Review of Literature
Tuberculosis is a global health problem whose morbidity and mortality is increasing. The main route of infection for the tubercle bacillus is the respiratory tract, in lungs, the alveolar macrophages phagocytose the bacterium providing the first line of defence. The bacteria replicate within the macrophage and induce cytokines that initiate the inflammatory response in the lungs. Macrophages and lymphocytes migrate to the site of infection and form a granuloma. The function of the granuloma is to segregate the infection to prevent spread to the remainder of the lung and to the other organs, as well as to concentrate the immune response directly to the site of the infection. The granuloma is maintained in a persistent infection, probably due to the chronic stimulation of the immune cells and forms the basis of tuberculous lesion (Verreck et al, 2002). Live bacilli have been isolated from the granulomas or tubercles in the lungs of the persons with clinically inactive tuberculosis (Opie et al, 1927; Robertson et al, 1933).

Immunological Response in Tuberculosis

Murine models have been used extensively to delineate the host factors that are key to controlling the initial infection with M. tuberculosis. Using either gene-deficient mice or neutralization with antibodies, various cytokines and cell types have been demonstrated to be essential in the control of M. tuberculosis. T cells both CD4+ and CD8+, participate in protection against tuberculosis (Flynn and Ernst, 2000). In humans, infection with human immunodeficiency virus (HIV) leads to a loss of CD4+
T cells, which is associated with an increased risk of tuberculosis. While an HIV-negative, PPD-positive person has a 10% lifetime risk of developing active tuberculosis, coinfection with *M. tuberculosis* and HIV carries 5-15% yearly risk of active tuberculosis (Selwyn *et al*, 1989). It is generally accepted a T<sub>H1</sub> response, characterised by the production of IFN-γ by CD4 T cells, is important in control of *M. tuberculosis* infection. Studies of *M. tuberculosis* infected with CD4<sup>+</sup> T-cell deficient mice did exhibit an early defect in overall IFN-γ production and macrophage activation (Caruso *et al*, 1999), however as the infection progressed other cells, most notably CD8 T cells were also capable of producing this cytokine in the lungs. The CD4<sup>+</sup> T-cell deficient mice still succumbed to the infection, suggesting that early IFN-γ production by CD4 T cells was crucial to the control of the infection.

![Diagram](www.brown.edu/.../Bio_160/Projects1999/tb/tb.gif)
Production of reactive nitrogen intermediates (RNI) by induction of nitric oxide synthase (NOS2) in macrophages is necessary to protect against tuberculosis in mice (Chan et al, 1995; MacMicking et al, 1997). A pivotal cytokine in the immune response is IFN-γ. Mice deficient in the IFN-γ gene are the most susceptible to fatal tuberculosis (Cooper et al, 1993; Flynn et al, 1993). This cytokine is responsible for the macrophage activation in tuberculosis (Dalton et al, 1993; Flynn et al, 1993), including the production of RNI, which is the only known mechanism by which macrophages can kill *M. tuberculosis* (Chan et al, 1992).

TNF-α is also a crucial cytokine for the control of acute tuberculosis in mice. Without this cytokine, the effective granuloma formation is diminished and bacterial numbers rapidly increase, resulting in the death of the mice (Bean et al, 1999; Flynn et al, 1995; Kindler et al, 1989).

Role of macrophage in *M. tuberculosis* infection

Macrophages are phagocytic cells which can internalize, digest and process the microbes. The processed antigens are then presented as peptides to antigen-primed T cells for the induction of acquired immune response. After the microorganism has been internalized, the lysosomal hydrolases which acts in an acidic pH break down the microbe into peptides. Reactive nitrogen intermediates (RNI) produced by macrophages are effective molecules against intracellular pathogens. The cytokines IFN-γ and TNF-α activate macrophages leading to enhanced production of RNI. High level of expression of iNOS (nitric oxide synthase) and RNI production was observed in alveolar macrophages lavaged from the lungs of tuberculosis patients (Choi et al, 2002). Nitric oxide plays important role in vasodilation,
neurotransmission and elimination of microorganisms. Three major nitric oxide synthases (NOS) are NOS1 (ncNOS), NOS2 (iNOS), NOS3 (eNOS). NOS1 and NOS3 are calcium-dependent and are constitutively expressed. NOS-2 mRNA and protein are present throughout the persistent infection (Flynn et al., 1998; Scanga et al., 2000). Inhibition of NOS2 activity with aminoguanidine in persistently infected mice led to a rapid increase in bacterial numbers in the lung, although there was little effect observed on bacterial numbers in liver and spleens of these mice. This was in contrast to the acute infection in NOS-2−/− mice where increases in bacterial numbers were observed in all three organs (Chan et al., 1995; MacMicking et al., 1995). Inhibition of NOS-2 activity in a long-term infected mice treated with antibiotics led to the reactivation of the infection (Flynn et al., 1998). These data indicate that continuos macrophage activation and production of reactive nitrogen intermediates (RNI) is important in preventing reactivation in the lungs.

Like nitric oxide, cytokine activation of macrophages leads to the production of reactive oxygen species (ROS) like hydrogen peroxide (H₂O₂), which is capable of killing intracellular mycobacteria (Nathan and Shiloh, 2000). However many reports indicate that ROS are not crucial for controlling intracellular mycobacteria (Wayne and Sohaskey, 2001). It is possible that Mycobacteria have devised ways to protect itself from the ROS-mediated killing since the lipoarabinomannan (LAM) and phenolic glycolipids present on the surface act as free radical scavengers and interfere with ROS-mediated killing (Zahrt and Deretic, 2002).

*M. tuberculosis* uses several receptors on the surface of macrophage while infecting it. These include the mannose receptor, Fc receptor and complement receptors (Aderem and Underhill, 1999). Entry of virulent mycobacteria through
complement and mannose receptors do not trigger an oxidative burst (Schlesinger, 1993). However infection through Fc receptors triggers oxidative burst but the mycobacteria still survived and replicated (Schwacha et al, 1993).

**Host-Pathogen Interactions in Mycobacterial infection**

The pathogenesis of tuberculosis is complex and its manifestations diverse, reflecting a lifetime of dynamic interactions between mycobacterial virulence factors and the immune system. Despite the ability of *M. tuberculosis* to cause disease in all organ systems and tissues of its human host, the bacillus exhibits specific cellular tropism for mononuclear phagocytes i.e. macrophages, monocytes and dendritic cells. From the initial infection of alveolar macrophages, to the lysis of these naïve macrophages and the resultant dissemination of the bacilli, to the restriction of bacillar proliferation by the immune system, the dynamics of interactions between tubercle bacilli and the immune system are the primary focus of pathophysiology.

*M. tuberculosis* have developed mechanisms to block or subvert normal cellular processes and thereby contributing to the pathogenesis and disease outcome. The phagosomal maturation involves a series of sequential fusion events with various vesicles from the endocytic pathway, by which nascent phagosomes attain microbicidal properties and become phagolysosomes. Phagolysosomes are acidic organelles that are rich in hydrolytic enzymes and which digest engulfed bacteria and other ingested particles. Immediately after the phagocytosis, the phagosome acquires markers, such as Rab5 and EEA-1 (early endosomal antigen 1) which direct the fusion of phagosomes with early endosomal vesicles. During the course of maturation, the phagosomes lose Rab5 and acquire Rab7, another GTPase, which also
functions in vesicular fusion. Late phagosomes acquire lysosomal markers, such as lysosome associated membrane protein (LAMP-1) and acid hydrolases, such as Cathepsin D, through fusion with lysosomal vesicles. Phagosomal maturation also involves the acquisition of vacuolar proton–ATPase molecules, which results in the acidification of phagolysosomes.

Pathogenic mycobacteria are directed to phagosomes that subsequently fail to fuse with lysosomes (Clemens et al, 2000). 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). Mycobacterial phagosomes are characterized by the presence of certain cellular proteins on their membrane including tryptophan-aspartate containing coat protein (TACO) (Ferrari et al, 1999) and certain small GTP-binding proteins (Deretic and Fratti, 1999). TACO is recruited to and retained on the membrane of the phagosomes that contain *M. bovis* BCG but not on the membranes of phagosomes that harbour killed mycobacteria, and is also absent from endosomal vesicles in uninfected cells. The stable association of TACO with mycobacterial phagosomes is thought to inhibit the fusion of these phagosomes with lysosomal vesicles. In phagosomes harbouring *M. bovis* BCG, the loss of Rab5 from the membrane which is seen in normal phagosome maturation does not take place, and Rab7 is selectively excluded from these phagosomes (Via et al, 1997).

One of the pathways best studied is the *M. tuberculosis*–induced inhibition of macrophage Ca\(^{2+}\) signalling. Upon ingestion of complement or antibody-opsonized organisms, macrophages respond with a significant increase in cytosolic calcium levels from a basal level of approximately 50-100 nM to a stimulated level of 500-
1000 nM (Malik et al., 2000; Malik et al., 2003). The evidence that Ca\(^{2+}\) signalling is required for phagosome maturation in macrophages came from the experiments where live, virulent *M. tuberculosis* infect human macrophages without triggering an increase in cytosolic Ca\(^{2+}\) levels (Malik et al., 2000; Malik et al., 2003; Malik et al., 2001). This block in macrophage Ca\(^{2+}\) signalling requires viable mycobacteria since virulent *M. tuberculosis* that are heat killed or \(\gamma\)-irradiated induced normal rise in the cytosolic Ca\(^{2+}\) level and their phagosomes matured to phagolysosomes. Furthermore, addition of an intracellular Ca\(^{2+}\) chelator, which prevents the rise in cytosolic Ca\(^{2+}\) by chelating, blocks the maturation of phagosomes containing killed *M. tuberculosis* (Malik et al., 2000; Malik et al., 2001). Addition of calcium ionophore which increases macrophage cytosolic Ca\(^{2+}\) concentration resulted in significant increases in both phagosome maturation and intracellular killing of tubercle bacilli.

The effects of alteration in calcium levels on phagosome maturation and intracellular viability of *M. tuberculosis* occurs through calcium-binding protein calmodulin (CaM). Increase in cytosolic Ca\(^{2+}\) levels results in the activation of CaM, the activated Ca\(^{2+}\)-CaM complex translocates from cytosol to the membrane of the nascent phagosome. The Ca\(^{2+}\)-dependent increase in phagosomal CaM causes localized activation of CaMKII on phagosomal surface (Malik et al., 2001). Similar to blockade of CaM, specific inhibition of CaMKII results in decreased phagosome maturation. In the case of live *M. tuberculosis* inhibition of macrophage Ca\(^{2+}\) signalling results in a lack of activation of cytosolic CaM and impairment of its recruitment to the phagosomal membrane (Malik et al., 2001). In the absence of the activated of Ca\(^{2+}\)-CaM complex, there is a lack of conversion of CaMKII from its inactive, unphosphorylated state to the activated, phosphorylated form of the enzyme.
that is required for subsequent phagosome maturation. Reversal of mycobacterial inhibition of Ca$^{2+}$-signalling by ionophore-induced elevation of cytosolic Ca$^{2+}$ results in restoration of the CaM recruitment to the membrane of phagosome containing live tubercle bacilli. Taken together, these studies implicate specific inhibition of a phagosome-localized Ca$^{2+}$/CaM/CaMKII signalling complex in the pathogenesis of tuberculosis leading to the promotion of the intracellular survival of \textit{M. tuberculosis} within human macrophages.

Recent evidence has further clarified the mechanisms by which the Ca$^{2+}$/CaM/CaMKII pathway regulates the maturation of phagosomes to phagolysosomes by linking it to the components of phosphoinositide-dependent signalling cascades. A phosphatidylinositol (PI)-specific PI-3 kinase, hVPS34, is recruited to the membrane of nascent phagosome (Vergne \textit{et al}, 2003), hVPS34 catalyzes the production of lipid second messenger, PI-3-phosphate, in the phagosome membrane. PI-3-phosphate is required for the recruitment of proteins, including early endosomal antigen -1 (EEA-1), which regulate the fusion of phagosomes with the vesicles of the endosomal-lysosomal pathway. Inhibitors of the PI-3 kinase, which block the generation of phagosomal PI-3-phosphate and the subsequent recruitment of EEA-1, inhibit phagosome maturation (Vergne \textit{et al}, 2003). Phagosomes containing \textit{M. bovis} BCG, or latex beads coated with LAM from \textit{M. tuberculosis}, do not accumulate EEA-1. These data are consistent with a model in which virulent \textit{M. tuberculosis} blocks the hVPS34/PI-3-P/EEA-1 cascade via inhibition of macrophage Ca$^{2+}$/CaM/CaMKII signalling pathway.

Macrophages that are invaded by potentially harmful bacteria activate their apoptotic programme to resolve the infection of macrophages. Mycobacteria-induced
macrophage apoptosis is a complex phenomenon that is modulated by mycobacterial virulence factors (Nigou et al, 2002) and mycobacteria are thought to influence the host apoptotic pathway through several ways. Mannose-capped lipoarabinomannan (Man-LAM) has been shown to antagonize mycobacteria-induced apoptosis in murine macrophages by preventing the increase in cytosolic Ca\(^{2+}\) concentration that follows mycobacterial entry or treatment with a calcium-ionophore (Rojas et al, 2000). Ca\(^{2+}\) is believed to facilitate apoptosis by increasing the permeability of mitochondrial membranes, thereby promoting the release of cytochrome c (Rojas et al, 2000) resulting in formation of apoptosome which in turn activates the effector proteins, the caspases.

Man-LAM also stimulates the phosphorylation of apoptotic protein Bad which prevents it from binding to the anti-apoptotic proteins Bcl-2 and Bcl-X\(_L\) (Maiti et al, 2001). Free cellular Bcl-2 prevents the release of cytochrome c from the mitochondria, inhibits caspase activity and functions as an anti-apoptotic regulator. The phosphorylation of Bad upon stimulation by Man-LAM involves phosphorylation and activation of Akt by protein kinase B thereby triggering the survival pathways.

*M. tuberculosis* also limits macrophage apoptosis by inducing the production of immunosuppressive cytokine IL-10 (Balczewicz-Sablinska et al, 1998). IL-10 was shown to block the synthesis of TNF-\(\alpha\) in infected macrophages. TNF-\(\alpha\) binds to the death receptors to activate the apoptotic program. IL-10 antagonizes the TNF-\(\alpha\) activity by inducing the release of soluble TNF-receptor 2 (TNFR2), which forms an inactive complex with TNF-\(\alpha\) that prevents the induction of TNF-\(\alpha\)-mediated apoptosis.
Modulation of MAP kinase and JAK/STAT pathways

Proinflammatory cytokines such as IL-1, IL-6, TNF-α and interferons, induce a cellular immune response when invading bacteria are detected. The release of these cytokines results in localized tissue damage and enhanced recruitment of phagocytic cells to the site of infection. The activation of signalling cascades such as mitogen activated protein kinases (MAPK) or JAK/STAT (Janus kinase/signal transducer and activator of transcription) results in production of proinflammatory cytokines and chemokines leading to the containment of infection. Pathogenic but not non-pathogenic mycobacteria have evolved mechanisms to suppress these signal transduction pathways and thereby attenuate cytokine-induced immune response.

MAP kinases are evolutionary conserved enzymes that are important in signal transduction. MAP kinases play a diverse role in cell proliferation, cell death, cytokine production and cell differentiation. Three main families of MAPKs are found in mammalian cells: c-Jun-N-terminal kinases (JNK 1, 2 and 3); the extracellular signal-regulated kinases 1/2 (ERK1/2); and the p38 MAPK (p38 α, β, γ and δ) (Johnson and Lapadat, 2002). ERK1/2 and p38 are phosphorylated on threonine and tyrosine at Thr-X-Tyrosine motif by the upstream kinases, the phosphorylated MAPKs then phosphorylate a number of transcription factors like ATF-2, Elk-1, CREB etc.

Mycobacteria modulate MAPK signalling to promote their survival in host cells. Studies on MAPKs has been done using virulent and attenuated strains. M. avium has two strains; smooth transparent (SmT) and smooth opaque (SmO) which represent a more virulent and less virulent phenotype respectively. Both SmT and SmO induced early phosphorylation of p38 upon infection, however only the
attenuated strain elicited sustained activation of p38 MAPK. In another study, it was found that the infection by virulent *M. avium* strain causes early activation of p38 and ERK1/2 pathways, which in case of infection with attenuated strain of *M. avium*, this dies out (Roach and Schorey, 2002).

*M. tuberculosis* modulates JAK/STAT signalling. *M. tuberculosis* infection of macrophages inhibits macrophage responses to IFN-γ. This inhibition is exerted at the level of transcription of IFN-γ responsive genes and not at the proximal steps of JAK/STAT signalling like STAT-1 phosphorylation, dimerization, nuclear translocation or DNA-binding. Rather infection with *M. tuberculosis* inhibits IFN-γ responses by directly or indirectly 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 IFN-γ responses by activating macrophage signalling 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.

**Role of Reactive Oxidative Species in cell signalling**

Binding of a ligand to a cell-surface receptor often stimulates an elaborate cascade of signal-transduction events. Rather than simply represent a linear transmission of information, proteins involved in signaling build complex intracellular networks in which signal propagation is controlled in a combinatorial
manner (Pawson and Saxton, 1999; Weng et al, 1999). The receptor ligand interaction at the cell surface generates the initial signal. This information is transmitted by a variety of signalling intermediates to the nucleus where the regulation of gene expression ends in a possible outcome of the initial signal. In addition to transmission, information processing also represents a key aspect of intracellular signalling. The diversity of signal output indicates the complexity and a tight modulation of the signalling pathways. However, it is still a long way to go before complete elucidation of the context-dependent regulation of the parameters that constitute variables in the signal outputs.

Sometimes scientific progression is not based on a discovery or the generation of new data but on a change of viewpoint that allows one to see a set of already existing data in a new light. The study of the role of reactive oxygen species (ROS) in the cell is such a case. ROS are a group of reactive oxygen species that include oxygen anions and radicals (\(\bullet O_2^-\) and \(OH\bullet\)) or the milder oxidants such as hydrogen peroxide (H\(_2\)O\(_2\)). There exists a huge body of data concerning the cell-damaging role of ROS (Finkel and Holbrook, 2000). The generation of ROS has been connected to stress response, apoptosis, ageing and cell death (Adler et al, 1999; Buttke and Sandstrom, 1995). In recent years, however, the “bad reputation” of H\(_2\)O\(_2\) and other ROS molecules has changed. These molecules are now recognized as molecules of life that are essential to the proper development and proliferation of cells. It has been known for some time that low doses of H\(_2\)O\(_2\) have mitogenic effects and can mimic the function of growth factors (Roth and Droge, 1987; Staal et al, 1994). Recently, however, has it become clear that these effects are not simply a reaction to an artificial exposure to ROS, but that upon stimulation by ligands, cells themselves
produce H$_2$O$_2$ and use it as a second messenger for signal transduction and signal amplification.

H$_2$O$_2$ shares several features with the well-studied second messenger calcium (Bootman et al, 2001). It is a small molecule that can diffuse locally inside the cell. It acts via the oxidation of proteins. Compared to the more aggressive ROS molecules, H$_2$O$_2$ is a rather mild oxidant that primarily targets cysteine residues in diverse proteins. Four oxidation states of cysteine can be generated: disulfide (-S-S), sulfenic acid (-SOH), sulfenic acid (-SO$_2$H) and sulfonic acid (SO$_3$H). Generation of the latter two states requires strong oxidants such as pervanadate and their formation is irreversible under physiological conditions (Huyer et al, 1997). In contrast, H$_2$O$_2$ oxidises the –SH group of cysteine to sulfenic acid, which is readily reduced to cysteine by various cellular reducing agents, including glutathione (GSH) and thioredoxin (Trx) (Nordberg and Arner, 2001). The fact that only certain proteins inside the cell carry an oxidizable cysteine at a critical position is the reason why such a small molecule like H$_2$O$_2$ can act as a specific second messenger.

Many receptors start signalling in a ligand independent manner when cells are treated with either H$_2$O$_2$ or even stronger oxidants such as pervanadate. This indicates that H$_2$O$_2$ can mimic the function of the ligand. There are several possibilities as to how H$_2$O$_2$ could activate a receptor. It could directly oxidize receptor components and thus generate aggregation, cross-linking in these receptors that lead to their activation. Alternatively, H$_2$O$_2$ could activate intracellular protein tyrosine kinases (PTKs) involved in signal transduction from these receptors. However, when PTKs are treated in vitro with H$_2$O$_2$, no increase in their kinase activity is detected (Wienands et al, 1996). The third possibility is that H$_2$O$_2$ inhibits protein tyrosine phosphatase (PTP)
activity and thus allows receptor to signal in a ligand independent fashion. According to simple signalling mathematics, the amount of phosphorylation in the cell equals kinase activity minus phosphatase activity. Therefore, tyrosine phosphorylation of intracellular proteins can also be increased by the inhibition of PTPs, which are prominent targets of $\text{H}_2\text{O}_2$-mediated oxidation and thereby shifting the balance in favor of kinase activation.

Activation of receptor tyrosine kinases (RTKs) is implicated in cell proliferation, motility, actin reorganization and chemotaxis. Tight regulation of RTK signaling is, therefore, crucial for eliciting an appropriate type and level of response. It has been proposed that PTPs negatively regulate RTK activity and downstream signaling (Ostman and Bohmer, 2001). Recent evidence has claimed a role for $\text{H}_2\text{O}_2$ as an intracellular messenger that regulates protein phosphorylation of tyrosine residues (Sundaresan et al., 1995 and Bae et al., 1997). All PTPs contain an essential cysteine residue in the signature active-site motif. Oxidation of the active-site cysteine of PTPs to the cysteine sulfenic derivative by $\text{H}_2\text{O}_2$ leads to an enzymatic inactivation. These observations suggest that oxidation of the cysteine residue of PTPs might occur in vivo in response to ROS or to an increase in redox potential. It is well known that ligand binding to RTKs increase their intrinsic tyrosine kinase activity (Heldin et al., 1998). Growing evidence suggests that inhibition of PTP activity occurs after RTK dimerization and, thereby, also contributes to the ligand-induced increase in RTK tyrosine phosphorylation (Ostman and Bohmer, 2001). Hence, it is likely that the redox inhibition of PTPs has an important role in RTK signalling, and that the rescue of the PTP catalytic activity after oxidation is followed by a dephosphorylation of activated receptor, thereby terminating the signal elicited from the receptor. Thus
ROS produced after RTK engagement is considered a second messenger that is involved in the signal-transduction machinery.

Neutrophils and macrophages are known to produce large amounts of H$_2$O$_2$ during the oxidative burst reaction. So far, it is thought that the only role of the oxidative burst is to kill engulfed bacteria with the generated ROS. Macrophages from mice deficient for the cysteine protease cathepsin G can no longer kill bacteria, although their oxidative burst is not impaired (Reeves et al., 2002; Bokoch, 2002). It is, therefore, possible that the oxidative burst is not primarily a killing device but rather a mechanism for activating the macrophage and neighbouring lymphocytes.

Redox signalling may help orchestrating the inflammatory response by inducing the synthesis of cytokines that affect macrophages and induce neutrophil influx. In fact, several studies have suggested that ROS can regulate the production of cytokines in macrophages through mechanisms that are dependent on NF-κB. LPS, which stimulates the production of TNF-α, induced the production of ROS via a pathway dependent on Rac1 and the activation of NF-κB through IκB kinase in murine macrophage cells (Sanlioglu et al., 2001). It was shown that exogenous H$_2$O$_2$ activates ERK1/2 in many cell types. Further studies showed that increased intracellular production of ROS also activated the ERK pathway (Sundaresan et al., 2000). Many stimuli that trigger the respiratory burst in phagocytes induce the activation of the ERK (Torres and Forman, 2002; Torres and Forman, 2003; Torres, 2003). It was also shown that H$_2$O$_2$ augmented phagocytosis mediated by FcγRI and FcγRII in human neutrophils and amplified receptor-triggered tyrosine phosphorylation of FcγR-associated immuno tyrosine activation motifs (ITAMs) and signaling elements (Pricop et al., 1999). Thus, it is speculated that redox signaling
plays an important role in the regulation of the inflammatory response and ultimately in development of lung disease.

**Secretory Antigens of *M. tuberculosis***

Secreted antigens have attracted particular attention as candidate antigens for subunit vaccines. The observation that live mycobacteria, but not heat-killed preparations, elicit protective immunity in animal models (Hortelano *et al.*, 2002), apparently indicated secretion of CFP components to be responsible for the greater efficacy of vaccination with live attenuated mycobacteria than that with killed organisms. 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). Secreted antigens have been extensively characterized and some have been shown to induce protection in animal models (Mustafa, 2002).

**ESAT-6 and MTSA-10**

The genome of *Mycobacterium tuberculosis* H37Rv has five copies of a cluster of genes known as the ESAT-6 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 transporters, ATP-binding proteins and other membrane-associated proteins (Gey Van Pittius *et al.*, 2001). A 10 kb genomic DNA region, designated 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. Two proteins encoded by RD1 region namely ESAT-6 and MTSA-10, elicited strong skin reactions. A cooperative functional relationship has been proposed between the proteins encoded in RD1 (Wards et al, 2000). Failure of M. bovis knockout mutant of the ATPase gene (Rv3871) in the RD-1 to sensitize guinea pigs to an ESAT-6 skin test supports this view. Recently ESAT-6 and CFP-10 have also been shown to form a tight, fully folded 1:1 complex, suggesting their activity as a complex (Renshaw et al, 2002). Restoration of RD1 region by gene knock-in technique has been done in Mycobacterium bovis BCG and in Mycobacterium microti. These BCG::RD1 and M. 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).

Recombinant CFP-10 was shown to be a potent T-cell antigen, eliciting proliferative responses and gamma interferon production from peripheral blood mononuclear cells (PBMC) in 70% of PPD-positive individuals without evident disease. This antigen has also been shown to be 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 (Colangeli et al, 2000). Additionally, it has been demonstrated that PBMC from TB patients produce IFN-γ 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).

**Antigen 85b-complex**

The three proteins of the antigen-85 (Ag85) complex (85a, 85b and 85c) are major secretory proteins of actively growing mycobacteria. Serum Ag85 levels can be correlated with active mycobacterial infections independent of host immunity, as median serum Ag85 levels are known to be 50- to 150-fold higher in patients with active tuberculosis compared to healthy controls or patients with active *M. avium-intracellulare* disease or patients with non tuberculous pulmonary disease (Bentley-Hibbert et al, 1999). 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). These antigens induced lymphoproliferation 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 (Erp), also known as P36, PIRG and Rv3810 is an ubiquitous extracellular protein found in most of the mycobacterial species (de Mendonca-Lima et al, 2001). Both in case of *M. tuberculosis* and *M. bovis* (BCG), disruption of the bacterial *erp* gene impaired multiplication of bacterium in cultured macrophages and mice. Furthermore, reintroduction of *erp* into these mutants restored their ability to multiply (Berthet et al, 1998a), 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 24-kDa *M. tuberculosis* complex specific secretory protein is absent from the four most commonly used BCG vaccine strains, and therefore, it has 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). Indeed, both the native as well as recombinant MPT-64 has been shown to distinguish between *M. tuberculosis* infection and BCG (Danish 1331)-vaccination (Oettinger et al, 1997). 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).

**Effectors in Macrophage Activation**

Macrophages are professional phagocytes and they provide the first line of defense against an invading pathogen. Macrophages get activated when encountered by any foreign agent and it is the activation state of macrophage that determines whether the pathogen will proliferate or perish in its host cells. Several pathogen-specific molecules and a number of cytokines and chemokines activate the macrophage, essential for mounting an effective immune response. Pathogens, in order to establish a successful infection, de-activate the macrophage by inhibition of several host cell processes and host cell signalling which otherwise will lead to activation of the macrophages and a protective immune response.

**Lipopolysaccharide**

Lipopolysaccharide (LPS) is the principal component of the outer membrane of gram-negative bacteria. Recent studies have elucidated how LPS is recognized by monocytes and macrophages of the innate immune system. Monocytes and macrophages are exquisitely sensitive to LPS and respond by expressing many inflammatory cytokines. LPS binds to LPS-binding protein (LBP) in plasma and is delivered to the cell surface receptor CD14. Next, it is transferred to the transmembrane signalling receptor toll-like receptor 4 (TLR4) and its accessory protein MD2. LPS stimulation of macrophages activates several intracellular signalling pathways including the I-kappa B kinase (IKK)-NK-κB pathway and the
MAPK pathways. These signaling pathways in turn activate a variety of transcription factors, which coordinate the induction of many genes encoding inflammatory mediators.

Several researchers have demonstrated the role of LBP and CD14 in LPS activation of macrophages and monocytes. Immunodepletion of LBP from whole blood lowers the sensitivity of monocytes to LPS by two orders of magnitude (Schumann et al, 1990). Similarly, antibodies to CD14 block LBP-dependent activation of macrophages by LPS (Wright et al, 1990). Conversely, expression of CD14 on 70Z/3 pre-B cells that are poorly responsive to LPS raises their sensitivity to LPS (Lee et al, 1992). Analysis of LBP-deficient mice showed that LBP was essential for the rapid induction of an inflammatory response by small amount of LPS or gram-negative bacteria (Jack et al, 1997). Moreover, LBP-deficient mice were significantly more sensitive to *S. typhimurium* infection, indicating that LBP is required to combat infection by gram-negative bacteria. These studies demonstrate that LBP and CD14 are required for monocyte/macrophage activation by LPS. Nevertheless, the absence of a transmembrane segment for the attachment of CD14 to the cell surface strongly suggested that CD14 was not the LPS signaling receptor. However, co-precipitation of CD14 along with protein tyrosine kinases (PTK) (Stefanova et al, 1993) and phosphorylation of several proteins on tyrosine residue(s) induced by LPS in macrophages (Weinstein et al, 1991) suggest an involvement of PTKs in the signal transduction pathways triggered by LPS. It was also reported that the Src kinase Lyn co-immunoprecipitates with CD14, and LPS transiently stimulates activity of Lyn as well as other Src kinases Hck and Fgr in monocytes (Stefanova et al, 1993).
Many receptors stimulate protein tyrosine phosphorylation following ligand binding, and this event is thought to be part of the signal transduction mechanism that mediates later cellular response (Ullrich and Schlesinger, 1990). It was shown that LPS treatment rapidly increases tyrosine phosphorylation of several proteins in macrophage cell line and resident peritoneal macrophages (Weinstein et al, 1991). It was also shown that myeloid specific Src members Lyn, Hck and Fgr are predominantly activated after LPS stimulation (Han et al, 1993; Liu et al, 1994) and chronic LPS exposure (24-48 hrs) further results in increased synthesis of Lyn and Hck (Boulet et al, 1992). However, macrophages derived from mice with three-combined deficiency of Hck, Fgr and Lyn still retain full LPS responsiveness (Meng and Lowell, 1997). This result not only highlighted that the activation of the myeloid-specific Src family members was not obligatory for LPS induced macrophage activation, but also raised the question of what was the compensating tyrosine kinase(s)? Recently it was demonstrated that Bruton’s tyrosine kinase (Btk) and cSrc are activated in LPS stimulated macrophages and contribute to TNF-α and nitric oxide production (Horwood et al, 2003; Leu et al, 2005).

A number of upstream activators, as discussed before, are involved in LPS signaling in macrophages and monocytes. These and other signaling molecules mediate activation of several downstream signalling pathways. Indeed, it was shown that LPS is a potent activator of all three MAPK pathways in monocytic cells (Sweet and Hume, 1996). Many studies have shown that LPS activates ERK1/2 in macrophages and monocytes (Swantek et al, 1997; Marie et al, 1999; Durando et al, 1998). Dominant negative repressors of both Ras and c-Raf inhibited LPS induction of TNF-α promoter in macrophages (Geppert et al, 1994), supporting a role of Ras-
Raf-MEK MAPK pathway in LPS induced TNF-α expression. However there was also evidence for LPS activation of the MEK-ERK1/2 pathway via c-Raf independent pathways (Buscher et al, 1995; Chen et al, 1993). On the other hand LPS stimulation of human and murine macrophage cells rapidly activates the JNK pathway (Hambleton et al, 1996). It was also shown that LPS, through Syk and PI3K-dependent pathways, induce JNK activation in neutrophils that is essential for LPS-induced monocyte chemoattractant protein-1 (MCP-1) expression (Arndt et al, 2004). The third MAPK pathway includes isoforms of p38 and stress activated protein kinase (SAPK) (Ono and Han, 2000). p38 was originally identified in LPS-stimulated murine macrophages (Han et al, 1994). It was shown that specific inhibition of p38 activation reduced LPS induction of IL-1 and TNF-α expression (Lee et al, 1994).

**Interferon-γ (IFN-γ)**

Produced mainly by CD4+ and CD8+ T cells as well as by NK cells, IFN-γ is the most crucial cytokine in control of *M. tuberculosis* infection (Lyadova et al, 1998). A mutation in IFN-γ or the IFN-γ receptor gene leads to the serious mycobacterial infections and IFN-γ gene knock-out mice are most susceptible to virulent *M. tuberculosis* till date (Cooper et al., 1993). The knock-out mice show necrotic granulomas, defective macrophage activation with low iNOS expression and uncontrolled bacillary growth upon infection with *M. tuberculosis* (Flynn et al, 1993). Mycobacterial components/live bacteria affected both the T cell dependent IFN-γ production as well as IFN-γ dependent macrophage activation. Tuberculosis patients show depressed serum IFN-γ levels (Lin et al., 1996). Furthermore, *M. tuberculosis*-
infected human macrophages show diminished IFN-γ signaling, probably due to the disruption of the association of the transcription activator STAT-1 with the transcriptional co-activators CREB (cAMP Response Element-binding Protein) and p300 (Ting et al., 1999).

**Tumor necrosis factor-α (TNF-α)**

TNF-α is an essential component of tuberculous granuloma and potent mediator of macrophage activation. TNF-α plays an important role in the host response against *Mycobacterium tuberculosis*. Mice deficient in TNF-α or the 55-kDa TNF receptor died quickly in case of *M. tuberculosis* infection and were found to harbor substantially higher number of bacilli in the lung compared to those observed in control mice (Flesch and Kaufmann, 1990). This cytokine is critical for control of acute *M. tuberculosis* infection as it synergizes with IFN-γ to induce iNOS expression. Mice inoculated with a low-dose of *M. tuberculosis* were found to maintain a stable bacterial load six months later. However, when treated with anti-TNF-α antibody leading to neutralization of TNF-α activity, the bacillary counts in the lungs increased significantly and resulted in 100% mortality of these animals (Flesch and Kaufmann, 1990). Anti-TNF-a antibody treatment of an arthritis patient also resulted in development of fatal disseminated tuberculosis (Maini et al., 1999). Intriguingly, TNF-α 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 TNF-α (Engele et al., 2002). Although presence of TNF-α 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 TNF-α has been reported to cause destructive inflammation (Bekker et al, 2000). At the same time, TNF-α is known to contribute to macrophage inhibition of *M. tuberculosis* growth by a mechanism that is dependent on apoptosis and independent of IFN-γ activity (Keane et al, 2002).

**Interleukin-12 (IL-12)**

IL-12 is secreted mainly by macrophages and mycobacteria are strong IL-12 inducers. Following their infection with mycobacteria, mice genetically deficient in IL-12p40-gene (IL-12p40−/−) were found to develop greatly increased bacterial burden and suffer from decreased survival time, possibly due to the substantially reduced IFN-γ production in IL-12p40−/− mice (Cooper et al, 1997; Cooper et al, 2002b). Humans with mutations in IL-12p40 or the IL-12 receptor genes were more susceptible to disseminated BCG and *M. avium* infections (Casanova, 2000). Exogenous administration of murine recombinant IL-12 decreased multiplication of *Mycobacterium tuberculosis* in infected mice (Lounis et al, 1999).

**Interleukin-10 (IL-10)**

IL-10, another cytokine secreted by the macrophages, is known to inhibit CD4+ T cell responses as well as antigen presentation by the cells infected with mycobacteria (Roach et al, 2001). This anti-inflammatory cytokine is continuously produced in experimental tuberculosis (Fietta et al, 2001) and favors disease development by downregulating IL-12 production and obstructing macrophage
activation (Jacobs et al., 2000). 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). Again, 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 TNF and IL-12p40, and a decrease in antigen-specific IFN-γ 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-6 (IL-6)**

IL-6 is required during initial innate response to the pathogen and has been shown to influence T cell differentiation and inflammatory response (Saunders et al., 2000). The IL-6−/− mice did succumb to infection with a high intravenous dose of *M. tuberculosis* inoculum that are still controlled by IL-6-competent mice. Spleen cells from IL-6−/− mice produced elevated levels of IL-4 and reduced levels of gamma interferon compared to the control mice (Ladel et al., 1997). 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).
Present Studies

In the context of the studies cited above and available literature, the proteins secreted by *M. tuberculosis* assumes significant importance. Two open reading frames of RD-1 region (which are consistently deleted in all BCG strains), Rv3874 and Rv3875 encode 10-kDa culture filtrate protein and 6-kDa early secreted antigenic target respectively (Cole *et al*, 1998) and are cotranscribed. Thus CFP-10 and ESAT-6 proteins are specific for *M. tuberculosis*. Our studies have focussed on the modulation of macrophage cell signaling by ESAT-6 and CFP-10. ESAT-6 was found to limit the activation of extracellular signal-regulated kinases 1/2 (ERK1/2) MAP kinase in the nucleus but not p38 MAP kinase. This effect was due to some putative phosphatase(s) present in the nucleus. The effect of ESAT-6 on the ERK1/2 activation antagonized LPS-induced *c-myc, IL-1β, ICAM-1, Bax* and *TNF-R1a* gene expression. We also studied the effect of CFP-10, ESAT-6 and CFP10:ESAT6 complex on the ROS production in macrophages. ROS plays an important role in host defense against bacterial infections; however, the role of ROS in context of mycobacterial infection has not been studied in detail. The present study indicates CFP-10, ESAT-6 and CFP10:ESAT6 complex downregulated ROS production in RAW264.7 cells. The effect is specific for ESAT-6 family proteins as other Mtb proteins like CFP-21 and Antigen 85b did not affected ROS production. CFP-10, ESAT-6 and CFP10:ESAT6 complex antagonized LPS-induced ROS production. CFP-10, ESAT-6 and the CFP10:ESAT6 complex downregulated LPS-induced ROS-dependent NF-κB transactivation in RAW264.7 cells. Therefore the effect of these proteins on the LPS-induced gene expression might help in the persistence of the tubercle bacilli inside the macrophages.