Chapter I

Introduction
1.1 The Bacteria

The genus Mycobacterium comprises mostly of soil dwelling saprophytes, and only a few members of this genus are pathogenic (Cosma et al., 2003). Pathogenic mycobacteria cause diseases of diverse nature and varying severity. The disease Tuberculosis (TB) is caused by Mycobacterium tuberculosis (Mtb) or the other closely related species M. bovis and M. africanum. Together these bacteria constitute the Mtb complex (MTC). Mtb is an obligate aerobic, slender, non motile bacillus approximately 2-4μm in length that can survive and multiply inside macrophages (MΦs) and other mammalian cells. Other members of pathogenic mycobacteria include M. leprae, which are the causative agent of the human disease leprosy, M. marinum, which causes granulomatous infection in frogs and fish as well as skin lesions in humans (called swimmer’s granulomas) and M. ulcerans, which causes Buruli ulcers. Finally, members of M. avium complex (M. avium subspecies avium, paratuberculosis and silvaticum and M. intracellulare) can occur as opportunistic pathogens especially in immuno-depressed patients (Cosma et al., 2003).

Mycobacteria has been classified as a Gram-positive bacteria, but unlike other Gram-positive bacteria they have evolved a very complex cell wall, comprising of arabinogalactan-peptidoglycan polymer covalently bound to mycolic acids, a large variety of extractable lipids (Barry et al., 1998; Daffe and Draper, 1998) and pore forming proteins (Niederweis, 2003). Most of the mycobacterial lipids are part of the cell envelope, acting as an extraordinarily efficient permeability barrier and making mycobacteria resistant to most drugs (Brennan and Nikaido, 1995). The cell wall consists of mycolic acid and other complex lipopolysaccharides, which gives it an acid-fast staining property. Mutants and treatments affecting mycolic acid biosynthesis and the production of extractable lipids had shown an increase in cell wall permeability and a drastic decrease of virulence, underlining the importance of the integrity of the cell wall for intracellular survival of Mtb (Barry et al., 1998).

1.2 The Disease

TB is a major health problem with up to one third of the world’s population infected with Mtb. It is responsible for 1.6 million deaths every year and each year 8.8 million new patients are diagnosed with active TB (Jossy et al.; 2009). One additional problem is that Mtb has the capacity to remain viable within infected hosts for a prolonged time. Mtb infection of a host is initiated following the inhalation of droplets (aerosols) containing a small number of bacilli (Kaufmann, 2001). Mtb is highly infectious: once inside the lung,
bacilli get internalized through phagocytosis by lung and alveolar macrophages. Activated alveolar macrophages can effectively transfer the phagocytosed Mtb to the lysosomes, but some bacilli are able to escape lysosomal delivery and survive within the macrophage (Armstrong and Hart, 1975; Kaufmann, 2001; Russell, 2001). Mycobacterial infection leads to formation of tubercules in the affected parts. These tubercules are formed by accumulation of defense cells of host around the bacilli in an attempt to destroy or contain these bacilli. The pathogen effectively exploits phagocytes; where in they invade, replicate and persist (Beatty and Russell, 2000).

Fig. 1: Schematic representation of the mycobacterial cell envelope, consisting of the inner membrane (IM) and the cell wall. This representation is based on the model proposed by Minnikin (1982). Mycolic acids are covalently linked to the arabinogalactan–peptidoglycan (AG-PG) co-polymer and are thought to contribute to the inner leaflet of the asymmetrical outer membrane (OM).

In most cases, the bacterium and host establish equilibrium and infected individuals can remain asymptomatic for several decades, if not for their entire life time (Kaufmann, 2006). However, only about one-tenth of the infected individuals develop the disease (World Health Organization, 2007). In several cases, this might be due to weakening of the host immune system caused by human immunodeficiency virus (HIV), malnutrition or old age shifting the equilibrium in favour of bacterium (Chan and Flynn, 2004). Mycobacteria prevent the normal phagosomal maturation (Beatty and Russell, 2000; Clemens and Horwitz, 1995; Armstrong and Hart, 1971) and fusion with lysosomes (Bach et al., 2008;
Beatty and Russell, 2000; Frehel et al., 1986). The mycobacterial phagosome also fails to acidify due to lack of accumulation of proton ATPase complexes (Beatty and Russell, 2000; Sturgill-Koszycki et al., 1996).

1.3 The Diagnosis

Diagnosis of TB is a trivial task, by the time patients come to a diagnostic facility the bacilli can be readily seen with a microscope in sputum smear using inexpensive stains that can differentiate acid-fast bacilli. Delays in diagnosis of 3–6 months are common because of the lack of sensitivity of microscopy (Madebo and Lindtjorn, 1999; Liam, and Tang, 1997), during which the disease can progress and cause severe destruction of the airways and transmission continues. At present, there are only three methods with proven clinical utility for the diagnosis of TB: microscopy, culture and nucleic acid amplification. None of these approaches are simple enough to be used in primary health clinics in the developing world. There has been a great deal of progress in developing better tools to detect latent TB (Lalvani, 2007; Menzies et al., 2007; Porsa et al., 2007; Pai et al., 2006), but tools to detect active disease will have greater impact in countries with highest burden of disease. Detection of mycobacterial antigens is an attractive approach to detect TB, with theoretical benefits of high specificity, correlation to mycobacterial burden and independence from immune function. Thus, developing simple point-of-care tests for TB requires either the dramatic simplification of one of the three current TB diagnostic methods or the successful development of tests based on the detection of diagnostic antibodies or TB-specific antigens. Both of these options present important technical challenges (Young et al., 2008).

1.4 Treatment

The cell wall components are the major target for most of the antibacterial therapeutics developed for mycobacteria. Ethambutol inhibits the polymerization step of arabinogalactan synthesis (Mikusova et al., 1995), Isoniazid being a prodrug is activated by mycobacterially encoded KatG catalase in the cell and upon activation inhibits mycolic acid synthesis (Zhang et al., 1992) and Ethionamide also a prodrug, inhibits fatty acid synthesis required for mycolic acid synthesis (Banerjee et al., 1994). The standard treatment for tuberculosis is 6 to 9 months of Isoniazid, Rifampin, Pyrazinamide and Ethambutol compounds developed in the 1950s and 1960s (Nguyen and Thompson, 2006). Apart from having low rate of diffusion through the cell wall, mycobacteria also uses strategies like efflux pumps, response regulators, antibiotic-modifying or -degrading enzymes such as β-
lactamase (Chambers et al., 1995, Wang et al., 2006), target modifying enzymes and decoys that mimic the drug target (Nguyen and Thompson, 2006) for its protection from drugs. While antimycobacterial therapy exists, currently available drugs are only partially effective because of the impermeable nature of the mycobacterial cell wall and the propensity of *Mtb* to develop resistance (Warner and Mizrahi, 2006). The duration and complexity of TB treatment causes nonadherence to treatment which leads to relapse, the emergence of drug resistant mycobacteria and continuous spread of the disease (Volmink and Garner, 2007). Adverse effects to anti-TB drugs are common which also contributes to the problem of nonadherence (Chan and Iseman, 2002; Volmink and Garner, 2007). Due to increasing incidences of multidrug-resistant (MDR; resistance to at least rifampin and isoniazid) and extensively drug-resistant (XDR; MDR resistance plus resistance to a fluoroquinolone and an aminoglycoside) TB is a serious concern (Boogaard et al., 2009).

Bacille Calmette-Guerin (BCG) is a live attenuated derivative of virulent *M. bovis*, a close relative of *Mtb*. BCG has been controversial since its first use in 1921 (Bloom, 1994). Since then, BCG has been administered to >3 billion people (Fine et al., 2002), with an excellent safety record (Casanova et al., 1996). However, in controlled clinical trials, some studies have reported no efficacy (Huebner, 1996) of BCG for preventing pulmonary TB. The widespread use of BCG appears to have no detectable influence on the prevalence of pulmonary tuberculosis. Apart from this the BCG vaccine could not be administered to the immunocompromised individuals. Development of a universally effective vaccine might be the only way to eliminate TB (Young et al., 2008) as there are large reservoir of latently infected individuals, which would be impossible to eliminate through prophylactic drug treatment (Comas and Gagneux, 2009). Chemotherapy and vaccination with the BCG are the most extensively used strategies to control *Mtb* but there is an urgent need to find newer targets for controlling TB.

1.5 Scope of study

TB research has been hampered by the fact that *Mtb* is a Biosafety level three pathogen with long generation time, making it slow and complex to culture. Chronic nature of the disease with extended period of latency during which the pathogen can not be isolated from the individual (Comas and Gagneux, 2009) makes it even more difficult. Even though the whole genome of *Mtb* H37Rv has been sequenced (Cole et al.; 1998) but not much progress has been made in past decade to win over the pathogen.
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Some of the virulence genes identified till date, encode for proteins that are involved in the structure and function of cell wall and thus demonstrating the importance of these surface determinants (Camacho et al., 1999; Cox et al., 1999). One of these virulent protein is Erp (Exported Repetitive Protein), also known as p36 (Bigi et al., 1995), Pirg or Rv3810 (Cole et al., 1998), has been shown to be a crucial virulence determinant (Berthet et al., 1998). The erp gene has been shown to be present not only in the pathogenic mycobacteria but also in saprophytic and environmental opportunistic pathogenic mycobacteria (De Mendonca-Lima et al., 2001). Virulence studies with Mtb and M. bovis erp mutants revealed that the growth of the bacteria is attenuated in cultured macrophages and in mouse model of infection (Berthet et al., 1998; Bigi et al., 2005). Erp deficient bacteria exhibited permeability defects in vitro, which may be responsible for their specific failure to survive in host macrophages (Cosma et al., 2006). Berthet et al. (1998), used immunoelectron microscopy to demonstrate that Erp is produced in Mtb phagosomes and suggested that it may traffic intracellularly.

Several lines of evidences indicate that secreted proteins from mycobacteria are essential in the protective immune response. It has also been observed that live bacteria are more protective than dead bacteria (Orme, 1988). The chronic nature of mycobacterial infections emphasizes the importance of identifying the secreted proteins and the proteins released from the bacterial surface upon contact with the intracellular environment of a macrophage (Beatty and Russell, 2000). Mycobacterial proteins might be actively released from the mycobacterial phagosome and traffic within the endocytic network of the host macrophage. In addition, released bacterial proteins may be incorporated into the phagosomal membrane and contribute to the modulation of phagosome maturation. Moreover, Mtb resides within a phagosome and avoids phagolysosome fusion (Vergne et al., 2004), promotes intracellular survival and growth of tubercule bacilli (Sturgill-Koszycki et al., 1996; Vergne et al., 2004; Clemens and Horwitz, 1996) and avoids immunological detection (Russell et al., 2002). Along with these facts there are reports of M. marinum (Stamm et al., 2005; Stamm et al., 2003), Mtb (Van Der Wel et al., 2007) and M. leprae (Van Der Wel et al., 2007) escaping the phagolysosome in an ESX-1 dependent manner and growing as cytosolic bacteria. Several mechanisms of host-pathogen interaction have been reported till date; of that one possible mechanism could be direct protein-protein interaction between the Mtb and host macrophage proteins. A mycobacterial secretory protein tyrosine phosphatase (PtpA) dephosphorylated host VPS33B protein and prevents phagolysosome fusion (Bach et al., 2008). Apart from this study not much work has been done till date to
study the host-\textit{Mtb} protein-protein interaction. Among all the mycobacterial proteins, there is very high likelihood of secretory proteins being involved in the interaction with macrophage proteins. Hence studies of Erp in the context of an intracellular infection, and its association with specific sub-cellular macrophage proteins, will help in identification and functional characterization of these specific interactions. Such an understanding may ultimately help in identification of specific biochemical pathways which might be targeted to control the deadly pathogen.