Introduction
1.1 Background

Tuberculosis (TB) is caused by infection with gram positive, aerobic, non motile, non capsulated, non spore forming, acid fast bacilli of several species of mycobacteria: Mycobacterium tuberculosis (M. tb.), M. bovis, M. africanum or M. microti. It continues to be a scourge to humankind (Snider, et al., 1994). There has been a rapid resurgence in infections caused by the genus Mycobacteria, TB being the most fatal amongst them (WHO Report, 2005). Eight million new cases of TB are reported, 3 million die every year, and estimates show that more than 90 million people are expected to die from it within the next 30 years (Zahrt, 2003). Estimates are that a third of the world’s population is infected with TB (Flynn and Chan, 2001), out of which 90% remain latent, 5% of the infected individuals progress rapidly to primary disease and 5% of those who initially suppress infection later reactivate leading to the disease sometime during their lifetime (Comstock, 1982). These appalling figures put TB along with other major killers, AIDS and malaria.

Faced by this stark reality World Health Assembly had adopted in 1991, the target of reaching 70 % case detection rate and 85 % cure rate for smear positive TB, globally by the year 2000 (Raviglione and Pio, 2002). These goals were not met but were reaffirmed with a target date of 2005. WHO in 1993 had declared it a global emergency due to an upsurge in TB infections in developing countries in the last decade of the 20th century (WHO Report, 1993). TB control program initiated by WHO as the Directly Observed Treatment Short-course (DOTS) that has to be observed for at least 6 months, had achieved decent success in keeping a control on this menace. It had been adopted by 155 nations and covered 69% of the world population by 2001 (WHO Report, 2003). To ensure prevention of monotherapy leading to drug resistance, WHO and International Union Against Tuberculosis and Lung Disease (IUATLD) have recommended the use of Fixed Dose Combination formulations (FDCs) with multiple drugs and drug regimens as a routine practice in treatment of TB (WHO Report, 2005). In India alone, TB remains the leading infectious cause of death, killing close to 500,000 people a year. India has far more cases of TB than any other country in the world: about 2 million new cases each year (Khatri and Frieden, 2002) and accounts for nearly one third of the global prevalent cases.
Two factors of particular concern in the epidemiology of TB are the emergence of multiple-drug resistance TB (MDR-TB) and the AIDS epidemic (Espinal, et al., 2001). MDR-TB is a man made development, the root cause being laxity on the part of the patient or the health service provider in completing the course of treatment. The treatment for MDR-TB involves second line anti-TB drugs which apart from being more costly, are toxic when compared to drugs used for treating drug susceptible TB. The treatment schedule so becomes more prolonged leading to a greater risk of patient non-complinace (Mukherjee, et al., 2004). The rates of HIV-related TB in India and the rest of Asia have risen so sharply in the last decade that they are at par with the sub-Saharan Africa (Dolin, et al., 1994; Yanai, et al., 1996).

Though TB infects almost all organs, pulmonary TB is the most common and lung the primary infection site from where it spreads to other organs (Flynn and Chan, 2001). One report estimates more than 80% of all TB cases to be that of pulmonary TB (Pandey and Khuller, 2005). The current Bacillus Calmette-Guérin (BCG) vaccine, an attenuated strain of the closely related wild type M. bovis is used against all forms of TB and has been given to more people than any other vaccine (more than 3 billion individuals and approximately 100 million newborns annually). But this vaccine provides variable and inadequate protection against adult pulmonary TB (Fine, 2001).

1.2 Initial Infection and Host Response

TB spreads through inhalation of moist droplets containing small numbers of bacilli, expelled through coughing by an infected person, to the lungs of a healthy person where they lodge in the pulmonary alveoli.

Our knowledge of the tubercle bacilli and their complex interaction with the human host has improved significantly lately, particularly with regard to the fact that they are intracellular pathogens and inhabit the alveolar macrophage (AMϕ) during early stages of infection (Enarson, et al., 2004).

During the early stages of infection, the control of intracellular M. tb. survival and proliferation is dependent upon the interaction between the host’s defensive microbicidal strategies and the pathogen’s survival plan. The large surfaces of the respiratory tract in the lungs are often the initial sites of contact between the
microbes and their hosts. Various innate defences protect the lungs from inhaled microorganisms, including the cough reflex, mucociliary clearance (Knowles and Boucher, 2002), and anti-microbial properties of the mucosal surface (Ganz, 2002; McCormack and Whitsett, 2002).

Any extraneous matter entering the pulmonary tract encounters APC's (antigen presenting cells) comprised of alveolar and interstitial Mφ and effector T lymphocytes (Agostini, et al., 1993). The phagocytosis of a microbial pathogen results in an initial induction of non-specific innate response by the Mφ (Aderem and Ulevitch, 2000). This includes production of reactive oxygen species (ROI), reactive nitrogen species (RNI) and cytokines to further regulate the acquired immunity. The successful execution of innate defence responses by the Mφ is critical to the eventual outcome of the microbial infection (Flynn and Chan, 2001). The adaptive immune response occurs via interaction of APCs, such as Mφ or dendritic cells, with T cells (Sompayrac, 2003). Such interactions stimulate these cells to further evoke bactericidal mechanisms and produce cytokines for clearance of the infection. Cytokines thus not only activate the innate host immunity but also drive the adaptive immune response down different pathways of differentiation (Romani, 2005). As a last resort, apoptosis of the host Mφ serves as a suicidal strategy adopted by the host cell to eliminate the intracellular environment required by the bacteria for its propagation (Keane, et al., 1997).

Mycobacteria have evolved to evade most of these host-defence mechanisms, enabling their intracellular survival in the host lungs and replication within Mφ phagosomes that do not fuse with lysosomes (Armstrong and Hart, 1971; Quinn, et al., 1996, 1997; Sinai and Joiner, 1997). Pathogenic mycobacteria survive and proliferate with in Mφs by modulating their function to their own advantage (Clemens and Horwitz, 1995; Zahrt, 2003).

At the stage of cell entry, they interact with the Mφ through a set of cell surface receptors that ordinarily transduce activation signals to the host cell (Ferguson, et al., 2004). Phagocytosis of pathogenic mycobacteria, however, does not lead to similar signal transduction, preventing the build up of ROS and RNI in their environment. Within phagosomes, they inhibit the insertion of proton pumps into the phagosome membrane, preventing acidification of its contents (Hackam, et
al., 1997; Rathman, et al., 1996). Most strikingly, they inhibit phagosome-lysosome fusion and thus escape degradation by hydrolytic enzymes. Very soon, they modify the entire pattern of gene expression in the infected Mφ, leading to a state that is now being defined as ‘alternatively activated’ (Goerdt and Orfanos, 1999; Kahnert, et al., 2006).

Once inside mycobacterial phagosomes, the mycobacteria can survive for extremely long times without encountering lysosomes. The onset of T-cell immunity causes bacteriostasis and initiates a period of bacterial persistence (clinical latency period) in the granulomatous lesions of asymptomatic hosts. Of these, only 10% lead to resumption of mycobacterial replication and cause active disease. Granulomas are poorly aerated lesions that develop over weeks to months after initial infection and consist of Mφ harbouring viable bacteria surrounded by T-lymphocytes. Various evidences suggest that the dormant bacilli survive in a nutrient-deprived state. Gradual O₂ depletion leads to non-replicating persistent state characterized by bacteriostasis and metabolic adaptations by the dormant mycobacteria (Wayne and Sohaskey, 2001).

Pathogenic mycobacteria can thus endure in the lungs of their victim for decades being "fenced off" by the immune system. When the immunity is weakened by diseases like AIDS, intake of corticosteroids, alcohol, old age and malnutrition the bacterium re-emerge in a highly contagious form that can spread to other people.

1.3 Drug Delivery in Tuberculosis

Chemotherapy of TB in developing countries includes first choice anti-TB agents often called primary or 'first-line' agents namely, isoniazid (INH), rifampicin (RFM), pyrazinamide (PZA), ethambutol (ETH), streptomycin (STR) and thiacetazone (THZ).

The two most efficacious bactericidal drugs are INH and RFM, which act against the metabolically dynamic mycobacteria that multiply perpetually and rapidly, and also against the quasi-dormant bacilli. Another benefit of RFM is that it acts at a very early stage of bacillary propagation. RFM is bactericidal in nature and has an in vivo sterilising activity as it inhibits initiation of RNA synthesis by barricading bacterial 'DNA-dependent RNA polymerase'. INH interferes
principally with nicotinamide nucleotide metabolism in mycobacteria and also with the synthesis of mycolic acids. INH has been found to be active against tubercle bacilli that are growing within cultured human Mφ (Crowle, et al., 1988).

Two other bactericidal anti-TB drugs of average efficacy and complimentary action are PZA and STR. PZA is active only at acidic pH and destroys intracellular semi-dormant mycobacteria in an acid environment, that are less affected by other drugs. STR is active only against extracellular bacilli as it cannot infiltrate the cell membrane.

Two other bacteriostatic antibiotics, ETH and THZ are used in conjunction with powerful bactericidal drugs to prevent the emergence of resistant mycobacteria. The secondary agents including other antibiotics of low efficacy are not used in short-course chemotherapy. These include ethionamide, kanamycin, capreomycin, the quinolones, cycloserine and p-amino salicylic acid. These are used if resistance or toxicity to first line drugs develops during therapy, but are generally less effective or more toxic (Chopra and Brennan, 1997)

Newer drugs include rifamycins such as rifabutin (RFB), rifapentine (RFP), benzo axazino-rifampicins. RFB is a semi-synthetic derivative of rifamycin that inhibits nucleic acid synthesis and shows antibacterial (tuberculostatic) activity (Martindale 2002).

Conventional therapy of TB consisting of oral administration of multiple drugs (to keep the MDR strain at bay) is prolonged, hepatotoxic, poorly compliant and very often inadequate in attaining optimal drug concentrations inside the AMφ (Girgling, 1978; Schreiber, et al., 1997; Teo, 1999; Zierski and Bek, 1980). Many patients also remain sputum smear-positive for Mtb despite ongoing chemotherapy which is chiefly due to failure of oral anti-TB drugs reaching the cavitary lesions in the lungs (Telzak, et al., 1997). Such chronic dosing is associated with persistent high blood levels of drug that may be neither necessary nor sufficient to kill the mycobacterium residing in the Mφs (Sharma, et al., 2001). Current therapeutic regimens relying on the above mentioned drugs generally succeed in uncomplicated tuberculosis infection. Drug resistance greatly complicates treatment of this disease which almost always turns out to be fatal (Dye, et al., 2002; Espinal, et al., 2001). Besides, the current drugs used in
the conventional chemotherapy were synthesized more or less four decades back (Grassi and Peona, 1995), and without many new active substances foreseeable in the near future, an alternative therapeutic strategy has to be established to outsmart this ancient and cunning pathogen. The reappearance of this worldwide epidemic has intensified research efforts to find a replacement to conventional therapy of TB. A targeted drug delivery system that could shorten the duration of TB chemotherapy, or overcome drug-resistant strains of the causative organism would be a welcome addition to the armamentarium available for treatment of TB.

Recent advances in targeted delivery consisting of particulate and vesicular systems such as liposomes, niosomes, polymeric microparticles (MP), nanoparticles and implants have altogether sparked a renewed interest in their potential application for the treatment of Mφ infections. These drug delivery systems purport to target lung Mφ harbouring the bacteria via the respiratory route, thus obviating drug dumping to non-target organs. Since the drug is targeted directly to the site of infection, the dose administered also decreases drastically, further lowering the toxicity (Suarez, et al., 2001). The above delivery systems could be targeted to the lung by ways of fluidizing the drugs as powders in dry powder inhalers (DPI), solubilising/ suspending in an aqueous medium and aerosilizing (liquid aerosolization) through a nebulizer and by using positive pressure using an insufflator.

Simply aerosolizing an anti-mycobacterial compound may not be enough for efficient killing of bacteria present in the Mφ (Ahsan, et al., 2002; Basu and Lala, 2004) as the intracellular drug concentration achieved will be due to only diffusion. Instead, a targeted multi-drug treatment, with the potentiality for extended release, may not only achieve high intracellular concentrations at low doses, but also increase patient compliance by allowing for smaller, less frequent dosing. It will also lead to less systemic toxicity of the drugs.

In order to achieve extremely specific targeting to macrophages, several researchers have designed delivery systems. Liposomes bearing anti-TB drugs have been formulated for targeting Mφ in the lungs (Khuller, et al., 2004; Owais and Gupta, 2005; Pandey, et al., 2004). Ligands have been tagged to liposomes which have the ability to seek out Mφ, and such liposomes have been
used for site specific drug delivery to the lungs (Agarwal, et al., 1994; Agrawal, et al., 2001; Deol and Khuller, 1997; Deol, et al., 1997; Gupta and Haq, 2005; Vyas, et al., 2005). Vyas et al (2004) had shown better localization of ligand appended liposomes to AMϕ compared with conventional liposomes. However, the large size, low drug loading especially for polar drugs, problems of burst release and stability and storage problems work against the adoption of such delivery systems into mainstream practice.

Niosomes or non-ionic surfactant vesicles were considered a better choice for Mϕ targeting due to enhanced retention capacity for drug molecules than liposomes, the absence of labile lipid molecules and also their smaller size. This form of delivery system was found to be biodegradable and non-immunogenic making it suitable for deep lung delivery. But again this delivery system had the inherent problem of low drug loading.

Polymeric delivery systems for deep lung delivery have several features that are suited for this application especially after biodegradable polymeric devices such as implants and bioresorbable surgical sutures entered the market two decades ago. Polymeric MPs have been in use to deliver anti-TB drugs by various routes such as injectable, oral and aerosol (Pandey and Khuller, 2004). Nanoparticles and MPs differ from liposomes and niosomes in that they do not have an aqueous core but a solid polymer matrix. They are prepared from polymers that are biodegradable, biocompatible and non-immunogenic (Dhiman, et al., 2000; Shive and Anderson, 1997) especially those made of PLA, poly (glycolide) (PGA) and the copolymer of lactide and glycolide referred to as poly (lactide-co-glycolide) (PLGA).

Targeting nanoparticles to the deep lung (Pandey, et al., 2003; Zahoor, et al., 2005) is more difficult due to their small size, which can lead to exhaling of particles during breathing and consequently lower drug targeting. Due to this nanoparaticles are targeted to deep lung by incorporating along with carrier particles (Azarmi, et al., 2006). The carrier particles transport the smaller particles adhered to their surface into the mouth and throat, where the large carrier particles, by virtue of their size, fall out of the air stream (Edwards and Li, 1997).
For ample targeting of MΦ the size of delivery system should be in the range of 1-5 μm which is most amenable to phagocytosis (Ayhan, et al., 1995; Tabata and Ikada, 1988). A microparticulate delivery system containing RFM to target host AMΦ on parenteral injection was reported towards the end of the millenium (Barrow, et al., 1998; Quenelle, et al., 1999). These authors observed a drastic decrease in the number of viable bacteria at 7 days after initial M. tb. infection in monocyte cell lines after treatment with RFM loaded microspheres. Suarez et al (2001) delivered PLGA microspheres again containing RFM to a post-treatment low level respiratory challenge Guinea pig infection model by insufflation or nebulization. They found a dose-effect relationship between insufflated RFM loaded microspheres and burden of mycobacteria in the lungs. The treated Guinea pigs also had a significantly smaller number of viable bacteria, reduced inflammation and lung damage than lactose control, PLGA or RFM treated animals. Sharma et al (2001) had used PLA as polymer to incorporate two anti-TB drugs INH and RFM to avert drug resistance but they encountered the formation of an adduct due to the interaction of the two drugs.

Thus, anti-TB drug loaded MP can be directly targeted to lungs for improved chemotherapy of pulmonary TB, as the particles reaching the lungs are rapidly phagocytosed by AMΦ. Phagocytosis of inhaled particles is a problem with regard to drugs administered for local action in bronchio-pulmonary system, but is an advantage in case of pulmonary TB as the mycobacteria reside and multiply within host AMΦ. Thus, the called for mechanism of action is uptake of drug loaded particles by MΦ confining the dose at the residence site of mycobacteria. A similar mechanism by which bacteria invade MΦ is exploited here for guiding MP to pathogens residing inside them. It is expected that drug-containing MP would co-localise with bacteria that reside inside the MΦ. This would result in the MP and pathogen lying in close proximity to each other ensuring that high drug concentrations would be maintained in the vicinity of the pathogen all the time. Apart from hematogenous dissemination, mycobacteria are carried to extrapulmonary sites according to the trafficking of MΦ like the secondary lymphoid organs. Drug targeting to MΦ might have the additional advantage of reaching out to these areas where conventional dosing is inconceivable. The need for any other homing mechanism in the form of carriers is also not required making it a
less complex and reliable delivery system. In addition, the intake of MP by Mφ is reported as an activation signal by itself, leading to downstream effects like respiratory burst and cytokine release. Thus, intracellular drug targeting by MP and the activation of host Mφ may be of synergistic significance in the therapeutic efficacy of drug-loaded MP for pulmonary TB.

1.4 Research Problem and Objective

The central objective of this thesis was to deliver defined doses of anti-TB drug combinations to laboratory animals via the inhalation route using biodegradable, inhalable MPs. The second objective was to evaluate the specificity and extent of targeting. Finally, it was sought to examine the effects of MP delivery on the pattern of cytokines secreted by lung Mφ.

During the course of the work, MPs were prepared by a standardized spray-drying process, and their drug content and size distribution established. Appropriate apparatus was standardized for inhalation delivery to guinea pigs and conditions for defined dose delivery were worked out. Work-elements relevant to these objectives are listed below:

- Preparation of inhalable microparticles containing anti-TB drug combinations
- Analytical method development and validation
- Drug content determination in MPs
- Establishment of geometric and aerodynamic size distributions of MPs
- Isolation of lung cells by various techniques and estimation of AMφ populations
- Standardization of an in-house inhalation apparatus for inhalation delivery of MP
- Standardization of inhalation exposure conditions using the in-house apparatus and a benchmark apparatus (In-Tox Products, Inc.) for optimal drug delivery to laboratory animals
- Investigation of dose delivery to AMφ using the standardized apparatus and conditions
- Confocal imaging of co-localization of bacteria and MP in AMφ
- Assessment of intracellular cytokines in infected and treated Mφ in vitro
The underlying principle for seeking the principal objective was to establish the dose administered as dry powder inhalation (DPI) to the lung Mφ. The relation between the amount of MP taken in the delivery apparatus, the airflow and particle fluidization achieved, and the time for which the animal is exposed, with the amount of dose delivered needed to be established. This would in turn be of assistance in deciding the dose that has to be administered to clear the lungs of the bacteria. The mass of aerosol inhaled is primarily a function of the animal's breathing pattern and the aerosol delivery system. Once inhaled, deposition is governed by factors related to the properties of the aerosol and the individual characteristics of the subject (e.g., particle size distribution, airway geometry, and residence time).

The administration of MP containing two drugs to the upper airways and lungs of laboratory animals by means of a “nose-only” inhalation apparatus has been reported earlier from our laboratory (Sharma, et al., 2001). The two drug chosen earlier had led to the formation of an adduct during MP preparation. The current study aimed to target MP containing two front line anti-TB drugs INH and RFB to AMφ of laboratory animals by inhalation therapy using an in-house inhalation apparatus as well as from a standard one. The intra cellular drug concentration were determined immediately after inhalation.

Most of the studies on Mφ targeting so far have focussed on various delivery system to target antibiotics and subesquently investigated the bacterial clearance from the target organ. Not much work has been done regarding the dose reaching the target site namely Mφ, employing a particular type of inhalation delivery tool. The rationale for measuring the concentration of drugs at potential sites of infection other than serum is that pathogens may be confined to sites which are separated from the blood by significant barriers to drug movement. The concentration within these sites may differ markedly from those observed in serum, and hence drug efficacy may be more directly related to the concentrations of drugs confined to the vicinity of the pathogen.

This problem gains importance in the light of the fact that apparatus for reproducible and accurate delivery of inhalations is not freely available. A simple, inexpensive apparatus for use in an Animal Biosafety Level-3 (ABSL-3) setting would provide much needed impetus to research in pulmonary drug
delivery in TB. Thus, ascertaining the dose delivered at the target site assumes significance with regards to the amount required initially to clear the lungs of bacteria.