Introduction
Introduction
1.1 Tuberculosis and its Treatment
Tuberculosis (TB) is a common and deadly infectious disease caused by *Mycobacterium tuberculosis* (*M. tb.*) and congeners. Over one-third of the world's population now has the TB bacterium in their bodies and new infections are occurring at a rate of one per second (WHO 2006). In 2004, 14.6 million people had active TB, there were 8.9 million new cases and 1.7 million deaths, mostly in developing countries (WHO 2006). A rising number of reactivation TB cases are observed in immunosuppressive disease, pharmacological immunosuppression or HIV/AIDS patients. Drug-resistant strains of TB have emerged and are spreading (MMWR 2006). TB is conventionally treated by a 6-month to 1-year regimen of 4 orally-administered drugs under direct observation (DOTS) as recommended by WHO, and implemented in large parts of India under the Revised National Tuberculosis Control Programme (RNTCP).

We (Sharma et al. 2001; Sen et al. 2003; Muttil et al. 2007; Sharma et al. 2007; Kaur et al. 2008; Verma et al. 2008) and others (O'Hara et al. 2000; Dutt et al. 2001; Suarez et al. 2001; Sethuraman et al. 2002; Ul-Ain et al. 2003; Makino et al. 2004; Tian et al. 2004; Yoshida et al. 2006; Hasegawa et al. 2007; Lu et al. 2007) have proposed an alternative drug delivery system, namely, microparticles containing anti-TB agents for pulmonary delivery to lung and alveolar macrophages. The extremely high efficacy of this drug delivery system (Suarez et al. 2001; Sen et al. 2003; Lu et al. 2007; O'Sullivan et al. 2007) can be best understood from the point of view of (1) efficient drug targeting to the exact site of infection, and (2) classical, bactericidal activation of the infected macrophage. The present work relates to closer investigation of macrophage activation in the presence of infection with *M. tb.* when the infection is treated with anti-tuberculosis drugs in soluble or microparticulate form. For this purpose, a closer understanding of the pathogen (*M. tb.*) and the host cell, the lung macrophage is necessary.
1.1.1 The Pathogen

*M. tb.* is a nonmotile and rod-shaped facultative intracellular parasite, an obligate aerobe which can survive and multiply inside macrophages (Mφs) and other mammalian cells. The size of the mycobacterium is 2-4 µm in length and 0.2-0.5 µm in width. *Mycobacteria* are not classified into Gram-positive or Gram-negative because they do not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall (Prescott LM et al. 1996). In phylogenetic studies, 16S rRNA sequencing showed that *Mtb* belongs to a group of ‘slow growers’, also known as ‘*M. tuberculosis* complex’ (Rogall et al. 1990). The generation time of mycobacterium is typically ~24 hours in solid medium. This genus includes six members: *M. tuberculosis* the causative agent of human tuberculosis cases; *M. africanum*, infecting humans in sub-Saharan Africa; *M. microti*, the causative agent of TB in some aquatic rodents; *M. bovis*, which infects a very wide variety of mammalian species including humans; BCG, an attenuated variant of *M. bovis*; and *M. canetti*, a smooth variant that is very rarely encountered but causes human disease. The important features shared by all members of the *Mycobacterium* genus include a cell wall composed of a complex outer cell wall, containing a large amount of cell wall lipid. It consists of several unique components such as lipoarabinomannan, lipomannan, pthiocerol dimycocerostate, mycocerostate, mycolic acid, trehalose dimycolate and sulpholipids (Brennan et al. 1990; Besra et al. 1994). These components are suggested to be responsible for mycobacterial hydrophobicity, ability to form clumps or cords, ability to survive intracellularly and it is the cell wall that gives acid-fastness, enabling it to retain basic dyes in the presence of acid alcohol.

Hundreds of clinical strains of pathogenic bacteria have been reported in the literature. Two laboratory strains are most commonly used to develop understanding of TB. The virulent H37Rv strain ATCC 25618 and its close relative, the attenuated H37Ra strain ATCC 25177. Virulent strains share the ability to invade macrophages and survive within them in maturation-arrested phagosomes, which do not fuse with lysosomes.
1.1.2 The Host Macrophage

The word ‘macrophage’ is Greek for “big eater”. Macrophages differentiate into their terminal phenotype from monocytes. Monocytes and macrophages are both phagocytic, with roles in both antigen-specific and non-specific (innate) defence responses. Macrophages engulf pathogens, and being classical antigen-presenting cells, process and present pathogen-derived peptides to T lymphocytes. Several biochemical and cell biological processes relate to the defence responses of macrophages. Some of these include:

(a) Acidification of the pathogen-containing phagosome by inserting proton pumps into the membrane, and assembling proteins on the surface to facilitate docking and fusion of lysosomes (phagosome maturation),

(b) Fusion of the mature phagosome with lysosomes to enable enzymatic lysis of the phagocytosed pathogen (antigen processing),

(c) Loading pathogen-derived peptides on major histocompatibility complex II molecules for presentation to T lymphocytes (antigen presentation),

(d) Secretion of chemokines and cytokines to attract T lymphocytes to the vicinity, and influence their differentiation to appropriate cytotoxic/helper phenotype,

(e) Generation of free radicals such as reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) to cause oxidative stress to the pathogen,

(f) Execution of an apoptotic programme designed to deny sanctuary to intracellular pathogens, and package these pathogens in apoptotic bodies for uptake by bystander antigen presenting cells (APC), etc.

During infection with TB-causing bacteria, all of the above are invoked in immunocompetent individuals, whereby most people infected with these pathogens manage to clear the infection without drug therapy. In susceptible individuals, one or more of the above responses is compromised, since the pathogen has evolved several biochemical mechanisms to subvert the host responses. Such interplay between the host and the pathogen biochemistry and cellular biology is increasingly being addressed by research into host-pathogen interactions.
1.2 Macrophage-mycobacterium interactions

Alveolar macrophages are the primary cell type involved in the initial uptake of *M.tb*. Along with these, dendritic cells and monocyte-derived macrophages also take part in the phagocytosis process (Henderson et al. 1997; Thurnher et al. 1997).

1.2.1 Pathogen Entry Into Host Cell

The phagocytosis of *M. tb.* involves different receptors on the macrophage, which either bind to nonopsonized bacteria or recognize opsonins on the surface of the pathogen. Complement receptors (CRI, CR2, CR3 and CR4), mannose receptors (MR), CD14, collectins, scavenger receptors, etc, mediate binding of the bacterium to the phagocyte surface (Schlesinger 1993; Gaynor et al. 1995; Hoheisel et al. 1995; Schlesinger 1996; Zamzami et al. 1996; Aderem et al. 1999). In the absence of CR3, phagocytosis of *M. tb.* by human macrophages and monocytes is reduced by approximately 70 to 80% in vitro (Schlesinger et al. 1990; Schlesinger 1993). Nevertheless, nonopsonized *Mycobacterium* can bind directly to CR3 (Hu et al. 2000) and CR4 (Zaffran et al. 1997). The best characterized receptor for non-opsonin-mediated phagocytosis of *M. tb.* is the macrophage mannose receptor (MR). The interaction between manose receptor is through mycobacterial surface glycoprotein lipoarabinomannan (LAM) (Schlesinger et al. 1991). Prostaglandin E2 (PGE2) and interleukin-4 (IL-4) up regulate CR and MR receptor expression and function while γ interferon (IFN-γ) decreases the receptor expression, resulting in diminished ability of the mycobacteria to adhere to macrophages (Cynamon et al. 1999). When uptake by CRs and MR is blocked, macrophages may also internalize bacterium through the type A scavenger receptor (Zimmerli et al. 1996). *M. avium* induces TNF-α and IL-10 by binding to the CD14 on human macrophages (Reiling et al. 2001). Collectins form a structurally related group of proteins that includes surfactant proteins and mannose-binding lectins (MBLs). Surfactant protein A (Sp-A) facilitates the uptake of *M. tb.* (Pasula et al. 1999), through binding to either macrophages (Vishwanath et al. 1997), type II pneumocytes (Weikert et al. 1997; Aderem et al. 1999), or neutrophils (Ernst 1998). Interestingly, it has been reported that human immunodeficiency virus infected individuals have increased levels of Sp-A in the lungs, and this results in a three fold-greater attachment of *M. tb.* to alveolar macrophages (Downing et al. 1995). Another surfactant protein,
Sp-D, has been found to block the uptake of pathogenic strains of *M. tb.* by macrophages (Ferguson et al. 1999). MBL recognizes carbohydrate patterns on a wide variety of pathogens (Reddy et al. 2002) and induces phagocytosis directly through a yet-undefined receptor or indirectly by activation of the complement system (Turner 1996). In a few studies high concentrations of MBL in the serum of TB patients have been reported (Garred et al. 1997), and genetic polymorphisms associated with increased production of MBL are a relative disadvantage in mycobacterial infections (Selvaraj et al. 1999). Fc receptors, which facilitate phagocytosis of particles coated with antibodies of the immunoglobulin G class probably play little role in TB (Cohn 1963).

*Mtb.* also interacts with nonprofessional phagocytic cells, such as alveolar epithelial cells (Byrd et al. 1993). This binding may involve fibronectin, a glycoprotein found in plasma and on the outer surface of many cell types (Ruoslahti 1988). Similar to *Mycobacterium leprae* (Byrd et al. 1993), *M. tb.* binds to epithelial cells since the bacterium produces and secretes the antigen 85 complex, a member of the fibronectin-binding protein family (Wiker et al. 1992). In addition, a 28-kDa heparin-binding adhesin, produced by *M. tb.*, binds to sulfated glycoconjugates on host cells (Menozzi et al. 1996). After entry, mycobacteria survive inside macrophage by inhibiting various natural pathogen-killing pathways operating in infected macrophages.

1.2.2 Phagosomal maturation inhibited by mycobacteria

Internalization of particles by the macrophage initially forms a phagosome, followed by a series of sequential fusion events with various vesicles from the endocytic pathway culminating in a phagolysosome. *M.tb.* survives inside host macrophages by inhibiting phagosome-lysosome fusion (Vergne et al. 2004), which is essential for killing the pathogen (Armstrong et al. 1971; Vergne et al. 2004). Incomplete acidification of the phagosome is due to inhibition of insertion of proton pumps in the vacuolar membrane (Sturgill-Koszycki et al. 1994) and absence of mature lysosomal hydrolases. The incomplete luminal acidification allows intracellular survival and growth of mycobacteria inside infected macrophages (Russell 2001).
1.2.3 Other host mechanisms inhibited by mycobacteria

Antigen processing

An important aspect of the mycobacterial phagosome is its inefficient antigen processing capacity (Pancholi et al. 1993; Ramachandra et al. 2001). It has been shown using purified phagosomes that the majority of peptide–MHC-II complexes are formed within phagosomes, by loading MHC-II molecules without prior export of bacterial antigens from phagosomes to conventional antigen processing compartments. In these assays, heat-killed mycobacteria were processed more readily than live *M. tb* (Ramachandra et al. 2001).

Reactive oxygen intermediates (ROI) and Reactive nitrogen intermediates (RNI)

Bactericidal mechanism inside phagolysomes of activated macrophage includes the production of ROI and RNI. Proteins secreted by *M. tb*. such as superoxide dismutase (SOD) and the catalase-peroxidase system are antagonistic to ROI (Dahl et al. 1996). Mycobacterial components such as sulphatides, LAM and phenolic- glycolipid I (PGL-I) are potent oxygen radical scavengers (Chan et al. 1989; Chan et al. 1991). Mycobacteria are resistant to killing in vitro by ROI such as superoxide and hydrogen peroxide (Chan et al. 1992). Hydrogen peroxide (H₂O₂), 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 et al. 1981). However, the ability of ROI to kill *M. tb*. has been demonstrated only in mice (Flesch et al. 1990) and remains to be confirmed in humans. Studies show that *M. tb*. infection induces the accumulation of macrophages in the lung and also H₂O₂ production (Selvaraj et al. 1988). Similar local immune response in tuberculous ascitic fluid has also been demonstrated (Swamy et al. 1988). However, the increased production of hydrogen peroxide by alveolar macrophages is not specific for TB (Selvaraj et al. 1988). Moreover, alveolar macrophages produced less H₂O₂, than the corresponding blood monocytes.

RNIs produced by activated mouse macrophages are major elements in antimicrobial activity (Nathan et al. 1991). The presence of RNIs in human macrophages and their potential role in disease has been the subject of controversy, but the alveolar macrophages of a majority of TB-infected patients exhibit inducible nitric oxide
synthase (iNOS) activity (Nicholson et al. 1996). Phagocytes, upon activation by IFN-γ and TNF-α, generate nitric oxide (NO) and related RNI via iNOS2 using L-arginine as the substrate. Infection of human alveolar macrophages with M. bovis BCG in vitro resulted in increased iNOS mRNA and inhibition of iNOS is followed by increased bacterial growth (Nozaki et al. 1997). In TB patients, alveolar macrophages show increased production of iNOS (Nicholson et al. 1996). However, whether iNOS gene expression leads to in vivo NO production remains uncertain, as in humans posttranslational modification of iNOS may be necessary for functional activity (Salh et al. 1998). Therefore, the exact contribution of RNI in human TB remains to be elucidated. Sustained intracellular growth of M. tb. may depend on its ability to avoid destruction by lysosomal enzymes, ROI, and RNI.

The significance of these toxic nitrogen oxides in host defense against M. tuberculosis has been well documented, both in vitro and in vivo, particularly in the murine system (Chan et al. 2001). In iNOS gene knock-out mice M. tb. replicates much faster than in wild type animals, implying a significant role for NO in mycobacterial host defense (MacMicking et al. 1997).

1.3 Immune evasion and cytokine signaling in TB
M. tb. is able to survive inside host by employing various immune evasion strategies, including modulation of antigen presentation to avoid elimination by T cells. M. tb. infected macrophages are diminished in their ability to present antigens to CD4⁺ T cells, which leads to persistent infection (Hmama et al. 1998). Another mechanism by which APC contribute to defective T cell proliferation and function is by the production of cytokines, including TGF-β, IL-10 (Rojas et al. 1999) or IL-6 (VanHeyningen et al. 1997).

Since M. tb. is an intracellular pathogen, antibodies are unlikely to play any protective role. Although many researchers have dismissed a role for B cells or antibody in protection against TB (Shiratsuchi et al. 1991), recent studies suggest that these may contribute to the response to TB (Bosio et al. 2000).
1.3.1 Cytokines Involved in the Immune response

Colonization of phagocytic cells by *M. tb.* leads to cell activation and cytokine production, which induces further activation and cytokine production in a complex process of autoregulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and active disease. Some key cytokines in the process include

**γ Interferon**

IFN-γ is produced primarily by both CD4+ and CD8+ T cells, as well as by NK cells in addition to macrophages themselves. IFN-γ is important for macrophage activation in TB, and is considered to be essential for effective host response against infection. The protective role of IFN-γ in TB is well established, primarily in the context of antigen-specific T cell immunity (Garred *et al.* 1997). IFN-γ is produced by T cells from healthy PPD+ subjects as well as those with active TB. Although some studies suggest that IFN-γ levels are depressed in patients with active TB (Nicholson *et al.* 1996; Cynamon *et al.* 1999), this cytokine may not be ideal as a marker to correlate protection against infection. A report that *M. tb.* has developed mechanisms to limit the activation of macrophages by IFN-γ (Oddo *et al.* 1998) suggests that the amount of IFN-γ produced by T cells may be less predictive of outcome than the ability of the cells to respond to this cytokine. IFN-γ is the major activator of macrophages in mouse, but not human, macrophages to inhibit the growth of *M. tb.* in vitro (Cooper *et al.* 1993). IL-4, IL-6 and GM-CSF could bring about *in vitro* killing of mycobacteria by macrophages either alone or in synergy with IFN-γ in the murine system (Blanchard *et al.* 1989). Humans defective in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including infection with *M. tb.* (Ottenhoff *et al.* 1998). IFN-γ knockout (GKO) mice are the most susceptible mouse strain to virulent strain H37Rv (Cooper *et al.* 1993). Macrophage activation is defective in IFN-γ knockout mice and also NOS2 expression is low. These factors probably contribute to the extreme susceptibility of and unchecked bacterial growth in GKO mice. However, the mean survival time for *M. tb*-infected NOS2-/- mice is at least twice that of GKO mice, suggesting that there are IFN-γ-dependent, NOS2-independent mechanisms of protection against TB (Flynn *et al.* 1996; MacMicking *et al.* 1997).
**Tumor Necrosis Factor (TNF-α)**

TNF-α is considered “pivotal” to the control of the infection. *M. tuberculosis* supresses TNF-α secretion in a strain-specific way. Avirulent strains are not able to inhibit TNF secretion, but virulent strains inhibit TNF secretion by infected macrophages (Riendeau et al. 2003). In mice deficient in TNF-α or TNF receptor, *M. tb*, infection resulted in rapid death of the mice, with substantially higher bacterial burdens compared to control mice (Bean et al. 1999). TNF-α in synergy with IFN-γ induces NOS2 expression (Liew et al. 1990). TNF-α is important for walling off infection and preventing dissemination. Convincing data on the importance of this cytokine in granuloma formation in TB and other mycobacterial diseases has been reported (Flesch et al. 1990; Flynn et al. 1995). TNF-α affects cell migration and localization within tissues in *M. tb* infection. During chronic infection, NOS2 expression in the lungs was reduced following TNF-α neutralization (Mohan et al. 2001). TNF-α influences expression of adhesion molecules as well as chemokines and/or their receptors and affects the formation of functional granuloma in infected tissues. TNF-α has also been implicated in immunopathologic response and is often a major factor in host-mediated destruction of lung tissue (Moreira et al. 1997). Increased levels of TNF-α were found at the site of lesion (pleural fluid), as compared to systemic response (blood) showing that the compartmentalized immune response must be containing the infection (Raja 2004). In response to *M. tb* infection, NOS2 expression in the granulomas of TNFRp55-/- mice was delayed (Flynn et al. 1995), although a similar delay was not observed in TNF-α -/- mice (Bean et al., 1999). The requirement of TNF-α in control of *M. tb* infection is complex, but it clearly is an important component for macrophage activation. TNF-α levels were generally higher in resistant individuals in comparison to susceptible individuals during TB infection. TNF-α also plays a major role in apoptosis induction, signaling through the death receptor (Riendeau et al. 2003).
**Interleukin-1β**

A second proinflammatory cytokine involved in the host response to *M. tuberculosis* is IL-1β. Like TNF-α, IL-1β is mainly produced by monocytes, macrophages, and dendritic cells. In TB patients, IL-1β is expressed in excess at the site of disease (Law et al. 1996). IL-1β is important in influencing the course of experimental TB in mice: IL-1α and -1β double-KO mice (Sugawara et al. 2000) and IL-1R type 1-deficient mice (which do not respond to IL-1β) display an increased mycobacterial burden and also defective granuloma formation after infection with *M. tb* (Leemans et al. 2001).

**Interleukin-2**

IL-2 has a crucial role in generating an immune response against specific antigen by inducing an expansion of the pool of lymphocytes. Therefore IL-2 secretion by the protective CD4 Th1 cells is an important parameter to be measured protection against specific antigen. Several studies have demonstrated that IL-2 can influence the course of mycobacterial infections, either alone or in combination with other cytokines (Blanchard et al. 1989).

**Interleukin-4**

IL-4 is a classic Th2 cytokine. The role of IL-4 in TB is still controversial. In TB patients a depressed Th1 response, but not an enhanced Th2 response was observed in isolated PBMC (Robinson et al. 1994; Nicholson et al. 1996; Ottenhoff et al. 1998; Cynamon et al. 1999). Elevated IFN-γ expression was detected in granuloma within lymph nodes of patients with tuberculous lymphadenitis, but little IL-4 mRNA was detected (Nicholson et al. 1996). These results indicated that in humans a strong Th2 response is not associated with TB. In mice, studies (Cooper et al. 1993) suggest that the absence of a Th1 response to *M. tb* does not necessarily promote a Th2 response and an IFN-γ deficiency, rather than the presence of IL-4 or other Th2 cytokines, prevents spread of infection. In a study of cytokine gene expression in the granulomata of patients with advanced TB by in situ hybridization, IL-4 was detected in 3 of 5 patients, but never in the absence of IFN-γ expression (Fenhalls et al. 2000). The presence or absence of IL-4 did not correlate with improved clinical outcome or differences in granuloma stages or pathology. The deleterious effects of IL-4 in intracellular infections (including TB) have been ascribed to this cytokine’s suppression of IFN-γ production (Powrie et al. 1993).
and macrophage activation (Appelberg et al. 1992). In mice infected with *M. tb*, progressive disease and reactivation of latent infection are both associated with increased production of IL-4. Similarly, over-expression of IL-4 is intensified tissue damage in experimental infection (Lukaes et al. 1997). Conversely, inhibition of IL-4 production does not promote cellular immunity. IL-4/- mice displayed normal instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of TB development (Erb et al. 1998; North 1998). In contrast, a study on IL-4 KO mice showed increased granuloma size and mycobacterial outgrowth after airborne infection (Sugawara et al. 2000). Compared with control mice, production of proinflammatory cytokines was increased in these animals and accompanied by excessive tissue damage.

**Interleukin-6**

This cytokine has multiple roles in the immune response, including inflammation, hematopoiesis and differentiation of T cells. Pro- and anti-inflammatory properties (VanHeyningen et al. 1997) of IL-6 are manifested early during mycobacterial infection and at the site of infection (Law et al. 1996). A potential role for IL-6 in suppression of T cell responses was reported (VanHeyningen et al. 1997). The role of IL-6 in mycobacterium infection is still not clear. Some reports state that IL-6 inhibits production of TNF-α and IL-1β and promotes *in vitro* growth of *M. avium* (Dahl et al. 1996). Other reports support a protective role for IL-6. IL-6-deficient mice display increased susceptibility to infection with *M. tb* (Ladel et al. 1997), which is related to a deficient production of IFN-γ early in infection, before adaptive T cell immunity has fully developed (Saunders et al. 2000).

**Interleukin-8**

IL-8 recruits neutrophils, T lymphocytes, and basophils in response to a variety of stimuli. It is released primarily by monocytes/macrophages, but it can also be expressed by fibroblasts, keratinocytes, and lymphocytes (Munk et al. 1995). IL-8 is a neutrophil-activating factor. Elevated levels of IL-8 in BAL fluid and supernatants from alveolar macrophages were seen in TB patients (Law et al. 1996). IL-8 gene expression level was also increased in the macrophages of infected as compared to healthy subjects. *In-vitro* experiments also demonstrated that intact *M. tb.* or LAM, but not deacylated
LAM, could stimulate IL-8 release from macrophages (Cynamon et al. 1999). Friedland et al., (1996) studied HIV positive patients, and reported that both plasma IL-8 and secretion of IL-8 after ex vivo stimulation of peripheral blood leukocytes with lipopolysaccharide remained elevated throughout therapy for TB. Other investigators had previously shown that IL-8 was also present at other sites of disease, such as the pleural space in patients with TB pleurisy (Ceyhan et al. 1996).

**Interleukin-10**

IL-10, an anti-inflammatory cytokine, is produced by macrophages after phagocytosis of *M. tb* (Robinson et al. 1994) and after binding of mycobacterial LAM (Dahl et al. 1996). T lymphocytes, including *M. tb*-reactive T cells, are also capable of producing IL-10. In patients with TB, expression of IL-10 mRNA has been demonstrated in circulating mononuclear cells, at the site of disease in pleural fluid, and in alveolar lavage fluid (Gerosa et al. 1999). IL-10 directly inhibits CD4+ T cell responses, as well as APC function of cells infected with mycobacteria (Rojas et al. 1999). IL-10 antagonizes the proinflammatory cytokine response by downregulation of IFN-γ, TNF-α, and IL-12 (Zamzami et al. 1996; Fulton et al. 1998). This cytokine is reported to downregulate IFN-γ level in infected macrophages by a STAT 4-dependent pathway. IL-10 transgenic mice with mycobacterial infection develop a larger bacterial burden (Murray et al. 1997) and IL-10-deficient mice show a lower bacterial burden early after infection in one report (Murray et al. 1999). However, normal resistance to bacterial survival was indicated in two other reports (Erb et al. 1998; North 1998). In human TB, IL-10 production is higher in patients displaying T-cell anergy, both before and after successful treatment, suggesting that *M. tb*-induced IL-10 production suppresses an effective immune response (Boussiotis et al. 2000).

**Interleukin-12**

IL-12 is a key player in host defense against *M. tb*. IL-12 is produced mainly by phagocytic cells, and phagocytosis of *M. tb* seems necessary and sufficient for its production (Fulton et al. 1996). IL-12 has a crucial role in the induction of IFN-γ production (O'Neil et al. 1998). In TB, IL-12 has been detected in lung infiltrates, in pleurisy, in granulomas, and in lymphadenitis. The expression of IL-12 receptors is also increased at the site of disease (Cynamon et al. 1999). The exogenous administration of...
IL-12 was reported to improve survival of BALB/c mice (Flynn et al. 1995). IL-12 knock mice are highly susceptible to mycobacterial infections (Cooper et al. 1997; Wakeham et al. 1998). IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria (Sieling et al. 1994; Trinchieri 1995) and which exerts its protective effects mainly through the induction of IFN-γ (Cooper et al. 1997) through the STAT 4 pathway (Raju et al. 2008).

**Transforming growth factor-beta (TGF-β)**

TGF-β is reported to suppress protective immunity to TB. Mycobacterial products induce production of TGF-β by monocytes and dendritic cells. TGF-β is also present in the granulomatous lesions of TB patients and is produced by human monocytes after stimulation with *M. tb* or lipoarabinomannan (Dahl et al. 1996). TGF-β has important anti-inflammatory effects, including deactivation of ROI and RNI production by macrophages (Ding et al. 1990). TGF-β also plays an important role in inhibition of T cell proliferation (Rojas et al. 1999), interfering with NK and CTL function and downregulation of IFN-γ, TNF-α and IL-1 release. When TGF-β is added to co-cultures of mononuclear phagocytes and *M. tb*, it inhibits both phagocytosis and growth in a dose dependent manner (Dahl et al. 1996). The ability of macrophages to inhibit mycobacterial growth may depend on the relative influence of IFN-γ and TGF-β in any given focus of infection.

**Chemokines**

Chemotactic cytokines (chemokines) are largely responsible for recruitment of inflammatory cells to the site of infection. About 40 chemokines and 16 chemokine receptors have been identified (Zlotnik et al. 2000). A number of chemokines have been investigated in TB. Several reports have addressed the role of IL-8, which attracts neutrophils, T lymphocytes, and possibly monocytes. Upon phagocytosis of *M. tb* or stimulation with LAM, macrophages produce IL-8 (Cynamon et al. 1999; Leemans et al. 2001). This production is substantially blocked by neutralization of TNF-α and IL-1β, indicating that IL-8 production is largely under the control of these cytokines (Cynamon et al. 1999). A second major chemokine is monocyte chemoattractant protein 1 (MCP-1), which is produced by and acts on monocytes and macrophages. *M. tb* preferentially induces production of MCP-1 by monocytes (Kasahara et al. 1994).
Deficiency of MCP-1 inhibited granuloma formation in a murine model (Tunctan et al. 1998). A third chemokine is RANTES, which is produced by a wide variety of cells and which shows promiscuous binding to multiple chemokine receptors. RANTES expression in murine macrophages was associated with development of \textit{M. bovis} induced pulmonary granulomas (Chiu et al. 2003). Apart from IL-8, MCP-1, and RANTES, other chemokines may be involved in cell trafficking in TB (Ragno et al. 2001). Inhibition of chemokine production may lead to an insufficient local tissue response.

1.3.2 Other factors involved in the immune response

The Toll-like receptors (TLRs)

TLRs are phylogenetically conserved mediators of innate immunity which are essential for microbial recognition on macrophages and dendritic cells (Belvin et al. 1996; Medzhitov et al. 1997; Visintin et al. 2001). Members of the TLR family are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The interactions between \textit{M. tb} and TLRs are complex. Distinct mycobacterial components interact with different members of the TLR family. \textit{M. tb} activates cells via either TLR2 or TLR4 in a CD14-independent, ligand-specific manner (Means et al. 1999). The cytoplasmic domain of TLRs is homologous to the signaling domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (Oddo et al. 1998).

\textit{M. tb} lysate or soluble mycobacterial cell wall-associated lipoproteins induce IL-12 production through TLRs (Brightbill et al. 1999). MyD88 (myeloid differentiation protein 88), a common signaling component that links all TLRs to IRAK (Oddo et al. 1998), was found to be essential for \textit{M. tb}-induced macrophage activation. A mutation of TLR2 specifically inhibited \textit{M. tb}-induced TNF-α production. This inhibition was incomplete, suggesting that besides TLR2, other TLRs may be involved (Underhill et al. 1999). In a transfection model using TLR-deficient Chinese hamster ovary (CHO) cells, expression of TLR2 or TLR4 conferred responsiveness to both virulent and
attenuated *M. tb.* (Means *et al.* 1999). TLR2, and not TLR4, was necessary for signaling of the mycobacterial LAM (Means *et al.* 1999; Underhill *et al.* 1999), while an undefined heat-labile cell-associated mycobacterial factor was found to be the ligand for TLR4 (Means *et al.* 1999). Mycobacterial infection and proinflammatory cytokines increase surface expression of TLR2 (Wang *et al.* 2000). Besides TLR2 and TLR4, other TLRs may be involved in immune recognition of *M. tuberculosis*: heterodimerization of TLR2 with TLR6 or TLR1 is necessary for signal transduction (Ozinsky *et al.* 2000; Bulut *et al.* 2001), and TLR9 binds CpG dinucleotides in bacterial DNA (Hajjar *et al.* 2001).

It appears that phagocytosis does not lead to immune activation in the absence of functional TLRs. Although TLR2 is recruited to phagosomes during phagocytosis (Underhill *et al.* 1999), cytokine production was eliminated by the expression of a mutant TLR2, but particle binding and internalization were unaffected. Furthermore, the expression of CD14 and TLRs did not alter uptake of *M. tb* in *in vitro* studies. TLR2 activation directly led to killing of intracellular *M. tb* in alveolar macrophages *in vitro* (Thoma-Uszynski *et al.* 2001).

### 1.3.3 Th1 and Th2 response in TB

T-cells can be classified into Th1 and Th2 phenotypes, based on the pattern of cytokines they secrete when stimulated. Th1 cells secrete, among others, IL-2, IFN-γ and TNF-α while Th2 cells secrete IL-4, IL-5 and IL-10. The balance between the two types of responses is crucial for effective host response to infection.

Th1 and Th2 cytokines play important roles in the case of susceptible or resistant individuals exposed to *M. tb*. Mice infected with virulent strain of *M. tb* initially show Th1 like and later Th2 like responses (Orme *et al.* 1993). There are contradictory reports in literature on preponderance of Th1 or Th2 type of cytokines, increase in both, decrease of Th1, but not increase of Th2 cytokines, etc. Th1 and Th2 responses vary between peripheral blood cells and those at the site of tubercular lesion; among the different stages of the disease depending on the severity, and between individuals. It has been reported that PBMC from TB patients, when stimulated *in vitro* with PPD, release lower levels of IFN-γ and IL-2, as compared to tuberculin positive healthy subjects (Huygen *et al.* 1988). Other studies have also reported reduced IFN-γ (Vilcek *et al.*
increased IL-4 secretion (Sanchez et al. 1994) or increased number of IL-4 secreting cells (Surcel et al. 1994). These studies concluded that patients with TB had a Th2-type response in their peripheral blood, whereas tuberculin positive patients had a Th1-type response. Cellular response at the actual sites of disease has also been examined. Zhang et al. (1994) studied cytokine production in pleural fluid and found high levels of IL-12 after stimulation of pleural fluid cells with M. tb. IL-12 is known to induce a Th1-type response in undifferentiated CD4+ cells and hence there is a Th1 response at the actual site of disease. Lin et al., (1996) observed that TB patients showed evidence of high IFN-γ production and no IL-4 secretion by the lymphocytes in the lymph nodes. There was no enhancement of Th2 responses at the site of disease in human TB. Robinson et al., (1994) found increased levels of IFN-γ mRNA in situ in BAL cells from patients with active pulmonary TB.

Some reports suggest that in humans with TB, the strength of the Th1-type immune response relates directly to the clinical manifestations of the disease. Sodhi et al., (1997) have demonstrated that low levels of circulating IFN-γ in peripheral blood were associated with severe clinical TB. Patients with limited TB have an alveolar lymphocytosis in infected regions of the lung and these lymphocytes produce high levels of IFN-γ (Nirmala et al. 2001). In patients with far advanced or cavitary disease, no Th1-type lymphocytosis is present.

1.4 P2X Receptor role in tuberculosis

Purinergic receptors such as P2X7 are cation-selective channel receptors gated by ATP. P2XR function correlates with the innate bactericidal response of the infected macrophage. Purinergic receptors play an important role against mycobacterial infection in the macrophage. Stimulating the purinergic P2X7 receptor induced ATP-dependent killing of intracellular mycobacteria. At low levels of ATP, P2X7 causes influx of Ca+ across the membrane, and at high concentrations, it induces the formation of a large, non-selective pore in the mitochondrial membrane, permeable to hydrophilic molecules (Gan et al. 2005). Signaling through P2Rs has been shown to induce apoptosis in infected macrophages. Avirulent H37Ra strains inhibit functional activity of P2R in infected macrophages and treatment with cyclosporine D, which binds cyclophilin, restored functional activity (Gan et al. 2005). The resultant alteration of the
intracellular environment leads to the activation of nuclear factor-κB and stress-activated protein kinase (SAPK/JNK) and caspases, culminating in apoptosis. We have shown earlier that microparticles induce calcium influx and oxidative free radicals in infected mouse macrophages (Sharma et al. 2007). Both these factors contribute to pro-apoptotic responses. The evidence on P2XR function adduced here supports the view that such treatment enhances the propensity of infected cells to undergo apoptosis.

1.5 Modulation of macrophage signaling by mycobacterium

The bactericidal action of the macrophage is altered by inhibiting various pathways which directly or indirectly involve host defense. Mycobacterium inhibits various pathways inside macrophage by interfering with host signaling mechanisms, with the help of some antagonist or inhibitors that it has evolved. These entities inhibit some metabolic pathways in the infected macrophage, and upregulate others. Alteration of the gene expression profile of the host is preceded by a change in gene expression by the infecting Mycobacteria (Cappelli et al. 2006; Waddell et al. 2007). Some gene products of M. tb. are required for inhibiting signaling pathways in infected macrophage, which ultimately leads to survival of intracellular pathogen. In previous studies, it was observed that gene expression by the mycobacterium is different inside macrophages as compared to the extracellular state (Waddell et al. 2007). The alteration of gene expression of bacteria inside host cells evades host bactericidal action.

Macrophages and bacteria both try to influence each other’s transcription profile. The outcome of this interaction is either elimination of the pathogen or infection of the host. If the host immune system prevails, the bacterium is not able to survive, or is sequestered in a dormant state in the infected host. If bacterial transcription takes the upper hand, various degrees of pathological symptoms appear in the infected host. The secretion of virulence mediator molecules is required for the modulation of host bactericidal responses (Rosenberger et al. 2003). A heterogeneous mixture of lipids and glycolipids is released from mycobacteria in a vesicle-bound form, into the host cytoplasm, where they accumulate in late endosomal/lysosomal organelles (Beatty et al. 2000). These molecules might interfere with host signaling pathways, leading to an
arrest of phagosomal maturation, modulation of host-cell apoptotic processes and suppression of the bactericidal response.

1.5.1 Mycobacteria alter host apoptotic pathways

Apoptosis helps to check spreading infection in the host. In mycobacterial infection, apoptosis induction is strain specific. Avirulent strains induce, while virulent strain inhibit apoptosis if present in low (<10) numbers in the host cell (Keane et al. 2000). Apoptosis of the infected macrophages has been directly linked to killing of intracellular mycobacteria, while necrosis of the host macrophage is unable to kill intracellular bacteria, and may even promote their growth in extracellular milieu (Molloy et al. 1994; Lee et al. 2006). At low MOI, apoptosis induction is caspase dependent but at at high MOI (>20) it is caspase independent (Lee et al. 2006). Keane et al. (2000) demonstrated that at low MOI, apoptosis induction is driven by TNF. Mycobacteria-induced macrophage apoptosis is a complex phenomenon that is modulated by mycobacterial virulence factors (Nigou et al. 2002), and mycobacteria are thought to modulate the host apoptotic pathway through several mechanisms. First, Man-LAM has been shown to antagonize mycobacteria-induced apoptosis in murine macrophages by preventing the increase in cytosolic Ca²⁺ concentration by inhibiting P2X receptor activity (Gan et al. 2005; Yadav et al. 2007) which helps in maintaining Ca²⁺ inside cytoplasm of cells. Ca²⁺ is believed to facilitate apoptosis by increasing the permeability of mitochondrial membranes, thereby promoting the release of pro-apoptotic elements such as cytochrome c (Szalai et al. 1999). Man-LAM also stimulates the phosphorylation of the apoptotic protein Bad, which prevents it from binding to the anti-apoptotic proteins Bcl-2 and Bcl-XL (Maiti et al. 2001). Free, cellular Bcl-2 prevents the release of cytochrome c from the mitochondria, inhibits procaspase 9 activation and functions as an anti-apoptotic regulator in many systems (Esdar et al. 2001), including mycobacteria-infected cells. M. tb also limits macrophage apoptosis by inducing the production of the immunosuppressive cytokine IL-10 (Balcewicz-Sablinska et al. 1998). IL-10 was shown to block the synthesis of TNF-α, a stimulator of apoptosis, in infected macrophages. TNF-α binds to death receptors to activate the apoptotic program. IL-10 inhibits TNF-α activity by inducing the release of the soluble TNF receptor type 2 proteins (TNFR2), which forms an
inactive complex with TNF-α and prevents the induction of TNF-mediated apoptosis. Mycobacterium also inhibits nitric oxide induction in infected macrophage, which is well known for bactericidal action in infected cells (Arriaga et al. 2002). In contrast to their inhibition of apoptosis during the early stages of infection, mycobacteria might induce apoptosis in the acute phase so as to infect neighboring cells.

1.6 Macrophage activation by phagocytosis

Macrophages play a dual role in the host defense. Mycobacterium infection inhibits macrophage activation via inhibiting different mechanisms. Macrophages represent an important part of the first line in innate immune response and are also important as APC in the adaptive immune response. Phagocytosis leads to macrophage activation. Phagocytosis of particulate chitin particles activates macrophages (Levitz et al. 1987). Sharma et al. reported that infected murine macrophages can be activated by the event of phagocytosis, which results in increased nitric oxide and reactive oxygen species (ROS) production, and alteration of cytokine secretion (Sharma et al. 2007). Phagocytosis of polyethylene particles alters the gene expression of U937 cells (Matsusaki et al. 2007).

1.7 Research Problems and Objective

The central objective of this work was investigation of changes in gene expression profiles of infected macrophage after treatment with drugs in solution, drug-containing microparticles or blank microparticles. The second objective was to investigate biochemical consequences, such as oxidative radical and cytokine production, apoptosis induction and bacterial survival, due to change in the gene expression profiles. The third and last objective was to examine responses of primary macrophages isolated from healthy volunteers after infection in vitro and different modes of treatment.

During the course of the work, microparticles were characterized for their particle size and morphology. The multiplicity of infection (MOI) and duration of exposure of H37Ra and H37Rv was standardized for optimal infection of macrophage for further studies. Macrophages were infected at 10 (H37Rv) or 20 (H37Ra) MOI for 3 & 2 hrs respectively. RNA was isolated at 0, 12 & 24 hrs after different treatments of infected cells. The same MOI was also used in experiments conducted to study biochemical consequences after different treatments.
Experiments relevant to these objectives are listed below:

- Characterization of microparticle size using particle size analyzer and morphology by scanning electron microscopy (SEM).
- Optimization of the percentage of THP-1-derived macrophages infected at different MOI using flow cytometer and microscopy.
- Assessment of cytotoxicity induced by mycobacterium at different MOI by crystal violet staining.
- Establishment of gene expression profiles of the THP-1 derived macrophage upon infection with virulent \( M. \) \( tb \). H37Rv, after different modes of drug and microparticle treatment, by microarray hybridization.
- Microarray results validation using real-time PCR and data analysis using clustering of significantly up or down regulated genes using Cluster and Tree View.
- Identification of differentially-regulated genes in different pathways by use of GenMAPP software.
- Delineation of Th1/Th2 cytokine profiles in supernatants of infected THP-1-derived macrophages after different treatment using cytokine bead array on flow cytometer.
- Study of early apoptosis in THP-1-derived macrophages infected with H37Ra or H37Rv after different treatments; in presence or absence of caspase 3 inhibitor by annexin V and propidium iodide staining.
- Study of late apoptosis by TUNEL assay, cell cycle arrest and DNA fragmentation.
- Estimation of specific activities of Caspase 3, 8 & -9 in cell lysates of infected macrophage after different treatments using fluorogenic substrates.
- Assay of P2R receptor functional activity by measuring rate of EtBr influx inside cells using flow cytometer.
- Investigation of mitochondrial membrane potential by flow cytometry using Rhodamine.
- Estimation of Nitric oxide release using Griess reagent.
- Assessment of survival of intracellular bacteria after different treatments in Bactec assays and by counting CFU after plating cell lysates.
- Examination of responses of primary macrophages derived from PBMCs of 5 healthy human volunteers.
The results obtained from these are described in the next section. It is important to view the results in the context of the high efficacy of pulmonary delivery of microparticles that target alveolar macrophages, as demonstrated by several authors (Dutt et al. 2001; Suarez et al. 2001; Sen et al. 2003).

The unifying theme of the experiments was to investigate whether equal amounts of drugs delivered in soluble form or in particulate form, or equal amounts of particles whether or not they contained drugs, differentially influenced gene expression, production of effector molecules, and biochemical or cell biological events in infected macrophages. It was also attempted to delineate whether these changes were due to the drugs, the event of microparticle phagocytosis or to both (in synergy). Thus, with the knowledge that M. tb. induces alternative activation (Gordon 2003; Kahnert et al. 2006) of the infected macrophage, it was investigated whether any of the methods of treatment used here rescues the infected macrophage from alternative activation and induces classical, bactericidal activation.