Chapter 1

Introduction
Tuberculosis (TB) is often regarded by paleomicrobiologists as one of the oldest diseases in human history (Donoghue et al., 2004). For example, ancient Egyptian doctors cataloged a chronic tuberculous node in the neck. Further, various archaeological art findings and human remains showed spinal TB, also known as Pott's disease (Bedeir et al., 2004). Though many old manuscripts described features of tuberculosis infection in humans, it was not until 1882 that Robert Koch identified its causative bacterial pathogen Mycobacterium tuberculosis (M. tuberculosis). Since then, TB has been a major cause of illness and death worldwide, especially in Asia and Africa. Globally, 9.2 million new cases (139 per 100 000 population) and 1.7 million deaths from TB occurred in 2006 (WHO TB Report, 2008). The global burden of TB can be seen in the light of latest statistics. There were an estimated 14.4 million prevalent cases of TB and an estimated 0.5 million cases of multidrug-resistant TB (MDR-TB) in 2006. An estimated 1.5 million deaths resulted from TB disease in human immunodeficiency virus (HIV)-negative people and 0.2 million among people infected with HIV in 2006 alone. In particular, the increasing prevalence of multidrug-resistant TB (MDR-TB) and even extremely drug resistant TB (XDR-TB) strains combined with co-infection with HIV have greatly contributed to the increasing difficulties in the control of TB. TB is a problem in both developing nations and developed countries, affecting immunocompromised patients such as AIDS (Acquired Immunodeficiency Syndrome), transplantation, immunosuppressive therapy such as anti-TNF (Tumor Necrosis Factor) treatment. Moreover, global migration is raising the likelihood of TB transmission from endemic to non-endemic areas (Das et al., 2006, Anderson et al., 2007).

Mycobacterium tuberculosis (M. tuberculosis) is an acid-fast bacillus and is transmitted solely by direct human-to-human spread via aerosols through inhalation (Iseman et al., 2000). The bacilli remain in the air for prolonged periods of time. After being exposed to M. tuberculosis, there are several ways in which the host responds to
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infection. A spectrum of possible clinical manifestations occurs at all stages of life in TB infected individuals. Fortunately, of the infected individuals only 5 to 10%, a relatively small subset, develop active TB within one or two years after infection (primary TB) (Van Crevel et al., 2002). When active TB develops, disease presentation (disease localization and severity) can be quite variable. Because the vast majority of infected individuals will not develop disease, the host immune system apparently is competent enough to contain or in rare cases even eradicate the pathogen. However, at least a significant proportions continues to harbor *M. tuberculosis* through their life span and are classified as latent TB infection (LTBI). However, these latently infected individuals carry a risk of reactivating the infection, resulting in the development of clinically active TB (post-primary TB). The estimated lifetime risk of reactivation for a 25-year old with LTBI has been estimated to be around 7%, at least in certain populations (Marks et al., 2000). Control of risk factors which can trigger reactivation of LTBI may be an important TB control strategy. As LTBI offers itself as an enormous reservoir for the pathogen and a potential risk of infecting other individuals, diagnosis and treatment have been recommended not only for active TB but also for LTBI (American Thoracic Society et al., 2000). There are a number of important questions that remain to be answered with respect to latent tuberculosis and vaccine development: How could the immune system be induced to eliminate, rather than just control, the tubercle bacillus? Otherwise, immune compromise can lead to reactivation of the infection. A clearer picture of the interactions between host and bacillus during latent or persistent infection is essential to answering these questions. A more complete understanding of the roles played by each component of the immune system in protection or exacerbation of tuberculosis, as well as of the bacterium’s weapons to evade those components, will enhance the development of preventive and therapeutic strategies against this enormously successful pathogen.

Breakdown of the immune response through various factors can result in reactivation and replication of the mycobacteria (Flynn et al., 2001). Currently complete information on how *M. tuberculosis* survives against a vigorous immune response is lacking and one probable explanation could be the presence of a lipid rich cell envelope, however *M. tuberculosis* might employ more than one factor to survive. Bacilli can also subvert the immune system to its advantage by the induction of a Th2
response as measured in terms of IL-4 production (Rook et al., 2005). Th2 cytokines are certainly detectable in TB patients and probably contribute to disease-associated immunopathology. Also, live bacilli have been isolated from granulomas or tubercles in the lungs of persons with clinically inactive tuberculosis, indicating that the organism can persist in a granulomatous lesion for many years (Opie et al., 1927; Robertson, H. E. 1933). Studies have demonstrated that specific proteins can be upregulated in specific in vivo growth conditions, for example, within phagosomes (Mattow et al., 2006). One key aspect of *M. tuberculosis* virulence is the secretion of around 250 protein antigens in axenic cultures. One of the many approaches involved in the generation of new vaccine design against TB is the utility of subunit vaccine(s), which in most cases are based on proteins secreted by *M. tuberculosis* (Andersen et al., 2005). Apart from their use as putative vaccine candidates these antigens have found use in the immunodiagnosis of TB because of their recognition by the sera of patients. But despite a wealth of information available on these antigens, their roles at the site of infection are yet to be ascertained and the physiological relevance of secretion of these antigens in the first place.

In this direction one of the important findings is the role of antigens encoded by the RD1 region of *M. tuberculosis* genome. RD1 region or region of difference-1 is a 9.5 kb region present in all the virulent strains of *M. tuberculosis* and *M. bovis* but is absent from the vaccine strain, i.e. BCG (Bacillus Calmette-Guérin) (Mahairas et al., 1996). RD1 region is a genetic locus encoding 9 ORFs named *Rv3871* through *Rv3879*. There are strong experimental evidences that the RD1 region contributes to the virulence of members of *M. tuberculosis* complex. It has been observed that deletion of RD1 region from virulent *M. tuberculosis* results in attenuation of its ability to grow inside human macrophages, to cause macrophage cytotoxicity, and to grow and cause pathology in the lungs and disseminate to the spleen of immunocompetent mice. Conversely, deletion of RD1 from *M. tuberculosis* results in decreased virulence that is similar to BCG (Lewis et al., 2003). It has also been demonstrated that RD1 complemented BCG has increased growth of the bacteria in the lungs and spleen of mice when compared with BCG (Pym et al., 2002). Therefore, the above studies point to a role of the RD1 region in *M. tuberculosis* virulence and indicate that deletion of this region from *M. bovis* caused the attenuation of *M. bovis* resulting in the birth of BCG.
Two ORFs in the RD1 region, *Rv3874* and *Rv3875* encode previously identified secretory proteins- Culture Filtrate Protein 10 kDa (CFP-10) also known as *Mycobacterium tuberculosis* Secretory Antigen 10 kDa (MTSA-10) and Early Secreted Antigenic Target 6 kDa (ESAT-6) which are targets of cellular immune response in mice, guinea pigs and humans infected with *M. tuberculosis* and serve as important immunodiagnostic markers for latent TB infection. These proteins are secreted in supernatants of *M. tuberculosis* cultures despite the lack of recognizable signal sequences. Further, ESAT-6 and CFP-10 interact with each other with high affinity to form a 1:1 heterodimer and their secretion is mutually dependent (Renshaw *et al.*, 2005, Renshaw *et al.*, 2002). It is believed that both proteins require other members of the RD1 region for their secretion. Individual disruptions of genes of RD1 region do not prevent synthesis of the protein; however, an intact RD1 region is mandatory for their secretion (Guinn *et al.*, 2004, Stanley *et al.*, 2003). All these arguments make RD1 region a compelling candidate as a virulence determinant and indicate towards the role of ESAT-6 and CFP-10 in immune evasion by *M. tuberculosis*.

Towards this end, the present body of work focuses on the immunomodulatory action of CFP-10. Since Dendritic cells (DCs) are instrumental in initiating primary T cell responses and thereby influence the overall immune response, we characterize the interactions of DCs with CFP-10 and the likely outcome of this interactions on subsequently generated immune responses to mycobacteria. In this context, work from our laboratory had demonstrated that CFP-10 induces the differentiation of mouse bone marrow precursor cells into DCs (hereafter CFP10-DCs). Characterization of these DCs showed that they are phenotypically and morphologically similar to DCs generated conventionally with granulocyte macrophage colony stimulating factor (hereafter GM-CSF-DCs) (Latchumanan *et al.*, 2002). Further, CFP-10 also induced the maturation of bone marrow derived and splenic CD8⁺ and CD8⁻ DCs. However, functional characterization of these DCs showed that unlike GM-CSF-DCs, CFP10-differentiated and CFP10-matured DCs induce suppressor responses to a challenge with *M. tuberculosis* cell extract (*M. tuberculosis* CE) in an IL-10 and TGF-β dependent manner (Balkhi *et al.*, 2004). It
was also observed that CFP-10 modulates the intracellular signaling machinery of DCs with CFP10-DCs showing an increased activation of PKC (Protein Kinase C) both during DC differentiation and subsequent activation by *M. tuberculosis* CE and live BCG, while GM-CSF-DCs induced a poor activation of PKC. On the other hand GM-CSF-DCs mounted good intracellular mobilization of calcium to *M. tuberculosis* CE stimulation, while CFP10-DCs induced a poor calcium influx. Further CFP10-DCs generated lower levels of reactive oxygen species which resulted in enhanced survival of mycobacteria in these DCs (Sinha *et al.*, 2006). This indicated that secretion of some of the antigens such as CFP-10 could be a strategy employed by *M. tuberculosis* towards downregulation of protective and pro-inflammatory responses at sites of infection.

A recent study from the lab showed that infection of CFP10-DCs with mycobacteria down-modulated RANTES and IP-10 levels. Pathway specific microarray analyses showed that in addition to RANTES and IP-10, mycobacteria infected CFP10-DCs showed reduced expression of many Th1 promoting chemokines and chemokine receptors. Importantly, T cells co-cultured with RANTES and IP-10 conditioned CFP10-DCs mediated killing of mycobacteria from infected macrophages. Similarly, T cells recruited by RANTES and IP-10 conditioned CFP10-DCs mediated significant killing of mycobacteria from infected macrophages. IFN-gamma treatment of CFP10-DCs restored RANTES and IP-10 levels and T cells activated by these DCs mediated significant killing of virulent *M. tuberculosis* inside macrophages. Adoptive transfer of either RANTES and IP-10 or IL-12 and IFN-gamma conditioned CFP10-DCs cleared an established *M. tuberculosis* infection in mice. The extent of clearance was similar to that obtained with drug treatment (Salam *et al.*, 2008). These results indicate that chemokine and cytokine secretion by DCs differentiated by *M. tuberculosis* antigens such as CFP-10 play major roles in regulating protective immune responses at sites of infection.

In the backdrop of previous work from our lab, the present study explored the key roles played by voltage gated calcium channels in CFP10-DCs. In parallel, we also employed DCs differentiated conventionally with GM-CSF, as this approach highlights the similarities and differences between CFP10-DCs and GM-CSF-DCs and brings out the significance of DC-differentiation by antigens such as CFP-10.