TlyA has an essential virulence role in *Mycobacterium tuberculosis* pathogenesis

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Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the department of Medical Microbiology, Infection Prevention and control

University of KwaZulu-Natal, South Africa

2015
As the candidate’s supervisor I agree to the submission of this thesis.

Signed:

Date: 17 Nov 2015
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1. *Mycobacterium tuberculosis* TlyA negatively regulates Th1 and Th17 differentiation and promotes tuberculosis pathogenesis.

2. *Mycobacterium tuberculosis* subverts the TLR-2-MyD88 pathway to facilitate its translocation into the cytosol.
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<tbody>
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<td>ADC</td>
<td>Albumins-Dextrose-Catalase</td>
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<td>Ab</td>
<td>Antibody</td>
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<td>AIDS</td>
<td>Acquired Immune Deficiency Syndrome</td>
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<td>APC</td>
<td>Antigen Presenting Cells</td>
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<td>ATCC</td>
<td>American Type Culture Collection</td>
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<td>BAL</td>
<td>Bronchoalveolar Lavage</td>
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<td>BM</td>
<td>Bone Marrow</td>
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<td>Bp</td>
<td>base pair</td>
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<td>BCG</td>
<td>Bacillus Calmette-Guerin</td>
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<td>Cytosine</td>
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<td>CD</td>
<td>Cluster of Differentiation</td>
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<td>CFU</td>
<td>Colony Forminsg Unit</td>
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<td>CSA</td>
<td>Complete Soluble Antigen</td>
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<td>DC</td>
<td>Dendritic Cell</td>
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<td>DC</td>
<td>Dendritic cell- Specific Intercellular adhesion molecule-3- grabbing Non</td>
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<td>Acronym</td>
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<tr>
<td>SIGN</td>
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<td>deoxynucleoside triphosphate</td>
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<td>DTH</td>
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<td>ELISA</td>
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<td>Early Secreted Antigenic Target protein -6kDa</td>
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<td>Guanine</td>
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<td>GMCSF</td>
<td>Granulocyte Macrophage Colony Stimulating Factor</td>
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<td>Hrs</td>
<td>Hepatocyte growth factor- regulated tyrosine kinase substrate</td>
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<td>HIV</td>
<td>Human Immunodeficiency Virus</td>
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<td>IRAK-1</td>
<td>Interleukin-1 receptor associated kinase-1</td>
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<td>kDa</td>
<td>Kilodalton</td>
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<td>KO</td>
<td>Knock out</td>
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<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>LT</td>
<td>Lymphotoxin</td>
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<tr>
<td>M</td>
<td>Molar</td>
</tr>
<tr>
<td>MDR</td>
<td>Multi-Drug Resistant</td>
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<tr>
<td>MFI</td>
<td>Mean Fluorescence Intensity</td>
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<td>Mg</td>
<td>Milligram</td>
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<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
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<td>Mins</td>
<td>Minsute</td>
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<td>miRNA</td>
<td>MicroRNA</td>
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<tr>
<td>Ml</td>
<td>Millilitre</td>
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<tr>
<td>MOI</td>
<td>Multiplicity of Infection</td>
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<tr>
<td>mRNA</td>
<td>messenger RNA</td>
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<tr>
<td>MTB</td>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td>MyD88</td>
<td>Myeloid differentiation protein 88</td>
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<tr>
<td>µg</td>
<td>Microgram</td>
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<td>µM</td>
<td>Micromolar</td>
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<tr>
<td>NKTcell</td>
<td>Natural Killer T cells</td>
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<tr>
<td>NF-κB</td>
<td>Nuclear Factor kappa-light-chain-enhancer of activated B cells</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>Ng</td>
<td>Nanogram</td>
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<td>OADC</td>
<td>Oleic acid-Albumins-Dextrose-Catalase</td>
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<tr>
<td>OD</td>
<td>Optical Density</td>
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<td>Open Reading Frame</td>
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<td>OVA</td>
<td>Ovalbumins</td>
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<td>PAGE</td>
<td>Polyacrylamide Gel Electrophoresis</td>
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<tr>
<td>PAMP</td>
<td>Pathogen Associated Molecular Patterns</td>
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<td>PBMC</td>
<td>Peripheral Blood Mononuclear Cells</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffer Saline</td>
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<tr>
<td>PE</td>
<td>Phycoerythrin</td>
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<td>Pictogram</td>
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<td>PPD</td>
<td>Purified Protein Derivative</td>
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<tr>
<td>PRR</td>
<td>Pattern Recognition Receptor</td>
</tr>
<tr>
<td>RBC</td>
<td>Red Blood Corpuscles</td>
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<tr>
<td>RD1</td>
<td>Region of Difference1</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducer and Activator of Transcription</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulphate</td>
</tr>
<tr>
<td>T</td>
<td>Thymine</td>
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<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
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<tr>
<td>TB</td>
<td>Tuberculosis</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>TCR</td>
<td>T Cell Receptor</td>
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<td>Transforming Growth Factor-β</td>
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<tr>
<td>Th1</td>
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</tr>
<tr>
<td>Th17</td>
<td>T helper cell type 17</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll Like Receptor</td>
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<tr>
<td>TNF-α</td>
<td>Tumour necrosis factor-α</td>
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ABSTRACT

*Mycobacterium tuberculosis* (*M.tb*), the causative agent of the disease tuberculosis, is an ancient pathogen and a major cause of death worldwide. Although various virulence factors of *M.tb* have been identified, its pathogenesis remains incompletely understood. TlyA is a virulence factor that is evolutionarily conserved in many gram-positive bacteria, but its function in the pathogenesis of infection with *M.tb* has not been elucidated. Here, we report that TlyA cause translocation of *M.tb* from phagolysosome into the cytosol in murine macrophages, which is the key to mycobacterial pathogenesis. In this study we also showed that TlyA mutant *M.tb* strain induces increased IL-12 and reduced IL-1β and IL-10 cytokine responses, which is in contrast to the immune responses induced by wild type *M.tb*. Mice infected with TlyA deficient mutant *M.tb* organisms exhibited increased host protective immune responses, reduced bacillary load, and increased survival compared with animals infected with wild type *M.tb*. Therefore it is likely that *M.tb* employs TlyA as a host evasion factor, thereby contributing to its virulence.
CHAPTER 1-INTRODUCTION

Tuberculosis (TB) is a chronic infection that is a major global public health problem, with approximately 9 million new cases and nearly 2 million deaths each year. According to World Health Organization [WHO], 2012 South Africa has the highest incidence of tuberculosis (TB) in the world, and the highest prevalence of drug resistant TB in Africa. The capacity of *Mycobacterium tuberculosis* (*M.tb*), to survive and cause disease is strongly associated to their ability to escape host immune defense mechanisms. *M.tb* has developed efficient strategies to ensure its successful survival and replication within phagocytes. These strategies include inhibiting phagolysosome fusion and reduction of the pH of the phagolysosomal compartment (Sinai *et al*., 2007; Sturgill-Koszycki *et al*., 1994), expression of virulence protein, inhibition of protective cytokines, evasion of antigen presentation (Baena A *et al*., 2009; Meena *et al*., 2010), and inhibition of apoptosis (Keane J *et al*., 2000). *M.tb* additionally down regulates the T helper Th-1 type immune response, conferring protection to the bacillus, and simultaneously up regulates the Th-2 type cytokine response which responsible for disease progression (Jozefowski S *et al*., 2008). *M.tb* with all these mechanism has been able to survive in the host for years. However, recently it has been studied that *M.tb*, *M. leprae* and *M. marinum* efficiently escape to the cytosol from the phagolysosome (van der Wel *et al*., 2007). Many intracellular pathogens have pore-forming proteins which inhibit phagolysosome acidification, allow access to nutrients from the cytoplasm and facilitate escapes to new host cells, thereby spreading the disease. ESAT-6, a protein encoded within the RD-1 region, has been shown to play an important role in release and spread of *M.tb* to neighboring cells (Smith *et al*., 2008). However, the mechanism by which *M.tb* can transmigrate from the phagolysosome to the cytosol remains controversial. It has been shown that avirulent *M. bovis* BCG, lacking the RD-1 region, is unable to translocate to the cytosol. Therefore, cytosolic escape could be another potential mechanism of virulence exerted by *M.tb*.

In this study, we identified a protein Rv1694 (TlyA) encoded by the *tlyA* gene in *M.tb*, which is present outside RD-1 (Esx-1) region. This protein may be responsible for the interruption of the phagosomal maturation process. We have observed that TlyA (Rv1694) is a non-conventional „haemolysin like” membrane damaging protein that self assembles into large oligomers upon contact with target membranes, such as the phagosomal membranes (Rahman *et al*., 2010). In
addition TlyA also contains motifs that align well with rRNA methyltransferase and RNA binding protein. Rehman et al in 2010 has confirmed the ribosomal RNA methylation activity of TlyA protein. Also, TlyA mediated methylation of rRNA results in capreomycin susceptibility (Courtney E.M et al 2005). TlyA is also present in M. leprae, a pathogenic mycobacterial species that causes leprosy. TlyA homologues encoding for pore forming haemolysin and host cell adherence factors have been found in other pathogenic bacterial species including Serpulina hyodysenteriae and Helicobacter pylori (Hyatt et al., 1994; Ter huume et al., 1994). From our initial experimental data we have observed that infection of macrophages with an E. coli strain that expressed this protein prevented the maturation process and evaded degradation by the phagosomal compartment. Since TlyA is known to have a membrane destabilizing property we sought to ascertain if it plays a role in M.tb pathogenesis by favoring the translocation of the bacterium from the phagolysosome into the cytosol. Our study showed that the TlyA deficient mutant of H37Rv, like BCG was unable to translocate from the phagolysosome to cytosol. We also found that TlyA is present on the cell wall of M.tb and its secretion is similar to the antigenic protein ESAT-6 through the RD1 region of M.tb. This supports a possible virulence role of TlyA in M.tb.

ESAT-6 has been extensively studied and is known to play a role in inducing an immune response, and is currently being studied as a potential subunit vaccine against M.tb infection. We extended the study to look at the role of TlyA protein on the immune system in an attempt to understand the effect of this protein on host immune response. Adaptive immunity against M.tb infection predominately consists of interferon (IFN)-γ–producing CD4^+ T lymphocytes that activate macrophages to restore phagolysosome activation and enhance autophagy (Jo EK., 2013; Sweeney et al., 2011; Rosenzweig et al., 2005; Dwivedi VP et al., 2012). IFN-γ is an essential component of the immunological defense against intracellular infections (Sweeney et al., 2011). Both mice and humans with genetic defects in IFN-γ signaling are highly susceptible to mycobacterial diseases (Rosenzweig et al., 2005). It has been established that T helper 1 (Th1) cells producing IFN-γ play a central role in host immunity against M.tb infection, and this type of immune response is generated in the presence of interleukin (IL)-12 secretion by infected macrophages (Rosenzweig et al., 2005). IFN-γ-induced autophagosomes target M.tb-containing phagosomes for lysosomal destruction.
Additionally, lysosomal degradation products are presented via MHC class II molecules to MHC class II-restricted CD4\(^+\) T cells, and these helper T cells orchestrate the specific immune response. On the other hand it is well known that *M. tb* promotes the differentiation of regulatory Th2 and Treg cells, and this is associated with inhibition of protective T cell responses in the host (Dwivedi VP *et al* 2012). Here we report that TlyA assists *M. tb* survival in a mouse infection model by inhibiting Th1 cytokines (IL-12 and IFN-\(\gamma\)). Furthermore, deletion of the TlyA gene in wild-type *M. tb* H37Rv impedes its pathogenicity in mice. Therefore, together our findings suggest that, TlyA is a virulence factor in the pathogenesis of *M. tb* and deserves a more in-depth study especially focused on designing of potential vaccines against *M. tb*. 
CHAPTER 2-REVIEW OF LITERATURE

2.1 Tuberculosis: Global Epidemic

One of the most widespread serious infectious diseases worldwide today is tuberculosis (TB). Killing 1.3 million people annually (WHO, 2013) and infecting 8.6 million in 2012 alone, it has become a major public health priority for most of developing countries (Fig 1). Among the estimated number of TB cases in 2012 Asia had (58%), African region (27%), smaller percentage occurred in Eastern Mediterranean Region (8%), European Region (4%) and the Region of America (3%). Among 75% of the total TB death in 2012, India and South Africa accounted for about one-third of global deaths (Daniel TM., 2006). *Mycobacterium tuberculosis* infection may not always lead to active disease. Active TB can occur due to perturbation of the immune response such as that which occurs during HIV infection.

![Global Tuberculosis Report](image)

Figure: 1 Global Tuberculosis Report (Adapted from WHO report 2013)

Globally, 3.7% of new cases and 20% of previously treated cases were estimated to have multi drug-resistant (MDR)-TB, defined as TB caused by strains of *M.tb* resistant to at least isoniazid and rifampicin (Chiang CY *et al.*, 2013; WHO 2012). TB control is further compromised by the
increasing spread of extensively drug resistance (XDR) strains of *M. tuberculosis* that displays additional resistance to the fluoroquinolones and the injectable drugs viz: amikacin, capreomycin or kanamycin. Totally drug resistant-TB displaying resistance to all available drug has also been reported. (Zhao Y et al., 2012; Udwadia et al., 2012). India and China are estimated to have the largest number of MDR-TB patients (Bogdan., 2000; Gonzalez-Navajas et al., 2012., WHO, 2013). The WHO aims to eradicate TB as a public health problem by 2050 under the Millennium Development Goals (Raviglione MC et al., 2006). One of the strategies touted to eradicate TB, is the development of an effective vaccine. Until now, the only vaccine used for TB is Bacilli Calmette-Guerin (BCG), which was first discovered in 1921. (Daniel TM., 2006). BCG is a live attenuated strain of *Mycobacterium bovis* (Liu J et al., 2009) and it is used to immunise newborns (Hesseling AC et al., 2009). Nearly 120 million children receive BCG every year worldwide (Dalmia et al., 2012). Through various studies it was found that, although BCG provides protection against severe forms of TB disease such as TB meningitis and other disseminsated forms in children its efficacy in adult pulmonary TB varied from 0 to 80% based on well-controlled field trials (Behr MA., 2002; Colditz GA et al., 1994). One of the proposed factors for decreased efficacy of BCG in adults is declining immunological memory. Other factors include strain difference in BCG, presence of co-infection, exposure to environmental bacteria in the host, and other nutritional and genetic factor. (Ottenhoff TH, Kaufmann SH., 2012). Recent reports have shown that helminthic infection plays an important role in increasing TB infection and reducing the efficacy of BCG vaccine (Hatherill M et al., 2009). It has been reported that HIV infected children have high risk of disseminsated infection from the attenuated *M. bovis* BCG strain, even if they were asymptomatic at the time of infection (Hesseling AC et al., 2007). Therefore, WHO Global Advisory Committee on Vaccine safety advises against the administeration of BCG in HIV-infected patients (Global advisory committee on vaccine safety, 2007). The overall efficacy of BCG is highly uncertain, therefore, there is an urgent need for a novel and effective vaccine against TB.

### 2.2 Mycobacterium tuberculosis complex

Robert Koch was the first person who isolated *Mycobacterium tuberculosis* and also revealed in the guinea pig that this slow growing bacterium was the causative agent of human diseases (Koch R., 1882). *Mycobacterium tuberculosis* along with other highly related bacteria forms a
tightly knit complex a single species as defined by DNA/DNA hybridization studies (Sreevatsan S et al., 1997). This complex consists of six members: *M.tb*, the causative agent of human TB, *M. africanum* an agent of human TB in sub-Saharan Africa, *M. bovis* which infects wide variety of mammals including humans, *M. microti* infects voles but avirulent in humans and mice and *M. canetti* rarely found but cause human disease (Cole ST., 2002). There are various other pathogenic mycobacteria which include *M. leprae*, causing leprosy in human, *M. marinum*, which causes granulomatous infection in frogs and fish as well as skin lesions in humans and *M. ulcerans*, which cause Buruli ulcers. Finally, members of *M. avium* complex (*M. avium* subspecies *avium*, *paratuberculosis* and *silvaticum* and *M. intracellulare*) can behaves as opportunistic pathogens especially in immuno-compromised patients.

### 2.3 Morphology of Mycobacterium tuberculosis

*Mycobacterium tuberculosis* complex is the member of genus *Mycobacterium*. The bacterium is non-motile, non-spore forming, and non-capsule forming. The cell envelope of *M.tb* is the most distinctive feature with very high lipid content in the cell wall. The cell wall consists of an inner peptidoglycan layer which is covered by hydrophilic branched polysaccharide, the arabinogalactan. The arabinogalactan layer is further covered by a network of long-chain hydrophobic fatty acids known as mycolic acids, which contributes for the impermeability of hydrophilic molecules (Barrera L., 2007). These three layers together contribute for the structural integrity of the bacterium. Certain glycolipids such as phosphatidyl myoinositol mannosides, lipomannan and lipoarabinomannan are attached to the plasma membrane and spread out to the outside of the cell wall crisscrossing the whole envelope.

### 2.4 Salient features of Mycobacterium tuberculosis

*Mycobacterium tuberculosis* is an aerobic bacterium with a long generation time 18-20 hrs. It is an extremely slow growing bacteria compared to other bacteria (generation time of *E.coli* is only 20-30 minss). This long generation time contributes to its virulence and lengthy treatment regimen. The thick cell wall of *M.tb* might partially account for the slow growth rate as it limits the uptake of nutrients. Harshey and Ramakrishnan recognized that the long generation time of *M.tb* is associated with RNA synthesis (Harshey R. M et al., 1977, J bacteriol) since the ratio of RNA to DNA and the RNA chain elongation rate are ten-fold less frequent in *M.tb* than *E. coli*
The genome of *M. tb* was sequenced in 1988 and shown to comprise 4,411,529 base pairs, ~4000 genes and average GC content of 65.6%. Re-annotation of the genome sequence was carried out in 2002 and the size changed from 4,411,529 to 4,411,532 (Camus J C *et al*., 2002). Re-annotation identified 4043 gene encode 3993 proteins and 50 stable RNAs (Cole, ST *et al*., 2002). Only 376 putative proteins share no homology with known proteins and presumably are unique to *M. tb* (Camus, Pryor *et al*. 2002). In total, there are ~250 different enzymes that participate in metabolism of fatty acid in *M. tb* whereas in *E. coli* only 50 enzymes. Almost 10% of the genome consists of motif PE and PPE found near N-terminus of the protein in most cases. These proteins help replication and survival of mycobacteria under different environmental condition (Marri P R *et al*., 2006). The availability of this type of information is important for identifying genes that code for virulence factors and antigens against which host immunity is directed. The genome sequence is also important for identifying targets for chemotherapy.

### 2.5 TB pathogenesis

*M. tb* is an air borne pathogen where infection occurs when few tubercle bacilli dispersed in the air from infected person with active TB. While lungs are the primary site of infection, this pathogen can also spread to other parts of the body. After the bacilli are inhaled, it reaches the alveoli of the lung. *M. tb* is phagocytosed by professional alveolar macrophages and mostly degrades the harboured pathogen due to innate immune response. However, if the bacilli survive, it starts replicating actively in macrophages and diffuses to nearby cells including epithelial and endothelial cells (Wolf A.J *et al*., 2008). During the early steps of infection, *M. tb* can diffuse to other organs through the lymphatic and by haematogenous disseminasion infecting other cells (Balasubramaniam *et al*., 1996). Once the adaptive immune response comes into play, neutrophils, lymphocytes and other immune cells migrate to the site of infection and form a cellular infiltrate called a granuloma (Ottenhoff, Kaufmann., 2012). The granuloma consists of macrophages and giant cells, T cells, B cells and fibroblasts. Bacilli remain encapsulated inside the granuloma thus protected from the host immune response. In latent infection *M. tb* remain dormant, a non-metabolically active state for years, decades or most often for lifetime. Lungs of patients with inactive tuberculosis found to contain live bacilli in their granulomas or tubercles, therefore it shows that the organism continue to live in granulomatous
lesions for several years (Opie and Aronson., 1928; Robertson, 1933), A dynamic equilibrium
appears to be maintained between the bacilli and the host immune responses and any event that
weakens the immune response may result in active TB leading to bacterial reactivation and
replication, with necrosis and damage to lung tissue.

2.6 Immunology of TB

The immune system has two branches, the innate and the adaptive. The innate system protects
the body non-specifically from all kinds of pathogens, while the adaptive components bring in
specificity. T and B lymphocytes are the major cells of adaptive immune system because it is in
these cells that the property of diversity, specificity and memory reside.

2.6.1 Innate immunity

2.6.1.1 Role of pattern recognition receptors (PRRs)

Microorganisms produce many structures known as pathogen associated molecular patterns
(PAMPs). Our innate immune cells express Pattern recognition receptors (PRRs) which are
capable of recognizing these PAMPs, and generate an innate immune response. M.tb produces
PAMPs, like lipoarabinomannan, phenolic glycolipids, phosphatidylinositol mannosidase, and
lipoproteins, which are recognized by pattern recognition receptors called the Toll like receptors
(TLRs) expressed on the surface of macrophage and DCs (Brightbill, Libratty et al. 1999). TLRs
are type-1 membrane proteins with an extracellular domain with leucine-rich repeats and a
cytoplasmic domain with homology to the interleukin (IL) receptor family (Medzhitov R., 1997
Nature). Ligation of PAMPs can trigger both protective and pathogenic immune responses
(Hawn, Dunstan et al. 2006). When M.tb interacts with TLRs, it does not cause direct ingestion
of mycobacteria, but leads to activation of phagocytosis. When TLRs interact with its ligands, it
forms dimers and then recruits the Toll/Interleukin 1 receptor (TIR) domain containing myeloid
differentiation protein 88 (MyD88) (D.M Underhill et al., 1999). This activates downstream
signalling through IL-1 receptor-associated kinases (IRAK), TNF receptor associated factor
(TRAF) 6, TGFβ-activated protein kinase 1 pathway leading to the activation of a transcription
factor viz: nuclear transcription factor (NF)-κβ (K.Takeda et al., 2004), This leads to
transcription of the gene which leads to production of proinflammatory cytokines like TNF, IL-
1β, IL-12 and nitric oxide (S.Akira., 2003). MyD88 plays an important role in the activation of
the innate immune response against \textit{M.\textit{tb}}. In has been observed that mice deficient of MyD88 are found to be more susceptible to \textit{M.\textit{tb}} infection than the wild type. (C.M Fremond \textit{et al.}, 2007). Out of all the TLRs, TLR-2, TLR-4, TLR-9 and possibly TLR-8 interacts with \textit{M.\textit{tb}} (Y.Xu., 2007; T.K Means \textit{et al} 1999). Signaling through TLR-2 and TLR-9 provides the strongest host resistance in TB (Bafica, Scanga \textit{et al.} 2005). TLR-2 forms heterodimers with either TLR1 or TLR6 and recognize various mycobacterial products (Takeda and Akira 2005). TLR-2 is known to bind directly to a 6- kDa secretory protein of \textit{M.\textit{tb}} known as early secreted antigenic target protein 6 (ESAT-6) (Pathak, Basu \textit{et al.} 2007) which is responsible for wide alterations of host immune response. TLR-2/1 recognizes the mycobacterial cell wall components like phosphatidylinositol mannosidase and the 19-kDa lipoprotein, and causes the production of pro-inflammatory cytokines, (Brightbill, Libraty \textit{et al.} 1999; Underhill, Ozinsky \textit{et al.} 1999; Hawn, Dunstan \textit{et al.} 2006) whereas recognition of lipoarabinomannan by TLR-2 induces production of anti-inflammatory cytokine IL-10, which suppresses the affective immune responses (Quesniaux, Nicolle \textit{et al.} 2004). TLR 2 stimulates the production of TNF\textalpha and IL-12 in macrophages (A, Bafica \textit{et al} 2005; L.pompei \textit{et al} 2007). Also, TLR-2/− mice were significantly more susceptible than control mice when challenged with high-dose aerosol infection of \textit{M.\textit{tb}} (N Reiling \textit{et al} 2002; M.B Drennan \textit{et al} 2004). This was probably due to decreased proinflammatory response.

TLR-4 can also be activated by heat shock protein 60/65 (Jo \textit{et al.}, 2007; Kleinnijenhuis \textit{et al.}, 2011). The role of TLR-4 in tuberculosis remains controversial, although some studies have shown less but not complete inhibition of TNF\textalpha production in macrophage of TLR-4\textsuperscript{−/−} mice. In addition to MyD88 pathway TLR-4 induces intracellular signals through a MyD88 independent pathway mediated by the adaptor molecule Toll/IL-1R (TIR) domain containing adaptor inducing interferon IFN-\textbeta (TRIF). It has been seen that TLR-4 induces activation of autophagy through TRIF dependent pathway an important role in phagosome-lysosome fusion (Y. Xu \textit{et al} 2007). It has been shown that mice deficient in TLR-4 are more prone to \textit{M.\textit{tb}} infection as compared to the wild type (N. Reiling 2002). Another study reported that mycobacterium survival was more in lungs, spleen and liver compared to wild type (B.Abel \textit{et al} 2002).

TLR-9 can recognize unmethylated CpG motifs present in bacterial DNA and results in production of cytokines by macrophages and DCs in infected mice. \textit{In vitro} studies showed TLR-9 induces production of IL-12 in DCs (A.Bafica \textit{et al} 2005). Also TLR-9\textsuperscript{−/−} mice are prone to infection when infected with high doses of \textit{M.\textit{tb}} compared to the wild type. Davila \textit{et al} (2008)
demonstrated high expression of the TLR-8 protein in BCG infected macrophages. Although the mechanism by which TLR-8 recognises *M.tb* remains unknown. TLR activation also increases the expression of the vitamins D receptor (VDR) and the vitamins D-1-hydroxylase genes converting pro-vitamins D into their active form. This leads to production of the antimicrobial peptides cathelicidin and β-defensin to kill intracellular mycobacteria (Liu PT, Stenger S *et al.*, 2006; Chocano-Bedoya *et al.*, 2009) (Fig. 3). There are few other specific members in addition to TLRs which helps in recognition of *M.tb* for innate immune response is Dendritic cell- Specific Intercellular adhesion molecule-3- grabbing Non integrin (DC-SIGN) mainly expressed in DCs which plays an important role in DC- *M.tb* interaction. It plays a role in DC migration and DC- T cell interaction, and therefore acts as both PRR and an adhesion receptor. DC-SIGN causes maturation of infected DCs and stimulates anti-inflammatory immune responses by induction of IL-10 production (T.B.H Geijtenbeek., 2003). The C-type lectin receptor (CLR) family includes dectin1, mannose receptor and minscle. This receptor is expressed mainly in macrophages, DC, neutrophils and T-cell subsets. A recent report showed that dectin-1 alone can generate innate immune response against recognition of *M.tb* and can induce Th1 and Th17 responses (F.LVan De Veerdonk *et al.*, 2010).These CLR genes have little or no effects on the course of infection.

Cytosolic pattern-recognition- receptors, NOD2 (nucleotide binding oligomerization domain protein 2) and NLRP3 (NOD-LRR and pyrin domain containing 3) recognize pepdtidoglycan subunit and one or more ESX- secreted substrate like ESAT-6 respectively. Therefore stimulation of these PRRs by specific PAMPs results in different intracellular signalling pathways which induces the expression of proinflammatory cytokines, selected chemokines cellular adhesion receptor leads to efficient activation of the innate host defense mechanism. (Cooper A.M *et al.*, 2011).

**2.6.2 Role of macrophages in the innate immune response**

Macrophages are the phagocytes at the forefront of the host immune defense against microbial pathogens, and constitute a potent antimicrobial component of both innate and cell mediated immunity. A number of studies report that complement receptors and complement opsonisation is the major route of entry of *M.tb* into host macrophages. Complement receptor 1 (CR1), CR3,
and CR4 promotes uptake of mycobacteria by macrophages (Schlesinger 1993; Hirsch, Ellner et al. 1994; Aderem and Underhill 1999). Non-opsonin-mediated phagocytosis of \textit{M.tb} is through the macrophage mannose receptor (MR), which recognizes terminal mannose residues on \textit{Mycobacteria} (Schlesinger 1993; Schlesinger, Kaufman et al. 1996) (Fig. 3). Besides these, Fc receptors and surfactant proteins such as collectins, which include mannose-binding lectins (MBLs), C1q, surfactant protein A (Sp-A) also mediate \textit{M.tb} adherence and uptake by phagocytes (Armstrong and Hart 1975; Gaynor, McCormack et al. 1995; Pasula, Wright et al. 1999). Ingested organisms are disintegrated after phagosome fuse with the lysosome to form phago-lysosome. The acidic environment of the lysosomal containing hydrolase enzymes destroys the microorganisms. The effector function by which macrophages mediate its degradation include phagolysosomal fusion, generation of reactive oxygen intermediates (ROI) by the oxidative burst, production of reactive nitrogen intermediates (RNI) via NOS2 dependent cytotoxic pathway mechanism and mechanism mediated by cytokines.

\textbf{2.6.2.1 Phagolysosomal fusion}

A phagosome is formed by phagocytosis of large particulate material and fusion with a lysosome. The lysosome is vacuole like structure and a complex organelle of the late endocytic pathway which contain hydrolytic enzymes capable of degrading microbes at acidic pH (4.5-5). Hart et al. (1972) first hypothesized that \textit{M.tb} survives inside macrophages by preventing this phagolysosomal fusion, a phenomenon observed only with phagosomes containing bacilli. Armstrong and Hart, coined the term „mycobacterial phagosome” because they observed \textit{M.tb} resides inside a unique phagosome with elevated pH of 6.2-6.3 (Deretic V., Singh S et al., 2006). Mycobacterial phagosomes inhibit further acidification by exclusion of vacuolar ATPase molecule (Sturgill-Koszycki et al., 1994) and lack the late endosomal marker Rab7 and the lysosomal marker such as LAMP 1 (Press et al., 1998). It retains Rab5 which is an early endosomal marker that plays a role in interaction between early endocytic compartments (Desjardins et al., 1994; Desjardins., 1995) and the phagosome. Fusion of phagolysosome is also known to be caused by mycobacterial sulfatides and lysosomotropic polyanionic glycolipids (Middlebrook, Coleman et al. 1959; Goren, BrokL et al. 1976; Goren, D'Arcy Hart et al. 1976). Furthermore, \textit{M.tb} has the ability to produce large amount of ammonia that neutralises the acidic
environment of the phagolysosome, thereby inhibiting the phagolysosome maturation (Gordon, Hart et al. 1980) Presence of the TACO (tryptophan aspartate-containing coat) protein on the mycobacterium phagosome is essential for the prevention of phagosome maturation (Ferrari et al., 1999). TACO, a 51-kDa protein, coats the phagosome containing live bacilli and is retained on the mycobacterial phagosomal membrane of BCG-infected macrophages but not on the phagosomes containing dead BCG bacteria and endosomal vesicles of uninfected cells. Thus, the viability of phagocytosed bacilli appears to be necessary for the retention of TACO by which mycobacteria inhibit phagolysosomal fusion and evade its destruction within the phagolysosome. Defective phagocytosis has been observed when macrophages isolated from MyD88−/− and TLR-2−/−×TLR-4−/− mice were infected with E.coli and Staphylococcus (Blander J.M and Medzhitov., 2004). Thus TLR signalling might control phagosome maturation. In contrast to this Yates and Russell have reported that maturation of the phagosome is independent of stimulation of TLR signalling. Although, evidence regarding TLR signalling in the phagosomal maturation is controversial, the TLR signal is important for determining the fate of the phagosome after phagocytosis.

2.6.2.2 Reactive Nitrogen Intermediates (RNI) and Reactive Oxygen intermediates (ROI)

One of the important antimicrobial mechanisms of macrophages is the production of nitric oxide (NO). The macrophages when activated by IFNγ and TNFα produce nitric oxide and other related RNI via inducible nitric oxide synthase (iNOS, macNOS, Type II NOS) (MacMicking et al., 1997; Shiloh and Nathan, 2000.). Nitric oxide plays an important role in vasodilation, neurotransmission and elimination of microorganisms (Blantz and Munger, 2002). Three major nitric oxide synthases (NOS) are NOS1 (neNOS), NOS2 (iNOS), NOS3 (ecNOS). The role of these toxic RNI in host defences against M. tuberculosis has been well established, both in vitro and in vivo in the murine model (Chan et al., 1992; MacMicking et al., 1997). It has been observed that M. tuberculosis replicates much faster in iNOS gene knock-out (GKO) mice than in wild type animals (MacMicking et al., 1995). High expression of inducible nitric oxide synthase (iNOS2) has been found in the alveolar macrophages of tuberculosis patients (Raghuwanshi, Sharma et al. 2010). In persistently infected mice, inhibition of NOS2 activity with aminosguanidine led to increased numbers of mycobacteria in the lung, liver and spleens (Flynn et al., 1998). These studies clearly depict the protective role of RNI in both chronic and
acute infection. TLR signalling from *M. tb* promotes induction of inducible (iNOS) and formation of NO products (Brightbill HD *et al.*, 1999). The production of RNI in human macrophages has been shown to be induced by 1, 25-dihydroxy vitamins D3, in concert with IFN-γ and TNF-α, which induces NOS2 in the human macrophage cell line HL-60. Combinations of vitamins D3, IFN-γ, and TNF-α successfully killed *M. tb* harboured by human macrophages. (Flynn and Chan 2001)

In addition to RNI, upon activation macrophages produce a large amount of reactive oxygen intermediates (ROI). One of the ROI generated by the oxidative burst is hydrogen peroxide (H₂O₂) (Walker *et al.*, 1981). The role of ROI in killing of *M. tuberculosis* has been elucidated in mice only (Flesch and Kauffman., 1987) and remains to be confirmed in humans. It has been shown that mice deficient in the NADPH oxidase complex are more susceptible to *M. tuberculosis* infection. (Adams *et al.*, 1997; Cooper *et al.*, 2000).

### 2.6.2.3 Apoptosis and Necrosis

Apoptosis also plays an important role in controlling the growth and dissemination of *M. tb* in infected cells (Keane, Balcewicz-Sablinska *et al*. 1997; Placido, Mancino *et al*. 1997). *M. tuberculosis* decreases the apoptosis of infected cells by producing IL-10 and decreases TNF-α activity (Keane, Balcewicz-Sablinska *et al*. 1997; Balcewicz-Sablinska, Keane *et al*. 1998). Lipoarabinomannan (LAM) prevents in vitro apoptosis of *M. tb* t-infected cells in a Ca²⁺-dependent mechanism (Rojas, Garcia *et al*. 2000). Infected human macrophages undergo apoptosis induced by the Fas-ligand resulting in decrease viability of *M. tb* in the cells (Oddo, Renno *et al*. 1998). Conversely, it has also been shown that high numbers of intracellular *M. tb* bacilli trigger a caspase-independent macrophage cell death pathway that could promote extracellular spread of infection, and contribute to the formation of necrotic lesions in tuberculosis (Lee, Remold *et al*. 2006). Natural killer cells (NK cells) are also mycobactericidal, producing immune-regulatory cytokines critical to early host defense. Reciprocal activation interactions occur between NK cells and DC cells via mechanisms dependent on cell-cell contact and soluble factors (Gerosa, Gobbi *et al*. 2005) Human neutrophils may also cause killing of *M.tb*. (Brown, Holzer *et al*. 1987; Jones, Amirault *et al*. 1990). Cytotoxic T cells and NK cells contain granulysin in their granules, which affects the mycobacterial membrane integrity, resulting in killing *M. tb*. (Stenger, Hanson *et al*. 1998)
Another component of innate immunity to mycobacterial infections are mucosal associated invariant T-cells (MAIT). (Porcelli et al., 1993) *M.tb*- reactive MAIT cells are present in both infected and uninfected individuals. (Gold and Lewinsohn., 2011) It has been observed that MAIT produces IFNγ to control the bacterium when cells are infected with *M.tb* ex-vivo.

### 2.6.3 Adaptive immune response to Mycobacterium tuberculosis

Infected macrophages and DC of the innate immune system, present antigens to T-lymphocytes and B-lymphocytes, resulting in the activation of the adaptive immunity. T lymphocytes expressing (α/β) T cell receptors, are of two major types: CD4⁺ and CD8⁺ T cells. CD4⁺ T cells, recognizes antigens processed in the phagosomes and presented by MHC II molecules on the surface of monocytes, macrophages or DCs and produces different soluble cytokines which then induces their target cells to perform different functions. Hence, the CD4⁺ T cells are also known as T helper (Th) cells. One the other hand, CD8⁺ T cells recognizes antigens processed in the cytosol and presented by MHC class I molecule, and produces effector molecules that lyses target cells. Hence CD8⁺ T cells are also known as cytolytic T cells (Tc).

CD4⁺ T helper cells can be further differentiated into Th1, Th2, Th17 and Treg cells. Th1 cells produce IFNγ, TNF α and IL-2 as signature cytokines that provide protection against mycobacterial infection. In contrast Th2 cells, predominately producing IL-4, IL-5, and IL-13 inducing susceptibility to TB infection (Kutlu et al., 2007; Rook, 2007; Rook et al., 2004). Many experiments in the animal model have confirmed the protective role of Th1 cells against TB infection, (Cooper et al., 1993; Flynn et al., 1993) eg: the role of IFNγ in protection against tuberculosis is well established. (Flynn et al., 1993) This was further strengthened by a study in IFNγ knockout (GKO) mice, which are the most susceptible to virulent M.tb (Cooper et al., 1993; Flynn et al., 1993). It has been seen that TNFα synergizes with IFNγ to induce the iNOS expression and hence is critical for control of the acute *M.tb* infection. (Flesch et al., 1990; Chan et al., 1992; Liew et al., 1990). TNFα is known to contribute to the inhibition of *M.tb* growth in macrophages by a mechanism that is dependent on apoptosis and independent of IFNγ activity (Keane et al., 2002). TNFα is known to play an important role in the regulation of inflammation and has a complex and multifaceted role in protection and immunopathology in tuberculosis infection. Tuberculosis disease is considered to be a delayed-type hypersensitivity (DTH)
generated by purified protein derivative (PPD). Hence TB is considered to be an IFN-γ mediated immune response. Therefore, we can say that susceptibility to TB increases with an increase in Th2 response (Rook GA et al., 2004). This assumption was further supported by the observation that IL-4-deficient mice are more resistance to M.tb infection (North, 1998; Saunders et al., 2000). In another study, expression of IL-4 was much higher in the granuloma of patients with advanced TB (Fenhalls et al., 2000). Through various studies we can say that production of IL-4 is associated with the immune response against tuberculosis and is responsible for disease progression in animals as well as humans (Dheda et al., 2005b; Ordway et al., 2005; Seah et al., 2000; Van Crevel et al., 2000). In addition, robust Th2 response have been observed in patients without BCG vaccine (Dlugovitzky et al., 1999; Rook et al., 2005). Therefore, it it is plausible that stimulating Th1 responses, whilst suppressing Th2 responses might provide resistance against TB. IL-12 is known to induce the development of Th1 cells from a naive CD4+T-cell precursor. IFN-γ is known to up regulate the IL-12 receptor enriching Th1 cells whilst inhibiting the growth of Th2 cells (O’Garra., 1998; Murphy K.M. et al., 1999; Trinchieri., 1995). IL-12 represents a family composed of IL-12p40, IL-12p70 and IL-23 or IL27). IL-12 production can be induced in macrophages and dendritic cells (Trinchieri., 1995; Macatonia., et al. 1995; Scheiche., 1995) by microbial products and/or CD40 ligation (Cella, M. et al. 1996; Koch., 1996; Reis e Sousa., 1997). IL-27 along with IL-12 initiates a Th1 response resulting in production of IFNγ during M.tb infection (Chen Q et al., 2000; Trinchieri G et al., 2003). A recent study has shown that IL-27 inhibits human macrophage activity (Robinson CM et al., 2012).

Th17 is the recently found T cell subset producing IL-17A, IL-17F, and in many cases IL-22 and TNF, as well as IFNγ in some cases. (Ottemhoff and Kaufmann., 2010) IL-17 induces resistance to certain bacterial and fungal infections (Jin, Zhang et al., 2008). There is no evidence for the protective role of IL-17 in primary immune responses during TB (Khader, Pearl et al. 2005; Cruz, Khader et al. 2006) but some studies have shown its role in the development of secondary immune responses against TB (Wozniak, Ryan et al. 2006; Khader, Bell et al., 2007; Chatterjee and Dwivedi VP et al., 2011). In BCG infected mice, a strong IL-17-dependent memory response is produced in the absence of IFN-γ and this memory response can successfully provide protection to the mice when infected later with M.tb (Wozniak, Ryan et al., 2006). Differentiation of Th17 cells requires presence of both IL-6 and TGF-β (Bettelli, Korn et al.,
IL-23, a closely related family member of IL-12, is known to induce growth of Th17-cells or Th17 memory cells (Sallusto F et al., 2009). Thus, Th1 and Th17 cells seem to provide protection against TB whereas Th2 cells contribute to disease progression. However, these roles of Th17-cells and IL-17 in TB have been challenged recently. Although studies have shown a protective role of IL-17, Th17-cells and Th17-derived cytokines also mediate inflammation and tissue damage in autoimmune diseases and infection (Khader and Cooper. 2008). IL-17 promotes granuloma formation in genetically susceptible mice, as well as neutrophil accumulation and tissue damage (Khader and Cooper., 2008).

Another phenotypically distinct T cell subset are T regulatory (Treg) cells, characterized as CD4+CD25+FoxP3+ T cells, known to be associated with immunosuppression (Fontenot and Rudensky., 2004). Tregs from tuberculosis patients not only suppress IFN-γ production but also inhibit IL-10 production by CD4+ T cells (Chen, Zhou et al. 2007). Tregs delays and inhibits adaptive immunity by inhibiting optimal priming of CD+ T cells, inhibiting the influx of CD+ T cells into lymph node and inhibits proliferation and expansion (Shafiani Set al., 2010). However, the attenuation of Tregs (when done with BCG vaccination) could be beneficial when the cytokine environment is detrimental to Treg generation, as in the presence of TGF-β, IL-6 and IL-21, while favoring the development of Th17 cells. The latter might play a potential role in initiation of Th1 responses at the site of M.tb infection (Jaron, Maranghi et al., 2008).

Therefore, there should be a dynamic balance between Th1, Th17 and Th2, Treg cells (Fig. 4).
Figure 3: Diagrammatic sketch to show the dynamic balance of Th cells in *M.tb* Infection.

CD8⁺T cells can secrete cytotoxic molecules like perforin, granzymes and granulysin. These can lyse the host cell and granulysin is also capable of directly killing *M.tb*. CD8⁺T cells can induce apoptosis to control *M.tb* infection (Keane J *et al.*, 2000). CD8⁺T cells can also produce Th1 cytokines like IFNγ, TNF and in some cases IL-2. Various studies in mice have shown that after *M.tb* infection CD8⁺T cells also migrate to the lungs in similar to CD4⁺T cells (Feng, Bean *et al.*, 1999; Serbina and Flynn., 1999). In the lungs of infected mice, *M.tb* infected dendritic cells after interaction with TCR of CD8⁺T cells produce IFN-γ in limited quantities (Serbina and Flynn., 1999). Thus, CD8⁺T cells directly contribute to protective immunity against TB.

Other than the conventional T-cells, we have a set of unconventional T cells that act as a bridge between innate and adaptive immunity. These express a unique TCR composed of gamma (γ) and delta (δ) chains known as γ/δ T cells (Hayday AC., 2000), a CD-1 restricted T cell. In TB γ/δ T cells produces cytokines IFNγ and IL-17 and may thus play a role in protection against *M.tb* (Cooper AM., 2009). BCG immunization induces human memory γ/δ T cells resulting in a more effective secondary response, thus suggesting its role in adaptive immune responses in *M.tb* infection in human (Spencer *et al.*, 2008). Because of the difference in the γ/δ T cells of humans and mice there is not much definitive information about the role of these cells. CD-1 restricted T cells recognize mycobacterial lipids (Constant P *et al.*, 1994). These are expressed on the surface of DCs but absent on macrophages. Cross priming can also help in antigen presentation in case of CD1 molecules for the unconventional T cells with the specificity for lipids antigens thus contributing to protection against TB. (Kronenberg M., 2005; Brigl M *et al.*, 2004)

2.7 Mycobacterium and the Host Macrophage

Once the *M.tb* is phagocytosed by macrophages, it can manipulate the cell to avoid its killing and create a favorable environment for its replication. The major success of *M.tb* pathogenesis is its ability to survive and replicate within the hostile environment of the macrophage. Mycobacteria modulate host trafficking pathways to create a protected niche for itself. The mycobacterial
phagosome or pathogen-friendly phagosome is central to tuberculosis infection, latency, disease activation, spread, and suppression of immunological detection by the host (Jeniffer chua et al., 2004). Inhibition of phagosomal maturation is therefore a much studied area.

### 2.7.1 Mycobacterial products interfering with phagosomal maturation

The mycobacterial cell wall is composed of peptidoglycan (PG), which is covalently attached with arabinogalactan (AG) and mycolic acids, which forms mycolyl arabinogalactan peptidoglycan (mAGP). Other lipid components on the cell wall of the *M.tb* are glycosylated phosphatidylinositol derivatives, which includes phosphatidylinositol mannose (PIM), lipomannan (LM) and lipoarabinomannan (LAM). A glycolipid, trehalose dimycolate (TDM) also known as cord factor is another cell wall component of *M. tuberculosis* known to be a virulent factors. Many components of mycobacterial lipid products are known to be responsible for modulation of membrane trafficking in the host cell.

#### 2.7.1.1 Lipoarabinomannan

It has been classified into three major groups on the basis of its capping, mannosylated LAM (ManLAM), phosphoinositol-capped LAM (PILAM) and arabinofuranosyl-terminsated LAM (AraLAM). ManLAM are mainly found in pathogenic mycobacterial strains including *M.tb, M. leprae* and *M. bovis*, which is known to inhibit phagosomal maturation (Gilleron et al., 1997, Fratti et al., 2001). ManLAM inhibits a PI3-kinase dependent pathway between the trans-golgy network (TGN) and the phagosome, which is important for maturation of phagosome (Fratti RA et al., 2003; Fratti RA et al., 2001). Furthermore, LAM interferes with the phagosomal maturation by inhibiting the recruitment of early endosomal auto antigen-1 (EEA1) (Rojas M et al., 2000; Simonsen et al., 1998). This inhibition of EEA1 is linked to the decrease cytoplasmic Ca$^{2+}$ rise, which may cause by the ManLAM (Lawe C et al., 2003). ManLAM is responsible for the inhibition of Ca$^{2+}$ rise because LAM does not have mannose capping (Malik ZA et al., 2000; Malik ZA et al., 2001). Although the exact mechanism of Ca$^{2+}$ rise by Man-LAM is still unknown. However, it has been suggested that macrophage phosphatase SHP-1 is activated by Man-LAM, which plays a role in blocking of Ca$^{2+}$ signaling (Knutson et al., 1998; Vergne et al., 2004). Other than the blocking of cytosolic Ca$^{2+}$ rise, ManLAM can interfere in phagosome
maturation by activating p38 mitogen activated protein kinase (p38 MAPK). Activation of p38 MAPK causes phosphorylation of GDP dissociation inhibitor (GD1) and stabilizes Rab5 in an inactive GDP-bound form (Cavalli et al., 2001; Vergne et al., 2004a). The p38 MAPK induction also decreases the recruitment of Rab5-effector protein EEA1 to the early endosome (Fratti et al., 2003). Additionally, it has been shown that during M. bovis BCG infection there is a significant increase in p38 MAPK ManLAM (Fratti et al., 2003; Vergne et al., 2004). However, a recent report showed that ManLAM, alone cannot induce activation of p38 MAPK and thus other mycobacterial components might be responsible for it (Welin et al., 2008). Lipid rafts present on the host cell membrane containing cholesterol and glycosphingolipids, is known to play a role in cell signaling (Simons & Toomre, 2000; Pike.,2009). Lipid rafts may fuse with LAM and interruption of this may cause disruption in phagosome maturation (Hayakawa et al., 2007). However, PI-LAM present in endomembranes of avirulent M. smegmatis neither prevent phagolysosome formation nor inhibit cytosolic Ca\(^{2+}\) rise (Vergne et al., 2003b; Welin et al., 2008). The fact that the macrophage mannose receptor (MMR) only recognizes the mannose-capped Man-LAM and but fails to recognize Ara-LAM or PI-LAM (Schlesinger et al., 1994), make it more evident that binding of Man-LAM to the MMR can inhibit the phagosome maturation, and thus be limited to the more virulent Mycobacterium spp. A recent study has confirmed that glycopeptidolipids from M. avium after interaction with MMR can delay phagosome maturation. MMR siRNA knockdown human monocyte-derived macrophage infection with M.avium resulted in increased phagosome maturation (Sweet et al., 2010). Apart of interruption of phagolysosome formation, ManLAM also acts as anti-inflammatory molecule and inhibits TNF-α and IL-12 production from macrophages, which again supports M.tb survival in the host (Nigou et al., 2001).

2.7.1.2 Phosphatidylinositol mannoside (PIM)

In contrast to the ManLAM, PIM has a distinct role in the phagolysosome maturation arrest (Vergne et al., 2004). PIMs can fuses with lipid rafts inhibiting LAM insertion (Ilangumaran et al., 1995; Welin et al., 2008). PIM can exclusively stimulate early endosomal fusion in an ATP-, cytosol-, and N-ethylmaleimide-sensitive fusion protein (NSF)-dependent manner, together with Rab. (Vergne et al., 2003). PI3K inhibition has no role in the stimulatory effect of PIM.
Therefore, PIM does not respond against LAM activity, it counteract the LAM inhibition of PI3K-dependent trafficking pathways by creating a bypass membrane fusion neo pathway which is independent of PI3K generation. Phagosomes containing PIM-coated latex bead showed increased delivery of recycling endosomal markers and resulted in escaping of phagosomal acidification (Vergne et al., 2004). PIMs do not reduce the recruitment of syntaxin-6, (Fratti et al., 2003; Vergne et al., 2004). It stimulated the acquisition of endosomal SNARE protein syntaxin-4 and the transferrin receptor, involved in iron delivery for preventing phagosomal maturation (Fratti et al., 2003; Vergnet et al., 2004).

Collectively, ManLAM inhibit phagosome maturation by arresting the recruitment of late endosomes and the delivery of lysosomes; In contrast, PIMs stimulate the fusion of early endosomes and thereby allowing the transfer of necessary nutrients for mycobacteria present in the phagosomal compartments (Kelley & Schorey, 2003; Vergne et al., 2004). Thus, there is a balance between ManLAM, which prevents maturation of phagosome and PIMs which stimulate early endosomal fusion to recover nutrients for the mycobacterial phagosome (Vergne et al., 2004).

2.7.1.3 Cord Factor

Cord factor another glycolipid, is chemically a trehalose di-mycolilate TDM, in which a trehalose sugar is esterified with two mycolic acid residues. This is a characteristic product of M.tb, capable of inhibiting the migration of polymorphonuclear neutrophils (Asano M et al., 1993). TDM inhibits fusion between two phospholipid bilayer compartments by facing the trehalose sugar in the aqueous interphase (Parsegian VA et al., 1979; Rand RP et al., 1984; Marsh D et al., 1989). Free mycolic acid and trehalose sugar are incapable of inhibiting fusion of vesicles. Therefore for the inhibition of vesicle fusion, intact cord factor is required (Spargo BJ et al., 1991). It has been reported that TDM also prevents Ca^{2+} mediated fusion between liposomes (Geisel et al., 2005; Indrigo et al., 2003). These studies were further supported by the observation that TDM coated latex beads showed a delayed phagosomal acidification (Axelrod S et al., 2008). Delipidation (a treatment that removes TDM, among other lipids) of M. tb allows it to enter into the acidified compartment (Axelrod S et al., 2008). However, the detailed mechanism of the TDM action remains unknown. TDM exposed macrophage cells produce
higher level of pro-inflammatory cytokines. Cells infected with delipidated \( M. \) \( tb \) produce decreased levels of IL-1\( \beta \), IL-6, TNF-\( \alpha \), and IL-12, and secretes higher level of IL-10 (Indrigo et al., 2002).

### 2.7.2 Modulation to inhibit phagolysosomal fusion

\( M. \) \( tb \) overcomes and modulates these pathways to survive within the phagosome and finally escapes to the cytosol of macrophages for survival and growth.

#### 2.7.2.1 Inhibition of Rab conversion

Rab proteins are low molecular weight GTPases that cycle between their active GTP and inactive GDP bound forms and helps promote vesicle fusion (Martinez and Goud., 1998). Rabs conversion is a highly coordinated and interdependent network that controls cargo delivery in the endosomal system from cell surface to the lysosomal fusion. Blocking the maturation of the mycobacterial phagosome was initiated with the analyses and conversion of endocytic Rabs. Blockage of phagosomal maturation takes place between the small GTP-binding proteins Rab5 and Rab7 (Via et al., 1997), which plays an important role in trafficking and maintaining the identity of endocytic organelle. Rab5 has been detected in early endosomes where it resides transiently and Rab7 is present at later stages of maturation in the late endosome fusion (Feng et al., 1995; Vitelli et al., 1997). A study has shown that Rab5 facilitates mycobacterial acquisition of iron by ensuring proper endocytic sorting and delivery to the mycobacterial phagosome (Kelley and Schorey., 2003). It has been observed that the mycobacterial phagosome does not recruit Rab7 even after seven days of infection. Additionally, another Rab 5 effector molecule called early endosomal antigen (EEA1) was also found missing. EEA1 contains two spatially separate Rab5 binding domains (Simonsen et al., 1999). In addition to its interaction with Rab, EEA1 has FYVE domain that binds to phosphatidylinositol 3- phosphate (PI3P) (Mills et al., 2001). Interestingly, PI3P is produced on endosomal membranes by hVPS34, a type III phosphatidylinositol 3-kinase (PI3K), which is also a Rab5 effector (Christopher Foridis et al., 1999). Another endosomal regulatory PI3P binding protein is known as hepatocyte growth factor- regulated tyrosine kinase substrate (Hrs) (Vieira et al., 2004). Alteration and reduction in
the recruitment of EEA1 and Hrs to mycobacterial phagosomes have implicated hVPS34 and PI3P in the mycobacterial phagosome maturation block (Fratti et al., 2003). Other Rab GTPases Rab14 and Rab22a are also found to be involved in the \textit{M.tb} containing phagosome maturation (Roberts EA et al., 2006; Kyei GB et al., 2006). Further studies are currently underway to investigate the roles of these Rab GTPases in phagolysosomal biogenesis to further understand how \textit{M.tb} evades its killing in the phagosome.

\subsection*{2.7.2.2 Interfering with host protein}

**Coronin1 or TACO:** The ability of \textit{M.tb} to survive within the host cells is not only because of their ability to produce virulence factors, but also because they can interfere with host signaling through various proteins. One such important protein called TACO (tryptophan aspartate containing coat protein) is also referred to as p57 or coronin1. Coronin1 is found not to be associated with any other subcellular organelles (Ferrari et al., 1999; Hasan et al., 1997). This protein is associated with phagosomes containing live bacilli, but is rapidly released from phagosomes containing killed mycobacteria. This suggests a possible role in the prevention of phagosome maturation and killing of mycobacteria inside macrophages (Gatfield et al., 2005). Mycobacteria in the kupffer cells of the liver that do not express coronin 1 are easily delivered to lysosome and therefore fail to survive (Ferrari et al., 1999). In mice deficient of coronin 1, both \textit{M. tb} and \textit{M. bovis} BCG are delivered readily to the lysosome in vivo. All these studies suggest a possible role of coronin 1 in mycobacterial trafficking. Coronin1 is an actin binding protein that associates with early phagosomes and in \textit{Dictyostelium doscoideum}, a coronin1 deficient mutant was shown to be defective in phagocytosis (Maniak M et al., 1995). Coronin1 dependent cytosolic influx of calcium in \textit{M.tb} infected macrophages induces the calcium-dependent phosphatase calcineurin. The activation of calcineurin can block the fusion of the phagolysosome (Jayachandran et al., 2007) and can further lead to several downstream signaling cascades responsible for endocytosis (Cousin and Robinson., 2001; Winslow et al., 2003).

\subsection*{2.7.2.3 Inhibition of Lipid mediated Signaling}

One of the survival tactics adopted by \textit{M.tb} for its successful survival in the host cell is the prevention of fusion of a phagosome with a lysosome. \textit{M.tb} has an unusual cell wall which is
known to play an important role in its virulence and loss of which reduces the survival capacity of bacteria inside the host cell (Glickman., 2003; Makinoshima and Glickman., 2005). Phosphatidylinositol 3-phosphate (PI3P) is a part of host membrane, produced by enzyme known as hVPS3. It is generated by phosphatidylinositol 3-kinase (PI3 kinase) on early endosomal and phagosomal membrane. PIP3 provides a docking site for many proteins such as early endosomal auto-antigen1 (EEA1) and hepatocyte growth factor- regulated tyrosine kinase substrate (Hrs) (Birkeland and Stenmark., 2004; Wurms et al., 1999). Preventing accumulation of PI3P on the phagosomal membrane can block the phagosome-lysosome fusion. Lipoarabinomannan (LAM) along with another enzyme called PI3P phosphatase, termed SapM prevents generation of PI3P and inhibits lysosomal delivery (Fratti et al., 2001; Kang et al., 2005; Saleh and Belisle., 2000; Vergne et al., 2005). Although killed mycobacteria fails to inhibit lysosomal transfer, it contains LAM, and therefore its role in blockage of trafficking remains unknown (Jean Pieters., 2008). SaPM is known to be released in the cytosol of the host cell after infection where it might play a role in blocking PIP3 on phagosomal membranes. Apart from lipid phosphatases, M. tb is also known to possess protein phosphatases PtpA and PtpB (Grundner et al., 2005) that may modulate vacuolar sorting of proteins, thus interfering with the host trafficking pathway.

2.7.2.4 Inhibition of p38 MAP Kinase

Previous studies have demonstrated that there is a connection between p38 mitogen-activated protein kinase (MAPK) stress signaling and the endocytic pathway (Cavalli V et al., 2001). Activation or phosphorylation of the p38 mitogen-activated protein kinase (p38 MAPK) decreases the level of GTP bound active Rab5 on early endosomes, and subsequently reduces the levels of EEA1 on endosomes (Cavalli V et al., 2001). The activated p38 MAPK by phosphorylating guanyl nucleotide dissociation inhibitor (GDI), stabilizes Rab5 in its inactive, GDP-bound form (Cavalli V et al., 2001). During mycobacterial infection activated p38 MAPK can also modulate the recruitment of EEA1 and prevent phagosome maturation (Fratti et al., 2003). EEA1 on mycobacterial phagosomes can be significantly increased by blocking p38 MAPK with SB20580 in infected macrophage which can ultimately lead to the recruitment of late endosome/phagosome markers, and also can cause acidification of the phagosomes (Fratti et al., 2003).
Both the Ca\(^{2+}\) and p38MAPK pathways diverge upon recruitment of EEA1, which is essential for the lysosomal delivery to the phagosome. *M.tuberculosis* infection in the macrophages blocks both these pathways, and this double latch mechanism inhibits the recruitment of EEA1 on the mycobacterial phagosome to ensure phagosomal maturation arrest.

### 2.7.2.5 Interfering with mycobacterial eukaryotic-like protein kinases

In bacteria, the major molecular system responsible for stimulus–response coupling involves a two-component system, consisting of histidine kinase sensors and their associated response regulators (Stock J B *et al*., 1989). In eukaryotes, by contrast, protein phosphorylation predominately results in phosphorylated serine, threonine or tyrosine residues, and the eukaryotic protein kinases and phosphatases are the backbone of signal transduction pathways. Recently, a number of bacterial species were shown to possess eukaryotic-like serine, threonine and tyrosine kinases. The *M.tuberculosis* genome is known to consist of 11 members of the eukaryotic-like serine/threonine kinases family (Cole *et al*., 1998). Most of these kinases are transmembrane molecules that play a role in mycobacterial physiology. Only two of the kinases PknG and PknK are soluble molecules. PknG is found in the genome of all pathogenic mycobacteria. PknG is required for the survival of *M.tuberculosis* once the bacteria have been internalized within the phagosome. It has been observed that PknG deficient bacteria are highly attenuated in immunocompetent mice, and when infection in immune deficient mice showed delayed mortality (Scherr *et al*., 2009). PknG helps in the survival of *M.tuberculosis* inside the host cell. A possible mechanism by which this is done is that PknG phosphorylates cellular protein and blocks the maturation of phagosomes, thus enabling the mycobacteria to survive in the host cells (Av-Gay Y *et al*., 1999, Cowley *et al*., 2004). In order to understand the mechanisms adopted by PknG for disruption of phagosomal maturation, it is important to find the cellular substrate for PknG.

### 2.7.2.6 Interfering with Ca\(^{2+}\) signaling

Change in the concentration of Ca\(^{2+}\) is another important factor that plays an important role in phagosomal maturation (Christoforidis S *et al*., 1999; Jaconi M E *et al*., 1990; Wurmser A *et al*., 1999). Cytosolic Ca\(^{2+}\) concentration affects the phagosomal maturation and *M.tuberculosis* is known to
exploit this feature thus affecting the fusion of phagosome with lysosome. Two important proteins a2+ dependent effector proteins Calmodulin and calcium/calmodulin-dependent protein kinase II (CaMKII) plays an important role in the Ca2+ signaling pathway (Malik ZA et al. 2001). Increase in concentration of Ca2+ in the cells causes the alterations in the conformation of calmodulin which then induces autophosphorylation and activation of CaMKII (Rich RC et al., 1998). CaMKII activation induces recruitment of EEA1 to phagosomal membrane and fusion with lysosome (Peter C and mayer A., 1998 Nature). Macrophages infected with killed or antibody opsonized M.tb showed increase in the concentration of cytosolic Ca2+ as compare to macrophages infected with live M.tb. Treatment of macrophages infected with live M.tb with calcium ionophore A23187 caused an increase in cytosolic Ca2+ and reducing the viability of M.tb also the macrophage membrane showed reduced amount of calmodulin and CaMKII (Malik ZA et al., 2000). Lysosomal delivery to the phagosome can be inhibited by Ca2+ chelation or by inhibition of calmodulin and CaMKII. Another lipid kinase, sphingosine kinase (SK) when inhibited can reduce cytoplasmic Ca2+ concentration (Malik Z A et al., 2003). SK phosphorylates sphingosine to form sphingosine-1-phosphate (S1P). Live bacteria and not heat killed bacteria, can inhibit SK activity decreasing the production of S1P therefore reduced Ca2+ concentration in the cytosol (Spiegel S and Milstein S., 2002 JBC).

2.7.2.7 TLR signaling

It has been reported that stimulation by TLR helps in maturation of phagosomes containing bacteria. In an experiment where macrophages isolated from MyD88−/− or TLR-2x4−/− mice were infected with Escherichia coli or Staphylococcus aureus, the phagosome did not fuse with the lysosome, whereas in uninfected macrophages phagosome fused with the lysosome (Blander JM and Medzhitov., 2004). Therefore TLR signaling may play a role in controlling phagosome maturation. However, the finding was challenged by Yates and Russell (2005). They reported that the phagosome maturation is independent of stimulation of TLR signaling. In their experiments they have shown that phagocytosis of mannose or IgG coated beads is followed by fusion with lysosome in both TLR-2−/− and TLR-4−/− monocytes macrophage as well as normal cells (Yates RM and Russell.,2005). Even though the role of TLR signaling in phagosome maturation is highly controversial, its signaling is critical for determining the fate of phagosome
after phagocytosis. In a recent study, Rehman et al showed that \textit{M.tb} downregulates TLR mediated signals to translocate into the cytosol (Rehman \textit{et al.}, 2014).

2.7.3 Macrophage activating cytokine

IFN-\(\gamma\) producing CD4\(^+\) T lymphocytes activate macrophages to induce phagolysosome formation and enhance autophagy. The inhibiton of the phagosome-lysosome fusion can be avoided by activating \textit{M.tb} infected macrophages with IFN-\(\gamma\) (Via LE \textit{et al.}, 1998). IFN-\(\gamma\) can control \textit{M.tb} by modulating various other mechanisms, such as leukocyte-endothelium cell interactions, antigen presentation, reactive nitrogen and oxygen intermediates, cell growth and apoptosis (Vie LE \textit{et al.}, 1998). Further, a small GTP- binding protein known as LRG-47, selectively up regulated upon activation of IFN-\(\gamma\) and is responsible for the restoration of phagosomal maturation block by \textit{M.tb}. LRG-47 enable the delivery of vacuoler H+ATPase subunits from trans-Golgi network (TGN) to the phagosome (Macmicking J D \textit{et al.}, 2003). On the other hand IL-10 secretion in response to \textit{M.tb} infection promotes mycobacterial phagosome maturation arrest allowing pathogen persistence (Seonadh O‘leary \textit{et al.}, 2011). Increased activation of P38MAP kinase is known to be responsible for increased 1L-10 in \textit{M.tb} infected macrophages. However inhibitory activity of IL-10 for phagosome maturation is independent of p38MAPK activity but essentially dependent on the signal transducer and activator of transcription 3 (STAT3) activity (Seonadh O‘leary \textit{et al.}, 2011).

2.7.4 Phagosomal Escape of \textit{M. tuberculosis}

It is strongly believed that \textit{M.tb} resides in membrane- enclosed vacuoles within macrophages and get their nutrients through endosomal trafficking. In the 1980s, it was reported that 60-100\% of bacteria that had escaped the phagosome and were in cytosol after 18-24 hours post infection (Leake \textit{et al.}, 1984; Myrvik \textit{et al.}, 1984). In the 1990s McDonough \textit{et al} noted that half of the bacteria infecting the J774 mouse macrophages escaped into the cytosol after 4 days of infection (McDonough \textit{et al.}, 1993). Subsequent studies by Xu \textit{et al} 1994 showed that H37Rv remain in a membrane –bound LAMP1 positive compartment for as long as 14 days after infection in murine macrophages (Xu \textit{et al.}, 1994). The differences in the findings of these 2 studies might be be explained by the different microscopic techniques used: Xu \textit{et al} used cryopreservation and McDonough \textit{et al} used organic solvents that have the potential to extract phagosomal
membranes resulting in an appearance of cytosolic localization (Hariff et al., 2012). In one of the studies, Fab fragments were injected into the cytoplasm of the macrophage infected with M.tb and the accessibility of M.tb to Fab fragments was measured. It was determined that the bacteria were unable to migrate into the cytosol of the macrophages (Clemens D L et al., 2002). Conversely, in another study, BCG was accessible to dextran molecules injected into the macrophage cytoplasm thereby gaining access to cytosolic nutrients or for cell to present antigens via MHC class 1 (Teitelbaum R., 1999). M. marinum is able to escape from the phagosome into the cytosol by using actin-based propulsion in a listeria like manner, and spreads into neighboring cells (Stamm L M et al; Stamn L M et al 2005). It was later found that (Region of differentiation 1) RD1 region was responsible for the escape of M. Marinum (Smith J et al., 2008). Debates on the intracellular localization of M.tb within phagocytes with respect to time of infection are ongoing. Van der Wel et al (2007) reported that M.tb infected human DCs and macrophages showed a large number of M.tb in the cytosol 48 hrs post infection using cryo-immunogold TEM (Van der wel et al., 2007). At a time point early in the course of infection, M.tb was found to be present within phagosome and it was characterized by the presence of CD63, Lamp1, Lamp2 and cathepsin D, while MHC-1, tlr and EEA1 were absent. At a later time point, M.tb but not BCG were found in the cytosol. They therefore concluded that ESX-1 TypeV III secretion is encoded in the RD1 region and absent in BCG is responsible for the translocation of M.tb into the cytosol (Van der wel et al., 2007). Much discussion and postulation regarding how M.tb antigen is presented via both via MHC class 1 and MHC class II has occurred. The ability of M.tb to escape into the cytosol allows them to access the MHC-1 processing pathway (Van der wel et al., 2007; Weerdenburg et al., 2010). Another explanation for the M.tb antigen presentation by MHC class I could be the interaction of mycobacterial phagosome and the endoplasmic reticulum (ER) i.e cross presentation. This leads to proteasome degradation and MHC class I presentation of M.tb antigen even when the bacteria is present inside the phagosome (Guermonprez P et al., 2003). The various factors that contribute to the conflicting results regarding the escape of M.tb to the cytosol could be viability of the host cell, preparation of inoculum and infection time. According to another study by Chastellier (2009), presence of M.tb in the cytosol is due to degeneration of phagosomal membrane when M.tb lyse the cell to escape and spread to neighbouring cells (De chastellier et al., 2009). Consistent with this, is the finding that H37Rv at a threshold number is capable of inducing necrosis in human
macrophages in an ESAT-6 dependent manner. Phagosomal escape followed by rapid necrosis and immediate clearance of cell debris makes it difficult to find bacilli in the cytosol of alveolar macrophages from TB patients (Mwandumba HC et al., 2004). Therefore we are yet to determine whether the escape of *M.tb* into the cytosol is a strategy adopted by *M.tb* to increase its replication rate and access to cytosolic nutrients, or whether it is simply because of the membrane lysis during cell death induced by *M.tb*. Although it is evident from all the previous studies that *M.tb* is indeed able to escape from the phagosome into the cytoplasm of the macrophage, the function and significance of *M.tb* escape is still not very clear. Therefore, further research on these issues is needed.

### 2.7.4.1 Factors for the phagosomal escape of *M.tb* in cytosol

**Exs1**: Recent evidence of *M.tb* escaping the phagolysosome into the cytosol (Van der wel at al., 2007) has revealed a new dimension for further research regarding the virulence mechanisms of *M.tb*. Many studies have suggested that at a later stage of infection, the pathogen is able to escape the phagolysosomal compartment into the cytosol of the host cells, which may provide them a better niche to survive (Simeone R et al., 2012; Houben D et al., 2012). The translocation of *M.tb*, in part, if not completely is responsible due to the Type VII secretion system that is also known as ESAT-6 secretion complex-1 (ESX-1) (Huoben D et al., 2012; Macgurn JA et al., 2007; Koo I C et al., 2008). Genomic analyses of mycobacterial species have revealed the presence of the region of differences (RDs) present in the genome of *M.tb*, but absent in the attenuated vaccine strain, BCG (Behr et al., 1999; Gordon et al., 1999). One such region that is found to be responsible for the virulence of *M.tb* and *M. marinum* is the region of differentiation 1 (RD1) region. RD1 region encodes the ESAT-6 secretion complex-1(ESX-1) secretion system that allows the secretion of two very important proteins: early-secreted antigenic target (ESAT-6), a 6-KDa protein and culture filtrate protein (CFP-10), a 10 KDa protein. ESAT-6 and CFP-10 are exported together in 1:1 complex to the cell wall of *M.tb* and is secreted by *M.tb* (Brodin P et al., 2006; Pym AS et al., 2002; Sorensen AL et al., 1995; Renshaw PS et al., 2002). Both these proteins exhibit host membrane lysis activity that helps spread of bacteria from one cell into another (de Jonge et al., 2007; Guimm et al., 2004). From the conclusion of different studies was that pathogenic mycobacteria like *M.tbs* and *M. leprae* were able to translocate into the
cytosol, whereas non-pathogenic species like *M. bovis* BCG, *M. avium* and *M. smegmatis* remain in the phagolysosome (Van der Wel et al., 2007; Houben D et al., 2012). Hence it is proved that cytosolic translocation is restricted to the pathogenic mycobacterial strain. Mutation in blocking the secretion of ESAT-6 and CFP-10 disables the *M. tb* to translocate into cytosol, (Van der wel et al., 2007) whereas introduction of the RD-1 region secreting ESAT-6 protein into *M. bovis* BCG enables cytosolic translocation in PMA activated THP-1 cells. BCG with the RD-1 region that has a functional ESX-1 secretion system also increases the virulence of the strain (Houben et al., 2012). However, even though *M. smegmatis* secretes ESAT-6 and CFP-10 that shares ~70 % amino acids homology with *M.tb* ESAT-6 and CFP-10 respectively, it fails to translocate into the cytosol (Converse SE et al., 2005). This may be due to the unique membrane interacting activity of ESAT-6. ESAT-6 secreted by *M.tb* causes leakage of membrane vesicles and undergoes significant conformational changes upon acidification whereas *M. marinum* ESAT-6 was inactive in membrane interactions and showed no conformational changes in response to acidification (Joaquin D L et al., 2012). Another reason for the inability of *M.smegmatis* to translocate into the cytosol could be lack of the C-terminus region of ESAT-6. It has been shown that *M. tuberculosis* H37Rv EsxAΔ84-95 that lacks 12 amino acids in ESAT-6 C-terminus when infected in THP-1 cells fails to translocate into the cytosol and remains in the phagolysosome (Brodin et al., 2005; Houben et al., 2012). Hence it is evident that C-terminus region of ESAT-6 is essential for its function in translocation. The mechanism of ESAT-6 in translocation remains to be understood.

**TlyA (a protein of interest for this study):** TlyA is a 268 amino acid polypeptide in *M.tb* that shows homology with the TlyA haemolysin/cytotoxin of the swine pathogen *Serpulina hyodysenteriae*. where TlyA was first characterized (Martino MC et al., 2001). Mycobacterial TlyA is present in virulent strains *M.tb*, *M. leprae*, *M. avium* and *M. bovis* BCG but absent in the non-pathogenic *M. smegmatis* strain. The TlyA homologue in some bacteria exhibits haemolytic activity by forming pores, as confirmed in *S. hyodysenteriae* (Wren BW et al., 1998) and TlyA in *H. pylori* functions as a hemolysin as well as an adherence factor for colonization of the gastric mucosa (Martino MC et al., 2001). The LsaA product of *Lawsonia intracellularis*, the TlyA homologue of this organism, lacks hemolytic activity but has been suggested to play a role in adherence and/or invasion (McCluskey J et al., 2002). Almost all TlyA homologues have K-D-K-E domain for 2'-hydroxy-ribose methylation in ribosomal RNA. It has been demonstrated that
when TlyA is introduced into non-haemolytic *M. smegmatis* strains, and when cloned into *E.coli*, it showed contact dependent haemolytic activity (B W Wren *et al.*, 1998). It has been previously shown that in H37Rv, the *tlyA* gene may be a part of an operon containing at least three other genes: *tlyA* (Rv1694), *ppnk* (Rv1695) and *RecN* (Rv1696), homologous to *E.coli* RecN (B W Wren *et al.*, 1998). Previous studies have suggested that the haemolytic activity of TlyA in *S. hyodysenteriae* is due to its capacity to form pores and not through proteolytic or enzymatic activity (Hyatt *et al.*, 1994). TlyA is also known to function as a ribosomal RNA methyltransferase (Johansen SK *et al.*, 2006, mol cell). It is known to methylate 50S and 30S ribosomal RNA and makes *M.tb* susceptible to the peptide antibiotic capreomycin (Monshupee T *et al.*, 2012). Further, recombinantly expressed *E. coli* purified TlyA protein showed hemolysis in a concentration dependent manner, and forms up to heptameric oligomerization on the membrane, which is the indicative of pore like structure on the cell membrane (Rehman *et al.*, 2010 BMC). TlyA also binds and oligomerizes on the isolated phagosomal membrane, indicates the possibility of pore formation. Interestingly, despite absence of the signal sequence, TlyA was shown to be exported on the cell wall of *E.coli* containing the *tlyA* gene (Rehman *et al.*, 2008). The ability of TlyA in altering the membrane of the bacterial containing phagosome may play a role in the escape of *M.tb* from phagolysosome. In this study, our aim was to understand the possible role of TlyA in the virulence of *M.tb*. 
Rationale of the study

In recent years, many essential mycobacterial virulence genes have been identified for the pathogenicity, virulence and persistent of mycobacteria. One such gene that was identified was tlyA gene of M.tb. TlyA protein have a “pore forminsg” haemolysin like membrane damaging activity.

The ability of pathogenic Mycobacteria to arrest phagosomal compartment in host macrophages was thought to be essential to their virulence. However, from our data we reported that virulent strain of M.tb H37Rv is able to translocate from the phagolysosomal compartment into the cytosol of macrophage whereas BCG, H37RvΔRD-1 and H37RvΔESAT-6 mutant strain of H37Rv were unable to transmigrate. And many researchers have established a clear link between mycobacterial translocation and virulence. Therefore, when we identified the protein TlyA of M.tb, our first aim is to identify TlyA has any role to escape M.tb from phagolysosome? With confocal and electron microscopic studies we observed that TlyA mutant of H37Rv similar to BCG and other mutant strain (H37RvΔRD-1 and H37RvΔESAT-6) was unable to translocate into the cytosol. These findings led us to ask the next question, the second objective of our study, why BCG and H37RvΔRD1 unable to translocate to the cytosol or act as avirulent, while TlyA is present. Furthermore, TlyA is known to destabilize the phagosomal membrane. For this activity it is necessary that TlyA must be present at or beneath the surface of mycobacterial cell envelope. Thus, in this study we also wanted to investigate, surface localization and secretion of TlyA from M.tb H37Rv, our third objective of our study. Translocation of M.tb is also known to be dependent on C-terminus of the early-secreted antigen ESAT-6, secreted via a RD1 secretion system (Houben et al., 2012). So, third objective of our study is to find if TlyA and ESAT6 is dependent or has independent role in M.tb pathogenesis. T cell responses, especially the Th1/ Th2 balance, are critical in pathogenicity of tuberculosis (Flynn and Chan 2001). As tuberculosis is understood to be a Th2 mediated disease, the polarization of T cells to Th1 or Th2 type by the absence of TlyA in H37Rv would be crucial in the understanding of disease susceptibility and host immunity. Therefore, our fourth objective was to study the role of TlyA in host immune response by studying the effects of TlyA in Th-cells activation and their role in subset differentiation and CFU in different organs of H37Rv and H37RvΔTlyA infected mice.
CHAPTER 3- MATERIALS AND METHODS

3.1 Ethical approval
All animal experiments conducted were approved by the Institutional Animals Ethics Committee at ICGEB (Approval Number: ICGEB/IAEC/IMM-22/2010 and ICGEB/AH/2013/01/IMM-34) New Delhi, India and are in accordance with the Department of Biotechnology (DBT) guidelines, Government of India. Non animal laboratory experiments were approved by the Biomedical Research Ethics Committee at the University of KwaZulu-Natal (Ref No: BCA274/09).

3.2 Mice and bacterial cultures
Wild type C57BL/6 mice for the experiments were obtained from the animal facility at the International Center for Genetic Engineering and Biotechnology (ICGEB, New Delhi, India). *M. tuberculosis* H37Rv, *M. bovis* BCG, H37RvΔESAT6, H37RvΔRD1, BCG:RD1 (Chaterjee et al., 2011) were all a kind donation from Prof. David Sherman (Seattle Institute of Biomedical Research, Seattle, USA) and H37RvΔTlyA strains was a kind donation from Tanya Parish, University of Washington (Parish T et al., 2000).

*M. tuberculosis* whole cell lysate (complete soluble antigen or CSA) was obtained from Dr. John Belisle, Colorado state University, USA under the National Institutes of Health, National Institute of Allergy and Infectious diseases.

3.3 Mice Immunization
Groups of 6-8 weeks old female BalB/c mice were immunized intraperitoneally with 50μg of purified TlyA protein (2mg/ml) (Rehman et al., 2010) and emulsified with Freund’s complete adjuvant. Mice were again boosted on 2nd, 4th and 8th week after primary immunization with 50μg of TlyA protein (Rehman et al., 2010) emulsified with Freund’s incomplete adjuvant intraperitoneally. Bloods were collected a day before primary immunization and at every two weeks after primary immunization.
3.4 Isolation of peritoneal macrophage

2 ml of 4% thioglycollate was injected into each wild type C57BL/6 mice intra peritoneal cavity. It was injected into the peritoneum to elicit the macrophages into the peritoneal cavity. After five days, macrophages were isolated from the peritoneal cavity by flushing the cavity with RPMI1640 medium supplemented with 10% fetal bovine serum. RPMI1640 should be kept in ice and process repeated two- three times. Cells were incubated overnight at 37°C, in 5% CO₂ and washed with RPMI supplemented with 10% FBS to remove non-adherent cells. Adherent monolayer cells were used as peritoneal macrophages.

3.5 Confocal microscopy

Bacteria were labelled with FITC dye according to standard methods (Watanabe et al., 2007), immediately before infection. The bacteria were grown up to mid-log phase (OD600~ 0.6). Bacteria were harvested and the pellet was washed twice with PBS (pH7.4). The pellet was resuspended with 0.1 M sodium carbonate buffer (pH 9.5) containing FITC at a concentration of 1mg/ml and incubated for 30 mins at room temperature on a rotar with very mild shaking. The bacteria were then spun at 3000 rpm for 5 mins at room temperature. The supernatant was discarded and pellet was washed three times with RPMI1640 (without antibiotics). The pellet was finally resuspended in RPMI1640 (without antibiotics), The FITC labeled bacteria were passed through a 26G needle 10 times to make a single cell suspension. Peritoneal macrophages were isolated and cultured in 12-well plates (~1x10⁶/well) tissue culture plate on glass cover slips. When macrophages are well adherent, cells are infected with bacteria stained with FITC dye at a multiplicity of infection (MOI) of 10:1. After 4hrs, cells were washed twice with RPMI 10% FBS and100μg/ml gentamicin was added and incubated for 1 hours to remove any extracellular bacteria. After gentamycin treatment, RPMI 10%FBS were added and cultured at 37°C in 5% CO₂. At different time points, the medium was discarded and cells were washed twice with PBS. Cells were fixed with 2% paraformaldehyde in PBS, pH 7.4 for 20-30 mins and washed three times with PBS. Cover slips were picked up carefully into fresh 12 well plates and stored at 4°C until used for staining. Cells were permeabilized with 0.2% Triton X-100 and labeled with primary antibodies rat anti-LAMP1 and mouse anti-Rab5 at 1:50 dilutions. Alexa Fluor 594-conjugated rabbit anti-rat IgG or Alexa Fluor 594 conjugated goat anti-mouse IgG were used as secondary antibodies. 10µl of Prolong Gold antifade reagent was dropped on the
glass slide and cover slip was placed on top of it and sealed using adhesives. Confocal microscopy images were acquired on a Nikon A-1R confocal microscope

3.6 Differential permeabilization using digitonin.
Peritoneal macrophages were isolated from wild type C57BL/6 mice and infected with *M.tb* strains labelled with FITC as explained earlier. Macrophages were then washed twice with KHM buffer (110 mM potassium acetate, 20 mM Hepes and 2 mM MgCl₂). Cells were treated with KHM buffer containing 25 μg/mL digitonin for 1 minute at room temperature. Cells were washed with KHM buffer without the detergent. Anti-*M.tb* antibody in KHM containing 3% BSA or with buffer alone were added at 30°C for 12 mins, after that cells were washed with PBS and fixed with 4% PFA. Primary antibodies were detected using secondary antibody Alexa 545-conjugated goat anti-rabbit IgG by incubating in 3% BSA in PBS for 30 mins. Controls without primary antibodies showed no fluorescence from the secondary antibodies alone. Images for colocalization were captured on a Nikon A-1R confocal microscope

3.7 Electron microscopy
Raw 264.7 (mouse leukaemic monocyte macrophage cell line) were cultured in 100 mm culture dishes in RPMI medium RPMI 10% FBS. Cells at 80 % confluency were counted and infected with the wild type and *M.tb* mutant H37RvΔTlyA at MOI of 10:1. Cells were incubated at 35°C in 5% CO₂. After the 48 hourss time point infected cells were washed three times in PBS. Following this cells were harvested and fixed using 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 24 hourss at 4°C and processed for sectioning as mentioned below. Ultra-thin sections were cut (50-60 nm) and collected on a nickel grid. Grids were double stained with an aqueous solution of uranyl acetate (BDH laboratory chemicals) for 10mins and Reynolds lead citrate (Reynolds. 1963) for 3mins. Electron microscopic images were taken using a transmission electron microscope (Jeol 1010 transmission electron microscopy) at the electron microscopy unit of the Westville Campus, University of KwaZulu-Natal.

**Processing for TEM samples:**

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<th>Primary fixation</th>
<th>24 hourss in 2.5% glutaraldehyde in 0.1M PBS (appendix)</th>
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<td>2X 2hourss Spurr resin</td>
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<td>Embedding</td>
<td>Orientate specimen in the mould in spurr resin and placed in oven for 8hourss at 70 degrees.</td>
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### 3.8 Immuno-staining of H37Rv and H37RvΔTlyA with TlyA specific immune mice serum

Mice polyclonal antibody was raised against purified TlyA. H37Rv and H37RvΔTlyA were harvested at late log phase, washed twice and bacteria were fixed with 2% paraformaldehyde in PBS, pH 7.4 for 20-30 mins. Bacteria were washed three times with PBS. Bacteria in PBS suspension was incubated with 1:50 dilution of TlyA immune mice serum for 1 hours at room temperature and washed thrice with PBS. Bacteria were resuspended in PBS and incubated with 1:100 dilution of Alexa Fluor-594 conjugated goat anti-mouse IgG for 45 mins and with DAPI for 2-3 mins, washed thrice with PBS. A thin smear of PBS suspended bacteria was made on the glass slide for observation with a confocal microscope.
3.9 Immuno-electron microscopy of wild type and mutants of M.tb with AntiTlyA
H37Rv and mutant of M.tb (BCG, H37RvΔRD1, H37RvΔESAT6, and H37RvΔTlyA) were harvested at log phase. Bacteria were washed twice with RPMI medium and fixed with 2% paraformaldehyde for 20 mins. After fixation bacteria were washed three times with RPMI medium and resuspended in 100μl of medium. Bacteria were stained with 1:50 dilution of antiTlyA antibody raised against purified TlyA and incubated for 1 hours at room temperature. Bacteria were washed thrice with RPMI medium and incubated with 1:50 dilution of 10 nm gold conjugated Protein A (TAAB: Code GEM020-10) for 1 hr at room temperature. Bacteria were washed three times with RPMI and the last wash with filtered water, and resuspended in water. A small drop of re-suspended bacteria was placed in nickel Grid. After 5 mins any excess of the resuspended bacteria were removed with a blotting sheet. Grids were dried under lamp and electron microscopic images were taken using a transmission electron microscope (Tecnai 12 BioTWIN, FEI, The Netherlands) at the electron microscopy facility of the ICGEB.

3.10 Isolation of genomic DNA and mRNA expression
Bacterial culture (50ml) were harvested at optical density of $A_{600}$ 0.5- 0.6 at 37°C by centrifugation at 4150 rpm for 7 mins. The pellet was resuspended by adding 6ml of freshly prepared chloroform-methanol (3:1) solution and vortexed until the bacteria were lysed as evident by a clear bottom layer. 6ml of Tris-buffered phenol (pH 8) was added and vortexed. 9ml of guanidinium thiocyanate buffer (GTC) solution (appendix) was added and vortexed. The sample was centrifuged at 10000x g for 10-15 mins and a clear supernatant was collected. DNA was precipitated out by adding equal volumes of isopropanol, mixed gently and centrifuged at 13-14,000 rpm for 10-15 mins. The pellet (sometime invisible) was suspended in 4 ml TE buffer and transferred to an eppendorf tube. The DNA was used for PCR with primers for tlyA gene (appendix).

3.11 mRNA expression
Total RNA was extracted from mid-exponential phase culture of different strain with an RNaseasy Minsi Kit (QIAGEN, Germany) according to manufacturer’s instructions. After DNase treatment, cDNA was synthesized using iScript cDNA synthesis kit (BioRad). Once cDNA is
synthesized, we used it as any other DNA as a template in PCR reaction (appendix) with gene specific primers (appendix).

3.12 Pull down assay
Bacteria were harvested at mid-log phase, washed and resuspended in PBS pH 7.4. Resuspended bacteria were lysed with bead beating technique (tubes with 250 µl of zirconia silica), as described by the manufactures protocol. Bacterial lysates were centrifuged at 14000 x g, 4°C for 20 mins. After centrifugation, supernatant was collected, protein concentration was checked using Bradford’s reagent (Bio-Rad, USA) and stored at -80°C until use. Equal amount (100 µg) of bacterial lysate were diluted in RIPA buffer. RIPA diluted bacterial lysate was pre-cleared with the protein A agarose beads for 20-30 minss at 4°C, followed by centrifugation and the supernatant were collected. For immunoprecipitation assay, anti-TlyA antibody (mouse raised antibody against purified TlyA) were added in a 1:50 fold dilution and incubated on ice for 30 minss. Immunocomplex were recovered by adding ProteinA agarose beads for 2 hourss with end-to-end rotating at 4°C. Beads were washed three times with RIPA buffer and pellets were re-suspended with 1xSDS containing sample loading buffer. This was boiled for 5minss at 100°C and resolved on 12% SDS-PAGE followed by western blot with anti-ESAT6 antibody.

3.13 Histology
Lung tissue samples were isolated from mice and washed thoroughly in PBS and fixed in 10% para-formaldehyde. Formaldehyde-fixed tissues were embedded in paraffin, and 5- to 6-µm sections were cut. Sections were stained with haematoxylin eosin (H&E) and Acid Fast Bacilli (AFB) stain and examinsed microscopically. The haematoxylin is a dark purple in color staining the chromatin within the nucleus and eosin stains is an orange-pink to red dye staining the cytoplasm material and connective tissue.

3.14 In Vivo M.tb infection of mice
Mice were infected with H37Rv as well as a mutant strain H37RvΔTlyA using a Madison aerosol chamber (University of Wisconsin, Madison, WI) pre calibrated to deliver a total of ~110 bacilli to per lung of each mouse. Stocks for various mycobacterial strains were taken out and thawed at room temperature. Each strain was ultra-sonicated to get a single cell suspension.
Strains were diluted with PBS to get 15 ml bacterial cell suspension containing $10 \times 10^6$ cells per ml. Suspension was attached with the nebulizer of the aerosol chamber that is already calibrated supply desired number of CFUs to the animals inside the chamber. After 24 hourss of aerosol infection three randomly selected mice were sacrificed to determinse the infectious dose in each experiment. To determinse viable number of CFUs, at various time points lungs and spleens were harvested and homogenized in 0.2 mm filtered PBS containing 0.05% Tween 80. Different dilutions (1:10, 1:100, 1:1000) of the lung and spleen homogenates were plated in duplicate on the 7H11 Middlebrook plates supplemented with 10% oleic acid, albumins, dextrose and catalase and incubated at 37°C for 15–21 days. Colonies were counted and CFU were estimated.

3.15 T cell proliferation assay

Spleens were isolated from uninfected, H37Rv and H37RvΔTlyA infected mice. Spleens were macerated by frosted slides in 10% RPMI 1640 and made into a single cell suspension. Red blood cells (RBCs) were lysed with RBC cell lysis buffer (appendix) and incubated at room temperature for three to five mins and washed with 10% RPMI 1460. Cells were counted and $0.1 \times 10^6$ cells per well were seeded in 96-well plates and induced with M.tb complete soluble antigen CSA at different concentrations. Cells were cultured for 48 hourss and then pulsed with tritiated thymidine ($^3$H-TdR, 1.0 μCi per well). One day later, cells were harvested on filter mats using a semi-automated cell harvester (Perkin–Elmer). Thymidine incorporation was determinsed by using a plate β-counter (Perkin–Elmer).

3.16 Flow cytometry: surface and intracellular staining

Spleens were isolated from H37Rv and H37RvΔTlyA infected mice and macerated by frosted slides in 10% RPMI 1640 and made into a single cell suspension. Red blood cells (RBCs) were lysed with RBC cell lysis buffer (appendix), incubated at room temperature for two to three mins and washed with 10% RPMI 1640. The cells were counted and $1 \times 10^6$ cells were used for surface staining. Cells were harvested and washed twice with PBS and stained with fluorescent antibodies directed against surface markers. For intracellular staining $1 \times 10^6$ cells were cultured per well in 24 well plates and activated with 50 ng/ml PMA (phorbol 12-myristate 13- acetate, and 750 ng/ml ionomycin overnight, and 10 mg/ml brefeldin A was added during the last 3 hourss of culture. After staining, cells were washed again with PBS and cells were fixed with
100 ml fixation buffer (appendix) for 15 mins, then re-suspended in 200 ml 1X permeabilization buffer (appendix) and stained with fluorescently conjugated monoclonal antibodies, anti-IL-4, anti-IL-17, anti-IFN-γ. Fluorescence intensity of fluorochrome-labelled cells was acquired and analysed by flow cytometry. Data analysis was performed by Flow Jo.

3.17 Generation of dendritic cells (DCs)
Mice were sacrificed with cervical dislocation and the femur bones were dissected out. Bone marrow cells from the femur were flushed out of the femur by using RPMI-1640 medium in a 2.0 ml syringe (26 gauge needle). The bone marrow cells were washed twice with PBS at 600xg for 5 mins at room temperature and then cultured in RPMI-1640 medium supplemented with 10% FCS. To differentiate bone marrow cells into myeloid DCs, 1 million cells were cultured in 24 well plate in the presence of complete RPMI media further supplemented with GM-CSF (40 ng/ml) and IL-4 (10 ng/ml). On day three, one third of the medium was discarded and replaced with fresh DC culture medium. On the fifth day, cells are washed with RPMI 1640 10% FCS to remove non adherent cells and adherent cells were collected as immature DCs. Flow cytometric analysis with CD11c-high and CD11b-low cells were >90% pure and were used infection purposes.

3.18 Detection of Cytokines
Bone marrow cells were isolated from mice (Balb/c), differentiated into immature DCs as mentioned above, and cultured in 24-well plates (1million cells/well). Cells were infected with H37Rv, H37RvΔTlyA at a multiplicity of infection of 1:10. Supernatants were collected at different time point 24 hourss, 48 hourss, and 72 hourss for cytokine analysis. Th1, Th2 and Th17 and dendritic cell secreted cytokines were assayed in the culture supernatant samples by a Luminex microbead-based multiplexed assay using commercially available kits according to the manufacturer’s protocol.

3.19 Quantitation of confocal images
Confocal microscopy images were captured using Nikon A-1R confocal microscope. For statistical data images were acquired using 60x objective and for the images 100x objective were used. Data was analysed using NIS element software and co-localization was studied after
background correction using the NIS element software. For one sample three slides were prepared, and for each slide at least 4 random fields containing ~10-15 cells were examined. For the kinetics, translocation of the bacteria into the cytosol of digitonin permeabilized cells was calculated. Bacteria in the cytoplasm of macrophages were counted from an average of 50 infected cells, which were accessible to the anti-M.tb antibody (red) and turned yellow after merging pictures. Experiments were run in triplicates and repeated three times.

3.20 Statistical analysis
All data were derived from at least three independent experiments. A value of p ≤0.05 was accepted as an indication of statistical significance. For all statistical analyses student”s t-test (two tales) was performed to compare two groups.
CHAPTER 4- RESULTS

4.1. Translocation of Virulent H37Rv from the Phagolysosome to the Cytosol in Murine Macrophages.

Macrophages where infected with FITC labelled virulent strain of M.tb H37Rv or BCG. After four hours, we observed that both H37Rv and BCG were phagocytised by macrophages in comparable numbers (Fig. 4A).

To investigate the localization of bacteria within the phagosomal compartment, cells were stained with antibodies against specific organelle endosomal marker Rab5 and the lysosomal marker, lysosomal associated membrane protein (LAMP-1) at different time points and analyzed for co-localization studies using confocal immunofluorescence microscopy. From the localization experiment we observed that all H37Rv and BCG co-localized with Rab5 or LAMP-1 during the first 24 hours, showing that they were present within phagosomal or phagolysosomal compartments. At 48 hours time point a small number of H37Rv did not show co-localization with either Rab5 or LAMP-1 or both, indicating that the bacteria were no longer present in phagolysosomal compartment, but had escaped to the cytosol (Fig. 4B-D). However, almost all BCG organisms showed co-localization with Rab5 and LAMP-1, suggesting that they remained within the phagolysosomal compartment (Fig. 4B-D). At 96 hours, about 30% of H37Rv had escaped into the cytosol of the macrophages and did not show staining with Rab5 or LAMP-1, whereas hardly any BCG were found in the cytosol (Fig. 4B).

To further confirm our hypothesis that these organisms were certainly present in the cytosol, we did differential permeabilization of macrophages with digitonin. H37Rv and BCG are stained with antibody against M.tb protein in the cell wall to detect the cytosolic bacteria as described by Collins and co-workers (Checroun et al., 2006; Collins et al., 2009). Digitonin selectively permeabilized the cell wall of macrophage keeping the membrane of the organelles intact, organisms if present in cytosol gets stained with anti M.tb antibody whereas M.tb in phagolysosome remains unstained.

After 72 hours, a large number of H37Rv bacilli were found bound to the M.tb cell wall protein antibody demonstrating their presence in the cytosol. In contrast BCG did not show any staining with the antibody as they remained within phagolysosomes making them inaccessible to the
antibody (Fig. 4E). The results of the differential permeabilisation with digitonin test, confirmed our hypothesis that these organisms were in the cytosol. We also compared the kinetics of *M.tb* escaping into the cytosol using this method. Similar to our findings with the Rab-5/LAMP-1 staining method, even in digitonin-permeabilized infected macrophages we were unable to detect any BCG in the cytosol (Fig. 4F). These observations suggest that transmigration from the phagolysosome to the cytosol is a characteristic feature of virulent *M.tb* strains.
Figure 4. Translocation of virulent H37Rv and not BCG from the phagolysosome to the cytosol in murine macrophages. (A) Average number of bacteria present per cell after 5 hours of infection. (B) Percentage of bacteria that did not co-localize with LAMP-1 and/or Rab5. Representative confocal images showing co-localization of *M. bovis* BCG with (C) LAMP-1 (red) or (D) Rab5 (red) but that some of the H37Rv organisms did not. (E) Digitonin permeabilized infected macrophages stained with rabbit anti-*M.tb* antibody followed by anti-rabbit IgG-Alexa594 (red). Bacteria that stained green in merged pictures are inaccessible to the antibody because they remain in the phagolysosomal compartment. DAPI (blue) stained the nucleus. The upper row showing H37Rv infected macrophages and the lower row *M. bovis* BCG. (F) Kinetics of the number of bacterial translocated into the cytosol of infected macrophages from 0 to 96 hours after infection.

4.2 Role of ESAT-6, within RD-1 Region, in translocation of *M.tb* to the cytosol.

Our results above show that virulent *M.tb* strain H37Rv can transmigrate from the phagolysosome into the cytosol whereas avirulent *M. bovis* BCG strain was unable to translocate. Translocation to the cytosol could be associated with the pathogenesis of *M.tb*. Genome analysis of *M.tb* and BCG has revealed that BCG possesses deletions of multiple genomic segments, which are known as regions of difference (RD). We therefore extended our
study to observe the translocation behaviour of H37RvΔRD-1 mutant of H37Rv within macrophages.

The translocation behavior of H37RvΔRD-1 mutants resembled that of BCG i.e. they were unable to escape to the cytosol showing co-localization with LAMP-1 and Rab5 (Fig. 5A-B). Similar results were found with digitonin-permeabilized infected macrophages, where H37RvΔRD-1 mutants could not bind with the M.tb cell wall protein antibody as they remained within the phagolysosomal compartment (Fig. 5C). Kinetics of digitonin-treated cells showed that after 96 hours 30-35% of the intracellular H37Rv had translocated from the phagosome to the cytosol, whereas H37RvΔRD-1 remained in phagolysosomal compartment (Fig. 5D). The RD-1 region is also responsible for secretion of two important mycobacterial secretory proteins viz: ESAT-6 and CFP-10. (Brodin et al., 2006; Guinn et al., 2004). Similar to the H37RvΔRD-1 mutant, a deletion mutant of ESAT-6 (H37RvΔESAT-6) resembled BCG in its biological functions, and was also responsible for mycobacterial virulence (Kaku et al., 2007; Tan et al., 2006). We therefore performed co-localisation studies with H37RvΔESAT-6 bacteria. H37RvΔESAT-6 bacteria showed co-localization with LAMP-1 and Rab5 suggesting that H37RvΔESAT-6 were also unable to escape to the cytosol (Fig. 5A-D).
Figure 5. H37Rv mutants H37RvΔESAT-6 and H37RvΔRD-1 unable enter the cytosol of macrophages. M.tb mutants H37RvΔRD1 and H37RvΔESAT6 showing co-localization (yellow after merge) with (A) LAMP-1 (red) or (B) Rab5 (red), after 72 hours of infection. H37Rv (green after merge) showed no co-localization (C) Infected cells selectively permeabilized with digitonin and stained with rabbit anti-M.tb antibody followed by anti-rabbit IgG-Alexa 594 (red). H37Rv in the cytosol accessible to the antibody staining red or yellow after merge. H37RvΔRD1 and H37RvΔESAT6 in phagolysosomes not stained by antibody remained green after merge. DAPI (blue) for staining nucleus. (D) Kinetics study of number of bacteria translocated to the cytosol of macrophages from 0 to 96 hours after infection.
4.3 TlyA helps M.tb to escape from phagosomal compartment to the cytosol

Recently, it was shown that the TlyA protein of M.tb has hemolytic activity which binds and oligomerizes on the membranes possibly to form heptameric pore like structures (Rehman et al., 2012). We therefore sought to explore if TlyA has any role in the translocation of M.tb from the phagolysosome into the cytosol. We observed that H37Rv showed no co-localization with Lamp-1 or Rab-5 (Fig 6A-B, Upper-row) whereas H37RvΔTlyA similar to BCG, showed co-localization with Lamp-1 and Rab-5, and were unable to escape from phagolysosome to cytosol (Fig 6A-B Lower-Row). These findings were further strengthened by our observation that in partially permeabilized macrophages, H37Rv that escaped from the phagolysosome to cytosol were available for staining with anti M.tb antibody (Fig 6C Upper row) whereas H37RvΔTlyA remain unstained due their presence in phagolysosome making them inaccessible to the anti M.tb antibody (Fig 6C Lower row). We observed that by 96 hours 25-30% of H37Rv escaped into the cytosol but no H37RvΔTlyA were observed in the cytosol (Fig 6D), making this evident that TlyA may play a role in facilitating the escape of M.tb from phagolysosome into cytosol.
Figure 6. TlyA helps *M*. *tb* to escape from phagosomal compartment to the cytosol. FITC labeled H37Rv and H37RvΔTlyA were infected on macrophages isolated from peritoneal cavity of C57BL/6 mice and after 72 hours of incubation, infected macrophage were observed using a confocal microscope to study *M*. *tb* localization within cells. (A) LAMP1 (Red) and (B) Rab5 (Red). (C) Digitonin treated partial permeabilized infected macrophage showed a population of *M*. *tb* outside the phagosome. Antibody against *M*. *tb* was used to stain cytosolic *M*. *tb* (Red). Yellow color in the “merged” section showed co-localization of the bacteria translocated to the cytosol. Upper panel of each section was H37Rv and the lower panel was H37RvΔTlyA infected macrophage. (D) Kinetics of bacterial translocation to the cytosol of infected macrophages from 0 to 96 hours after infection. This was calculated from confocal studies with digitonin-permeabilized cells.

To further confirm the role TlyA in translocation of *M*. *tb* from phagolysosome to the cytosol, we used electron microscopy. Consistent with the results obtained with confocal microscopy,
electron microscopy also demonstrated the escaping of H37Rv from phagolysosome to cytosol (Fig. 7 A) whereas H37RvΔTlyA remained in phagolysosome (seen inside a membranous structure) (Fig. 7 B).

Figure 7. TEM images of Raw 264.7 macrophage infected with H37Rv and H37RvΔTlyA for 72hrs and then fixed and prepared for TEM. (A) H37Rv in cytosol (B) H37RvΔTlyA inside phagolysosome. Red box show magnification of selected area showing presence or absence of phagosomal membrane.
4.4. Surface localization and secretion of TlyA from M.tb H37Rv

To ascertain the localization and secretion of TlyA from *M.tb* we performed immunostaining of live H37Rv and H37RvΔTlyA with polyclonal antibody against TlyA raised in mice. We observed the TlyA on the cell wall of H37Rv whereas absent in H37RvΔTlyA (*Fig 8*).

![Immuno-staining](image)

**Figure 8. TlyA expressed and localized on the surface/ cell wall of H37Rv.** Immuno-staining of live H37Rv with polyclonal antibody against TlyA raised in mice and Alexa-fluor 595 nm Conjugated Anti-Mouse IgG. TlyA was present on the cell wall of H37Rv (Red, Upper panel) and absent in mutant H37RvΔTlyA. DAPI is blue stained for genomic DNA.

We also examined the surface localization of TlyA with immune-electron microscopy. Both H37Rv and H37RvΔTlyA were cultured, fixed and then immune labeled for anti TlyA antibody with 10 nm gold particles. TEM images clearly show the presence of TlyA on the cell wall and its surrounding (implying secretion) in H37Rv, (*Fig 9. A*) whilst this was absent in H37RvΔTlyA (*Fig 9. B*)
Figure 9. Immuno-electron microscopy of paraformaldehyde fixed wild type H37Rv and mutants of *M. tb* H37RvΔTlyA with anti-TlyA and 10 nm gold conjugated protein A and observed under TEM. (A) TlyA was present on the cell wall and secreted from the H37Rv, whereas (B) Absence of TlyA from H37RvΔTlyA

We observed the surface localization of TlyA in BCG and other mutants of *M. tb*. As expected neither BCG nor H37Rv mutants ΔESAT6, ΔRD1 showed any secretion of TlyA from their cell wall (Fig. 10 A, B & C).
Figure 10. Immuno-electron microscopy of paraformaldehyde fixed mutants of *M.tbc* H37RvΔESAT-6 (A) and H37RvΔRD-1 (B) and BCG (C) with anti-TlyA and 10nM gold conjugated protein A and observed under TEM.

4.5. Insertion of RD1 region in *M. bovis* BCG restored TlyA secretion

We performed the localization study using a live strain of *M.bovis* BCG with a recombinant RD1 region reintroduced (BCG::RD1). Electron microscopy images revealed that after reintroduction of RD1 region in BCG, it was able to secrete TlyA similar to H37Rv making it evident that RD1 region is responsible for the secretion of TlyA (Fig. 11A).
Figure 11. (A) RD1 region complemented in BCG was restored TlyA secretion. Live BCG::RD1 was fixed with paraformaldehyde and immuno-stained with anti-TlyA showed secretion of TlyA from the intact bacterial surface as seen under the transmission electron microscope. (B) FITC labeled BCG::RD1 was infected on macrophage isolated form peritoneal cavity of C57BL/6 mice and after 72 hr of incubation, infected macrophage was observed on confocal microscope to studies its localization within cells. BCG::RD1 infected macrophage stained for the LAMP-1 (Red, upper panel) and Rab5 (Red, middle panel), colocalization of FITC labeled bacteria with LAMP1 and Rab5 was observed in merge (right panel). (C) Digitonin treated partial permeabilized infected macrophage showed a population of M.tb outside the phagosome (lower panel). Antibody against M.tb was used to stain cytosolic M.tb (Red). Yellow color in the “merged” section showed co-localization of the red fluorescent labeled molecules with FITC labeled bacteria. Images are representative of three independent experiments.

We also performed co-localization studies after 72 hours of infection with BCG::RD1. We observed BCG::RD1 was able to translocate from phagolyosome into the cytosol showing co-
localization with LAMP1 and Rab 5 (Fig. 11 B). Also, BCG::RD1 showed staining with the anti-Mtb antibody indicating its presence in cytosol (Fig. 11C).

4.6 Interaction between TlyA and ESAT-6

Our results demonstrated the presence of tlyA gene and its mRNA expression in H37Rv and its mutants, except in H37RvΔTlyA mutant (Fig. 12A).

Figure 12. (A) DNase-treated total RNA was extracted from mid-exponential-phase culture of (1) M.tb H37Rv, (2) M. bovis BCG, (3) H37RvΔRD-1, (4) H37RvΔESAT-6 and (5) H37RvΔTlyA to show mRNA expression of TlyA from tlyA-specific RT-PCR. Genomic DNA was also extracted from the same culture of Mycobacterium sp. and mutants to carry out PCR and show the presence of tlyA gene in their respective genome DNA (100 ng) of (6) M.tb H37Rv, (7) M. bovis BCG, (8) H37RvΔRD-1, (9) H37RvΔESAT-6 and (10) H37RvΔTlyA. tlyA gene was present in genome and showed its mRNA expression in all the Mycobacterium sp. and mutants except in H37RvΔTlyA mutant. (B) Pull-down assay of ESAT6 with TlyA was shown in mid-log-phase growth of H37Rv, H37RvΔRD-1 and H37RvΔTlyA bacterial cell lysate. Immunoprecipitation with anti-TlyA and immunoblot with anti-ESAT6 antibody (Upper panel). Lower panel of (E) showed the expression of ESAT6 in H37Rv, H37RvΔRD-1 and H37RvΔTlyA bacterial cell lysate.
We also performed a pull down assay ie: a method to ascertain which proteins or group of proteins interacts with one another. We used TlyA as the bait to pullout interacting proteins from bacterial lysates of H37Rv, H37RvΔRD-1 and H37RvΔTlyA. We did the immunoprecipitation with anti-TlyA and then immunoblotting with an anti ESAT6 antibody (Fig. 12B). Our pull down assay and immunoprecipitation results suggested an interaction between TlyA and ESAT-6.

4.7 H37RvΔTlyA exhibit dramatically lower granulomatic lesion
We harvested the lungs and spleen of mice infected with \textit{M. tb} H37Rv and H37RvΔTlyA for analysis of bacterial load and cytokine expression at different time points. We found that mice infected with H37RvΔTlyA developed lower numbers of granulomatous lesions than mice infected with H37Rv (Fig. 13A). To further strengthened our findings we did histological analysis and found that the granulomatous regions in lungs of mice infected with H37RvΔTlyA, were less compared with H37Rv (Fig. 13B). The number of granulomas in the lungs (Fig. 13C) as well as, the presence of acid fast bacilli in the lung sections was also lower in H37RvΔTlyA (Fig.13D). When we compared the CFU in the lungs and spleens of H37Rv and H37RvTlyA infected mice sacrificed at different time points after infection,, we observed that during the first phase of the infection both H37Rv and H37RvTlyA replicated to a similar extent; but, at later time points growth of H37RvΔTlyA bacilli gradually reduced both in lungs and spleen (Fig. 13E&F).
Figure 13. General analysis of lungs and spleen of H37Rv- and H37RvΔTlyA-challenged Balb/c mice. Balb/c mice were challenged with H37Rv or H37RvΔTlyA by the aerosol route and
lungs and spleen of the infected mice were harvested at different time points. (A) Gross pictures of lungs of uninfected, H37Rv-infected and H37RvΔTlyA-infected mice. (B) Histology of the lung tissue sections at 45 days post infection stained with haemotoxylin & eosin. (C) Numbers of granulomas in lungs at different time points after infection. (D) Acid fast staining of Bacilli. (E) CFU from the lung homogenates of H37Rv- and H37RvΔTlyA-infected mice at different time points. (F) CFU from spleenocytes of mice that were infected with H37Rv or the H37RvΔTlyA mutant. The results shown are representative of three independent experiments with five mice per group per time point.

4.8 H37RvΔTlyA induces enhanced antigen-specific Th cell activation

In our previous experiment we showed that only during the late phase of infection, H37RvΔTlyA mutants exhibited enhanced clearance in the lung and spleen of the infected mice, suggesting that TlyA plays a role in inhibiting the adaptive immune responses during disease progression. We therefore examined the status of the adaptive immune components in animals infected by H37RvΔTlyA. As expected, we observed significantly higher T cell proliferative responses upon a in-vitro challenge with complete soluble M.tb antigen (CSA) in mice infected with H37RvΔTlyA, as compared with H37Rv (Fig. 14A). This was supported by the higher prevalence of activated CD4+ T cells and CD8+ T cells in H37RvΔTlyA-infected mice, as extrapolated from CD69 expressing cells. These results suggested that deletion of TlyA in H37Rv promotes activation and proliferation of antigen-specific CD4+ T cells in infected animals (Fig. 14B&C). It is now clear that Th1 and Th17 cells play host-protective roles whereas Th2 and Treg cells potentiate disease progression. We therefore evaluated whether H37RvΔTlyA induces a biased Th response. We found that H37RvΔTlyA-infected animals produced dramatically higher numbers of IFN-γ- and IL-17-producing cells, whereas IL-4-producing cells were significantly reduced as compared with H37Rv-infected mice (Fig. 14D&E). We also observed that H37RvΔTlyA induced a significantly reduced Treg responses compared to H37Rv (Fig. 14F).
Figure 14. T cell proliferation and FACS analyses of T cell subsets. (A) T cell proliferation from spleen of H37Rv- and H37RvΔTlyA-infected mice. (B&C) FACS analysis shows the percentage of CD4+ T cells, CD8+ T cells and activation marker CD69- and CD25-positive cells in M.tb-infected mice. (D&E) T cells secreting IFN-γ, IL-4 or IL-17 among spleenocytes of M.tb infected mice. (F) Percentage of Treg cells (FoxP3+ CD4+CD25+ cells) among spleenocytes of M.tb-infected mice. The results shown are representative of three independent experiments with three mice per group per time point.
4.9 H37RvΔTlyA induces both Th1- and Th17-mediated immune responses

From the previous experiments, we concluded that deletion of the TlyA gene from H37Rv results in significantly higher Th1 and Th17 cytokine-producing cells, indicating that TlyA directly or indirectly regulates these host protective immune responses.

To further understand the mechanism whereby the deletion of TlyA induces Th1 and Th17 differentiation, we compared the cytokines produced by dendritic cells (DCs) when infected with H37Rv or H37RvΔTlyA. DCs are characterized with various markers- CD11c, CD11b, CD80, CD86, and MHC class II markers. We found that DCs infected with H37Rv produced significantly higher amounts of IL-1β, IL-10, and TNF-α than DCs infected with H37RvΔTlyA (Fig. 15). Additionally, we observed significantly higher amounts of IL-12p40, a key cytokine for Th1 cell differentiation, in the supernatant of DCs infected with H37RvΔTlyA, compared with DCs infected with H37Rv (Fig. 15). In addition, we observed that both H37Rv and H37RvΔTlyA induced equal amounts of IL-6 and TGF-β (Fig. 15), a cytokine that orchestrates Th17 cell differentiation. These findings imply that deletion of TlyA from H37Rv makes for an environment that is conducive for the differentiation of both Th1 and Th17 cells. We therefore concluded that TlyA inhibits Th1 and Th17 cell differentiation, either directly or indirectly.
Figure 15. Cytokine profiling of H37RvΔTlyA-infected dendritic cells. Dendritic cells (DCs) of mice were infected with H37Rv or H37RvΔTlyA and cytokines from culture supernatant were assayed at different time points, and compared to uninfected DCs. Luminex assay showing the cytokine concentration of IL-1β, IL-12p40, IL-6 and TNF-α and TGF-β in the culture supernatants of H37Rv- (■) or H37RvΔTlyA-infected (▲) DCs compared with uninfected DCs (◆). The results shown are representative of at least three independent experiments with three replicates.
CHAPTER 5-DISCUSSION

Virulence of *M. tb* is highly complex and not clearly understood. Leake *et al.* (1984) were the first to show that destruction of the phagosome is one of the virulent mechanisms exhibited by H37Rv after they are ingested by alveolar macrophages whereas BCG infected alveolar macrophages did not exhibit this phenomenon (Leake *et al.*, 1984). This mechanism of virulence of *M. tb* has been ignored for many years. However there are reports of bacteria from different species like *Listeria monocytogenes, Shigella flexneri, Burkholderia pseudomallei, Francisella tularensis* and *Rickettsia spp.*, that have exploited translocation as their virulence mechanism (Ray *et al.*, 2009). Recently, a group of scientists have shown that *M. tb* can escape from the phagolysosomes into the cytosol in DCs (van der Wel *et al.*, 2007). However it is known that DCs are not the primary site for *M. tb* replication, nor do they play any role in killing bacteria by producing nitric oxide (NO) and reactive oxygen intermediates (ROI). In contrast, in another experiment it was observed that a small number of *M. marinum*, a virulent strain of *Mycobacterium* species found in fish, can translocate into the cytosol of macrophages (Smith *et al.*, 2008; Stamm *et al.*, 2003; Stamm *et al.*, 2005). Therefore, we revisited these issues with primary macrophages which are not only the primary site for *M. tb* replication, but also exhibit bactericidal activity by producing ROI and NO. We observed that during the initial 24 hours after infection of macrophages, nearly all mycobacterial strains, irrespective of their virulence status, were present in phagolysosomal compartments. At later time points of infection almost 30% of H37Rv were present within the cytosol while the majority of the BCG bacilli remained in phagolysosome. It has been well documented that RD-1 is the key region, present in *M. tb* but absent from the attenuated BCG vaccine, responsible for the loss of virulence in BCG (Brodin *et al.*, 2006; Lewis *et al.*, 2003). It has also been shown that RD-1 deleted H37Rv resembles BCG in many biological functions, including infectivity and survival in the host (Lewis *et al.*, 2003). Additionally, ESAT-6 within the RD1 region is secreted via the ESX-1 secretion system can cause lysis of the plasma membrane, allowing *M. tb* to spread into uninfected macrophages (Smith J *et al.*, 2003; Gao *et al.*, 2004; Guinn *et al.*, 2004; Koo *et al.*, 2008; Junqueira-kipnis *et al.*, 2006). Furthermore, ESAT-6 is involved in translocation of *M. marinum* organisms from the phagolysosome to the cytosol (Smith *et al.*, 2008). These findings prompted us to evaluate the translocation of H37RvΔRD1 and H37RvΔESAT-6 mutant of H37Rv within macrophages. We found that like BCG, H37RvΔRD1 and H37RvΔESAT-6 mutants were unable to translocate into
the cytosol, suggesting that ESAT-6 plays an important role in the transmigration of *M. tb* into the cytosol. We therefore established that escaping to cytosol is one of the virulence mechanism adopted by H37Rv.

The mycobacterial protein TlyA is a virulence determinsant of many pathogenic bacteria. It is present in virulent strains but absent from avirulent strains of *M. tb*. It is also known to assemble into ordered oligomers capable of permeabilizing the membrane that comes in contact with it (Rehman *et al.*, 2010). However, the role of TlyA in *M. tb* pathogenesis has not been previously investigated. TlyA protein is expressed intra-cellularly, as well as on the cell envelope of *M. tb*. The extra cellular form behaved like a non-conventional membrane damaging protein and was found to exhibit contact-dependent lysis of human and rabbit erythrocytes (Rehman *et al.*, 2010). We therefore explored the virulence of this protein in terms of its capacity to translocate *M. tb* into the cytosol. Our confocal and electron microscopy data is suggestive that TlyA seems to play a role in the escape of *M. tb* contributing to its virulence. We can therefore predict that ESAT-6 and TlyA capable of lysing the cell or membrane may causes disruption of phagosomal membrane leading to free cytosolic *M. tb*.

Protein secretion is one of the important mechanisms involved in bacterial functioning in their natural environment. It has been previously shown that TlyA when expressed in *E. coli* was present on the outer cell wall (Rehman *et al.*, 2010). Therefore, for validating the role of TlyA of *M. tb* in destabilizing the phagosomal membrane, it is important that TlyA be present on the surface of the mycobacterial envelope or secreted out. Immunostaining of live H37Rv and H37RVΔTlyA with polyclonal antibody against TlyA revealed that TlyA is present on the cell envelope, as well as secreted out from the cell wall. However, no secretion of TlyA was observed in *M. bovis* BCG, H37RvΔRD-1, H37RvΔESAT-6 and H37RvΔTlyA strains. This further supports the role of TlyA in the translocation of *M. tb*.

The RD-1 region present in *M. tb* and absent in *M. bovis* BCG is responsible for delivering virulence proteins during infection. We demonstrated that when the RD1 region is inserted into *M. bovis* BCG, TlyA secretion was restored and it triggered the cytosolic translocation of bacteria, which in turn increases the virulence of the bacterium. Collectively, our data, demonstrates that TlyA may be present outside the RD-1 region but is secreted via RD-1 secretion system, and along with ESAT-6 it destabilize the membrane resulting in the escape of *M. tb* into the cytosol.
We found the presence of tlyA gene as well as its mRNA expression in H37Rv, *M. bovis* BCG, H37RvΔRD-1 and H37RvΔESAT-6 strains. This indicates that there is a presence and expression of the tlyA gene in all the strains mentioned above but they are unable to secrete TlyA due to lack of the RD1 secretion system. It has been well documented that ESAT-6 is secreted via a RD1 region and is responsible for the virulence of *M.tb* (Guinn KI et al., 2004; Lewis KN et al., 2003). We also previously showed that both TlyA and ESAT-6 leads to translocation of *M.tb*. Therefore we wanted to investigate any interaction between TlyA and ESAT-6. The pull down assay with TlyA followed by immune-precipitation with anti-TlyA allows us to establish that TlyA interacts with ESAT-6 and may contribute in the the pathogenesis of *M.tb*. However the exact mechanism of this interaction is still to be resolved.

Furthermore, we extended our study to elucidate the mechanism behind the virulence associated with TlyA. Virulence may be determined by growth of *M.tb* inside the host, histopathology and survival of the host after bacterial infection. We infected a group of mice with both H37Rv and or H37RvΔTlyA. Gross inspection of the spleen and lung from the H37Rv-infected and H37RvΔTlyA-infected mice, and the histopathology data, showed less granulomatous lesions and bacilli in the lung of mice infected with H37RvΔTlyA than the mice infected with H37Rv. We also observed that the growth of H37RvΔTlyA in mice was similar to the H37Rv strain during the initial phase of infection, but that the bacteria were rapidly cleared at later stages of infection. This observation suggests that TlyA plays a role in inhibiting the adaptive immune responses during disease progression. H37RvΔTlyA infected mice also showed enhanced antigen specific Th cell proliferation. It has been documented that immunity against *M.tb* in the host depends on Th1 and Th17 cells while Th2 and Treg cells leads to enhanced susceptibility to *M.tb* infection. Cytokine analysis revealed that H37RvΔTlyA mutant induces enhanced host protective Th1 and Th17 responses, suggesting that TlyA inhibits such responses during *M.tb* infection to subvert adaptive immunity. This modulation of adaptive immunity is mediated by cytokine regulation in infected cells. H37RvΔTlyA selectively alters expression of IL-12, suggesting that TlyA was evolutionarily acquired by mycobacteria to combat host immunity. Our findings in this study reveal that TlyA plays an important role in the survival of *M.tb* bacilli during infection. Therefore TlyA warrants consideration in the design of therapeutic strategies and antibodies for the control of *M.tb* infection.
CHAPTER 6- CONCLUSION

*Mycobacterium tuberculosis* (*M.tb*) is one of the most ancient human pathogens known to have co-evolved along with the human immune system to successfully persist within its host. *M.tb*, the etiological agent causing tuberculosis infects one third of the world’s population claiming 1.5 million lives annually. *M.tb* genome encodes for nearly 4000 proteins; functions and mechanism of action of many of them are not known. The secreted proteins of *M.tb* have been shown to play an important role in disease pathogenesis with some even acting as virulence factors. Among the many unexplored gene products of *M.tb*, TlyA was recently identified as a possible virulence gene in *M.tb*.

Our result described here, indicated that TlyA interacts with ESAT-6, another virulent secretory protein of *M.tb* and together they mediate cytosolic translocation, a pathogenic process, by directly interacting with the host membrane. In this study we have established that TlyA is expressed and localized on the surface or cell wall of virulent strain H37Rv but absent in avirulent BCG and its secretion is dependent on RD-1 region.

We further demonstrated that the TlyA protein contributes to the pathogenesis of *M.tb* by inhibiting host-protective, adaptive immune responses. Cytokine analysis revealed that TlyA inhibits host protective Th1 and Th17 responses. Furthermore we observed absence of TlyA in *M.tb* resulted in a marked decrease of virulence with bacterial counts and reduced pathology in the lung and spleen when compared with the WT strain in a murine model. Taken together our data suggest that TlyA could be a crucial virulence factor for intracellular survival of *M.tb*. Therefore elucidation the role of TlyA in TB pathogenesis is indeed on of the major advancement in providing a better understanding of host-pathogen interaction and also capable of designing new therapeutics and vaccines against *M.tb* infection.
REFERENCES


de Chastellier, C., et al. (2009). "Mycobacterium requires an all-around closely apposing phagosome membrane to maintain the maturation block and this apposition is re-established when it rescues itself from phagolysosomes." *Cell Microbiol* **11**(8): 1190-1207.


APPENDIX

REAGENTS

Thioglycollate Brewer modified BD, FITC -Sigma-Aldrich, mouse anti-Rab5- Cat. No. 610282; BD bioscience, rat anti-LAMP1- Cat. No.553792; BD bioscience, Alexa Fluor 594 conjugated goat anti-mouse IgG Cat. No. A-11032; Invitrogen, and Alexa Fluor 594-conjugated rabbit anti-rat IgG- Cat. No. A-21211; Invitrogen, Gold antifade reagent Cat. No. P36934; Invitrogen, digitonin D141, Sigma-Aldrich, anti-*M.tb*, ab905, Abcam, Alexa 545-conjugated goat anti-rabbit IgG Invitrogen, Carlsbad, CA, 10nm gold conjugated Protein A TAAB: Code GEM020-10. Luminex kits were purchased from Bio-Rad or Millipore. Recombinant mouse GM-CSF, IL-2 and IL-4 both were obtained from R&D Biosystems, USA. Fluorescence-tagged antibodies against mouse CD4, CD8, CD25, CD28, CD69, CD80, CD11b, CD11c, MHC class II. IFN-γ, IL4, IL-17, Foxp3, and IgG2a isotype controls were from BD Biosciences, eBiosciences and Biolegend. Recombinant mouse GM-CSF and IL-2 was from R&D Systems. Purified anti-IL1β, anti-TNFα, anti-IL6, anti-IL-12p40 and IL-10 were obtained from eBiosciences Ltd. Tritiated thymidine was purchased from PerkinElmer Inc. Multiplex cytokine detection kits were procured from Millipore and BioRad. RPMI-1640 medium purchased from Invitrogen life technologies. Fetal Calf serum (FCS) was purchased from Hyclone. Middlebrook 7H9 liquid media, 7H11 agar, Albumins-Dextrose-Catalase (ADC) and Oleic acid-Albumins-Dextrose-Catalase (OADC) supplement, were obtained from Difco-Becton-Dickinson. Phorbol 12-myristate 13-acetate (PMA) and Ionomycin were procured from Sigma Aldrich. Primers from sigma, cDNA synthesis kit (Omniscript), SYBR green enzyme, Random hexamers, RNase inhibitor, DNA oligonucleotides were obtained from MBI Fermentas. Fine chemicals, primers and antibiotics used in the study were procured from Sigma Aldrich.

GTC solution: 4 M Guanidium thiocyanate, 100 mM Tris pH 7.5, 1% β-mercaptoethanol (add freshly), 0.5% Sarcosyl (w/v)

RIPA buffer: 50mM Tris-HCl pH 8.0, 150mM NaCl, 1% NP40, 1mM PMSF, 5mM EDTA, 1X protease inhibitor cocktail).
**Phosphate Buffer Saline (PBS, pH 7.4):** 13.7 mM NaCl, 0.27 mM KCl, 10 mM Na$_2$HPO$_4$ and 0.2 mM NaH$_2$PO$_4$.

**RBC lysis Buffer:** 1.6gm NH$_4$Cl, 0.2gm KHCO$_3$, 0.03gm EDTA were dissolved in 100ml distilled autoclaved water. The solution is filtered sterile and can be stored for one month maximum.

**Wash Buffer:** 0.5% BSA, 0.1% sodium azide in PBS, filtered to remove particulates.

**Fixing Solution:** 2% Paraformaldehyde solution in PBS, filtered to remove particulates.

**Permeabilization Buffer:** 0.1% saponin, 1% fetal calf serum (FCS) in PBS.

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Dilute the combined 1M stock solutions to 1 liter with distilled H₂O

**Primer used PCR of TlyA gene:**

TlyF- 5’GATCCTCGGGTGGTGTC

TlyR- 5’CCTCAAACTGCGGCTTCACC

**Composition of PCR mixture and PCR conditions were**

- Polymerase buffer (10X) - 2.0 μL
- Forward primer (10 μM) - 0.5 μL
- Reverse primer (10 μM) - 0.5 μL
- Template DNA - 4 μl (50 ng)
- dNTPs (10 mM each) - 0.5 μL
- Pfu DNA polymerase (1U/ μL) - 0.5 μL
- Water (double distilled) - 12.0 μL

Total 20.0 μL

Conditions used for PCR were as follows, 95°C- 3 mins, 95°C- 30 sec, 55°C-30 sec, 72°C- 1 mins for 40 cycles in a Bio-Rad Industries Gradient PCR machine.

**Composition of cDNA synthesis reaction**

Set up three reactions in three separate PCR tubes on ice.
10X Reaction buffer (Exiqon kit) - 2μl

dNTPs (5mM) - 2μl

Random hexamers (10μM) - 2μl

RNase inhibitor - 1μl

Reverse transcriptase - 1μl

Template RNA 200ng - 3μl

Water (double distilled) - 9μl

Incubate the reaction for 60 mins at 42°C, 5 mins at 95°C. Store the reaction at -20°C.

Conditions used for PCR were similar as mentioned above.