower chamber. It was observed that there was increased migration of THP-1 cells from the upper chamber to the lower chamber containing 50 nM and 100 nM rZmp1 as compared to control without rZmp1 (Figure 23A). The fold changes in the migration of THP-1 cell line in the presence of 50 nM and 100 nM rZmp1 were 1.55±0.20 and 1.81±0.21 fold, respectively as compared to untreated, exhibiting a dose dependent increase in the cell migration (Figure 23B). Taken together the gap closure assays and Boyden chamber assays confirmed involvement of rZmp1 in migration of cells.

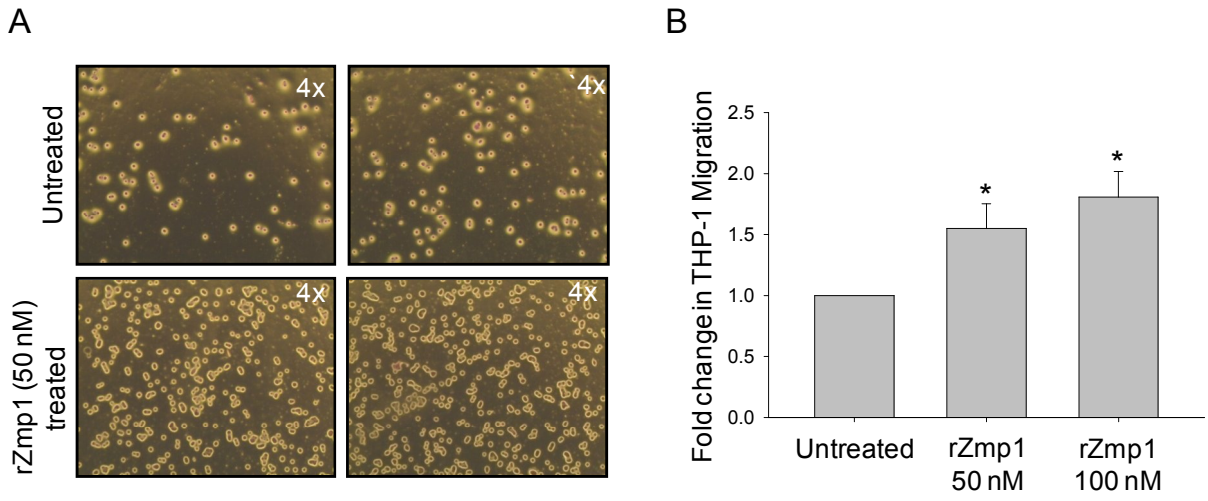

Figure 23: rZmp1 helps in THP-1 cell migration as confirmed by Boyden chamber assay. (A) Representative microscopic image of Giemsa Staining showing THP-1 cells migrated to the lower chamber upon 50 nM rZmp1 treatment. THP-1 cells were seeded into the upper chamber of Boyden chamber and the lower chamber contains media along with buffer control or 50 nM rZmp1 and incubated for 24 hr. 50 nM rZmp1 containing lower chamber showed more THP-1 cells migrated from upper chamber. (B) Bar graph represents the fold change in THP-1 migration in untreated or rZmp1 treated cells. The number of cells migrated to the lower chamber were counted using TrypanBlue live cell staining assay and plotted the increase in fold change in THP-1 cells migrated from upper chamber to lower chamber.

We further checked if rZmp1 treatment of THP-1 cells resulted in release of chemotactic chemokines, such as MCP-1, MIP-1β and IL-8 that are known to recruit monocytes, neutrophils, memory T cells, and dendritic cells to the sites of infection (Taub, Proost et al. 1995; Gerszten, Garcia-Zepeda et al. 1999; Bystry, Aluvihare et al. 2001; Deshmane, Kremlev et al. 2009). THP-1 cells upon incubation with rZmp1 at 50 nM and 100 nM showed increased levels of all the three cytokines compared to the untreated cells (Figure 24A, 24B and 24C). The cytokine levels in the culture supernatants of THP-1 cells untreated or rZmp1 treated were, MCP-1 (Untreated:

57.97±11.2 pg/mL; 50nM rZmp1: 654.82±118.97 pg/mL; 100 nM rZmp1: 1473.54±443.12 pg/mL; $p<0.05$) (Figure 24A), MIP-1β (Untreated: 38.51±15.71 pg/ml; 50nM rZmp1: 478.28±42.06 pg/mL; 100 nM rZmp1: 548.58±149.59 pg/mL $p<0.05$) (Figure 24B) and IL-8 (Untreated: 78.42±79.08 pg/mL; 50nM rZmp1: 1495.44±25.88pg/mL; 100 nM rZmp1: 1626.99±130.99 pg/mL; $p<0.05$) (Figure 24C).

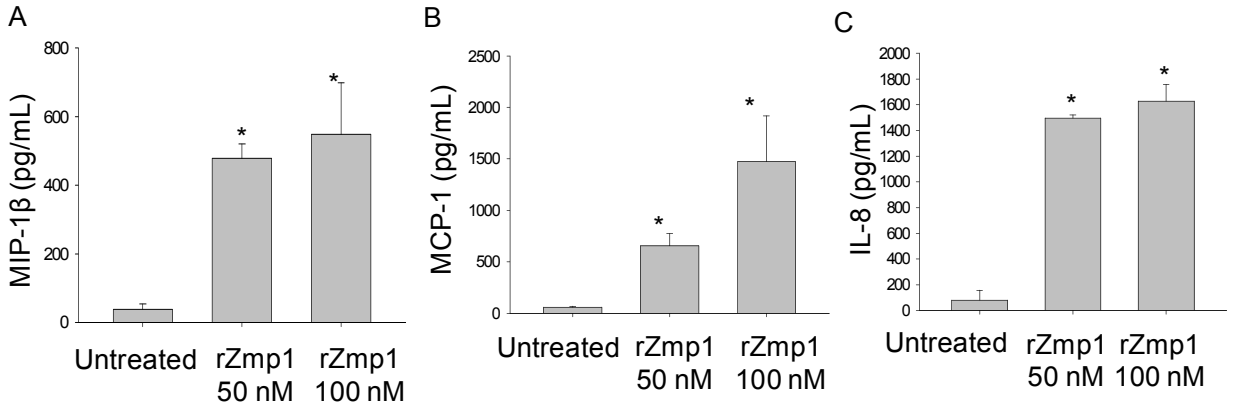

Figure 24: Induction of chemokines by rZmp1. The culture supernatants of rZmp1 (50 nM and 100 nM) treated cells were assayed for cytokines implicated in cell migration or chemotaxis, such as, (A) MIP-1β, (B) MCP-1 and (C) IL-8 using capture ELISA method. All the experiments were performed at the least three times. The error bars represent standard deviation from mean. * represents statistical significance with $p<0.05$.

Summing up the results so far, rZmp1 that induced necrotic cell death at higher concentration, promoted cell migration and secretion of chemotactic chemokines by THP-1 cells upon exogenous treatments at sub-toxic levels. One can thus speculate that upon causing necrosis, *M. tb* Zmp1 induces secretion of chemotactic chemokines that would assist migration of monocytes and dendritic cells to the necrotic site, causing fresh rounds of infections of these cells and thereby help dissemination of mycobacteria.

3.3.5 rZmp1 helped in dissemination of mycobacteria:

To examine our hypothesis that Zmp1 indeed helped in mycobacterial dissemination, we used Zebrafish as a model organism which was infected with fluorescently labeled *Mycobacterium marinum* (TdM. marinum) which harbored pTEC27 plasmid having Td-Tomato gene. Although Zmp1 of *M. marinum* is 84% similar to *M. tb* Zmp1, unlike *M. tb* Zmp1, *M. marinum* Zmp1 is

predicted to be a cytosolic protein (Kapopoulou, Lew et al. 2011). Therefore, to simulate the presence of secreted Zmp1 in the vicinity of mycobacteria, *M. marinum* was either mixed with rZmp1or with buffer alone before injections into Zebrafish. We injected 200 CFU of TdM. marinum either mixed with buffer or with various concentrations of endotoxin-free rZmp1 (25 nM, 50 nM, 100 nM and 100 nM of heat-inactivated rZmp1) into the yolk sac of 1dpf Zebrafish larva (refer experimental procedures) (Figure 25A). Initially toxicity of injected rZmp1 protein on Zebrafish was checked and found that rZmp1 was not toxic at any of the concentrations of 25 nM, 50 nM and 100 nM used. Survival of the Zebrafish after rZmp1 treatment (ranging from 25 nM to 100 nM) alone without any TdM. marinum was evaluated by injecting the fish and monitored the fish over the course of 5 days (Table 4). After injections with TdM. marinum with or without rZmp1, the fishes were monitored for 5 days and images were taken using fluorescence microscope on 0 day and 5th day (Figure 25A). Then we determined the spread of TdM. marinum from the site of injection to the tail region using two parameters. Firstly, we calculated the percentage of fish in a category with fluorescence in tail region and secondly, we measured the percentage of fluorescence intensity in tail region with respect to per whole fish fluorescence intensity (Figure 25B).

Injected with	Total Number of Zebrafish larvae injected	Number of Zebrafish larvae alive after 5 days
25 nM rZmp1	20	16
50nM rZmp1	20	16
100nM rZmp1	10	9
100nM heat-inactivated rZmp1	10	10

Table 4: Table represents the survival of Zebrafish for rZmp1 exogenous treatment. Survival of Zebrafish which was evaluated by injecting the fish with rZmp1 (ranging from 25 nM to 100 nM) alone without any TdM. marinum and monitored the fish over the course of 5 days.

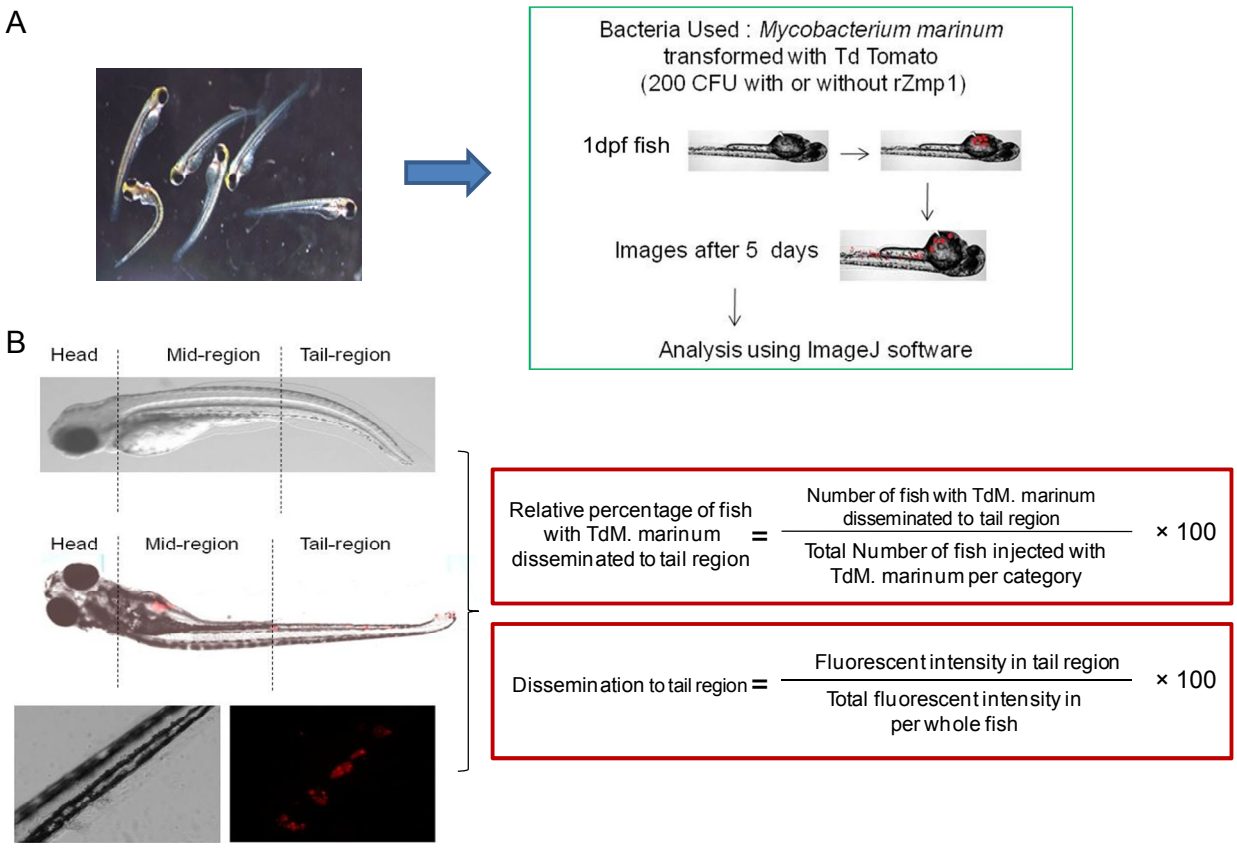

Figure 25. Schematic representation of *M. marinum* dissemination assay in Zebrafish. (A) 1 day post fertilization fish were taken for the experiments and injected with 200 CFU of Td-Tomato labeled *M. marinum* (TdM. marinum) along with either only buffer control or rZmp1 (25, 50 and100 nM) into the yolk sac. The fish were then incubated in E3 medium for five days, followed by visualization of TdM. marinum under fluorescent microscope on 5th day to evaluate for bacterial dissemination to tail region. (B) To determine the bacterial dissemination to tail region, firstly each fish was divided into three regions, head, mid-region and tail-region as depicted in the figure. Fish were then scanned under fluorescence microscopy for the presence of TdM. marinum in the whole organism and images were taken accordingly. Fluorescence intensity of TdM. marinum present in the fish was quantified using ImageJ software. Total fluorescence was calculated by taking count of all the regions in a whole fish. Dissemination of TdM. marinum towards tail region was calculated by two parameters and plotted: 1) Relative percentage of fish with TdM. marinum disseminated to tail region and 2) Dissemination to tail region.

Using these parameters, we observed that there was an increase in dissemination of fluorescently labeled *M. marinum* to the tail regions from the site of injection in the infected Zebrafish with rZmp1 than in infected Zebrafish without rZmp1 in a dose dependent manner ranging from 25 nM to 100 nM (Figure 26).

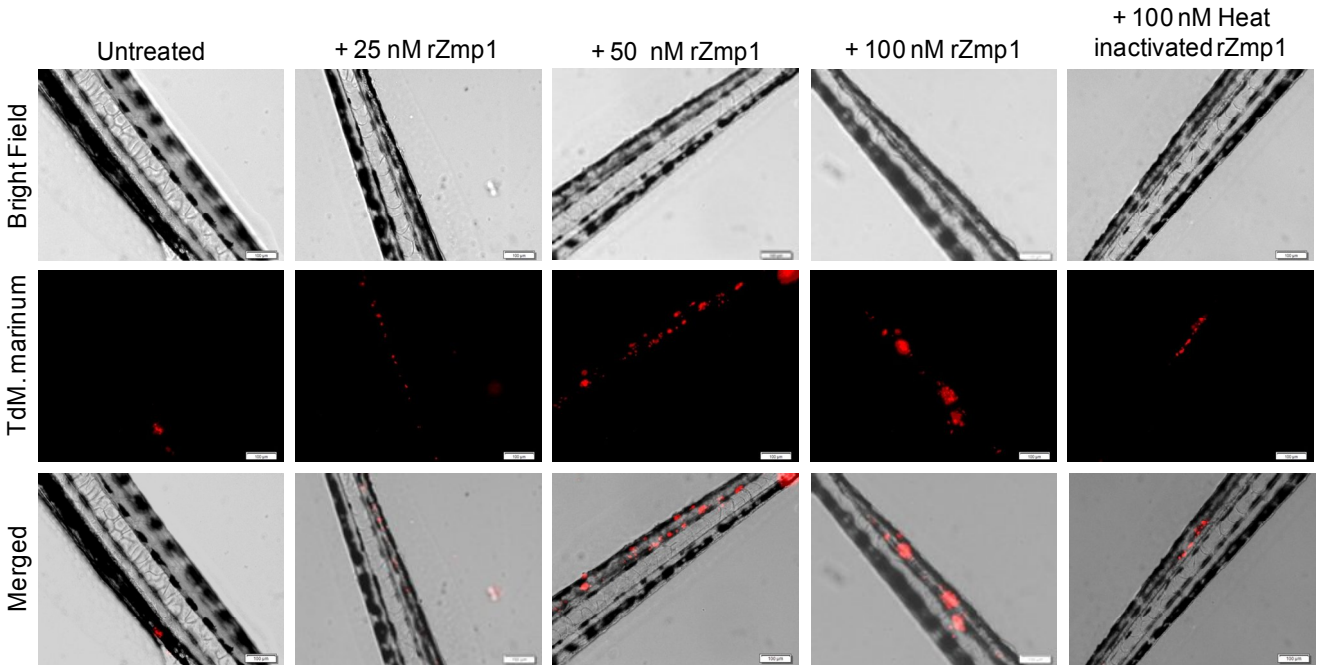

Figure 26: Zmp1 helps in the dissemination of *M. marinum* in Zebrafish model. Representative images showing the presence of fluorescently labeled *M. marinum* (TdM. marinum; Red) in the tail region of the Zebrafish. 1 dpf (days post fertilization) Zebrafish were injected with TdM. marinum either with buffer control (Untreated) or rZmp1 (25 nM to 100 nM) or heat-inactivated rZmp1 (100 nM). The dissemination of TdM. marinum to the tail region was then observed under fluorescence microscope on 5th day post infection. Fish were scanned for the presence of TdM. marinum in the whole organism and images were taken accordingly.

It was observed that 54.84% of control fish showed fluorescent TdM. marinum in tail region (n=31), while 76.67% of fishes injected with TdM. marinum +25 nM rZmp1 (n=30), 90% of fishes injected with TdM. marinum + 50 nM rZmp1 (n=20) and 93.1% of fishes injected with TdM. marinum + 100 nM rZmp1 (n=29) showed fluorescent TdM. marinum in the tail region (Figure 26). This indicated that rZmp1 helped in the dissemination of TdM. marinum from the site of injection to the tail region in a dose-dependent manner (Figure 27). In order to confirm that the impact of rZmp1 on mycobacterial dissemination is specific, we heat inactivated rZmp1 by denaturing the protein at 100°C for 10 minutes. The 100nM of heat-inactivated rZmp1 was mixed with TdM. marinum and injected into Zebrafishes similar to other groups. Only 45.45% of

fishes (n=22) showed fluorescent TdM. marinum in the tail region, which was similar to untreated control group (Figure 27).

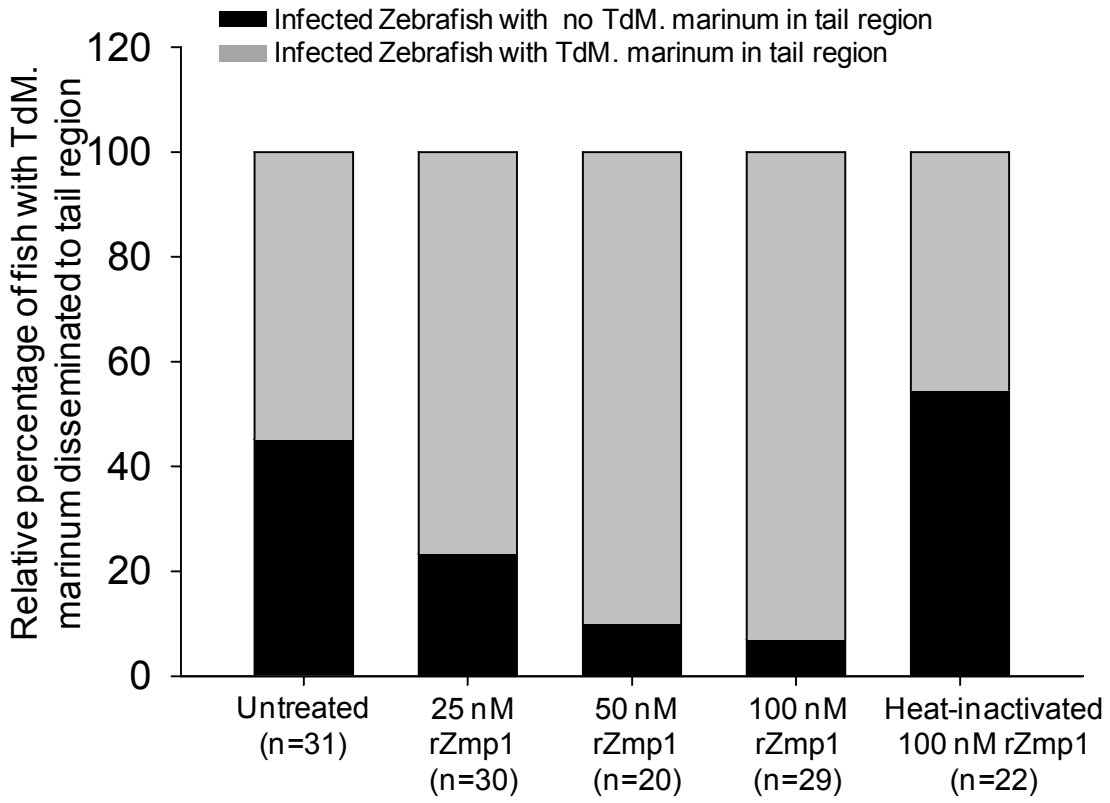

Figure 27: Bar graph representing the relative percentage of fish with TdM. marinum disseminated to tail region. Fluorescence intensity of TdM. marinum presence in the fish was quantified using ImageJ software. Total fluorescence was calculated by taking count of all the regions in a whole fish. Dissemination of TdM. marinum towards tail region was calculated by two parameters and plotted: 1) Relative percentage of fish with TdM. marinum disseminated to tail region = (Number of fish with TdM. marinum disseminated to tail region / total number of fish injected with TdM. marinum in the category) × 100. All the experiments were performed more than three times. n represents the number of fish used per category. * represents statistical significance with $p<0.05$.* represents statistical significance with $p<0.05$.

We then measured the fluorescence intensity of TdM. marinum per fish and calculated the ratio of fluorescence intensity in the tail region with respect to the whole fish intensities. With this, we observed that the control group infected with TdM. Marinum without rZmp1 (Median: 2.66%; IQR: 0-16.55) showed significantly lesser percentage of fluorescence intensity in tail region compared to Zebrafish category infected with TdM. marinum+100nM rZmp1 (Median: 23.91%;

IQR: 12.1 – 30.59) further confirming that rZmp1 helped in the enhanced dissemination of TdM. marinum from the site of injection to the tail region (Figure 28). Further, Zebrafish group injected with TdM. marinum+100nM heat-inactivated rZmp1 (Median: 0%, IQR: 0 – 19.31) showed dissemination similar to the rZmp1 untreated control group (Median: 2.66%; IQR: 0-16.55) (Figure 28). These analyses confirmed the role of Zmp1 in mycobacterial dissemination in Zebrafish model.

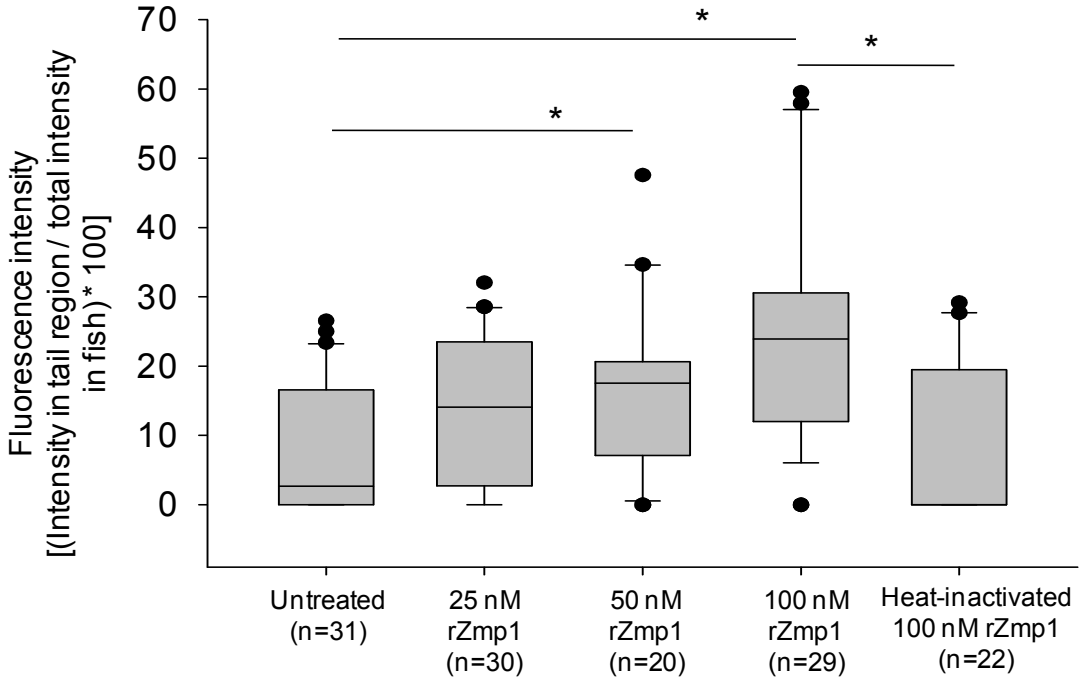

Figure 28: The box plot represents the percentage of dissemination to tail region. All the experiments were performed more than three times. Dissemination to tail region = (Fluorescence intensity in tail region / total fluorescence intensity in whole fish) × 100. All the experiments were performed more than three times. n represents the number of fish used per category. * represents statistical significance with $p<0.05$

3.4 DISCUSSION

With this study we added a new physiological function to the multi-faceted *M. tb* Zmp1. We observed that, exogenous treatment of macrophages with high concentration resulted in necrotic cell death, at the same time, sub-toxic levels stimulated cells to migrate and release chemotactic chemokines. Together, these properties resulted in rZmp1 facilitating dissemination of mycobacteria, as observed in Zebrafish infected with fluorescence labeled *M. marinum*.

Previous reports involving Zmp1 deletion mutants of *M. tb* advocated that Zmp1 is implicated in suppression of inflammasome activation. In these studies it was observed that deletion of Zmp1 resulted in improved pro-inflammatory response and antigen presentation (Master, Rampini et al. 2008). Complementing these inferences, we also observed that exogenous treatment of PBMCs with Zmp1 biased the immune response towards Th2 (Chapter 2) (Vemula, Ganji et al. 2016). It is well established that when Th2 wing of immune response gets activated it tends to suppress the pro-inflammatory Th1 response.

Earlier studies performed using a peptide array identified bradykinin, neurotensin, neuropeptide FF and many other neuropeptides as substrates (Petrera, Amstutz et al. 2012), possibly implying role for Zmp1 in TB meninges. In this study, we observed that rZmp1 possess caseinase and mucinase activity. From the mucinase activity of Zmp1 one may speculate that it probably help in colonization of mycobacteria in the lungs by degrading tracheal mucin and thus, help in the establishment of infection. Broad spectrum protease activity of Zmp1 may contribute to its varied physiological roles in the survival of the pathogen. High concentration of rZmp1 is toxic to macrophages and caused necrotic cell death. In accordance, rZmp1 treatment induced secretions of cytokines TNFα, IL-6 and IL-1β (Figure 18A, 18B and 18C) which are well-documented to be associated in inducing necrotic cell death (Vanden Berghe, Kalai et al. 2006; Kaczmarek, Vandenabeele et al. 2013; Moriwaki, Bertin et al. 2015). One may then argue how *M. tb* Zmp1 on one hand suppresses inflammasome activation and pro-inflammatory response as a whole can also induce necrosis. To explain the same, in our earlier experiments in chapter 2 (Vemula, Ganji et al. 2016), we had observed that Zmp1 treatment of PBMCs and THP-1 cells resulted in release of high titers of IL-4, IL-6 and IL-1β, along with high TNFα. It is known that concurrent high levels of TNFα with IL-4 increases toxic potentiality of TNFα, causing necrotic tissue damage, which may result in liquefaction of TB granuloma, hence dissemination and

relapse (Fenhalls, Wong et al. 2000; Rook 2007). TNFα, known to protect against apoptosis, however, at higher levels in an autocrine or paracrine mode induces necrosis through RIP1/RIP3 kinase pathway (Roca and Ramakrishnan 2013). Also it is reported that increase in IFN-γ levels restricts dissemination of bacteria(Cooper, Dalton et al. 1993). Many secreted bacterial Zinc-metalloproteases across various species have been reported to be cytotoxic in nature and help in bacterial pathogenesis (Hase and Finkelstein 1993). In general, necrosis of the *M. tb* infected cells has been associated with the dissemination of bacilli and further infection of fresh cells. Careful evaluations with complementary experiments are further required to understand the exact mechanism of necrotic death caused by rZmp1.

Zinc containing metalloproteases are widely distributed among prokaryotes and eukaryotes. They are known to play a critical role in bacterial pathogenesis and in various physiological processes such as mammalian cells' adhesion, migration and extracellular matrix remodeling and cell–cell interactions (Kearns, Bonner et al. 2002). For example, Zmp1 protein of *Clostridium difficile* has been shown to degrade the fibrin network of fibroblasts and thus help in bacterial infection and dissemination (Cafardi, Biagini et al. 2013). Pap6 of *Vibrio harveyi* and Hemagglutinin/protease from *Vibrio cholerae* have been shown to digest various ECM components such as fibronectin, collagen and gelatin (Booth, Boesman-Finkelstein et al. 1983; Finkelstein, Boesman-Finkelstein et al. 1983; Teo, Zhang et al. 2003). Digestion of fibronectin network by proteases results in fibroblasts losing adherence property and thus may lead to cell migration (Cafardi, Biagini et al. 2013). StcE, a secreted protease of *Escherechia coli* (*E. coli*), expressed during infection and contributes to virulence by affecting crucial neutrophil recruitment, migration and also oxidative burst production (Szabady, Lokuta et al. 2009). SslE, another extracellular zinc metalloprotease of *E. coli* through its mucinase activity help bacterial translocation through mucin-matrix allowing its penetration through the mucosal surface and reach host epithelium to make a niche inside the host (Nesta, Valeri et al. 2014; Valeri, Rossi Paccani et al. 2015). Based on the evidences that Zinc metalloproteases help in cell adhesion and migration, we sought to check the effect of rZmp1 on the cell migration. We employed both Gap closure assays and Boyden chamber assays on CHO cells and THP-1 cells respectively and observed that rZmp1 treatments showed improved cell migration compared to control treatments (Figure 22 and Figure 23). Though Gap closure assays using CHO cells confirm the effect of rZmp1 on migration, Boyden chamber assays using THP-1 cells, which create a concentration

gradient between upper and lower chamber, can be inferred in the context of either cell migration and/or chemotaxis (Chen 2005; Liang, Park et al. 2007). We also learnt that rZmp1 treatment induced secretion of chemotactic chemokines, MCP-1, MIP-1β and IL-8 in the extracellular milieu (Figure 24A, 24B and 24C).

Hypothesizing that together with induction of necrosis, release of chemotactic chemokines and induction of cell migration, Zmp1 may influence dissemination of mycobacteria from the site of infection, we used Zebrafish to confirm the same. For a detailed study of *M. tb* infection, various animal models such as mouse, guinea pig and other non-human primates were used with their own limitations. Mouse model is advantageous for immunological and genetic studies whereas guinea pigs and rabbits produce necrotic granulomas. Although non-human primates mimic tuberculosis infection condition, many ethical issues along with less cost effectiveness are observed. These differences play critical role in understanding the cell mediated immunity in all mammalian experimental models. For a better understanding, a close genetic aide of *M. tb*, *M. marinum* is being used to study pathogenesis of mycobacteria. *M. marinum* causes systemic granulomatous infection in natural hosts such as fish and frogs and peripheral chronic granuloma in humans. In leopard frogs, asymptomatic non-necrotic granulomas are observed whereas in zebra fish and gold fish necrotizing granulomas are observed leading to lethality of the organism

Zebra fish is a widely used animal model in the laboratory due to its transparent embryos and their ability to enhance genetic studies using knock down experiments. Zebra fish genome contains at the least one orthologues for around 70% of the human genome (Howe, Clark et al. 2013). Microarray analysis, drug screening with the whole fish can be easily performed zebrafish are infected with *M. marinum* and develops the disease. Embryo stage of zebrafish is mostly used as they are transparent and early events of infection such as macrophage aggregates and granuloma formations can be well studied. Zebrafish, as a model, has been, so far successfully used to study the dissemination of various pathogens like *Mycobacterium, Salmonella, Burkholderia, Staphylococcus, Shigella* and *Candida* (Torraca, Masud et al. 2014). The model has provided several insights into the understanding of tuberculosis infections. For example, understanding the role of granuloma in pathogenesis (Swaim, Connolly et al. 2006; Davis and Ramakrishnan 2009; Ramakrishnan 2013), role of bacterial efflux pumps in drug resistance (Adams, Takaki et al. 2011), role of LTA4H in mediating inflammation against mycobacteria (Tobin, Vary et al. 2010), induction of necrotic death of infected cells mediated by TNFα (Roca

and Ramakrishnan 2013) and to study the manipulation of macrophage recruitment by mycobacteria (Cambier, Takaki et al. 2014). Several therapeutic strategies against tuberculosis were employed or underway based on the insights from Zebrafish model (Torraca, Masud et al. 2014). In the current study, we have used the Zebrafish to elucidate the role of rZmp1 in the dissemination of *M. marinum* and we have observed that upon simultaneous injection of TdM. marinum and rZmp1 resulted in dissemination of TdM. marinum to the tail region compared to the TdM. marinum injected alone or with heat-inactivated rZmp1 (Figure 18, 19 and 20). Based on our quantitative analyses, we could infer that the presence of rZmp1 not only helped dissemination of TdM. marinum to the tail region per fish but also the number of fishes showing increased dissemination to the tail region were also high (Figure 20). Overall, the observations confirmed the possible role of rZmp1 in dissemination of *M. marinum* in Zebrafish.

With the present observations on rZmp1 inducing necrosis and the release of chemotactic chemokines from macrophages, we propose that when Zmp1 is released by mycobacteria in granuloma, may lead to both necrotic damage of the cells and induce release of chemotactic factors from surrounding infected macrophages. This would attract the uninfected immune cells like monocytes, dendritic cells and lymphocytes towards the site of necrosis. These cells then get freshly infected by *M. tb* released from necrotic cells, thus assisting mycobacterial dissemination. The study has added a new dimension to the understanding of molecular basis of mycobacterial pathogenesis. One may further explore how the concentration gradient of Zmp1 is decisive in necrosis on one hand and a possible systemic dissemination on the other or if secretion of Zmp1 into acellular milieu of granuloma causes granuloma dissolution owing to Zmp1 proteolytic activity and hence possible role in reactivation. In-depth studies in these lines would help in designing new anti-mycobacterial strategies to confront the emerging problem of drug resistance in *M. tb*.

Chapter 4

Summary and future prospects

4.1 Summary and Future prospects

The study was intended to characterize mycobacterial zinc metalloprotease 1 (Zmp1) and its physiological role under infection conditions.

Based on the previous reports and mutant studies suggesting a role for Zmp1 in inflammasome inhibition and phagosome arrest, we were interested in understanding the role of Zmp1 in the survival of pathogen. Also reports suggest its ability to degrade neuropeptides indicating Zmp1 to have many roles inside the host and mycobacteria. With this background, we observed that exogenous treatment by high concentration of purified recombinant Zmp1caused necrotic cell death of THP1 cells, while sub-toxic levels promoted cell migration and release of chemotactic chemokines. In agreement with the reports that necrotic cell death during TB infection is an imperative precondition for bacterial dissemination, we further established, using zebrafish infected with fluorescently labeled *Mycobacterium marinum* that exogenous (secretory) rZmp1 helped in dissemination of mycobacteria. With this study, along with the earlier observations from our laboratory and others, *M. tb* Zmp1 was further explored for its direct and indirect role in helping pathogen to evade the host mechanisms. In this regard, we studied the biochemical and physiological functions of Zmp1 using cell culture studies and immunological responses against Zmp1 using TB patient samples suggesting a pivotal role for this protease in containing infection.

With *M. tb* Zmp1 reported as virulent factor holding the properties of immunomodulation, high immunogenicity and proteolysis of synthetic neuropeptides, we further extended the study on the possible role of Zmp1 in pathogenesis of *M. tb* and evaluated the humoral response to *M. tb* H37Rv Zinc metalloprotease-1, Zmp1, a protein present in the culture filtrate (de Souza, Leversen et al. 2011). This study is focused on understanding the mechanistic details of Zmp1 in helping the survival of pathogen inside the host intracellular environment during active infection or for the establishment of infection. The study points to the potential of further exploration of mycobacterial proteins, such as Zmp1, as antigen candidates for prime-boost vaccination strategies or as markers for disease progression.

Two objectives were deliberated

1. Immunological characterization of secreted *M. tb* Zinc Metalloprotease-1 (Rv0198c) to explore the Tuberculosis-specific Humoral Immune Response by this secreted antigen.
2. Biochemical and physiological characterization of Zmp1 to attempt identifying its physiological function, especially as and exogenous (secretory) protease from *M. tb.*

4.2 Objective 1: Immunological characterization of secreted *M. tb* Zinc Metalloprotease-1 (Vemula, Ganji et al. 2016)

Under this objective, I deliberated on the immunomodulatory function of the secreted *M. tb* protein Zmp-1 (Rv0198c). Surface-exposed or secreted proteins of *M. tb* are customarily the targets of immune responses in the infected host. Several *M. tb* proteins, including culture filtrate proteins have been evaluated for their immunogenic properties, such as CFP10, ESAT-6, Ag85B, ICDs etc (Banerjee, Nandyala et al. 2004; Sinha, Kosalai et al. 2005; Malen, Softeland et al. 2008; Floss, Mockey et al. 2010). *M. tb* GlcB (malate synthase), MPT51 (FbpC1) and HSPX (alpha crystalline) have also been evaluated for humoral response in clinically asymptomatic Health-care workers with latent infections, suggesting the possibility of these responses to be protective (Reis, Rabahi et al. 2009).

***M. tb* Zmp-1 showed high antigenicity indices:** To begin with, the antigenic index of Rv0198c was analyzed by Protean software (Protean 5.00, DNASTAR Inc.). Protein sequence analyses by Jameson-Wolf plot using Protean software, which recorded high antigenicity indices based on surface probability and hydrophobicity of its amino-acid sequence of Zmp1 supported the notion that apart from an active enzyme, it can also be a B-cell stimulant. To study the same, Rv0198c (Zmp1) from pathogenic mycobacteria *Mycobacterium tuberculosis* strain H37Rv, was cloned, expressed and purified. The purified recombinant protein (rZmp1), was made endotoxin free and used for further characterization.

Zmp1 protein was secreted under granuloma like *in-vitro* growth conditions by *M. tb* H37Rv: Zmp1 was identified as one of the culture filtrate proteins of in vitro grown H37Rv metalloprotease-1, Zmp1 (Rv0198c), a protein present in the culture filtrate (de Souza, Leversen et al. 2011). We extended the study to check if the same holds true for granuloma-like

conditions. To study the same, H37Rv was grown under different stress conditions known to simulate acellular caseous environment of TB granulomas, that is, acidic pH 5.5, H_2O_2 induced oxidative stress, nutrient deprivation and iron deficiency(Stallings and Glickman 2010) and detected the presence of Zmp1 protein in culture supernatants using Western blotting with in-house generated anti-Zmp1 antibody. CFP10 and GroEL1, which were probed with their respective antibodies, were used as positive and negative controls respectively.

rZmp1 stimulated PBMCs to release Th2 class of cytokines: We next evaluated the immunostimulatory potential of Zmp1 in terms of release of Th1/Th2 cytokines from exogenously treated PBMCs derived from healthy volunteers to elucidate the association of Zmp1with Cell mediated immunity or Antibody mediated immunity. Lipopolysaccharide (LPS), a known strong immunostimulant of PBMCs, was used as a positive control (Jansky, Reymanova et al. 2003). The culture supernatants were then collected to assay for the levels of a minimal battery of cytokines, that is, TNF-α, IL-1β, IFN-γ, IL-12p70, IL-4 and IL-10. A comparative analyses and a ratio of IFN-γ to IL-10, suggested the secreted *M. tb* Zmp1 should incline the immune system towards Th2 response, promoting humoral immunity.

Zmp1 elicited a strong B-cell response which was specific for Tuberculosis (TB) infection: With the above experiments indicating that Zmp1 induces Th2 response, we next assessed if this is manifested in the form of anti-Zmp1 antibody production in TB patients. The humoral response of the host against Zmp1 was scored in a study population comprising 286 subjects. This included four groups, TB patients, Healthy controls, House hold contacts of TB patients and volunteers with non-specific infections. Purified rZmp1 was used as bait antigen in the indirect ELISA to score for the anti-Zmp1 antibodies in the serum samples of healthy (n=53), TB patients (n=121), house hold contacts (n=89) and non-specific infection control samples (n=23) (Figure 1A). We observed that there was a significant increase ($p<0.001$) in the absorbance at 450nm corresponding to the anti-Zmp1 antibody titers in TB patient sera as compared to all other categories. Similarly, we observed that rZmp1 ELISA was highly specific for TB cases, both Pulmonary TB and Extra-pulmonary TB response independent of mycobacterial load.

Zmp1 elicits strong B-cell response independent of mycobacterial load in both Pulmonary (PTB) and Extra-pulmonary tuberculosis (EPTB) cases: This was one of the most significant observations made from this study. We observed that the median values for both PTB (representing detectable high bacillary load) and EPTB (representing low bacillary load) cases were approximately similar, suggesting that anti-Zmp1 titers in patients were high regardless of bacterial load. This observation was significant, as it suggested that Zmp1 could elicit a strong humoral response even in paucibacillary EPTB cases.

We revealed hitherto unknown immunogenic property of *M. tb* Zmp1. Zmp1 is a strong *M. tb* specific immune-stimulant, the properties of which can be further explored both as a potential vaccine candidate or a disease marker. The possibility of taking the study on antigenicity of Zmp1 to the next level by using larger cohort, blinded samples and multi-centric study is very stimulating from both scientific point of view and translational research.

4.3 Objective 2: Biochemical and functional characterization of Zmp1: possible role in mycobacterial dissemination (manuscript under review).

Under this objective, we performed biochemical and functional assays to characterize Zmp1. To study the same, we used the purified recombinant Zmp1 (Rv0198c) from pathogenic mycobacteria *Mycobacterium tuberculosis* that was made endotoxin free by passing through treated with polymyxin-B agarose beads.

rZmp1 was proteolytically active: Protease activity of rZmp1 was confirmed using general substrates like casein and mucin. Casein activity was checked by observing the cleaved product on SDS-PAGE and colorimetric based mucinase activity using bovine submaxillary mucin as substrate, where degraded mucin showed decrease in the absorbance at 492nm.

High concentration of rZmp1 caused necrotic cell death: Toxicity of rZmp1 was tested and it was found to be toxic at higher concentrations of 500 nM and 1 μM. High concentrations of rZmp1 caused necrotic cell death upon exogenous treatment of macrophages (THP-1 cells) as

observed using propidium iodide staining and lactate dehydrogenase (LDH) assays. In accordance, rZmp1 treatment of THP-1 cells induced secretions of cytokines TNFα, IL-6 and IL-1β which are well-documented to be associated in inducing necrotic cell death (Vanden Berghe, Kalai et al. 2006; Kaczmarek, Vandenabeele et al. 2013; Moriwaki, Bertin et al. 2015). The toxicity of rZmp1 was confirmed using zebra fish model using groups of 12 zebra fish embryos exposed at doses from 1μM to 0.001μM of rZmp1.

rZmp1 induced cell migration and release of chemotactic chemokines: As necrotic cell death of the *M. tb* infected cells has been linked to mycobacterial dissemination (Tundup, Mohareer et al. 2014), the ability of rZmp1 to promote cell migration was assayed using sub-toxic level of rZmp1 to avoid excessive cell death. Gap closure assays and Boyden chamber assays on CHO cells and THP-1 cells respectively confirmed that rZmp1 promoted cell migration. Further, it induced secretion of chemotactic chemokines like MCP-1, MIP-1β and IL-8.

rZmp1 helped in dissemination of mycobacteria: Hypothesizing that together with induction of necrosis, release of chemotactic chemokines and induction of cell migration, Zmp1 may influence dissemination of mycobacteria from the site of infection. To examine our hypothesis that Zmp1 indeed helped in mycobacterial dissemination, we used Zebrafish as a model organism which was infected with fluorescently labeled *M. marinum* (TdM. marinum) which harbored pTEC27 plasmid having Td-Tomato gene. We observed that there was an increase in dissemination of fluorescently labeled *M. marinum* to the tail regions from the site of injection in the infected Zebrafish with rZmp1 than in infected Zebrafish without rZmp1 in a dose dependent manner ranging from 25 nM to 100 nM. Based on our quantitative analyses, we could also infer that the number of fishes showing increased dissemination to the tail region were also high. In order to confirm that the impact of rZmp1 on mycobacterial dissemination is specific, we heat inactivated rZmp1 by denaturing the protein at 100°C for 10 minutes. The 100nM of heat-inactivated rZmp1 was mixed with TdM. marinum and injected into Zebrafishes similar to other groups. Only 45.45% of fishes (n=22) showed fluorescent TdM. marinum in the tail region, which was similar to untreated control group. Overall, the observations confirmed the possible role of rZmp1 in dissemination of mycobacteria in host, here *M. marinum* in Zebrafish.

Model of possible mode of action by secreted *M. tb* Zmp1: With the observations on rZmp1 inducing necrosis and release of chemotactic chemokines from macrophages, we propose that when Zmp1 is released by mycobacteria in granuloma (as shown on objective-1), may lead to both necrotic damage of the cells and release of chemotactic factors from surrounding infected macrophages. This would attract the uninfected immune cells like monocytes, dendritic cells and lymphocytes towards the site of necrosis. These cells then get freshly infected by *M. tb* released from necrotic cells, thus assisting mycobacterial dissemination.

The study has added a new dimension to the understanding of molecular basis of mycobacterial pathogenesis. One may further explore how the concentration gradient of Zmp1 is decisive in necrosis on one hand and a possible systemic dissemination on the other or if secretion of Zmp1 into acellular milieu of granuloma causes granuloma dissolution owing to Zmp1 proteolytic activity and hence possible role in reactivation. In-depth studies in these lines would help in designing new anti-mycobacterial strategies to confront the emerging problem of drug resistance in *M. tb*

4.4 Future prospects:

Overall in this thesis, we observed that exogenous treatment by high concentration of purified recombinant Zmp1caused necrotic cell death of THP1 cells, while sub-toxic levels promoted cell migration and release of chemotactic chemokines. In agreement with the reports that necrotic cell death during TB infection is an imperative precondition for bacterial dissemination, we further established, using zebrafish infected with fluorescently labeled *Mycobacterium marinum* that exogenous (secretory) rZmp1 helped in dissemination of mycobacteria.

4.4.1 Leads from the study

Apart from the mechanisms described in the thesis

<u>From immunological characterization</u>

The humoral response to rZmp1 in TB patients in a sectional study was discussed in chapter 2; this can be extended for further research in the terms of diagnostics:

i. This study can be further extended by using different categories of TB patients such as bone TB, TB meninges and sought the humoral response based on their bacterial load.
ii. As serological diagnostics are less known for their specificity, improving the specificity and sensitivity of this study will help in easier handling and sampling techniques rather than laborious methods such as FNAC (Lawn and Zumla 2012).
iii. Other body fluids such as pleural effusions, lymphatic fluids from infected patients also can be checked and compared with their blood samples to see the specificity of sample to rZmp1.
iv. Mycobacterial proteins with high antigenic properties can be screened by computational methods and further checked for the humoral responses in the patients which would help in developing novel diagnostic targets and vaccine candidates.

Overall, the study provides several new leads which can be pursued to understand the antigenic responses of rZmp1 with larger cohort, blinded samples and multi-centric study would be useful for translational research

From biochemical and physiological characterization

i. Zmp1 has been known to have protease activity with varying substrates. In this regard, along with earlier observations showing neuropeptides as substrates for Zmp1, it could be explored further for the role of Zmp1 in TB meninges cases.
ii. rZmp1 doesn't inhibit IL-1b levels thereby the inflammasome inhibition which is in accordance with Nigericin, a positive inducer of inflammasomes (Pelegrin and Surprenant 2007), shows that the molecular mechanisms behind inhibition of inflammasomes by Zmp1 is an indirect one mediated by other protein of either host or mycobacteria. Mutant studies and immunoprecipitation assays would help in identifying these proteins.
iii. Exogenous treatments of rZmp1 have shown that it tends towards necrosis, therefore exploring the necrotic pathway and mechanism of action of rZmp1 in activating this pathway will open up ways to evade pathogen.

iv. Dissemination studies have shown that rZmp1 helps in spread of infection, but whether this is in consortium with other proteins or Zmp1 alone can be deliberated using knock out studies

v. Concentration gradient of Zmp1 is decisive in necrosis on one hand and a possible systemic dissemination on the other or if secretion of Zmp1 into acellular milieu of granuloma causes granuloma dissolution owing to Zmp1 proteolytic activity and hence possible role in reactivation.

Overall this study has shown the various physiological functions of Zmp1 which require further attention and need to be pursued to understand the underlying molecular mechanisms of mycobacteria for its survival inside the host and confront the emerging problem of drug resistance in M. tb.

Annexures

Annexure I: *The table represents QuantiFERON®-TB Gold (QFT®)* ELISA *assay details of healthy control samples*

Subject ID	Nil	TB Ag	Mitogen	TB Ag-Nil	Mitogen-Nil	Result	OD_{450nm}
ID 1	0.18	0.28	> 10	0.1	> 10	NEGATIVE	0.4713
ID 2	0.17	0.16	> 10	-0.01	> 10	NEGATIVE	0.7868
ID 3	0.11	0.13	> 10	0.02	> 10	NEGATIVE	0.8048
ID 4	0.11	0.15	> 10	0.04	> 10	NEGATIVE	0.7232
ID 5	0.14	0.21	> 10	0.07	> 10	NEGATIVE	0.9852
ID 6	0.13	0.12	> 10	-0.01	> 10	NEGATIVE	0.7408
ID 7	0.22	0.58	> 10	0.36	> 10	POSITIVE	0.6178
ID 8	0.17	0.17	> 10	0	> 10	NEGATIVE	0.5143
ID 9	0.14	0.12	> 10	-0.02	> 10	NEGATIVE	0.6565
ID 10	0.13	> 10	> 10	> 10	> 10	POSITIVE	0.7678
ID 11	0.11	0.17	> 10	0.06	> 10	NEGATIVE	0.5215
ID 12	0.18	0.2	> 10	0.02	> 10	NEGATIVE	0.6148
ID 13	0.11	0.13	> 10	0.02	> 10	NEGATIVE	0.4772
ID 14	0.15	0.14	> 10	-0.01	> 10	NEGATIVE	0.7852
ID 15	0.12	0.15	> 10	0.03	> 10	NEGATIVE	1.2812
ID 16	0.33	0.29	> 10	-0.04	> 10	NEGATIVE	0.4808
ID 17	0.14	0.13	> 10	-0.01	> 10	NEGATIVE	0.4095
ID 18	0.11	0.12	> 10	0.01	> 10	NEGATIVE	0.6452
ID 19	0.19	0.13	> 10	-0.06	> 10	NEGATIVE	0.6998
ID 20	0.15	0.13	> 10	-0.02	> 10	NEGATIVE	0.7875
ID 21	0.11	0.11	> 10	0	> 10	NEGATIVE	0.6885
ID 22	0.11	0.14	> 10	0.03	> 10	NEGATIVE	0.7075
ID 23	0.13	0.17	> 10	0.04	> 10	NEGATIVE	0.6803
ID 24	0.19	0.13	> 10	-0.06	> 10	NEGATIVE	0.8693
ID 25	0.12	0.15	> 10	0.03	> 10	NEGATIVE	1.1065
ID 26	0.16	0.13	> 10	-0.03	> 10	NEGATIVE	0.4458
ID 27	0.11	0.12	> 10	0.01	> 10	NEGATIVE	0.7765
ID 28	0.11	> 10	> 10	> 10	> 10	POSITIVE	0.9548
ID 29	0.17	2.67	> 10	2.5	> 10	POSITIVE	0.5645
ID 30	0.12	0.1	> 10	-0.02	> 10	NEGATIVE	0.8065
ID 31	0.19	5.78	> 10	5.59	> 10	POSITIVE	0.5158
ID 32	0.13	1.1	> 10	0.97	> 10	POSITIVE	0.9575
ID 33	0.23	0.2	> 10	-0.03	> 10	NEGATIVE	0.8182

ID 34	0.17	0.22	> 10	0.05	> 10	NEGATIVE	0.9182
ID 35	0.13	0.28	> 10	0.15	> 10	NEGATIVE	1.1005
ID 36	0.17	0.13	> 10	-0.04	> 10	NEGATIVE	0.9568
ID 38	0.13	0.23	> 10	0.1	> 10	NEGATIVE	0.9195
ID 39	0.12	0.13	> 10	0.01	> 10	NEGATIVE	1.1518
ID 40	0.12	0.12	> 10	0	> 10	NEGATIVE	0.9368
ID 41	0.13	0.13	> 10	0	> 10	NEGATIVE	0.6948
ID 42	0.13	0.17	> 10	0.04	> 10	NEGATIVE	0.4375
ID 43	0.15	0.15	> 10	0	> 10	NEGATIVE	0.5675
ID 44	0.12	0.14	> 10	0.02	> 10	NEGATIVE	0.4825
ID 45	0.16	0.15	> 10	-0.01	> 10	NEGATIVE	0.7048
ID 46	0.12	0.11	> 10	-0.01	> 10	NEGATIVE	0.2902
ID 47	0.26	0.33	> 10	0.07	> 10	NEGATIVE	0.383
ID 48	0.13	0.12	> 10	-0.01	> 10	NEGATIVE	0.893
ID 49	0.14	0.16	> 10	0.02	> 10	NEGATIVE	0.635
ID 50	0.2	0.17	> 10	-0.03	> 10	NEGATIVE	0.752
ID 51	0.13	0.17	> 10	0.04	> 10	NEGATIVE	0.2215
ID 52	0.14	0.13	> 10	-0.01	> 10	NEGATIVE	0.2315
ID 53	0.11	0.2	> 10	0.09	> 10	NEGATIVE	0.816
ID 54	0.18	0.21	> 10	0.03	> 10	NEGATIVE	1.5013
ID 55	0.44	0.29	7.99	-0.15	7.55	NEGATIVE	0.3343
ID 56	0.14	0.59	> 10	0.45	> 10	POSITIVE	0.5397
ID 57	0.13	0.21	> 10	0.08	> 10	NEGATIVE	1.1853
ID 58	0.16	0.21	> 10	0.05	> 10	NEGATIVE	0.4513
ID 59	0.13	> 10	> 10	> 10	> 10	POSITIVE	0.4267
ID 60	0.12	0.14	> 10	0.02	> 10	NEGATIVE	1.2813
ID 61	0.15	0.14	> 10	-0.01	> 10	NEGATIVE	0.3753
ID 62	0.14	2.68	> 10	2.54	> 10	POSITIVE	1.0883

The table represents the results of TB Interferon Gamma Release Assay of the healthy donors used in this study. QuantiFERON®-TB Gold (QFT®) ELISA kit (Reference# 0594-0201) was used and the results were analysed using QuantiFERON-TB Gold Analysis software (Version 2.62) as per the manufacturer's instructions. The subjects tested positive in TB IGRA test were shaded.

Annexure II -- *The table represents the details of Pulmonary Tuberculosis patient samples*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	F	40	16	NO	1.726
ID2	M	28	25	NO	1.0123
ID3	M	45	20	YES	0.808
ID4	F	49	25	YES	1.274
ID5	F	28	30	YES	0.810
ID6	M	36	16	YES	1.047
ID7	M	24	20	YES	0.878
ID8	M	31	25	YES	0.855
ID9	F	30	14	NO	1.107
ID10	F	49	20	NO	0.812
ID11	F	38	25	NO	1.280
ID12	F	22	25	YES	1.152
ID13	F	26	30	NO	1.179
ID14	F	39	12	NO	0.771
ID15	M	36	25	YES	0.734
ID16	F	21	14	NO	1.056
ID17	F	35	18	NO	1.401
ID18	F	29	18	NO	0.956
ID19	F	36	10	YES	1.345
ID20	F	32	25	YES	1.253
ID21	F	29	25	YES	0.718
ID22	M	38	25	YES	0.782
ID23	F	29	12	NO	1.313
ID24	F	39	30	YES	1.178
ID25	F	25	20	YES	1.071
ID26	M	35	20	YES	1.06
ID27	M	32	25	NO	0.731
ID28	F	30	25	NO	1.127
ID29	F	35	30	YES	0.963
ID30	F	32	14	NO	0.730
ID31	F	22	20	YES	0.991
ID32	F	18	20	NO	0.719
ID33	F	31	20	NO	0.741
ID34	F	49	20	YES	0.979

ID35	F	29	20	NO	1.250
ID36	M	32	10	NO	0.720
ID37	M	35	14	NO	1.320
ID38	M	21	20	NO	0.895
ID39	M	39	10	NO	1.090
ID40	F	41	20	YES	0.834
ID41	F	22	14	NO	1.261
ID42	F	39	25	YES	1.264
ID43	F	22	14	NO	1.297
ID44	F	23	25	YES	1.407
ID45	M	38	16	NO	1.565
ID46	M	35	20	YES	1.607
ID47	M	22	20	NO	1.716
ID48	M	49	20	YES	1.737
ID49	F	32	25	NO	1.773
ID50	F	27	20	NO	1.800
ID51	M	31	16	NO	1.811
ID52	M	19	12	YES	1.936
ID53	M	28	25	NO	1.947
ID54	F	35	25	NO	1.978
ID55	M	29	25	NO	2.148
ID56	M	32	20	NO	2.317
ID57	F	28	20	YES	2.334
ID58	M	19	20	NO	2.379
ID59	M	28	18	NO	2.380
ID60	F	29	25	NO	2.380
ID61	F	31	25	YES	2.487
ID62	M	39	25	NO	2.516
ID63	M	31	20	YES	2.569
ID64	F	36	20	NO	2.641
ID65	F	21	15	NO	2.666
ID66	F	23	30	NO	2.67

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure III -- *The table represents the details of Extra-Pulmonary Tuberculosis patient samples*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	F	19	16	NO	1.01
ID2	F	22	16	NO	0.986
ID3	F	28	10	NO	0.933
ID4	F	35	NIL	NO	1.0767
ID5	F	29	14	NO	1.169
ID6	F	22	20	YES	1.214
ID7	F	31	10	YES	0.941
ID8	M	18	10	NO	2.595
ID9	F	50	12	NO	1.187
ID10	F	17	NIL	YES	2.432
ID11	M	60	10	YES	1.977
ID12	M	33	10	NO	0.901
ID13	F	18	12	NO	2.693
ID14	F	18	NIL	YES	0.853
ID15	F	36	10	YES	2.045
ID16	F	16	10	YES	2.27
ID17	M	22	20	YES	0.949
ID18	F	32	10	NO	1.360
ID19	F	35	20	NO	0.671
ID20	M	18	NIL	NO	2.268
ID21	M	43	14	YES	2.832
ID22	F	21	10	YES	0.767
ID23	F	20	NIL	YES	2.142
ID24	F	25	16	YES	1.728
ID25	F	26	10	YES	2.231
ID26	M	27	10	NO	2.435
ID27	M	39	12	NO	1.668
ID28	F	32	18	YES	0.938
ID29	M	41	14	NO	2.718
ID30	F	34	NIL	NO	1.168
ID31	M	56	NIL	YES	1.615
ID32	M	30	14	YES	2.303
ID33	F	22	10	YES	1.05
ID34	F	15	NIL	NO	1.846

ID35	F	32	16	NO	2.505
ID36	M	47	10	NO	2.405
ID37	F	22	20	NO	0.69
ID38	M	16	16	NO	2.609
ID39	M	31	12	YES	1.884
ID40	M	24	20	NO	2.826
ID41	F	35	16	YES	1.948
ID42	M	18	25	YES	1.469
ID43	F	26	14	NO	2.710
ID44	M	32	20	NO	0.977
ID45	F	20	16	YES	1.992
ID46	F	20	25	YES	1.4205
ID47	F	15	NIL	YES	0.687
ID48	F	28	NIL	YES	1.023
ID49	F	19	16	YES	0.777
ID50	F	25	NIL	NO	0.713
ID51	F	37	NIL	NO	0.944
ID52	F	19	12	YES	1.477
ID53	F	38	10	NO	1.082
ID54	F	35	10	YES	1.204
ID55	F	29	12	NO	1.146

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure IV-- *The table represents the details of household infection contacts of Pulmonary Tuberculosis patients*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	M	28	10	YES	0.657
`	F	42	NIL	YES	0.249
ID3	M	30	30	NO	0.532
ID4	M	26	12	YES	0.409
ID5	M	26	25	YES	0.222
ID6	M	50	14	NO	0.316
ID7	M	27	10	YES	0.555
ID8	M	27	16	NO	0.442
ID9	F	55	20	NO	0.386
ID10	M	28	20	YES	0.397
ID11	F	35	20	NO	0.769
ID12	F	25	15	NO	0.883
ID13	M	32	20	YES	0.939
ID14	F	35	18	YES	0.568
ID15	F	26	20	NO	0.552
ID16	M	26	14	NO	0.629
ID17	M	50	25	YES	0.590
ID18	M	30	20	YES	0.573
ID19	F	36	13	NO	0.615
ID20	F	50	NIL	YES	0.587
ID21	F	35	18	NO	0.889
ID22	M	35	16	NO	0.614
ID23	M	65	17	NO	0.640
ID24	F	28	14	NO	0.614
ID25	M	27	20	NO	0.687
ID26	F	50	24	YES	0.343
ID27	M	23	NIL	YES	0.779
ID28	F	41	14	YES	0.386
ID29	F	55	20	YES	0.625
ID30	F	20	25	NO	0.387
ID31	F	35	18	YES	0.322
ID32	F	50	20	NO	0.538
ID33	F	40	10	NO	0.572
ID34	F	35	14	YES	0.351

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure V -- *The table represents the details of household infection contacts Extra-Pulmonary Tuberculosis patients*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	F	37	20	NO	0.787
ID2	M	20	>30	NO	0.583
ID3	M	50	20	NO	0.793
ID4	F	25	25	NO	1.001
ID5	F	40	1	NO	0.874
ID6	M	26	20	NO	0.809
ID7	F	22	16	YES	0.942
ID8	M	38	15	NO	0.755
ID9	M	40	18	NO	0.783
ID10	F	42	30	YES	0.879
ID11	M	28	16	YES	0.767
ID12	F	19	25	YES	0.72
ID13	M	24	25	NO	0.565
ID14	F	18	16	NO	1.035
ID15	F	30	18	NO	0.592
ID16	F	40	16	NO	0.484
ID17	M	30	14	NO	0.313
ID18	M	44	20	YES	0.717
ID19	M	21	14	YES	0.869
ID20	M	49	NIL	NO	0.663
ID21	F	32	18	YES	0.649
ID22	F	28	20	NO	0.509
ID23	F	32	20	NO	0.809
ID24	F	48	NIL	NO	0.810
ID25	F	35	18	NO	0.979
ID26	F	33	16	NO	0.824
ID27	F	20		NO	0.782
ID28	F	45	16	YES	0.398
ID29	M	45	NIL	NO	0.723
ID30	F	35	20	NO	0.854
ID31	F	24	16	YES	0.524
ID32	F	35	NIL	NO	0.557
ID33	F	32	25	YES	0.247

ID34	F	44	20	YES	0.679
ID35	F	24	18	NO	0.277
ID36	M	21	15	NO	0.606
ID37	F	35	8	YES	0.428
ID38	M	45	16	YES	0.487
ID39	F	40	8	YES	0.666
ID40	F	18	18	NO	0.323
ID41	M	25	NIL	YES	0.750
ID42	F	35	20	YES	0.586
ID43	F	25	20	NO	0.396
ID44	F	35	14	NO	0.807
ID45	M	35	20	YES	0.588
ID46	F	24	18	YES	0.639
ID47	M	45	20	YES	0.577
ID48	M	45	10	YES	0.868
ID49	M	25	15	YES	0.179
ID50	F	31	16	NO	0.605
ID51	F	45	14	YES	0.865
ID52	F	45	25	NO	0.428
ID53	F	30	16	NO	0.527
ID54	M	18	10	YES	0.616
ID55	F	20	11	NO	0.513

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure VI -- *The table represents the details of Extra-Pulmonary Tuberculosis patient samples with pleural effusion*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	F	31	10	YES	0.941
ID2	M	22	20	YES	0.949
ID3	F	35	20	NO	0.671
ID4	F	22	10	YES	1.05
ID5	F	22	20	NO	0.69
ID6	F	19	16	YES	0.777
ID7	F	38	10	NO	1.082
ID8	F	29	12	NO	1.146
ID9	M	43	14	YES	2.832
ID10	F	20	NIL	YES	2.142
ID11	M	27	10	NO	2.435
ID12	M	56	NIL	YES	1.615
ID13	F	15	NIL	NO	1.846
ID14	F	32	16	NO	2.505
ID15	M	47	10	NO	2.405
ID16	M	31	12	YES	1.884
ID17	M	24	20	NO	2.826
ID18	M	18	25	YES	1.469
ID19	M	32	20	NO	0.977
ID20	F	20	25	YES	1.421

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure VII -- *The table represents the details of the household infection contacts of Extra-Pulmonary Tuberculosis patient samples with pleural effusion*

Subject ID	Gender	Age	MX	BCG Scar	OD_{450nm}
ID1	F	37	20	NO	0.787
ID2	M	20	>30	NO	0.583
ID3	M	50	20	NO	0.793
ID4	F	25	25	NO	1.001
ID5	F	40	1	NO	0.874
ID6	M	26	20	NO	0.809
ID7	F	22	16	YES	0.942
ID8	M	38	15	NO	0.755
ID9	F	18	16	NO	1.035
ID10	F	30	18	NO	0.592
ID11	F	40	16	NO	0.484
ID12	M	21	14	YES	0.869
ID13	F	32	18	YES	0.649
ID14	F	28	20	NO	0.509
ID15	F	32	20	NO	0.809
ID16	F	35	18	NO	0.979
ID17	F	33	16	NO	0.824
ID18	F	45	16	YES	0.398
ID19	M	45	NIL	NO	0.723
ID20	F	50	15	YES	1.259

MX represents Monteaux test; **BCG** scar represents BCG vaccine obtained in childhood.

Annexure VIII -- *The table represents the details of the Non-Specific infection control samples*

Subject ID	Gender	Age	Infection	OD_{450nm}
ID1	F	22	Diabetic and leukocytosis	0.213
ID2	M	62	Viral and Neutrophilia	0.276
ID3	F	23	Eosinophilia	0.282
ID4	F	55	Diabetic and Neutrophilia	0.320
ID5	M	28	Mild Eosinophilia	0.336
ID6	M	23	Typhoid	0.390
ID7	F	30	Neutrophilia	0.407
ID8	F	45	Mild Leukocytosis	0.420
ID9	F	50	Diabetic and Eosinophilia	0.426
ID10	F	55	Viral and Neutrophilia	0.533
ID11	M	20	Viral fever	0.536
ID12	F	22	Neutrophilia	0.539
ID13	F	59	Diabetic and Eosinophilia	0.544
ID14	M	21	Leukocytosis	0.576
ID15	M	35	Eosinophilia	0.59
ID16	F	29	Mild Eosinophilia	0.678
ID17	M	24	Viral fever	0.690
ID18	F	27	Diabetic and Neutrophilia	0.714
ID19	F	25	Fever	0.714
ID20	F	30	Diabetic and Eosinophilia	0.752
ID21	F	25	Diabetic and Eosinophilia	0.878
ID22	M	34	Eosinophilia	0.892
ID23	M	42	Fever	1.02

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Mycobacterium tuberculosis Zinc Metalloprotease-1 Elicits Tuberculosis-Specific Humoral Immune Response Independent of Mycobacterial Load in Pulmonary and Extra-Pulmonary Tuberculosis Patients

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Conventionally, facultative intracellular pathogen, *Mycobacterium tuberculosis*, the tuberculosis (TB) causing bacilli in human is cleared by cell-mediated immunity (CMI) with $CD4^+$ T cells playing instrumental role in protective immunity, while antibody-mediated immunity (AMI) is considered non-protective. This longstanding convention has been challenged with recent evidences of increased susceptibility of hosts with compromised AMI and monoclonal antibodies conferring passive protection against TB and other intracellular pathogens. Therefore, novel approaches toward vaccine development include strategies aiming at induction of humoral response along with CMI. This necessitates the identification of mycobacterial proteins with properties of immunomodulation and strong immunogenicity. In this study, we determined the immunogenic potential of *M. tuberculosis* Zinc metalloprotease-1 (Zmp1), a secretory protein essential for intracellular survival and pathogenesis of *M. tuberculosis*. We observed that Zmp1 was secreted by *in vitro* grown *M. tuberculosis* under granuloma-like stress conditions (acidic, oxidative, iron deficiency, and nutrient deprivation) and generated Th2 cytokine microenvironment upon exogenous treatment of peripheral blood mononulear cells PBMCs with recombinant Zmp1 (rZmp1). This was supported by recording specific and robust humoral response in TB patients in a cohort of 295. The anti-Zmp1 titers were significantly higher in TB patients ($n = 121$) as against healthy control ($n = 62$), household contacts ($n = 89$) and non-specific infection controls ($n = 23$). A significant observation of the study is the presence of equally high titers of anti-Zmp1 antibodies in a range of patients with high bacilli load (sputum bacilli load of 300+ per mL) to paucibacillary smear-negative pulmonary tuberculosis (PTB) cases.

This clearly indicated the potential of Zmp1 to evoke an effective humoral response independent of mycobacterial load. Such mycobacterial proteins can be explored as antigen candidates for prime-boost vaccination strategies or extrapolated as markers for disease detection and progression.

Keywords: tuberculosis (TB), extra-pulmonary tuberculosis (EPTB), pulmonary tuberculosis (PTB), Rv0198c, humoral immunity

INTRODUCTION

Mycobacterium tuberculosis, the tuberculosis (TB) causing bacilli are facultative intracellular parasite, residing primarily in phagocytic cells like alveolar macrophages and monocytes, but may also colonize other cells, such as alveolar epithelial cells, bones, meninges, peritoneal linings of the intestines, etc. (Golden and Vikram, 2005). Traditionally, like all intracellular pathogens, protective immunity to *M. tuberculosis* has been credited to the cell mediated immunity (CMI) with $CD4^+$ T cells playing a crucial role in granuloma formation while antibody mediated immunity (AMI) is considered non-protective (van Crevel et al., 2002). The argument that *M. tuberculosis* is strictly intracellular, however, is debatable as *M. tuberculosis*, during some point of its infectious cycle, has also been observed in extracellular spaces (Grosset, 2003), where in principle they can be vulnerable to antibody action. There are a number of reports where despite being intracellular pathogen, antibodies have been shown to modulate the immune response in favor of the host against pathogens (Casadevall, 2003). Studies with antibodies, monoclonal or otherwise, have now demonstrated passive protection for several microbes, such as *Candida albicans* (Han and Cutler, 1995), *Listeria monocytogenes* (Edelson et al., 1999), *Leishmania mexicana* (Anderson et al., 1983), *M. tuberculosis* (Teitelbaum et al., 1998; Zhao et al., 2011) etc., though experiments with immune serum have provided inconsistent results. Similarly, a considerable expanse of data propose that defense against intracellular and extracellular pathogens are not stringently restricted to either Th1 (promoting CMI) or Th2 (promoting AMI) responses. Citing a few examples, humoral immunity have been shown to be protective against intracellular pathogens like Plasmodium or Mycobacteria, while protective immunity against extracellular parasitic flatworm Schistosoma was due to CMI triggered by Th1 response (Abebe and Bjune, 2009; Greenhouse et al., 2011; Wen et al., 2011; Dups et al., 2014). Recently, Modified Vaccinia Ankara 85A (MVA85A) failed to clear the phase 2b trial, where *M. tuberculosis* major secreted antigen complex 85A (Ag85A) that induces a strong Th1 immune response in BCG-primed host was used (Tameris et al., 2013). Therefore, though CMI may remain the mainstream immune response, the role of AMI in conferring protection against intracellular pathogens, including *M. tuberculosis*, cannot be dismissed.

Serological studies advocate that *M. tuberculosis* infection, beside CMI, also evokes a strong humoral response in patients against a variety of mycobacterial antigens (Steingart et al., 2009). Corroborating these observations are other studies where *M. bovis* BCG vaccination led to generation of mycobacterial antigen specific IgG and IgM (Beyazova et al., 1995; Brown et al., 2003; de Valliere et al., 2005). Some anti-*M. tuberculosis* antibodies enhanced both innate and CMI responses during mycobacterial infection (de Valliere et al., 2005). Antibodies, through a range of mechanisms, including simple opsonization to complicated FcR activation can regulate the fate of intracellular pathogens. Some vaccine trials have included induction of AMI to transduce protection against fungal diseases (Vecchiarelli et al., 2012). A recent study has evaluated the feasibility of using humoral immunity in vaccine development against *M. tuberculosis* by comparing immunoglobulin titers (IgG and IgA) with a variety of clinical and immunological parameters (Niki et al., 2015). While these studies strongly support the inclusion of evoking AMI alongside CMI in TB vaccine development program, there is a need for systematic investigation of mycobacterial antigens for a strong and specific humoral response that can be employed against TB.

Surface-exposed or secreted proteins of *M. tuberculosis* are customarily the targets of immune responses in the infected host. Several *M. tuberculosis* proteins, including culture filtrate proteins have been evaluated for their immunogenic properties, such as CFP10, ESAT-6, Ag85B, ICDs etc. (Banerjee et al., 2004; Sinha et al., 2005; Malen et al., 2008; Floss et al., 2010). *M. tuberculosis* GlcB (malate synthase), MPT51 (FbpC1), and HSPX (alpha crystalline) have also been evaluated for humoral response in clinically asymptomatic Health-care workers with latent infections, suggesting the possibility of these responses to be protective (Reis et al., 2009). In this study, we have evaluated the humoral response to *M. tuberculosis* H37Rv zinc metalloprotease-1, Zmp1 (Rv0198c), a protein present in the culture filtrate (de Souza et al., 2011). Extracellular zinc-containing metalloproteases are ubiquitously present, quite a few of them from pathogenic bacteria function as exotoxins, such as, Clostridial neurotoxins, Anthrax toxins, Botulinum neurotoxin, *Bacillus* sp. thermolysin etc. (Hase and Finkelstein, 1993; Miyoshi and Shinoda, 2000). These zinc-metalloproteases are also known to elicit a strong and specific humoral response, for which the inactivated toxin (toxoid) function as vaccine candidate (Hase and Finkelstein, 1993; Miyoshi and Shinoda, 2000). The annotated zinc-metalloproteases from *M. tuberculosis* H37Rv are, namely, Rv0198c (zmp1), Rv0563 (htpX), Rv2467 (pepN), Rv2869c (rip) and Rv3610c (ftsH), Rv1977 (Stewart et al., 2002; Griffin et al., 2011; Kelkar et al., 2011; Mazandu and Mulder, 2012; Schneider et al., 2014). Mutant studies of Zmp1 in *M. tuberculosis* strain H37Rv and *M. bovis* BCG suggested that it is essential for the intracellular survival of the bacteria and possibly impairs inflammasome activation and phagosome maturation (Master et al., 2008; Johansen et al.,

2011). With these cues suggesting Zmp1 as an immunomodulator and upon recording high antigenic index by Jameson-Wolf plot using Protean software, we hypothesized that Zmp1 could be a strong immunostimulant and provoke an effective humoral response during infection. Presence of anti-Zmp1 antibodies in TB patient sera confirmed that it is indeed expressed during infection. We established that Zmp1 is secreted by H37Rv under granuloma-like *in vitro* growth conditions and is capable of orienting the immune response toward Th2 cytokine microenvironment. Further, we compared the humoral response to Zmp1 in various TB patient categories, including smear-negative Extra-pulmonary TB cases. Presence of high titers of anti-Zmp1 antibodies in smear-negative extra-pulmonary tuberculosis (EPTB) cases similar to pulmonary tuberculosis (PTB) patients that ranged from low to high bacillary load indicated the potential of Zmp1 to evoke an effective humoral response independent of mycobacterial load. The study points to the potential of further exploration of mycobacterial proteins, such as Zmp1, as antigen candidates for prime-boost vaccination strategies or as markers for disease progression.

MATERIALS AND METHODS

Cloning, Expression, and Purification of Zmp1 (rZmp1) Protein

The antigenic index of Rv0198c was analyzed by Protean software (Protean 5.00, DNASTAR, Inc.; **Figure 1A**). Rv0198c *(zmp1)* gene was amplified from the genomic DNA of H37Rv using specific forward (5′-ataGGATCCgtgacacttgccatcccctcgg-3′) and reverse (5′-agtCTCGAGgtcttagcctagttccagat-3′) primers. The amplicon was cloned into *Bam*HI and *Xho*I sites of pET28a vector. The positive clones were confirmed by sequencing. pET28a-*Rv0198c* construct was expressed in BL-21 DE3 cells and N-terminal Histidine tagged recombinant Zmp1 (rZmp1) was purified under native conditions (50 mM Tris-HCl buffer, 300 mM NaCl, pH-8.0) by affinity chromatography using cobalt based resin. The purified protein was dialyzed against dialysis buffer (50 mM Tris-HCl buffer pH-8.0, 100 mM NaCl, 4% glycerol, 1 mM PMSF) at 4°C. Dialyzed protein was treated with polymyxin-B agarose beads to remove endotoxins (**Figure 1B**). The endotoxin-free rZmp1 protein was checked for functional activity using casein as a substrate (Rowland et al., 1997; Coffey et al., 2000; Grandgenett et al., 2007). Proteolytic degradation of casein can be assessed on SDS-PAGE by monitoring for cleavage products at lower molecular weights or apparent shift in the casein band to lower molecular weight after proteolytic digestion. Hydrolysis of casein by rZmp1 was performed at 37°C for 1 h in Tris-HCl buffer (50 mM Tris-HCl pH-8.0 and 100 mM NaCl). The reaction was stopped by adding Laemmli's buffer to the reaction mix followed by fractionation on SDS-PAGE. As anticipated, hydrolysis of casein by rZmp1 has yielded two bands corresponding to apparent molecular weights of 27 and 20 kDa (**Figure 1C**). Band corresponding to 20 kDa is the cleaved product of casein which was observed when casein was incubated with rZmp1 suggesting that the purified protein was functionally active (**Figures 1B,C**).

In Vitro Mycobacterial Growth Conditions and Western Blots

The mycobacterial strain used was *M. tuberculosis* H37Rv. The growth of mycobacteria was performed as described earlier (Ganji et al., 2016). The mycobacteria were plated on 7H10 agar media supplemented with 10% oleic acid, albumin, dextrose, and catalase (OADC) and incubated at 37°C. The colonies were picked into the 7H9 broth media supplemented with 10% OADC and incubated at 37°C at 180 rpm until the $OD_{600\ nm}$ reached 0.5–0.6. The culture was checked for any contamination using Ziehl-Neelsen (ZN) staining procedure. The culture was then centrifuged at 3700 rpm for 7 min. The culture pellet was washed with phosphate buffered saline (PBS) pH 7. The pellet was then resuspended in Sauton's media under granuloma-like stress conditions, such as acidic pH 5.5, oxidative stress (10 mM H_2O_2; Voskuil et al., 2011), Iron deprivation and Nutrient starvation for 36 h. For Nutrient stress the culture was resuspended in PBS. For Iron deprivation, the glassware and the media were made Iron-free as described earlier (Hall and Ratledge, 1982).

Sample Collection

A total of 295 subjects in the age group of 15–60 years were recruited at Mahavir Hospital and Research Centre (MHRC), Hyderabad and University of Hyderabad (UH) after taking prior ethical committee approvals (ECR/450/Inst/AP 2013 and UH/IEC/2014/36) and written consents from the subjects. 2–5 mL of blood was collected in vacutainers with EDTA and later sera were separated for the experiments. The study population was divided into four categories, namely, Clinically Healthy donors ($n = 62$), TB patients ($n = 121$), household contacts ($n = 89$), and non-specific infection controls ($n = 23$). Clinically healthy donors had no symptoms of any disease at the time of sera collection and were tested for TB-interferon-gamma release assays (TB-IGRAs) using QuantiFERON-TB Gold (QFT) ELISA kit (Reference# 0594-0201) and the results were analyzed using QuantiFERON-TB Gold Analysis software (Version 2.62) as per the manufacturer's instructions (Supplementary Table S1). For TB patients, the sputum microscopy for AFB was performed as per Revised National Tuberculosis Control Programme (RNTCP), government of India, guidelines with confirmed diagnosis of sputum, culture, and chest X-ray in patients (http://tbcindia.nic.in/view.php?lid=3143&type=1). Tuberculin skin test (TST) was performed in all the subjects. TB patients were further categorized into PTB ($n = 66$) and EPTB ($n = 55$) cases. EPTB patients were defined atleast with one culture-positive specimen from an extra-pulmonary site, or histological or radiological, or strong clinical evidence consistent with active extra-pulmonary TB. Household contacts of the respective patients were those who resided in house of the TB patient during 3 months period for atleast seven consecutive days prior to the diagnosis of tuberculosis. Mostly they were siblings and spouses. The household contacts, though, were clinically asymptomatic but many of them were Mantoux positive (Out of 89 household contacts, 60 subjects were Mantoux positive with diameter of Induration >15 mm). Non-specific infection controls comprised of patients with random infections other than TB

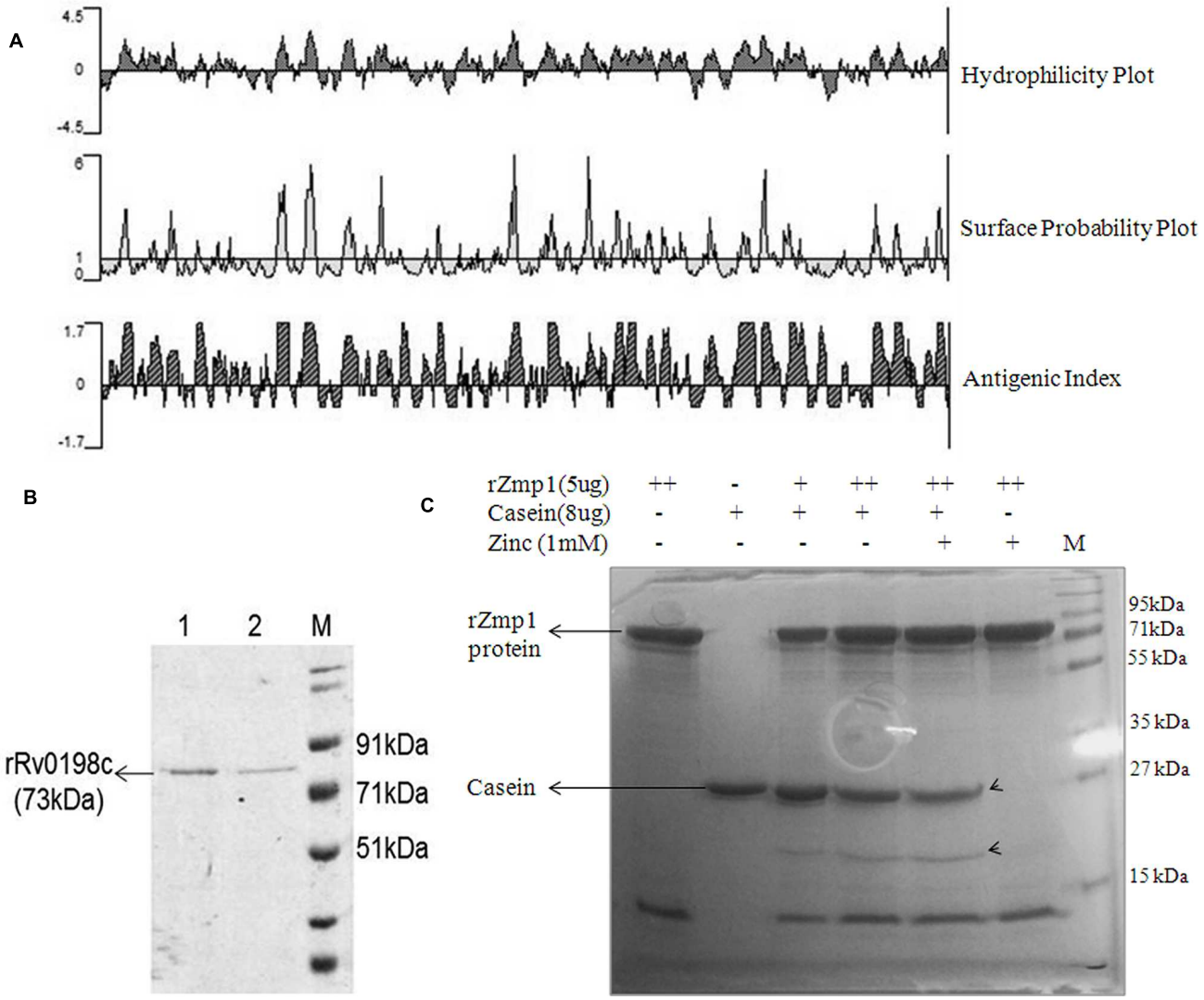

FIGURE 1 | Rv0198c (Zmp1) protein is antigenic and purified recombinant Zmp1 (rZmp1) was functionally active. (A) Antigenicity prediction of Rv0198c using PROTEAN software. Kyte-Doolittle plot for hydrophilicity; Emini plot for Surface probability and Jameson-Wolf analyses for Antigenic index. **(B)** SDS-PAGE showing purified recombinant Rv0198c protein (rRv0198c), M: marker. Rv0198c was cloned in pET28a (+) with N-terminal 6× Histidine tag and expressed in *Escherichia coli* BL21-DE3 and purified using Cobalt-based affinity chromatography. **(C)** SDS-PAGE depicting the functional activity of purified rRv0198c using casein as substrate. Endotoxin-free rRv0198c was incubated with casein in presence or absence of Zinc ions. Casein was hydrolyzed by rZmp1 yielding two bands (marked by arrowheads) corresponding to apparent molecular weights of 27 and 20 kDa.

such as viral, bacterial, and parasitic infections. Pregnant women, terminally ill patients, immunocompromised patients, patients undergoing any chemotherapy or with chronic illness were not included in the study.

Enzyme Linked Immunosorbent Assay

The peripheral blood mononulear cells (PBMCs) were isolated from blood collected from healthy donors using Ficoll gradient. They were either left untreated or exogenously treated with 50 and 100 nM of purified, endotoxin-free rZmp1 or 0.5 μg/mL of LPS in RPMI media supplemented with 10% FBS and kept at 37°C for 24 h. For measurement of cytokine levels we have used BD OptEIA enzyme linked immunosorbent assay (ELISA) sets and performed as per manufacturer's instructions. For measurement of anti-Zmp1 antibody titers, 100 ng of purified rZmp1 in 100 μL of coating buffer was coated per well at 4°C overnight. The plates were then washed three times with PBST (PBS containing 0.05% Tween-20) before blocking with 120 μL of blocking solution (3% BSA in PBS) at 37°C for 1 h. After blocking, plates were washed thrice with PBST. 1:100 times diluted (100 μL/well) sera samples were added and incubated at 37°C for 1 h followed by five washes with PBST. Anti-Human IgG conjugated with HRP (Sigma) was used as the secondary antibody in 1:10000 dilution (100 μL/well) and incubated at 37°C for 1 h. After seven washes with 1X PBST, 100 μL/well of chromogenic substrate (Tetramethylbenzidine) was added and

kept for incubation at 37°C for 30 min. The reactions were stopped using 100 μL of 2 N H_2SO_4. The absorbance was measured at 450 and 570 nm in multi-well plate reader (Biotek). To check for the cross-reactivity of rZmp1 with other anti-TB antibodies, we have performed Western blotting for the rZmp1 using anti-ESAT6 antibody (**Supplementary Figure S1**). rZmp1 is not detected by the anti-ESAT6 antibody in the Western blot (**Supplementary Figure S1**) and thus confirms the specificity of rZmp1 and fidelity of rZmp1-based ELISA method to determine the anti-Zmp1 antibody titers in the sera samples.

Graphs and Statistical Analyses

Statistical analyses were carried out using SigmaPlot software version 11.0.0.77 (Systat Software, Inc., USA). For cytokine data, One-way ANOVA was performed with Holm–Sidak multiple pair-wise comparison method and the threshold for significance was set at $p < 0.05$. The error bars represent the ± standard deviation (SD) from the mean of at least three independent experiments. For statistical analyses of anti-Zmp1 antibody titers measured from the blood samples, One-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method. The threshold for significance was set at $p < 0.05$. They were represented as box plots using SigmaPlot. Within the plots, the upper quartile of the box represents the 75th percentile and the lower quartile for the 25th percentile. The line inside the box represents the median. The whiskers arising from either side of the upper half and the lower half of the box correspond to 1.5 times the interquartile range (IQR; Benjamin et al., 2013). Any datum to the further extreme of the whiskers is termed as outlier.

RESULTS

Zmp1 Protein is Expressed during Infection and is Secreted under Granuloma-Like *In Vitro* Growth Conditions by *M. tuberculosis* H37Rv

The functionally active, endotoxin free, purified recombinant Zmp1 (rZmp1) was used as antigen to capture anti-Zmp1 antibodies in the sera samples of TB patients (results presented and discussed later). The presence of anti-Zmp1 antibodies in *M. tuberculosis* infected patients confirmed that Zmp1 was indeed expressed by *M. tuberculosis* during infection. Zmp1 was identified as one of the culture filtrate proteins of *in vitro* grown H37Rv (de Souza et al., 2011). We extended the study to check if the same holds true for granuloma-like conditions. To study the same, H37Rv was grown under different stress conditions known to simulate acellular caseous environment of TB granulomas, that is, acidic pH 5.5, H_2O_2 induced oxidative stress, nutrient deprivation and iron deficiency (Stallings and Glickman, 2010). Bacteria were grown to mid-log phase and then subjected to various stresses for 36 h. The mycobacterial cells were then harvested and the culture supernatants separated. Culture supernatants were then precipitated using 10% TCA and the precipitate was used to detect presence of Zmp1 protein using Western blotting with in-house generated anti-Zmp1 antibody (**Figure 2A**, upper panel). CFP10 and GroEL1, which were probed with their respective antibodies, were used as positive and negative controls respectively (**Figure 2A**, middle and lower panel). CFP10 is a known mycobacterial secretory protein (Malen et al., 2007, 2008) and hence was used as a positive control for culture supernatant preparations while GroEL1 is an intrabacterial, membrane associated protein which is not secreted out (de Souza et al., 2011; Malen et al., 2011) and is used as negative control. Absence of GroEL1 in the culture supernatants indicated absence of cell lysis products in the culture supernatant (**Figure 2A**, lower panel). The presence of band corresponding to Zmp1 in Western blots suggested secretion of the protein under all the tested stress conditions (**Figure 2A**, upper panel). The assay was a qualitative check to confirm the secretion of Zmp1, though it is possible that the levels of secreted Zmp1 in culture supernatants may vary with stress conditions. **Figure 2B** represents the Western blots with the whole bacterial lysates as control experiment. This suggested that Zmp1 is indeed a secreted protein, possibly secreted within granuloma of infected host.

rZmp1 Stimulated PBMCs to Release Th2 Class of Cytokines

We next evaluated the immunostimulatory potential of Zmp1 in terms of release of Th1/Th2 cytokines from exogenously treated PBMCs derived from healthy volunteers to elucidate the association of Zmp1 with CMI or AMI. To do the same, functionally active, endotoxin free rZmp1 was used for stimulatory assays.

Peripheral blood mononulear cells were treated with rZmp1 at 50 and 100 nM for 24 h. LPS, a known strong immunostimulant of PBMCs, was used as a positive control (Jansky et al., 2003). The culture supernatants were then collected to assay for the levels of a minimal battery of cytokines. TNF-α and IL-1β are the cytokines of innate response that stimulate the acute phase reaction and represent initial stimulation of immune cells. High titers of TNF-α (Untreated: 91.98 ± 27.67 pg/mL; rZmp1 100 nM: 408.71 ± 52.96 pg/mL) and IL-1β (Untreated: 205.62 ± 65.63 pg/mL; rZmp1 100 nM: 634.34 ± 51.72 pg/mL; **Figures 3A,B**) upon exogenous treatment of PBMCs with rZmp1 established that rZmp1 is indeed a strong immunostimulant, comparable with LPS (TNF-α: 255.89 ± 107.67 pg/mL; IL-1β: 485.46 ± 111.66 pg/mL; **Figures 3A,B**). We next measured the levels of pro-inflammatory cytokines IFN-γ and IL-12p70 and regulatory cytokines IL-4 and IL-10. It was observed that upon treatment of PBMCs with rZmp1 protein, there is an increased secretion of regulatory cytokines, IL-10 (Untreated: 562.41 ± 244.02 pg/mL; rZmp1 100 nM: 1218.77 ± 270.60 pg/mL) and IL-4 (Untreated: 168.91 ± 60.62 pg/mL; rZmp1 100 nM: 292.31 ± 49.13 pg/mL; **Figures 3C,D**) and while no change was observed in the levels of IL-12p70 (Untreated: 46.32 ± 2.15 pg/mL; rZmp1 100 nM: 45.23 ± 8.31 pg/mL) and IFNγ (Untreated: 81.84 ± 31.44 pg/mL; rZmp1 100 nM: 52.49 ± 19.43 pg/mL; **Figures 3E,F**). When the ratio of IFNγ to IL-10 was considered, rZmp1 treatment

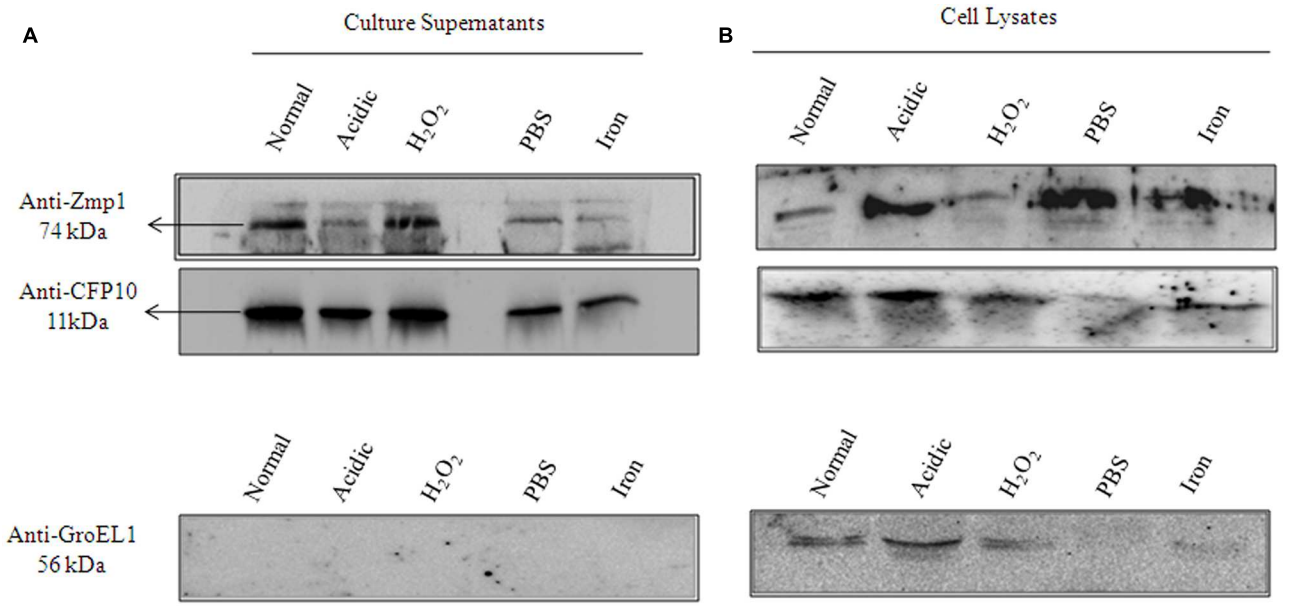

FIGURE 2 | Zmp1 is expressed and secreted by *in vitro* grown *Mycobacterium tuberculosis* H37Rv under granuloma-like conditions. (A) The culture supernatants of *in vitro* grown *M. tuberculosis* H37Rv under normal conditions and under various stress conditions, such as, acidic pH5.5, H_2O_2 induced oxidative stress, nutrient starvation and iron depletion, were precipitated by 10% trichloroacetic acid and the resultant precipitate was subjected to Western blot using Mouse anti-Zmp1 antibody (1:1000 dilution; Upper panel). Rabbit anti-CFP10 antibody (1:1000 dilution) against CFP10, a culture filtrate protein 10, was used as a positive control (Middle Panel) and Mouse anti-GroEL1 antibody (1:1000 dilution) against GroEL1, a cytoplasmic chaperone was used as a negative control (Lower Panel) to evaluate for cell lysis products in supernatants. The absence of band corresponding to GroEL1 in the supernatant fractions suggests the purity of culture filtrate preparations. **(B)** Figure represents the Western blots with the whole bacterial lysates as control experiment.

showed lower IFNγ:IL-10 ratio of 0.30 ± 0.07 as compared to untreated (1 ± 0.13) or LPS (0.54 ± 0.10) treated (**Figure 3G**). This indicated that rZmp1 promoted a Th2 immune response. Summing up, these experiments indicated that the secreted *M. tuberculosis* Zmp1 should incline the immune system toward Th2 response, promoting humoral immunity.

With the above experiments indicating that Zmp1 induces Th2 response, we next assessed if this is manifested in the form of anti-Zmp1 antibody production in TB patients. To verify the same, we measured anti-Zmp1 antibodies in the sera samples of TB patients using rZmp1 as the bait antigen in ELISA based assays.

Zmp1 Elicited a Strong B-cell Response Which Was Specific for Tuberculosis (TB) Infection

The humoral response of the host against mycobacterial secretory protein, Zmp1 was scored in a study population comprising 295 subjects. This included four groups, TB patients, Healthy controls, Household contacts of TB patients and volunteers with non-specific infections. Purified rZmp1 was used as bait antigen in the indirect ELISA to score for the anti-Zmp1 antibodies in the serum samples of healthy ($n = 62$), TB patients ($n = 121$), household contacts ($n = 89$), and non-specific infection control samples ($n = 23$; **Figure 4A**). We observed that there was a significant increase ($p < 0.001$) in the absorbance at 450 nm corresponding to the anti-Zmp1 antibody titers in TB patient sera as compared to the healthy or household contacts or non-specific infection controls (**Figure 4A**). The median values for the Healthy was 0.702 (IQR: 0.506–0.893), TB patients was 1.264 (IQR: 0.961–1.982), household contacts was 0.606 (IQR: 0.486–0.78) and non-specific infection control was 0.539 (IQR: 0.394–0.708) showing distinct difference of TB patients from other groups. The non-reactivity of rZmp1 to sera samples of non-specific infection controls clearly indicated that the rZmp1 did not cross-react with the antibodies generated due to other infections in human host. In addition, negligible absorbance observed in sera of healthy donors and Household contacts strongly points to the specificity of rZmp1 to TB infection. This clearly indicated that Zmp1 could elicit a strong humoral response that was specific to TB patients and could clearly distinguish TB patient category from all other categories, including asymptomatic household contacts.

Zmp1 Elicits Strong B-cell Response Independent of Mycobacterial Load in TB Patients

Having seen that rZmp1 stimulated release of Th2 cytokines over Th1 in PBMCs and accordingly Zmp1 elicited a strong B-cell response that could be detected in terms of high anti-Zmp1 IgG titers in TB patients, we next checked if these titers differed between PTB ($n = 66$) and EPTB ($n = 55$) cases. This was important as all EPTB patients were sputum smear-negative but recorded presence of mycobacteria by acid-fast bacilli (AFB)

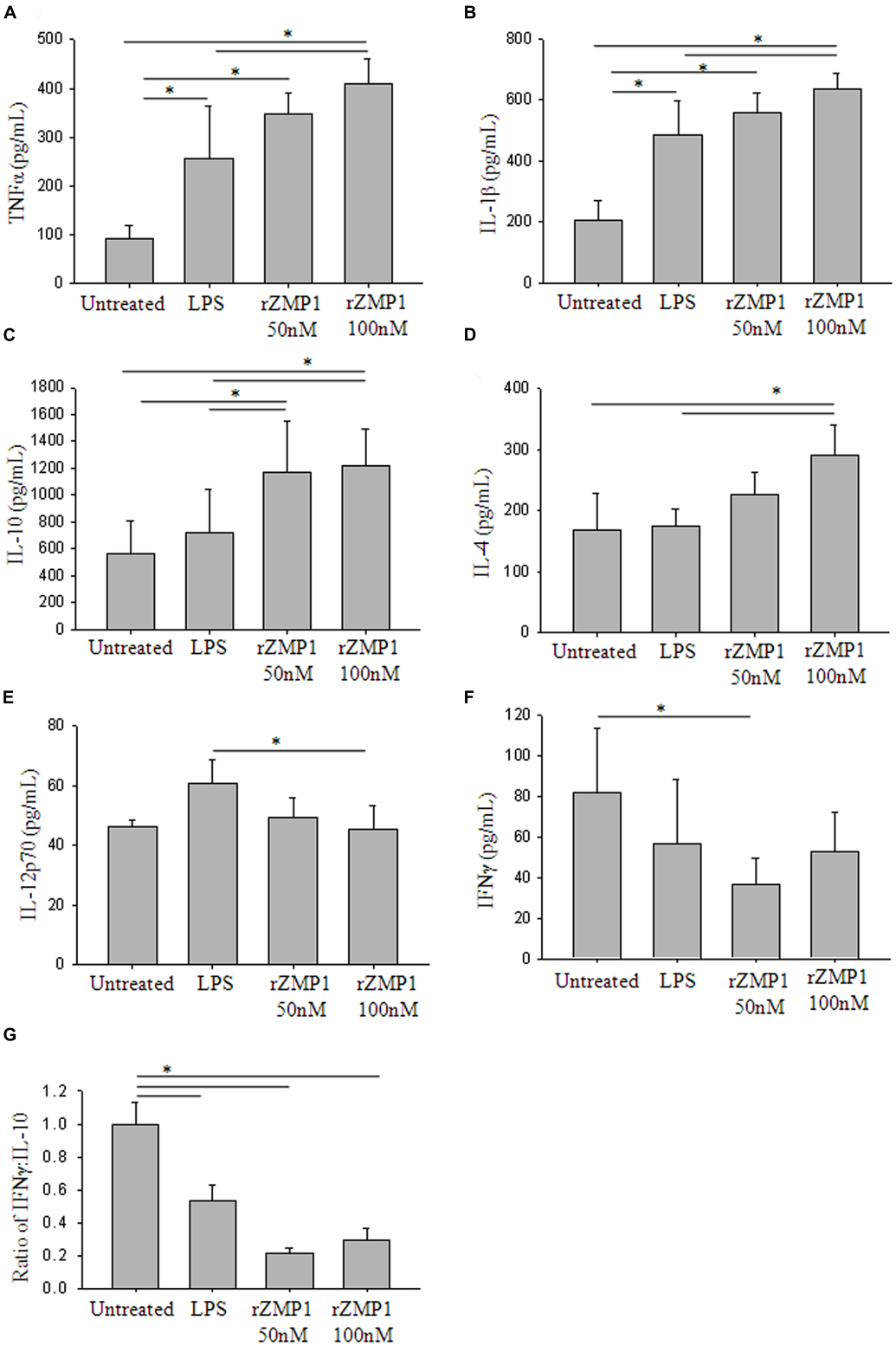

FIGURE 3 | Treatment of PBMCs with recombinant Zmp1 protein inclines the immune status to Th2 response. Cytokine profiles of the PBMCs either untreated or treated with LPS, 50 nM rZmp1 and 100 nM rZmp1 was estimated. **(A)** TNFα, **(B)** IL-1β, **(C)** IL-10, **(D)** IL-4, **(E)** IL-12p70, **(F)** IFNγ titers were measured using capture ELISA. **(G)** Ratio of IFNγ:IL-10. All the experiments were performed more than three times. Statistical analyses were done using one-way ANOVA with Holm-Sidak multiple pair-wise comparison method. Error bars represent ±SD (standard deviation). * Represents $p < 0.05$.

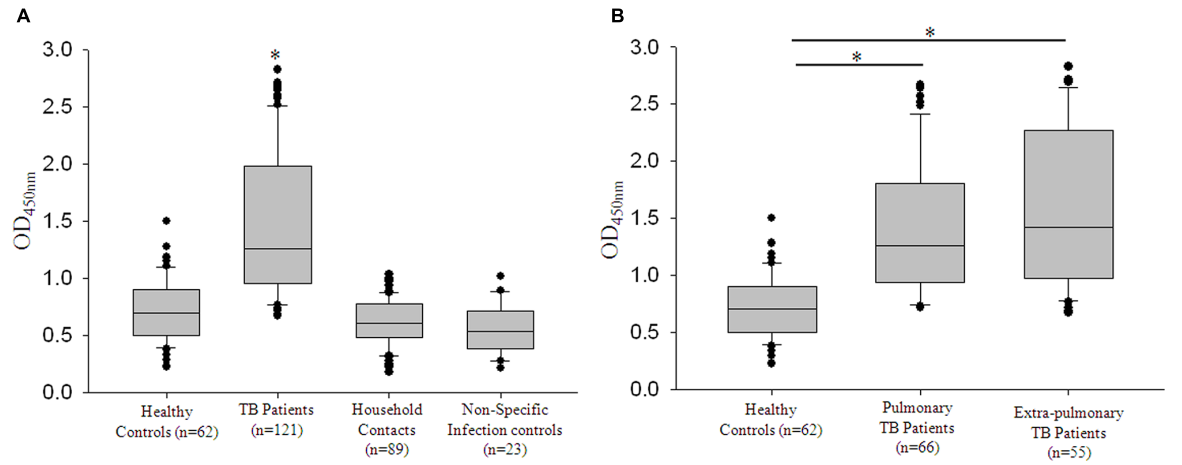

FIGURE 4 | rRv0198c ELISA was specific for tuberculosis (TB) infection. (A) Box plots representing titers of anti-Rv0198c antibody titers as indicated by values at Absorbance 450 nm in Healthy, TB, Household or patient contacts and Non-specific infection control. *The differences in Abs450 values for TB vs. Healthy; TB vs. Household contacts and TB vs. non-specific infection controls were highly significant and a *p*-value of <0.001 was observed in all the three cases. **(B)** The box plots representing the anti-Rv0198c antibody titers in Healthy controls, Pulmonary TB patients and Extra-pulmonary TB patients. Statistical analyses were done using one-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method. * Represents $p < 0.05$.

staining and caseous necrosis in their biopsy samples, suggesting localized mycobacterial load. We observed that the median values for both PTB and EPTB cases were approximately similar, viz.; for PTB it was 1.257 (IQR: 0.956–1.8) and for EPTB, it was 1.421 (IQR: 0.979–2.259; **Figure 4B**). We then compared the anti-Zmp1 titers in PTB patients with various gradients of mycobacterial load. **Table 1** lists the representative ELISA readings of PTB patients ranging from a high bacilli load (3+) to smear negative, synonymous with extremely low bacilli titers, clearly showing humoral response to Zmp1 was independent of *M. tuberculosis* load. This observation was significant, as it suggested that Zmp1 could elicit a strong humoral response even in paucibacillary PTB cases and can detect EPTB cases significantly.

Humoral Response to Zmp1 Was Detected Only in Active TB Cases and Not in Their Household Contacts

Some of the reports have indicated antibodies against specific *M. tuberculosis* antigens in the sera of clinically healthy, latently infected Health-care workers (Reis et al., 2009). With the anti-Zmp1 antibodies detected in active EPTB and even in active paucibacillary PTB cases (**Table 1**), we wanted to check if anti-Zmp1 titers were also detectable in the respective household contacts. These household contacts had stayed with active patients for at the least seven consecutive days during the 3 months prior to the diagnosis of TB. They were expected to be exposed to *M. tuberculosis* though it is reported that EPTB patients, specifically those with tissue TB, are unlikely to transmit the bacilli. Most of these clinically asymptomatic contacts were tested positive for Mantoux's test (Out of 89 household contacts, 60 subjects were Mantoux positive with diameter of Induration >15 mm) and hence may represent possible cases of latent TB. To evaluate that, ELISA readings of EPTB ($n = 55$) and their respective household contacts ($n = 55$) and PTB ($n = 66$) and their household contacts ($n = 34$) were plotted (**Figures 5A,B**). It was observed that compared to EPTB patient contacts (Median: 0.663; IQR: 0.524–0.808) or PTB patient contacts (Median: 0.570; IQR: 0.387–0.630), titers of anti-Zmp1 antibody were distinctly high in EPTB patients (Median: 1.421; IQR: 0.979–2.259; $p < 0.001$) or PTB patients (Median: 1.257; IQR: 0.956–1.8; $p < 0.001$), respectively (**Figures 5A,B**), suggesting that Zmp1 humoral response is restricted to active infection cases as against asymptomatic household contacts including Mantoux positive cases under the category which may be possible latent subjects.

DISCUSSION

Till recently, TB vaccine program was driven by the belief that protective immunity against *M. tuberculosis* infection is chiefly because of CMI generated by Th1 microenvironment. The majority of candidate vaccines were focused on improving CMI either by engineering the present BCG vaccine or using mycobacterial antigens that elicited Th1 responses as boosters after BCG priming. Interestingly, the vaccine strategies that use the whole cell (such as *M. indicus pranii*) has reached phase III of clinical trials (Weiner and Kaufmann, 2014; Tye et al., 2015). The success of this may be owing to a balanced stimulation of both CMI and AMI wings rather than trying to bias the same toward CMI. Additionally, with monoclonal antibodies conferring passive immunity against several intracellular pathogens, including *M. tuberculosis*, humoral response to TB is being explored with a new enthusiasm.

In this study, we deliberated on the immunomodulatory function of a secreted *M. tuberculosis* protein Zinc

TABLE 1 | Representative table to show that pulmonary TB (PTB) cases with varying loads of bacilli load in sputum does not show variation in their $Abs_{450\ nm}$ when detected by rZmp1 ELISA test.

Sample no.	AFB (Bacilli load)	Abs_{450}
PTB# 1	PI -VE	1.260667
PTB# 2	PI -VE	1.297333
PTB# 3	PI -VE	1.407333
PTB# 4	PI -VE	1.716
PTB# 5	PI -VE	1.936333
PTB# 6	PI -VE	2.3335
PTB# 7	PI 1+	1.127333
PTB# 8	PI 1+	1.178333
PTB# 9	PI 1+	1.725667
PTB# 10	PI 1+	1.978333
PTB# 11	PI 1+	2.317333
PTB# 12	PI 2+	1.249667
PTB# 13	PI 2+	1.319667
PTB# 14	PI 2+	1.773
PTB# 15	PI 2+	1.800333
PTB# 16	PI 2+	2.487
PTB# 17	PI 3+	1.107
PTB# 18	PI 3+	1.274333
PTB# 19	PI 3+	1.564667
PTB# 20	PI 3+	2.67

PI 1+ indicates more than 100 acid-fast bacilli (AFB) in sputum, PI 2+ indicates more than 200 acid-fast bacilli in sputum, PI 3+ indicates more than 300 acid-fast bacilli in sputum from 100 microscopic fields, PI -VE indicates no acid-fast bacilli in sputum.

metalloprotease-1. *M. tuberculosis* Zmp1 is a well characterized protein that alters phagosome maturation and is considered essential for intracellular survival of *M. tuberculosis*. With the X-ray structure available, this protein has been screened for small molecule inhibitors to evaluate it as a potential drug target (Ferraris et al., 2011; Mori et al., 2014). Zmp1 works optimally under slightly acidic conditions with neuropeptides as possible substrates (Petrera et al., 2012). It was also shown to be involved in inflammasome activation (Master et al., 2008), suggesting that it definitely has immunomodulatory function besides its role in regulating phagosome maturation. Protein sequence analyses by Jameson-Wolf plot using Protean software, which recorded high antigenicity indices based on surface probability and hydrophobicity of its amino-acid sequence of Zmp1 further supported the notion that apart from an active enzyme, it can also be a B-cell stimulant (**Figure 1A**).

We confirmed the secretory nature of Zmp1 in granuloma-like *in vitro* growth conditions and concluded that Zmp1 could indeed be released out in the extracellular milieu when *M. tuberculosis* is growing in acidic, nutrient deprived and oxidatively stressed acellular environment at the center of TB granulomas (**Figure 2**). In addition to that, we also observed that it is a strong immune-stimulant and could stimulate mono-nuclear cells to release high titers of TNF-α and IL-1β, the shock inducing cytokines that also start the innate mechanisms toward acute phase reaction. However, subsequently, it was not the pro-inflammatory, but Th2 cytokines that were pre-dominantly released upon rZmp1 stimulation of PBMCs (**Figure 3**). Corroborating the Th2 response, in a cohort of about 121 TB patients, high titers of anti-Zmp1 antibodies could be recorded (**Figure 4**). This study revealed yet another facet of *M. tuberculosis* Zmp1 as a highly immunogenic mycobacterial antigen that could elicit strong and specific humoral response in TB patients.

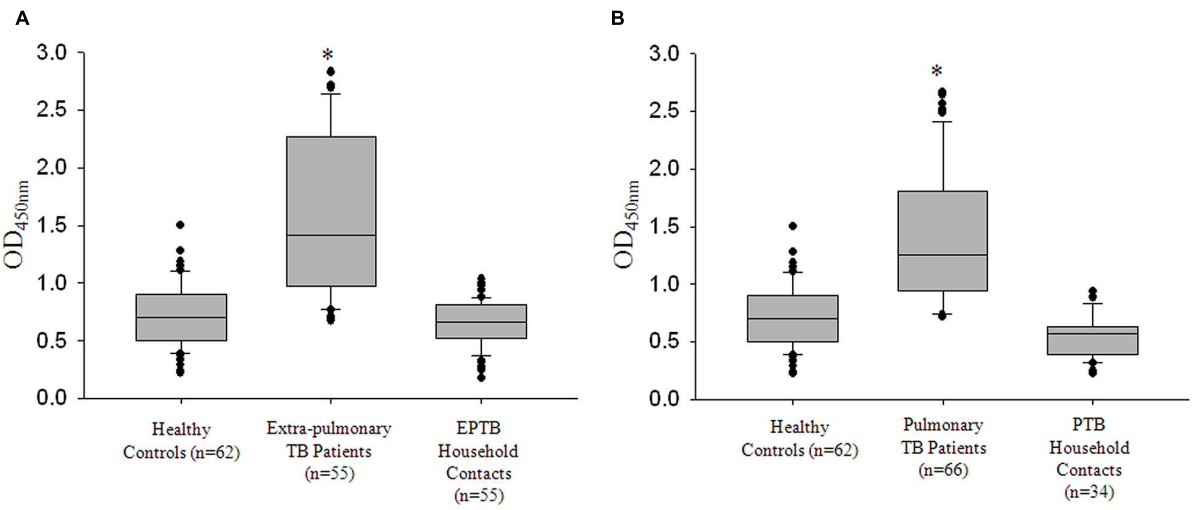

FIGURE 5 | rRv0198c ELISA detected all active TB cases with high specificity and sensitivity. (A) Box plots representing titers of anti-Rv0198c antibody titers as indicated by values at Absorbance 450 nm in Healthy, extra-pulmonary TB (EPTB), EPTB patient contacts (Household contacts of EPTB patients). **(B)** Box plots representing titers of anti-Rv0198c antibody titers as indicated by values at Absorbance 450 nm in Healthy, pulmonary TB (PTB), PTB patient contacts (Household contacts of PTB patients). *The differences in Abs450 values for PTB vs. Healthy; PTB vs. PTB Household contacts; EPTB vs. Healthy; EPTB vs. EPTB Household contacts were highly significant and a *p*-value of <0.001 was observed in all the cases. Statistical analyses were done using one-way ANOVA on ranks was performed with Dunn's method for pair-wise comparison method.

There are many reports analyzing the levels of IgG antibody titers against *M. tuberculosis* antigens, secretory or otherwise, in different clinical stages (Wu et al., 2010; Baumann et al., 2014, 2015). The studies also claim that these antibodies are present during active disease, but reduce upon decrease in bacterial load with treatment (Singh et al., 2005). That would mean antibody titers are bacterial load dependent and a low bacterial infection may not sufficiently trigger a good humoral response. To check the same, we compared the anti-Zmp1 antibody titers in patients with high or low bacillary loads. To our surprise, anti-Zmp1 titers were equally high in paucibacillary cases as in patient with sputum bacilli score of 3+ (**Figure 5B**; **Table 1**). This suggested that a very low dose of *M. tuberculosis* Zmp1 could induce very strong and specific B-cell response, a property that can be suitably explored for prime–boost vaccination strategy. In total, with the evidence that Zmp1 is secreted by active *M. tuberculosis* in granuloma-like growth conditions and stimulated PBMCs to release Th2 class of cytokines, one may hypothesize that very low concentration of this protein when released in the granulomas of infected host, stimulate the neighboring mono-nuclear cells to eventually generate a Th2 immune response that supports B-cell specific immunity toward Zmp1 resulting in high titers of anti-Zmp1 antibodies in patients. However, unlike reported humoral response to GlcB (malate synthase), MPT51 (FbpC1) and HSPX (alpha crystalline) in latent TB cases (Reis et al., 2009), anti-Zmp1 antibodies could not be detected significantly in healthy household contacts who were asymptomatic, Mantoux's positive and possibly represent latently infected population in this study.

Likewise, we observed that rZmp1 ELISA was highly specific for TB cases, both PTB and EPTB (**Figure 4B**). The group comprising of non-specific infections were used to rule out if, Zmp1 cross-reacts with antibodies generated against other bacterial or viral proteins. This group had patients with random infections other than TB such as viral, bacterial, and parasitic infections. Non-reactivity of rZmp1 with antibodies in the sera of non-specific infection controls as indicated by low-absorbance values (**Figure 4A**) points to the specificity of rZmp1 to distinctly differentiate TB cases from all other categories. This suggests the potential of rZmp1 ELISA as a disease marker which can be further explored. Identification EPTB disease remains challenging for reasons like diffused symptoms, low *M. tuberculosis* load at the site of infection and difficulties in obtaining clinical specimens from deep-seated organs (Lawn and Zumla, 2012). Dependence on cultures for EPTB frequently leads to substantial delays, compromising patient care and spread of infection to others. Though serological tests are less acceptable in the field of TB detection, the problem possibly lies in the selection of an antigen that shows a good serological response even when infection load is less. In comparison with microscopy and cultures, ELISA based serological tests offer several advantages such as in terms of time, infrastructure and ease of sample collection in the form of peripheral blood.

In this study, we revealed *hitherto* unknown immunogenic property of *M. tuberculosis* Zmp1. Zmp1 is a strong *M. tuberculosis* specific immune-stimulant, the properties of which can be further explored both as a potential vaccine candidate or a disease marker. The possibility of taking the study on antigenicity of Zmp1 to the next level by using larger cohort, blinded samples and multi-centric study is very stimulating from both scientific point of view and translational research.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: MV, GR, SB. Performed the experiments: MV, RS, KJ, GR, SP. Analyzed the data: MV, GR, RS, KJ, SG, SP, SB. Contributed reagents/materials/analysis tools: SG, SP, SB. Contributed to the writing of the manuscript: MV, GR, SG, SB. We declare that all the authors have approved the article for submission, its contents, order of authorship and that there are no competing interests.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00418

FIGURE S1 | Cross-reactivity check of anti-Zmp1 antibody and rZmp1. **(A)** Recombinant mycobacterial antigens, ESAT6 (5 μg), CFP10 (5 μg), and Zmp1 (5 μg) were loaded on to 15% SDS-PAGE followed by Western blot with Mouse anti-Zmp1 antibody. The corresponding Ponceau S stained blot showing transferred protein is provided. **(B)** Recombinant mycobacterial antigens, ESAT6 (5 μg) and Zmp1 (5 μg) were loaded on to 15% SDS-PAGE followed by Western blot with rabbit anti-ESAT6 antibody (1:1000 dilution). The corresponding Ponceau S stained blot showing transferred protein is provided.

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Proteomics approach to understand reduced clearance of mycobacteria and high viral titers during HIV–mycobacteria co-infection

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Summary

Environmental mycobacteria, highly prevalent in natural and artificial (including chlorinated municipal water) niches, are emerging as new threat to human health, especially to HIV-infected population. These seemingly harmless non-pathogenic mycobacteria, which are otherwise cleared, establish as opportunistic infections adding to HIV-associated complications. Although immune-evading strategies of pathogenic mycobacteria are known, the mechanisms underlying the early events by which opportunistic mycobacteria establish infection in macrophages and influencing HIV infection are unclear. Proteomics of phagosome-enriched fractions from *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) mono-infected and HIV–*M. bovis* BCG co-infected THP-1 cells by LC-MALDI-MS/MS revealed differential distribution of 260 proteins. Validation of the proteomics data showed that HIV co-infection helped the survival of non-pathogenic mycobacteria by obstructing phagosome maturation, promoting lipid biogenesis and increasing intracellular ATP equivalents. In turn, mycobacterial co-infection up-regulated purinergic receptors in macrophages that are known to support HIV entry, explaining increased viral titers during co-infection. The mutualism was reconfirmed using clinically relevant opportunistic mycobacteria, *Mycobacterium avium*, *Mycobacterium kansasii* and *Mycobacterium phlei* that exhibited increased survival during co-infection, together with increase in HIV titers. Additionally, the catalogued proteins in the study provide new leads that will significantly add to the understanding of the biology of opportunistic mycobacteria and HIV coalition.

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Introduction

Mycobacterium tuberculosis complex comprises of phylogenetically close groups of *Mycobacterium* species that infect humans causing tuberculosis (TB), a disease that despite being curable has re-emerged as a global pandemic (Kaufmann and McMichael, 2005). Multiple factors, including lack of effective vaccine, failure of early detection, emergence of drug resistance and, of late, the co-epidemics with HIV/AIDS, had limited the success of TB disease management. In the recent times, AIDS epidemic has had an immense bearing on the clinical presentation, transmission and epidemiology of TB worldwide (Pawlowski *et al.*, 2012). TB is predominantly caused by the pathogenic mycobacteria *M. tuberculosis*; however, non-tuberculous or attenuated strains of mycobacteria such as *Mycobacterium avium*, *Mycobacterium kansasii*, *Mycobacterium fortuitum*, *Mycobacterium xenopi* and *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) cause opportunistic infections in HIV patients (Juffermans *et al.*, 1998; Smith *et al.*, 2001; Bachmeyer *et al.*, 2002; Karakousis *et al.*, 2004; Serra *et al.*, 2007; Singh *et al.*, 2007; Azzopardi *et al.*, 2009; Hesseling *et al.*, 2009) even before T-cell depletion is apparent (Sonnenberg *et al.*, 2005). More than 90 species of *Mycobacterium* had been reported to inhabit natural (air, water, soil or other organisms) and artificial (chlorinated municipal water) reservoirs (Primm *et al.*, 2004). These are emerging as potential opportunistic pathogens in human, especially in immunocompromised HIV patients (Herzmann *et al.*, 2011), resulting in an increased risk of disease progression from both the pathogens in the individuals concurrently infected with mycobacteria and HIV as compared with those carrying single infections (Goletti *et al.*, 1996).

Several molecular mechanisms have been attributed to the synergistic impact of pathogenic mycobacteria on HIV propagation. Mycobacterial components directly or indirectly increase the expression of transcription factors

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The PE16 (Rv1430) of *Mycobacterium tuberculosis* Is an Esterase Belonging to Serine Hydrolase Superfamily of Proteins

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Abstract

The PE and PPE multigene families, first discovered during the sequencing of *M. tuberculosis* H37Rv genome are responsible for antigenic variation and have been shown to induce increased humoral and cell mediated immune response in the host. Using the bioinformatics tools, we had earlier reported that the 225 amino acid residue PE-PPE domain (Pfam: PF08237) common to some PE and PPE proteins has a ''serine α/β hydrolase'' fold and conserved Ser, Asp and His catalytic triad characteristic of lipase, esterase and cutinase activities. In order to prove experimentally that PE-PPE domain is indeed a serine hydrolase, we have cloned the full-length Rv1430 and its PE-PPE domain into pET-28a vector, expressed the proteins in *E. coli* and purified to homogeneity. The activity assays of both purified proteins were carried out using *p*-nitrophenyl esters of aliphatic carboxylic acids with varying chain length (C2–C16) to study the substrate specificity. To characterize the active site of the PE-PPE domain, we mutated the Ser199 to Ala. The activity of the protein in the presence of serine protease inhibitor- PMSF and the mutant protein were measured. Our results reveal that Rv1430 and its PE-PPE domain possess esterase activity and hydrolyse short to medium chain fatty acid esters with the highest specific activity for pNPC6 at 37°C, 38°C and pH 7.0, 8.0. The details of this work and the observed results are reported in this manuscript.

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Introduction

The complete genome of the *Mycobacterium tuberculosis* H37Rv strain comprises about 4000 genes, of which 250 genes are involved in fatty acid metabolism [1]. The cell wall structure of *M. tuberculosis* deserves special attention because it is unique among prokaryotes and is a major determinant for virulence of the bacterium. The mycobacterial cell wall contains complex peptidoglycan and lipids [2]. Apart from the expected richness of genes involved in fatty acid metabolism, two novel gene families, PE and PPE were identified in H37Rv genome encompassing 10% of coding regions [1]. These two large unrelated families of acidic, glycine-rich proteins are often based on multiple copies of the polymorphic GC-rich repetitive sequences (PGRSs), and major polymorphic tandem repeats (MPTRs). The names PE and PPE are derived from the motifs Pro–Glu (PE) and Pro–Pro–Glu (PPE) often found at the N terminus. The PE and PPE proteins have the characteristic 110 amino acid and 180 amino acid conserved domains towards the N-terminus, respectively [1,3].

Since their revelation, experiments have been conducted that assigned several physiological roles to different ORFs belonging to these novel families as mentioned below. Transcriptomics revealed that these proteins are expressed under different conditions with 128/169 PE and PPE genes differentially regulated [4]. It is reported that some PE proteins (Rv0746, Rv1759c, Rv1818c) play a role in immune evasion and antigenic variation [1,5–7], Some members of the PE and PPE families (Rv1818c, Rv1917c, Rv3873) are associated with the cell wall [2,8]. The PE family protein, Rv1818c influences the interactions of mycobacteria with macrophages [9]. The PPE protein, Rv2430c induces a strong B-cell response [10] and some members of the PE and PPE families (Rv1787, Rv2430c, Rv3018c) are associated with virulence [10–12]. Some of the PE/PPE members exist as gene pairs that are co-regulated, co-expressed and interact functionally [13]. Several PE/PPE genes were found to be upregulated only during macrophage infection and in host granulomas supporting their role in pathogenesis of mycobacteria [4]. Despite these efforts, several ORFs in PE/PPE gene clusters are largely unannotated with regard to their biochemical activity with the exception of Rv3097c (LipYtub). The C-terminal domain of Rv3097c shares homology with the hormone-sensitive lipase family characterized by the conserved GDSAG active-site motif and was shown to hydrolyze extracellular lipids [14–17]. Combined with the facts that these are multigene families with a high potential for functional redundancy as well as diversity, are unique to mycobacteria, have evolutionarily expanded preferentially in pathogenic mycobacteria [18] and are absent in human host, make them ideal for diagnosis and drug targeting for the design of new anti-tuberculosis drugs.

Nearly 50% of these proteins comprise only the characteristic N-terminal conserved domain, while other members comprise C-terminal extensions. Based on the composition of the C-terminal extensions, these were further classified into various subfamilies by Cole et al., 1998. The PPE family proteins comprise (i) the NxGxGNxG, major polymorphic tandem repeats (MPTR)

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by Mani Harika Vemula

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