Review of Literature:

Tuberculosis and its control

Tuberculosis is one of the most important infectious diseases in the developing and underdeveloped countries of the world with about one-third of the world’s population infected, resulting in about 1.5 million deaths annually (WHO report, 2010). Tuberculosis and its associated diseases are caused by mycobacteria belonging to *Mycobacterium tuberculosis* complex (MTC). These are a group of highly related mycobacteria including *Mycobacterium tuberculosis*, which causes human pulmonary tuberculosis and *Mycobacterium bovis* which causes bovine tuberculosis in cattle, and others namely *M. pinnipedii*, *M. microti* and *M. caprae* infecting seals, voles and goats, respectively. Although treatment for human pulmonary tuberculosis is available, the long duration of the chemotherapy regimen results in poor patient compliance and hence poor control of the disease.

A study by Kochi in 1991 reviewed the tuberculosis situation and suggested that 1.7 billion people, i.e. a third of the human population were estimated to be infected with the tubercle bacillus and of these, an estimated 8 million were new cases of TB and 2.9 million succumbed to the infection; unfortunately, more than 95% of all the new cases and all TB-related deaths occurred in the developing world (Kochi, 1991). These studies provided a new impetus to global tuberculosis research and in 1993 WHO declared tuberculosis a "global emergency" which was an unprecedented step in the history of public health. The framework for effective TB control was launched in 1994 which defined five essential elements of TB control policy: (i) government commitment to sustainable TB control; (ii) diagnosis through sputum-smear microscopy mainly among symptomatic patients self-referring to health services; (iii) standardized short-course chemotherapy provided under proper case management conditions, including direct observation of treatment (DOT); (iv) a functioning drug supply system; and (v) a recording and reporting system allowing assessment of treatment results.
This program was named DOTS and was rapidly implemented by many countries. Unfortunately, there were many constraints in implementing the DOTS strategy, such as, weak political will and commitment, lack of required financial support, lack of trained human resources, lack of good management at the program level, poor quality and supply of anti TB drugs and finally weakness of the information system (Raviglione, 2003). Also, BCG vaccine, the only available vaccine against tuberculosis is ineffective in providing any protection against adult pulmonary tuberculosis in several parts of the world. Both M.tb and BCG induce an immune response that can contain infection at least partially, but ultimately fails to prevent the development of pulmonary disease. BCG protects efficiently against leprosy (Fine and Rodrigues, 1990) as well as against the childhood manifestations of TB (miliary or meningeal) (Rodrigues et al., 1993), whereas the efficacy in preventing pulmonary TB in adults is very limited. The factors responsible for inefficacy of BCG vaccine are: inappropriate storage of vaccine, inadequate strains of BCG (Fine, 1995), loss of certain genes responsible for protection due to attenuation (Behr et al., 1997), phenotypic and genotypic variation in strains of BCG (Behr, 2002), deficits of certain fundamental immunological character such as lack of an effective stimulation of the optimal blend of T cell populations and in particular induction of CD8 T cells, may explain the insufficient levels of immunity promoted by the BCG vaccines (Hess et al., 1999). Also the lack of important T cell antigens in BCG has been suggested as an important factor (Mahairas et al., 1996). Also, exposure to large quantities of environmental mycobacteria has been suggested as a possible factor interfering with the efficacy of BCG vaccination (Fine, 1995; Palmer and Long, 1966). Hence, there is a need to develop a more efficient vaccine than the present BCG vaccine.

Immunobiology of tuberculosis

It is estimated that one third of the world population is infected with M. tuberculosis but the infection does not usually culminate into active disease. The individuals remain asymptomatic and noninfectious in most of the cases of latent infection. Hence, the immune system is generally successful in containing the
infection, although not in eliminating the pathogen. Reactivation of the latent infection into active disease results from perturbations in the immune response, which leads to necrosis and damage to the lung tissue. However, in many cases of active tuberculosis, the obvious immunodeficiency is not found. On the contrary, the organism has developed mechanisms for evading immune responses. Thus, a constant battle between the mycobacterium and the host is being waged, and the outcome depends on many factors.

**Role of Macrophages in tuberculosis infection and immunity**

*M. tb* usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought to be with the resident macrophages. Macrophages are the primary habitat of *M. tb*; unlike other microbial pathogens that depend on the avoidance of phagocytosis to survive, *M. tb* preferentially targets the macrophage vacuoles. *M. tuberculosis* resides within macrophage and persists in a granuloma which consists of macrophages and giant cells, T cells, B cells and fibroblasts. Macrophages are the phagocytes at the front line of the host immune defense against microbial pathogens, which constitutes a potent antimicrobial component of both innate and cell mediated immunity. However, the precise mechanisms by which these cells mediate killing or inhibition of bacterial pathogens are not clearly understood. The effector functions by which macrophages mediate antimicrobial activity include phagolysosome fusion, generation of ROI by the oxidative burst, production of RNI via the NOS2-dependent cytotoxic pathway and mechanisms mediated by cytokines.

**Phagolysosome fusion:** Phagosomes formed by phagocytosis of large particulate materials including microbes, fuse with lysosomes containing potent hydrolytic enzymes capable of degrading a whole range of macromolecules including microbes at acidic pH of 4.5-5. Phagocytosed microorganisms are subject to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion (Cohn, 1963). Hart *et al.* in 1972 first hypothesized that *M. tuberculosis* survived inside macrophages by preventing this phagolysosomal fusion, a
phenomenon observed only with phagosomes containing live bacilli. These phagosomes do not undergo further acidification, due to the absence of proton-ATPase molecules from the vacuolar membrane, and this reduced level of acidification allows the intracellular survival and growth of mycobacteria (Sturgill-Koszycki et al., 1994). These observations have been supported by many subsequent studies that included identifying specific markers for phagosome and lysosome and observing their distribution in various endocytic compartments (Russell, 1995; Deretic and Fratti, 1999). It has been reported that mycobacterial sulfatides (Goren et al., 1976), derivatives of multi-acylated trehalose 2-sulfate (Goren et al., 1976a), a lysosomotropic polyanionic glycolipid (Middlebrook et al., 1959), have the ability to inhibit phagolysosomal fusion. In vitro studies have demonstrated that ammonia generated by M.tb inhibits phagolysosomal fusion by affecting salutatory movement of lysosomes and by alkalizing the intralysosomal compartment (Gordon 1980; Hart et al., 1983). However, the precise mechanism by which ammonia prevents phagolysosomal fusion is not yet known. Also, the GTPases of the Ras family, known to play a role in the interaction between various endocytic compartments (Desjardins et al., 1994; Pfeffer, 1992; Zerial et al., 1993), have been a target of investigation aimed at understanding the inability of mycobacterial phagosomes to mature to phagolysosomes (Via et al., 1997). Thus, mycobacterial phagosomes retain Rab5, which directs the interaction between early endocytic compartments and phagosomes (Desjardins et al., 1994; Desjardins et al., 1995), and exclude Rab7 (Press et al., 1998), a GTPase that regulates late endosomal membrane trafficking. Presence of TACO (tryptophan aspartate-containing coat) protein on the phagosomal membrane containing live mycobacteria provides a direct explanation for the inability of mycobacteria-containing phagosomes to fuse with lysosomes (Ferrari et al., 1999). The 50-kDa host cell protein specific for phagosomes containing live bacilli is recruited and is retained on to the mycobacterial phagosomal membrane of BCG-infected macrophages there but not to the phagosomes containing dead BCG bacteria and endosomal vesicles of uninfected cells. Thus, viability of phagocytosed bacilli is the prerequisite to the
retention of TACO by which mycobacteria inhibit phagolysosomal fusion and evade potent lysosomal antimicrobial functions of macrophages. Elucidation of the mechanisms by which \textit{M. tuberculosis} contains phagosomes and retains TACO will provide insight into the pathogenesis of the tubercle bacillus.

**Reactive Nitrogen Intermediates (RNI):** Nitric oxide (NO) production by activated macrophages is an important antimicrobial mechanism. These macrophages when activated by IFN\(\gamma\) and TNF\(\alpha\) produce nitric oxide and other related RNI via inducible nitric oxide synthase (iNOS, macNOS, Type II NOS) using L-arginine as the substrate (MacMicking \textit{et al.}, 1997a; Shiloh and Nathan, 2000.). Nitric oxide plays important role in vasodilation, neurotransmission and elimination of microorganisms (Blantz and Munger, 2002). Three major nitric oxide synthases (NOS) are NOS1 (ncNOS), NOS2 (iNOS), NOS3 (ecNOS). NOS1 and NOS3 are calcium-dependent and are constitutively expressed. iNOS is a soluble enzyme that, unlike ecNOS and ncNOS, does not require elevated intracellular Ca\(^{2+}\) levels for activation. NOS-2 mRNA and protein are present throughout the persistent infection (Flynn \textit{et al.}, 1998; Scanga \textit{et al.}, 2000). In murine systems, the role of these toxic RNI in host defense against \textit{M. tuberculosis} has been well documented, both \textit{in vitro} and \textit{in vivo} (Chan \textit{et al.}, 1992; MacMicking \textit{et al.}, 1997). \textit{M. tuberculosis} replicates much faster in genetically altered iNOS gene knock-out (GKO) mice than in wild type animals, indicating a significant role for NO in mycobacterial host defense (MacMicking \textit{et al.}, 1995). In persistently infected mice, inhibition of NOS2 activity with aminoguanidine led to increased numbers of mycobacteria in the lung, liver and spleens of these mice (Flynn \textit{et al.}, 1998). Also, in case of acute infection, increased bacterial numbers were observed in all the three organs in NOS2\(^{+}\) mice (Chan \textit{et al.}, 1995; MacMicking \textit{et al.}, 1997). These studies clearly depict the protective role of RNI in both chronic and acute infection. Inhibition of NOS2 activity in the experimentally infected mice that had been treated with antibiotics, led to reactivation of the infection (Flynn \textit{et al.}, 1998). These data indicate that continuous macrophage activation and production of reactive nitrogen
intermediates (RNI) is important in preventing reactivation of \textit{M. tb} infection in the lungs. Even in human studies, high level of expression of iNOS and RNI production was observed in alveolar macrophages lavaged from the lungs of tuberculosis patients (Choi \textit{et al.}, 2002). Yet another study has shown increased levels of exhaled NO in tuberculosis patients (Wang \textit{et al.}, 1998).

**Reactive Oxygen intermediates (ROI):** Hydrogen peroxide (H$_2$O$_2$), one of the ROI generated by macrophages via the oxidative burst, was the first identified effector molecule that mediated mycobactericidal effects of mononuclear phagocytes (Walker \textit{et al.}, 1981). Activation of macrophages by cytokines leads to the production of reactive oxygen species (ROS), which is capable of killing intracellular mycobacteria (Nathan and Shiloh, 2000). However, the significance of toxic oxygen species in the control of tuberculosis remains controversial as many reports indicate that ROS are not crucial for controlling intracellular mycobacteria (Wayne and Sohaskey, 2001). The ability of ROI to kill \textit{M. tuberculosis} has been demonstrated only in mice (Flesch and Kauffman, 1987) and remains to be confirmed in humans. Despite the demonstration that H$_2$O$_2$ generated by cytokine-activated macrophages was mycobacteriocidal (Walker \textit{et al.}, 1981), the ability of ROI to kill \textit{M. tuberculosis} remains to be confirmed (Flesch \textit{et al.}, 1987; Chan \textit{et al.}, 1992). Indeed, mycobacteria are capable of evading the toxic effect of ROI by various means (Zahrt and Deretic, 2002). Mycobacterial components lipoarabinomannan (LAM) (Chan \textit{et al.}, 1991) and phenolic glycolipid I (PGL-I) are potent oxygen radical scavengers (Chan \textit{et al.}, 1989) and mycobacterial sulfatides interfere with the oxygen radical–dependent antimicrobial mechanism of macrophages. Despite these findings, a role of ROI in defense against the tubercle bacillus cannot be entirely excluded as reports have shown that mice deficient in the NADPH oxidase complex exhibit enhanced susceptibility to \textit{M. tuberculosis} infection (Adams \textit{et al.}, 1997; Cooper \textit{et al.}, 2000).
Role of Cytokines against *M. tuberculosis*

**Interferon-γ (IFNγ):** IFNγ is produced by both CD4 and CD8 T cells in tuberculosis (Lyadova *et al.*, 1998; Lalvani *et al.*, 1998; Serbina *et al.*, 1999), by *M. tb* infected alveolar macrophages in IL-12 dependent manner (Wang *et al.*, 1999; Fenton *et al.*, 1997) and by NK cells. The protective role of IFNγ in tuberculosis is well established (Flynn *et al.*, 1993), primarily in the context of antigen-specific T-cell immunity. IFNγ plays a key role in control of *M. tuberculosis* infection. IFNγ knockout (GKO) mice are the most susceptible to virulent *M. tuberculosis* (Cooper *et al.*, 1993; Flynn *et al.*, 1993). IFNγ GKO mice, upon *M. tuberculosis* infection show uncontrolled bacillary growth, necrotic granulomas, and defective macrophage activation with low NOS2 expression (Flynn *et al.*, 1993; Dalton *et al.*, 1993). However, the mean survival time for *M. tuberculosis*-infected NOS2⁺ mice is at least twice that of GKO mice (MacMicking *et al.*, 1997; Flynn *et al.*, 1993), suggesting that there are IFNγ-dependent, NOS2-independent mechanisms of protection against tuberculosis. Studies in humans have shown that individuals with mutations in genes for IFNγ or the IFNγ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* (Ottenhof *et al.*, 1998). Some studies have demonstrated that IFNγ levels are depressed in the serum of patients with active tuberculosis (Lin *et al.*, 1996; Zhang *et al.*, 1995). Moreover, *M. tuberculosis* and its components can prevent macrophages from responding adequately to IFNγ by inhibiting IFNγ signaling in human macrophages by disrupting the association of the transcription activator STAT1 with the transcriptional coactivators, namely CREB-binding protein (CBP) and p300 (Ting *et al.*, 1999).

**Tumor Necrosis Factor-α (TNFα):** TNFα plays an important role in the host response against *Mycobacterium tuberculosis*. TNFα is secreted by macrophages, dendritic cells and T cells upon activation with *M. tuberculosis*. TNFα is an essential component of tuberculous granuloma and a potent mediator of macrophage activation. In case of mice deficient in TNFα or the 55-kDa TNF receptor, *M. tuberculosis* infection resulted in a deficient granulomatous
response followed by rapid death of mice with substantially higher number of bacilli in the lung compared to those observed in the control mice (Flynn et al., 1995; Fenhalls et al., 2000). TNFα synergizes with IFNγ to induce the iNOS expression and hence is critical for control of the acute *M. tuberculosis* infection (Flesch et al., 1990; Chan et al., 1992; Liew et al., 1990). Nitric oxide production in the granulomas of TNFRp55−/− as well as TNFα−/− mice was delayed in response to *M. tuberculosis* infection (Flynn et al., 1995; Bean et al., 1999). Mice inoculated with a low-dose of *M. tuberculosis* were found to maintain a stable bacterial load even six months later. However, neutralization of TNFα with anti-TNFα antibody resulted in increased bacillary counts in the lungs followed by 100% mortality of these animals (Flesch and Kaufmann, 1990). Anti-TNFα antibody treatment of an arthritis patient also resulted in development of fatal disseminated tuberculosis (Maini et al., 1999). Intriguingly, TNFα has also been shown to permit the multiplication of intracellular bacteria in human alveolar macrophages. Treatment of these *M. tuberculosis* infected macrophages with neutralizing anti-TNF antibody reduced the growth rate of intracellular bacteria, whereas bacterial replication was augmented by addition of exogenous TNFα (Engele et al., 2002). Although presence of TNFα is not an absolute requirement for necrosis of lung tissue, it is considered to be a major factor in the host-mediated destruction of lung tissues (Aung et al., 2000). In fact, recombinant BCG expressing very high levels of TNFα has been reported to cause destructive inflammation (Bekker et al., 2000). At the same time, TNFα is known to contribute to the inhibition of *M. tuberculosis* growth in macrophages by a mechanism that is dependent on apoptosis and independent of IFNγ activity (Keane et al., 2002). Thus, TNFα has an important role as a modulator of inflammation and has a complex and multifaceted role in protection and immunopathology in tuberculosis infection.

**Interleukin-12 (IL-12):** IL-12, produced mainly by phagocytic cells, is a key player in the host defense against *M. tuberculosis*. IL-12 is important in controlling *M.tb* infection and is strongly induced after phagocytosis of *M.*
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tuberculosis bacilli by macrophages and dendritic cells (Ladel et al., 1997; Henderson, 1997). It helps in the development of TH1 response leading to production of IFNγ. In tuberculosis, IL-12 has been detected in lung infiltrates (Casarini et al., 1999; Taha et al., 1997), in pleurisy (Zhang et al., 1994), in granulomas (Bergeron et al., 1997), and in lymphadenitis (Lin et al., 1996). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al., 1999). IL-12 when administered during the early phase of M. tuberculosis infection in BALB/c mice, results in decreased bacterial numbers and increased mean survival time (Flynn et al., 1995a). Also, IL-12p40 gene deficient mice produce low amounts of IFNγ and therefore, are observed to be more susceptible to M. tuberculosis infection with increased bacterial numbers and decreased survival time as compared to control mice, suggesting a protective role of IL-12 in M. tuberculosis infection (Cooper et al., 1997). In humans, studies have shown that humans having mutations in IL-12p40 or the IL-12 receptor genes and are more susceptible to disseminated BCG and M. avium infections due to lower production of IFNγ by T cells, although M. tuberculosis infections were not reported (Ottenhof et al., 1998; Casanova, 2000). The IL-12 encoding plasmid DNA when administered to mice with a chronic M. tuberculosis infection, results in reduced bacterial numbers (Lowrie et al., 1999), suggesting that IL-12 induction may play an important role in the design of a tuberculosis vaccine.

Interleukin-6 (IL-6): IL-6 has an important implication in inflammation and T cell differentiation in response to M. tuberculosis (Saunders et al., 2000). Suppressive role of IL-6 on T cell responses has been demonstrated in BCG-infected macrophages (vanHeyningen et al., 1997). Spleen cells from IL 6− mice produced elevated levels of IL-4 and decreased levels of gamma interferon compared to the control mice (Ladel et al., 1997a). However, upon development of acquired immunity, IL 6− mice successfully controlled the infection, retaining the memory response to subsequent aerosol challenge (Saunders et al., 2000). However, if a high intravenous dose of M. tuberculosis inoculum was given, the IL 6− mice did succumb to infection compared to the IL-6-competent control
mice, perhaps due to overwhelming of the defective innate response by introduction of large bacterial numbers (Ladel et al., 1997a).

Interleukin-10 (IL-10): IL-10 is an anti-inflammatory cytokine produced by macrophages after phagocytosis of M. tb (Shaw et al., 2000) and T cells during M. tuberculosis infection, possesses macrophage-deactivating properties, including downregulation of IL-12 production, which in turn decreases IFNγ production by T cells. IL-10 is known to inhibit CD4 T cell responses as well as antigen presentation by the cells infected with mycobacteria (Rojas et al., 1999a, Rojas et al., 1999). Although, IL-10−/− mice were not more resistant to acute M. tuberculosis compared to wild type mice, transgenic mice constitutively expressing IL-10 were less capable of clearing a BCG infection (North, 1998; Murray et al., 1997). Also, transgenic mice over-expressing IL-10 showed no increase in the susceptibility to M. tuberculosis during the early stages of infection, but showed evidence of reactivation of tuberculosis during the chronic phase of the infection with a highly significant increase in bacterial numbers within the lungs associated with the formation of macrophage, dominated lesions, decreased mRNA production for TNFα and IL-12p40, and a decrease in the antigen-specific IFNγ secretion (Turner et al., 2002). Attenuation of in vitro T cell proliferation by macrophages from tuberculosis patients was partially reversed by IL-10 inhibition. Together, these data suggests that IL 10 plays a pivotal role during the chronic/latent stage of pulmonary tuberculosis.

Interleukin-1 (IL-1): IL-1, along with TNFα, plays an important role in the acute phase response such as fever and cachexia, prominent in TB. In addition, IL-1 facilitates T lymphocyte expression of IL-2 receptors and IL-2 release. The major antigens of mycobacteria triggering IL-1 release and TNFα have been identified (Wallis et al., 1990). IL-1 has been implicated in immunosuppressive mechanisms, which is an important feature in tubercular immunity (Fujiwara et al., 1986).
Transforming Growth Factor-β (TGFβ): This anti-inflammatory cytokine has been implicated in the suppression of T cell responses in tuberculosis patients (Hirsch et al., 1997). TGFβ is present in the granulomatous lesions of tuberculosis patients and is produced by human monocytes after stimulation with *M. tuberculosis* (Toossi et al., 1995) or lipoarabinomannan (Dahl et al., 1996). Reportedly it inhibits the T cell responses to *M. tuberculosis* (Hirsch et al., 1997; Rojas et al., 1999a) as well as participates in the macrophage deactivation by inhibiting the IFNγ-induced NOS2 production (Ding et al., 1990). Regulation of this cytokine is very complex and occurs at various levels. The in vivo role of TGFβ in protection or pathology in tuberculosis has not been directly tested.

Role of T cell Subsets in Protection against Mycobacterial Infection
The protective response to *M. tb* requires the cell-mediated immunity. This pathogen is intracellular within the host, usually residing within macrophages, and thus T-cell effector mechanisms, rather than antibody, are required to control the infection. Within 1 week of infection with virulent *M. tb*, the number of activated CD4 and CD8 T cells in the lung-draining lymph nodes increases (Feng et al., 1999; Serbina et al., 2000). Between 2 and 4 weeks post-infection, both CD4 and CD8 T cells migrate to the lungs, and demonstrate an effector/memory phenotype (Serbina et al., 1999). This indicates that activated T cells migrate to the site of infection and interact with antigen-presenting cells. The granuloma contains both CD4 and CD8 T cells (Randhawa et al., 1990; Flynn et al., 1992) that likely participate in containing the infection within the granuloma and preventing its spread or reactivation.

Importance of CD8 and CD4 T Cells
The protective role of CD4 T cells can be determined with Class II gene-deleted mice, which are incapable of the positive and negative selection of CD4 T cells and of presenting antigen to these cells. These cells are of utmost importance in protection against *M. tb*. CD4 T cells primarily produce IFNγ to activate mycobacterial macrophages which control intracellular bacterium (Tascon et al., 1998; Caruso et al., 1999). Also, CD4 T cells play a crucial role in apoptosis of
infected cells, which has been suggested to be important in controlling *M.tb* infection (Keane *et al.*, 1997; Balcewicz-Sablinska 1998). The significance of CD4 cell can be assessed from the observation that in the absence of Class II-dependent immunity, infection remains progressive and is quickly lethal while in the absence of Class I-dependent immunity, lung infection progresses to a 1 log higher level than in the wild type mice but can then be controlled at a stationary level for a long period of time (Mongues *et al.*, 2001). To counter its destruction by the host immunity, the bacterium downregulates the surface expression of MHC Class II molecules on the macrophages, as well as the production of cytokines, such as TGFβ, IL-10 or IL-6, which can reduce T-cell stimulation (Gong *et al.*, 1996; Hirsch *et al.*, 1997; Rojas *et al.*, 1999a). However, infection of DCs with *M.tb* did not result in diminished MHC Class II cell surface expression (Henderson *et al.*, 1997; Stenger *et al.*, 1998; Bodnar *et al.*, 2001). In mice as in humans, the sterile eradication of *M.tb* is rarely achieved, suggesting that long-term CD4 memory T cells must continually enlist the aid of macrophages to maintain bacterial dormancy.

The way CD8 T cells contribute to anti-*M.tb* immunity is not fully understood. In vitro evidence suggests that these cells function via the secretion of IFNγ to activate macrophages to a mycobacteriostatic state by lysing *M.tb* infected macrophages or by secreting products that can directly kill *M.tb*. Given that CD8 T cells generated in response to *M.tb* infection are both cytolytic and capable of synthesizing IFNγ, it is possible that they contribute to immunity by performing both functions. Some antigens from the phagosome could enter the cytoplasm and be presented on MHC Class I molecules to CD8+ T cells in humans and in mice, and antigens recognized by these cells have been identified (DeLibero *et al.*, 1988; Silva *et al.*, 1994; Stenger *et al.*, 1997; Tan *et al.*, 1997; Zhu *et al.*, 1997; Mohagheghpour *et al.*, 1998; Lewinsohn *et al.*, 1998; Lewinsohn *et al.*, 2000). Previous studies have already indicated that CD8+ T cells are required to control *M.tb* infection (Flynn *et al.*, 1992; Behar *et al.*, 1999; Sousa *et al.*, 2000; VanPinxteren *et al.*, 2000; Rolph *et al.*, 2001). CD8 cells are induced during the
infection, and perhaps better induction, activation, or effector function by these cells could enhance protection against tuberculosis. CD8 T cells also recognize various antigens from \textit{M.tb} that are presented by non-classical MHC like Class Ib molecules which primarily present lipid antigens from \textit{M.tb} to CD8 T cells, thereby increasing the possible antigen source greatly. Cytokines produced by CD8 T cells in \textit{M.tb} infection include IFN\textsubscript{\gamma} and TNF\alpha, although there are likely more produced as well. IFN\textsubscript{\gamma} production by CD8 T cells probably participates in activation of macrophages (Caruso \textit{et al.}, 1999; Scanga \textit{et al.}, 2000). It also induces apoptosis of infected cells which prevents the release of bacteria from granuloma to other tissues. However, in humans, CD8 T cells also produce granulysin, which enters the macrophage via the perforin pore. Granulysin was shown to be directly toxic to \textit{M.tb} and represents a mechanism by which CD8 T cells can contribute to killing of the bacilli within cells (Stenger \textit{et al.}, 1997; Stenger \textit{et al.}, 1998).

Modulation of MAP kinase and JAK/STAT pathways

The immune system when comes in contact with the invading bacteria, it induces a cell mediated immune response by producing proinflammatory cytokines such as IL-1, IL-6, TNF\alpha and interferons. These cytokines cause localized tissue damage and enhanced recruitment of phagocytic cells when released at the site of infection. These cytokines and other effector molecules are produced as a result of activation of signaling cascades such as mitogen activated protein kinases (MAPK) or JAK/STAT (Janus kinase/signal transducer and activator of transcription) eventually leading to the containment of infection. However, pathogenic mycobacteria have evolved mechanisms to suppress these signal transduction pathways and thereby attenuate cytokine-induced immune response whereas this suppression is not seen in nonpathogenic mycobacteria. Recently, \textit{in vitro} studies have shown that secretory proteins of \textit{M.tb} such as, ESAT-6 and MTSA-10, downregulate the LPS-induced activation of signal transduction pathways which eventually result in downregulation of effector
responses of macrophages (Trajkovic et al., 2001; Ganguly et al., 2007; Ganguly et al., 2008).

Mitogen activated protein kinase (MAPK) pathways are highly conserved modules (Lewis et al., 1998; Widmann et al., 1999). MAP kinases are signal transduction molecules which play a diverse role in cell proliferation, cell death, cytokine production and cell differentiation. MAPK pathways are composed of three tier kinase modules in which a MAPK is activated upon phosphorylation by a Mitogen activated protein kinase kinase (MAPKK). This in turn is activated upon phosphorylation by a MAPKKK. To date six distinct groups of MAPKs have been characterized in mammals; Extracellular signal regulated Kinase (ERK)1/2, ERK3/4, ERK5, ERK7/8, c-Jun-N-terminal kinase(JNK)1/2/3 and p38 isoforms (p38 α, β, γ and δ) (Dhillon et al., 2007). The MAP kinases are activated by dual phosphorylation at the tripeptide motif Thr-Xaa-Tyr. The sequence of this tripeptide motif is different in each group of MAP kinases: ERK (Thr-Glu-Tyr); p38 (Thr-Gly-Tyr); and JNK (Thr-Pro-Tyr), the phosphorylated MAPKs then phosphorylate a number of transcription factors like ATF-2, Elk-1, CREB etc. Studies on MAPKs have shown that mycobacteria modulate MAPK signaling to promote their survival in the host cells. M. avium has two strains; smooth transparent (SmT) and smooth opaque (SmO) representing a more virulent and less virulent phenotype respectively. Initially, both SmT and SmO induced early phosphorylation of p38 MAPK and ERK1/2 upon infection, but a sustained activation was seen only in the attenuated strain SmO, whereas the activation died in the case of more virulent strain SmT (Tse et al., 2002). These results were also supported by a study on the avirulent and virulent strains of M. avium (Roach and Schorey, 2002).

An important development in understanding the mechanism by cytokines rapidly activate gene expression has been the identification and characterization of molecules termed signal transducers and activators of transcription, or STATs. One or more STAT molecules are activated by each member of the hematopoietin receptor superfamily and the related set of receptors for
interferon-related molecules (Darnell, 1997). Elegant experiments utilizing mutant cell lines that lack specific JAK kinases have shown that JAK activation is required for STAT activation (Velazquez et al., 1992). Thus, the STAT activation pathway is often referred to as the JAK/STAT pathway. *M. tuberculosis* modulates JAK/STAT signaling pathway upon infection. *M. tuberculosis* infection inhibits macrophage responses to IFNγ by inhibiting transcription of IFNγ responsive genes. The inhibition is not at the proximal steps of JAK/STAT signaling like STAT-1 phosphorylation, dimerization, nuclear translocation or DNA-binding, rather *M. tuberculosis* inhibits IFNγ responses by disrupting the essential interaction of STAT-1α with transcriptional coactivators, CREB-binding protein (CBP) and p300 (Ting et al., 1999). This might be accomplished by *M. tuberculosis* in two ways; firstly, *M. tuberculosis* might indirectly inhibit IFNγ responses by activating macrophage signaling pathway that requires CBP and/or p300 and thereby restrict the availability of these coactivators for use by STAT-1α. Secondly, *M. tuberculosis* could directly target the domains of either STAT-1 or CBP that are involved in protein-protein interactions.

**Secretory protein antigens of Mycobacterium tuberculosis**

*Mycobacterium tuberculosis* H37Rv, the most widely used strain in tuberculosis research was isolated in 1905. The complete genome sequence and annotation of this strain was published in 1998 (Cole et al., 1998; Cole, 2002). Since the completion of the genome sequence of the H37Rv strain of *M.tb*, a vast body of information about the bacterium’s proteome has become available. For 4006 proteins, genes have been identified in *Mycobacterium tuberculosis* H37Rv genome of which only half have been assigned specific functions. Since only live mycobacterial vaccines provide protection against tuberculosis infection (Mustafa, 2002; Orme, 1988), it seems that the secretory proteins of *Mycobacterium tuberculosis* play a major role in stimulating the protective immunity. This indicates that secretion of these extracellular proteins might be responsible for the greater efficacy of vaccination with live attenuated mycobacteria than that with killed organisms. Hence, these secretory proteins
have attracted particular attention as candidate antigens for subunit vaccines against *M. tuberculosis*. Secreted antigens play a major role in the virulence, pathogenesis as well as the protection against the tuberculosis. These antigens may be crucial at the early stages of infection being available for processing and presentation to T cells prior to the accessibility of cytoplasmic or cell-wall antigens from dead bacteria (Anderson, 1997). Recent studies have indicated that secretory antigens may play role in activating macrophages, dendritic cells (DC) and mast cells to produce proinflammatory molecules. Conversely, they might be also responsible for mycobacterial evasion of immune mechanisms by downregulating macrophage NO production and B7.1 expression or by impairing DC induced Mtb-specific Th1 responses. Secreted antigens have been extensively characterized and constant research is being carried out to unravel their role in tuberculosis infection.

**ESAT-6 and MTSA-10 (CFP-10)**

ESAT-6 and MTSA-10 belong to the *RD1* locus which encompasses 9 genes from Rv3871 to Rv3879 in *Mycobacterium tuberculosis*. The *Mycobacterium tuberculosis* H37Rv genome has five copies of a cluster of genes known as the ESAT-6 locus, another name for *RD-1* locus. These clusters contain members of the CFP-10 (*lhp*) and ESAT-6 (*esat-6*) gene families encoding secreted T-cell antigens (that lack detectable secretion signals) as well as genes encoding secreted, cell-wall-associated subtilisin-like serine proteases, putative ATP-binding-cassette (ABC) transporters, ATP-binding proteins and other membrane-associated proteins (Gey van Pittius et al., 2001). The 10 kb genomic DNA region designated as Region of Difference-1 (*RD-1*), that is present only in virulent and clinical strains of *M. tuberculosis* and *M. bovis*, has been shown to be deleted in all the vaccine strains of bacillus Calmette Guerin (BCG) and many environmental mycobacterial species (Brusasca et al., 2001). Two of the genes within RD1 region, *esat-6* and *cfp-10* (Early secretory antigenic target-6 kDa and culture filtrate protein of 10 kDa) encode secreted proteins ESAT-6 and CFP-10 (also known as MTSA-10). Both proteins have been investigated for their utility in diagnosing tuberculosis (Pollock et al., 1997) and for differentiating latent
infection from BCG vaccination (Lalvani et al., 2001). The ESAT-6 and CFP-10 genes are cotranscribed in \( M. \) \( \text{tb} \) (Berthet et al., 1998) and have been shown to form a tight, fully folded 1:1 complex, suggesting their activity as a complex (Renshaw et al., 2002). Both these proteins are highly immunogenic and are found in the early culture filtrates of \( M. \) \( \text{tb} \) after short periods of growth and in the absence of obvious autolysis. A cooperative functional relationship has been proposed between the proteins encoded in RD1 (Wards et al., 2000). Failure of \( M. \) \( \text{bovis} \) knockout mutant of the ATPase gene (Rv3871) in the RD1 to sensitize guinea pigs to an ESAT-6 skin test supports this view. Though none of the nine open reading frames (ORFs) that comprise RD1 have a biochemically assigned function, this region has been carefully scrutinized \textit{in silico}. The predicted functions of several RD1 region genes suggest they may have roles in protein translocation. Further, as CFP-10 and ESAT-6 lack clear secretion signals, they may require a novel secretion machinery for export, and components of RD1 may form that machinery (Cole et al., 1998; Tekaia et al., 1999; Gey van Pittius et al., 2001; Pallen, 2002; Lewis et al., 2003). This notion was proved true when disruption of individual genes (Rv3870, Rv3871 and Rv3877) within this locus prevented secretion of ESAT-6 and CFP-10, providing the first genetic evidence that this region encodes for a secretion system. Cox and others showed that disruption of individual RD1-region genes did not prevent production of ESAT-6 or CFP-10. However, an intact RD1 region was required to ensure that these proteins were secreted by the bacterium (Stanley et al., 2003; Pym et al., 2003). Restoration of RD1 region by gene knock-in technique has been done in \textit{Mycobacterium bovis} BCG and in \textit{Mycobacterium microti}. These \( BCG::RD1 \) and \( M. \) \( \text{microti}::RD1 \) knock-ins grew more vigorously than controls in immunodeficient mice, inducing extensive splenomegaly and granuloma formation. Increased persistence and partial reversal of attenuation were also observed when immunocompetent mice were infected with the \( BCG::RD1 \) knock-in, whereas BCG controls were cleared. Knocking-in five other RD loci did not affect the virulence of BCG (Pym et al., 2002).
Studies have shown that recombinant CFP-10 is a potent T-cell antigen that elicits proliferative responses and gamma interferon production from peripheral blood mononuclear cells (PBMC) in 70% of PPD-positive individuals without evident disease. Colangeli et al. (2000) have shown that this antigen is capable of eliciting delayed-type hypersensitivity in *M. tuberculosis*-infected guinea pigs but not in *M. bovis* BCG infected or *M. avium*-infected guinea pigs. It has been demonstrated that PBMCs from TB patients produce IFNγ in response to recombinant CFP-10 (Skjot et al., 2000). Paradoxically, CFP-10 has also been shown to modulate macrophage functions (Trajkovic et al., 2002). Also, CFP-10, ESAT-6 and CFP10: ESAT-6 complex of *Mtb* have been shown to downregulate the signal transduction pathways responsible for immune activation of macrophages (Ganguly et al., 2007; Ganguly et al., 2008). A lot of work has been carried out to elucidate exact function(s) of CFP-10 and ESAT-6 in particular in *M.tb* virulence and RD1 region in general, but the picture remains unclear.

**Antigen 85b-complex**

Antigen 85b (Ag85b)-complex is an immunogenic group of secreted proteins, recognized by antibodies and T cells in infected individuals. It consists of three homologous proteins (85a, 85b and 85c), each being approximately of 30 kDa. All the three are encoded by 3 different genes located at separate chromosomal loci and share 70-80% identity with each other (Eiglmeier et al., 1993). These three proteins are present in the cell wall as well as in the culture medium, but their relative amounts vary in both the compartments (Wiker et al., 1991). All three proteins catalyze synthesis of trehalose dimycolate, an essential component of the bacterial cell wall, by transferring the fatty acid mycolate from one trehalose monomycolate to another (Kremer et al., 2002). Serum Ag85 levels are found to be 50- to 150-fold higher in patients with active tuberculosis compared to healthy controls or patients with active *M. aviumintracellulare* disease or patients with non-tuberculous pulmonary disease, showing that serum Ag85 levels can be correlated with active mycobacterial infections independent of host immunity (Bentley-Hibbert et al., 1999). These antigens induced lympho-
proliferation and gamma interferon production in peripheral blood mononuclear cells from healthy tuberculin reactors. All three proteins induced strong cellular and humoral immune responses in infected experimental animals and human, although responses to the Ag85b and Ag85a proteins by the patients were significantly greater than responses to the Ag85c protein (Lim et al., 1999). In patients with active tuberculosis, Ag85 forms complexes with plasma fibronectin and IgG and hence not excreted out with urine (Bentley-Hibbert et al., 1999). Epitope mapping shows that peptide 91-108 of Ag85b is the major focus of the CD4 response to mycobacterial antigens in peripheral blood mononuclear cells and in T cell lines from PPD responders (Valle et al., 2001).

**Exported Repetitive Protein (Erp)**

Exported repetitive protein (Rv3810), also known as P36 and PIRG is an ubiquitous extracellular protein found in most of the mycobacterial species (de Mendonca-Lima et al., 2001). Disruption of the bacterial erp gene in *M. tuberculosis* and *M. bovis* (BCG) both, impaired multiplication of bacterium in cultured macrophages and mice. The ability to multiply is restored by reintroduction of erp into these mutants (Berthet et al., 1998), indicating significant contribution of Erp to the virulence of *M. tuberculosis*. The predicted Erp protein has repeated amino acid motifs, a typical N-terminal signal sequence and a hydrophobic domain. These predicted structural features are similar to the cell-wall-associated surface proteins of Gram-positive bacteria (Berthet et al., 1995).

**MPT-64**

This is a 24-kDa molecule present mainly in *M. tuberculosis* complex, but is absent from the four most commonly used BCG vaccine strains (Harboe et al., 1986; Yamaguchi et al., 1989; Li et al., 1993). Hence this protein was thought to have the potential of being used in the development of a diagnostic test to discriminate between BCG vaccination and infection with *M. tuberculosis* (Roche et al., 1996), as both the native and recombinant MPT-64 have been shown to distinguish between *M. tuberculosis* infection and BCG (Danish1331)-vaccination
MPT-64 as well as ESAT-6 from *M. tuberculosis*, elicited delayed-type hypersensitivity (DTH) skin responses in out-bred guinea pigs infected with *M. tuberculosis* by the aerosol and intravenous routes but not in those sensitized with *M. bovis* BCG or *M. avium*. A 15-residues single DTH-inducing epitope has been detected at the carboxy terminal region of MPT-64 using overlapping synthetic peptides. MPT-64 DNA vaccination has also been shown to enhance immunity against *M. tuberculosis* (Wu et al., 2001).

**MPT 70**

MPT 70 (*Rv2875*) and its identical *M. bovis* homologue MPB70 are 163-residue polypeptides, secreted from mycobacterial cells following cleavage of a 30-residue signal peptide (Cole et al., 1998; Hewinson et al., 1993). MPB 70 of *M. bovis* is a major serodominant antigen, which stimulates a strong, delayed type hypersensitivity response and cellular immune responses during infection in cattle (Billman-Jacobe et al., 1990; Fifis et al., 1991, Fifis et al. 1994; Pollock et al., 1994).

**Differential expression of MPB/MPT 70 in Mycobacteria**

Virulent *M. bovis* strains secrete high levels of MPB 70 when grown in culture, but the protein is produced at much lower levels by *M. tuberculosis* and by a number of *M. bovis* BCG strains such as Pasteur, Copenhagen and Glaxo (Harboe et al., 1998; Miura et al., 1983; Harboe et al., 1984; Hewinson et al., 1996). Further studies demonstrated that all other members of the *Mycobacterium tuberculosis* complex including *M.tb*, *M. africanum* and *M. microti*, were low producers of MPB/MPT 70. Interestingly, *M. bovis* BCG strains also show variation in the levels of MPB 70 production (Wiker et al., 1996). Early strains of BCG, obtained from Pasteur Institute before 1927 are high producers of MPB 70 in vitro, whereas the late strains obtained in or after 1931 are low producers. Those that express high levels of MPB 70 are BCG Tokyo, Moreau, Russia, Sweden, Birkhaug (Bergen), Romania (Cantacuzinho strain), and those that express low levels are BCG Pasteur, Danish 1331, Glaxo, Tice and Beijing (Wiker, 2009). The difference in the expression level of MPB 70 between the two
groups of BCG strains is due to a mutation in \textit{sigK}, the gene encoding the extracytoplasmic function (ECF) sigma factor K (Charlet et al., 2005). Moreover, it has been shown that the difference in the expression level of MPB/MPT 70 between \textit{M. bovis} and \textit{M. tuberculosis} is due to mutations in a regulatory gene, \textit{Rv0444c}, which encodes anti-sigK factor RskA (Said-Salim et al., 2006). \textit{M. bovis} lacks a functional anti-SigK which fails to repress the SigK resulting in constitutive expression of MPB/MPT 70. Based on these studies, Said-Salim and colleagues (2006) have proposed this model for \textit{mpt70} regulation: in \textit{M. tuberculosis} H37Rv, RskA binds to SigK through its N-terminal domain and inhibits its activity in vitro. Upon sensing an in vivo stimulus through its extracellular C-terminal domain, RskA releases SigK, which is set free to bind to the promoter of its target genes (\textit{mpt70} and \textit{mpt83}) and initiate their transcription. In \textit{M. bovis}, RskA is rendered dysfunctional due to two SNPs in its encoding gene, \textit{Rv0444c}. Consequently, SigK is always switched 'on' in \textit{M. bovis} directing transcription of \textit{mpt70} (and \textit{mpt83}), even in the absence of any stimulus. However, the signal to which RskA senses and responds to is yet to be determined. They also suggest that SigK-RskA target genes are restricted to just the \textit{sigK} and \textit{mpt70/mpt83} regions which is in contrast to other regulons controlled by other ECF sigma factors, which are quite extensive.

Though there is a significant difference in MPB70/MPT 70 expression, \textit{M. bovis} and \textit{M. tuberculosis} stimulate a strong immune response to MPB 70 upon infection, suggesting that expression of the protein is up-regulated by \textit{M. tuberculosis} in vivo (Roche et al., 1994; Hewinson et al., 1996). It is shown that serum from mice infected with live \textit{M. tuberculosis} react strongly to these antigens, that is, MPT 70 and MPT 83 (Hewinson et al., 1996). Another study described the transcriptional profile of \textit{M. tuberculosis} inside stimulated macrophages and found that \textit{mpt70} and \textit{mpt83} are both significantly induced in macrophages, with \textit{mpt83} induction also confirmed in the infected mice (Schnappinger et al., 2003). The shut down in expression of MPB 70 and MPB 83 by BCG Pasteur can be explained in a way that there was a cost to produce these proteins in high levels during prolonged in vitro passage in the absence of
host pressures. Finally, it has been shown that a DNA vaccine encoding MPB 70 has a significant therapeutic effect on M. tb infected mice (Lowrie et al., 1999). Even though MPT 70 is highly immunogenic, the function of this protein is still unknown. However, the solution structure of MPT 70 has been reported by Carr et al. (2003).

Solution structure of MPB/MPT 70
The structure reveals a complex and novel bacterial fold, which has clear structural homology to the two C-terminal third and fourth FAS1 domains of the cell adhesion protein fasciclin I, known to be involved in mediating interactions between cells and the extracellular matrix (Kim et al., 2000, Billings et al., 2002). This carboxyl terminal half is also homologous to osteoblast specific factor-2 (OSF-2) and human transforming growth factor beta-induced gene product (βih-3). This fold is a characteristic of eukaryotic proteins containing FAS1 domains and was not seen in bacteria previously.

Fig. 1: Ribbon representation of the backbone topology of MPB 70 (Carr et al., 2003).
The structure suggests that it contains two functional surfaces on opposite faces, which are probably involved in binding to host cell proteins (Fig. 1). MPB/MPT 70 also contains a single disulfide bond between Cys-40 and Cys-174. The protein consists of a seven-stranded β-barrel (β, β2, β3, β4, β5, β6, and β7) and eight α-helices (α1, α2, α3, α4, α5, α6, α7, and α8) that pack together on one side of the barrel. The β-barrel is closed by one hydrogen bond between β2 and β3, forming a β-sandwich with strand 6 coiling to form part of two sheets (β6C-β7-β1-β2 and β3-β4-β5-β6N). All the strands are anti-parallel to each other except β1 and β7, and the shear number is 10 (the residue offset for one transverse around the barrel). The circumference of the barrel is wider at the bottom than at the top, with its base plugged by the side chain of Val-112 (in the loop between β2 and β3) and the top by Leu-159 (from the C terminus of the protein). Sheet 1 (β6C-β7-β1-β2) forms the hydrophobic core of MPB 70 with its side chains being either part of the β-barrel interior or involved in the packing of helices α1, α2, α3 and α5 against the strands. In contrast, the opposite face of the barrel (sheet β3-β4-β5-β6N) is entirely solvent-exposed. Helices α2, α3 and α4 run in a zigzag manner, with α2 and α3 packing against the face of strands β1, β6, and β7, and α4 packing against α6, α7, and α8, which are positioned in a U-shaped arrangement at the top of the β-barrel (the tapered end). The N-terminal helix (α1) is perpendicular to α2 (both being parallel to the bottom rim of the barrel) and anchored to the β-barrel via a disulfide bond to strand β6 (Cys-8 to Cys-142). Strand β6 forms the bottom rim of the barrel in this region and contains two β-bulges in its hydrogen bonding network with β7 at Val-141 to Cys-142 and Gly-144 to Val-145; α5 runs anti-parallel to the direction of the α2-α4 zigzag and connects the N-terminal helical region to the first β-strand of the barrel.
Fig. 2: Multiple sequence alignment showing sequence similarity between MPB 70, MPB83, FAS1 domains 3 and 4 from fasciclin I and FAS1 domain 4 of βig-H3. The secondary structure elements are indicated above the alignment for MPB70 and also by colour coding of the sequences for both fasciclin I (domains 3 and 4) and MPB 70, with α-helices in red and β-strands in green. '*' indicates absolutely conserved residues across the group of sequences and ':' indicates residues conserved within close structural groups (Carr et al., 2003).

Homology between MPB/MPT 70 and MPB/MPT 83

Mycobacteria from Mycobacterium tuberculosis complex express another protein MPB 83 in M. bovis and MPT 83 in M. tuberculosis, which is highly homologous to MPB/MPT 70 and also stimulates a strong protective immune response upon infection (Morris et al., 2000; Chambers et al., 2002). The genes encoding
MPB/MPT 70 and MPB/MPT 83 form a part of an operon encoding five genes (Rv2871 to Rv2875 in *M. tuberculosis*) and are expressed under identical conditions. The mature MPT 83 (Rv2873) and its identical *M. bovis* homologue MPB 70 is 196-residue long polypeptide, of which residues 33-195 are 80% homologous to full length MPB 70 with 74% identity over 163 residues. The multiple sequence alignment shown in Fig. 2 highlights the sequence similarity between MPB 70, MPB 83, FAS1 domains 3 and 4 from fasciclin l and FAS1 domain from βig-H3. MPB 83, apart from having a disulfide bond analogous to MPB 70, is also glycosylated at T24 and T25 and lipoylated at the N-terminus. This feature is not seen in MPB 70 as MPT 70 is secreted outside the cell and MPB 83 is anchored to the cell wall via the N-terminal lipid group and hence is localized to the cell surface indicating similar structures but having distinct functional roles.

As the structure suggests, it may interact with some host protein and affect the functioning of the host cell or it may interact with certain cell receptors and modulate the host cell immune response through changes in signal transduction mechanisms. The present study was aimed to study some of the putative effects of MPT 70 on macrophages, the natural host cell for *M.tb*. 