INTRODUCTION

Tuberculosis (TB) is primarily a chronic lung infection that is one of the most potent and wide-spread human infections today, and a major cause of death from bacterial pathogens (Lawn, S.D., et al., 2011). Historically, TB disease has killed more human beings than any infectious disease. In 2012, WHO estimated 1.4 million deaths from TB and 8.7 million new TB cases, which mostly (80%) affect vulnerable populations in 20 high-burden countries (WHO report, 2012). Although TB is a serious global health problem, several medical advances have been made in the past 150 years to facilitate prevention and control of TB. The discovery of Mycobacterium tuberculosis (M. tb.) as the etiological agent of TB was done by Robert Koch in 1882. The Bacillus Calmette Guerin (BCG) vaccine was introduced in 1921 and has been administrated in over 4 billion doses worldwide. In addition, the first anti-TB drugs were introduced to the market in 1944, when streptomycin was successfully used to treat TB disease (Daniel, T.M., et al., 2006). Only during the 20th century, TB mortality started to decrease in most industrialized countries, probably due to improved nutrition and living conditions (Lienhardt, C., et al., 2012). But, emergence of drug resistant M. tb. strains and resurgence in HIV/AIDS patients, TB re-emerged during the 1990s both in developing and several industrialized countries (Raviglione, M.C., et al., 1995; Dheda, K., et al., 2010 and Bloom, B.R., et al., 1993). Pharmacological management of TB is extremely resource intensive, especially in developing countries and treatment of multidrug-resistant TB (MDR-TB) increases the costs several fold compared to drug-susceptible TB (Schnipple, K., et al., 2013). Also, additional resources are needed to achieve higher treatment completion rates by more intensive follow up programs (Steffen, R., et al., 2010). Therefore, protective host responses that are accountable for control of M. tb. infection in order to develop effective therapy against TB need to be explored to address the disease progression in human TB.
Mycobacteria:

Over 100 mycobacterial species have been identified, but the majority of these species are non-pathogenic. The disease-causing *mycobacteria* in mammals with close genetic similarity are categorized in the *M. tb*-complex, which comprises seven mycobacterial species: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. canettii*, *M. caprae*, *M. microti* and *M. pinnipedii* (Forrellad, M.A., et al., 2012). Mycobacteria are aerobic, non-motile, hydrophobic, rod-shaped, facultative intracellular bacteria with a size of 2-4 µm. The pathogenic species typically replicate slowly with a doubling time of 12 to 24 hours (Sakamoto, K., 2012), resulting in lengthy cultures of clinical specimens (4-8 weeks) that often cause delays in TB diagnosis.

The lipid-rich cell wall of *M. tb* is complex and consists of peptidoglycans, unique mycolic acids, arabinogalactan and lipoarabinomannan (LAM) as well as free lipids, and scattered proteins. Interestingly, the mycobacterial cell wall is about twice as thick compared to gram-positive and gram-negative bacteria. The very unique properties of the thick mycobacterial cell wall make it impermeable to many toxic compounds and also deliberate acid-fastness, which can be used for detection of *mycobacteria* in clinical specimen such as sputum, cell- or tissue samples (Lawn, S.D., et al., 2011).

Fig. 0.1 Microscopic image (x125) of acid-fast stained bacilli (red rods) (provided by Internet).
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*M. tb.* has evolved to escape immune mechanisms and actually survives within macrophages (Pieters, J., 2008). Mycobacteria owe their success to their ability to persist, without symptoms, within the host for prolonged times, in an assumed viable but non-replicating (dormant) state. Upon immune suppression they are able to replicate and cause disease (Chao, M.C. and Rubin, E.J., 2010).

**IMMUNE RESPONSE IN TB**

Upon inhalation, *M. tb.* is phagocytosed by resident alveolar macrophages (MΦ) in close vicinity to the small airways. The macrophage may instantly kill the bacteria, or may secrete pro-inflammatory cytokines to recruit monocytes and neutrophils from the blood stream to the site of infection. In the tissue, monocytes differentiate into macrophages and dendritic cells (DC). Infected dendritic cells will further migrate to a regional lymph node to present the mycobacterial antigens to T cells that will then proliferate. Once proliferation is triggered, the DC then migrate back into the infected tissue.

**THE ROLE OF CYTOKINES IN TB**

Cytokines are soluble signalling molecules that the immune system uses for intercellular communication. Cytokines are produced and secreted by distinct cells in the body in response to a stimulus and exert their immunomodulatory functions by binding to definite receptors (Jafari, C., et al., 2008). Cytokines are the major mediator of immune responses towards pathogens. Production of cytokines by leukocytes in response to *M. tb.* plays a crucial role in the inflammatory response, and the balance between pro-inflammatory and anti-inflammatory cytokines is crucial in the determination of immune activation and disease clearance (Flynn and Chan, 2001). Based on their cytokine production, effector T cells differentiate into one of the subsets known as Th1, Th2, Th17 or T<sub>reg</sub> (T regulatory). Here, Th1 cells are involved in cellular immunity, Th2 cells induce humoral immune responses, Th17 cells are involved in mucosal immunity and autoimmune inflammation and T<sub>reg</sub> cells participate in the regulation of inflammatory immune responses.
Cytokines first assist to recruit such innate cell populations as macrophages, neutrophils, dendritic cells and NK cells to the site of inflammation, where cytokines then provide the cellular signals for these populations to grow. As these populations arrive and grow, a different set of cytokines activates and matures these cells, inducing effector mechanisms that include killing the pathogen, killing infected cells, antigen presentation to adaptive immune cells and production of cytokines that supplement either innate or adaptive immune cells. The following cytokines are especially important for the generation of an immune response against *M. tb*.

**IFN-γ:** The protective role of IFN-γ in tuberculosis is well established (Flynn, J., et al., 1993), primarily in the context of antigen-specific T-cell immunity (Andersen, P., 1997). Mycobacterial antigen-specific IFN-γ production *in vitro* can be used as a surrogate marker of infection with *M. tb*. (Van Crevel, et al., 1999). Owing to its well known protective effects, IFN-γ has been widely studied as a potential biomarker or correlate of immune protection in TB. However, the number of IFN-γ secreting T cells does not always correlate with enhanced immune control (Leal, I.S., et al., 2001, Majlessi, L., et al., 2006), which may suggest that more complex immune signatures are required to define protective immunity in human TB.

**TNF-α:** Stimulation of monocytes, macrophages (Briken, V. et al., 2004), and dendritic cells (Metcalfe, J.Z., 2011) with *mycobacteria* or mycobacterial products induces the production of TNF-α, a pro-inflammatory cytokine. TNF-α plays a key role in granuloma formation (Jassal, M. and Bishai, W.R., 2009), induces macrophage activation, and has immunoregulatory properties (Elias, D., et al., 2001, Corleis, B., et al., 2012). In mice, TNF-α is also important for containment of latent infection in granuloma (Andersson, M. et al., 2012). In tuberculosis patients, TNF-α production is present at the site of disease (Solovic, I., et al., 2010). TNF-α secretions may account for unwanted inflammatory effects like fever. To control the deleterious effects of TNF-α, systemic production of TNF-α is downregulated (PrayGod, G., et al., 2012, Nicholson, S., et al., 1996).

**IL-12:** It 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 for its
production (Fulton, S.A., et al., 1996). IL-12 has a crucial role in the induction of IFN-γ production (O’Neill, L.A., and Greene C., 1998). In tuberculosis, IL-12 has been detected in lung infiltrates (Taha, R.A., 1997). The protective role of IL-12 can be inferred from the observation that IL-12 KO (knock out) mice are highly susceptible to mycobacterial infections (Cooper, A. M., 1997, Wang, J. et al., 1999). IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria (Trinchieri, G., 1995) and it exerts its protective effects mainly through the induction of IFN-γ (Cooper, et al., 1997).

The precise role of Treg cells in TB has not yet been elucidated, since inhibition of inflammation can both be beneficial and detrimental (Dheda, K., et al., 2010). A key role of IL-17 in intracellular M. tb. infection is to promote recruitment and accumulation of IFN-γ producing CD4+ T cells in the M. tb. infected lung (Khader, S.A., et al., 2010). IL-17 has also been shown to be particularly important to induce chemokine production and recruitment of neutrophils that take part in initial granuloma formation upon mycobacterial infection. IL-17 is a polyfunctional cytokine that can be produced by many cells including MQs, follicular DCs, B cells, fibroblastic reticular cells, epithelial cells, keratinocytes, endothelial cells and smooth muscle cell. Production of IL-17 is induced by IL-1, IFN-γ, and TNF-α and is essential for T cell survival and homeostasis (Khader, S.A., et al., 2007).

**IL-2** has a central role in regulating T cell responses to M. tb. Recovery from TB depends, in part, on the generation of an effective cell-mediated immune response against the pathogen. Effective T cell function is key in controlling M. tb. infection. IL-2, a cytokine produced by activated T lymphocytes, has a central role in the activation and expansion of T cells. In murine models of M. leprae, IL-2 has been shown to limit mycobacterial replication, possibly by macrophage activation via interferon-mediated pathways or directly by the development of cytotoxic T lymphocytes recognizing mycobacterial antigens (Bermudez, L.E. and Young, L.S. 1988).

**IL-10** is produced by macrophages after phagocytosis of M. tuberculosis. IL-10 antagonizes the pro-inflammatory cytokine response by downregulation of production of IFN-γ, TNF-α, and IL-12 (Fulton, S. A., et al., 2000).
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Th2 cytokines are typically involved in antibody-mediated humoral immunity with limited protective effects in intracellular M. tb. infection (Rook, G.A., 2007). **IL-4** and **IL-13** have been shown to be detrimental in the control of intracellular M. tb. infection, as these Th2 cytokines suppresses IFN-γ production and IFN-γ mediated effects including MQs activation (Van Crevel et al., 2002 and Ghadimi, D., et al., 2010). IL-4 impairs antimicrobial activities by reducing TNF-α mediated apoptosis of infected cells, decreasing RNI expression and increasing iron availability to support the growth of intracellular M. tb. (Rook, G.A., 2007). Eventually, increased Th2 responses in the lung augments immunopathology by initiation of pulmonary fibrosis and cavitation, which compromises function of lungs in TB patients (Rook, G.A., 2007).

**OXIDATIVE STRESS**

Oxidative stress occurs when reactive oxygen species (ROS)/reactive nitrogen species (RNS) production and antioxidant defences become imbalanced. Redox regulation via ROS/RNS and the antioxidant defences represents a tightly controlled system that can have both deleterious and beneficial effects within the cellular environment. Oxidative stress generated by M. tb. infected activated macrophages produces a substantial amount of toxic oxygen and nitrogen radicals with the ability to kill the bacillus. H2O2 and O2 are two common forms of ROI that M. tb. encounters inside phagocytes. Nitric oxide (NO), which is produced upon activation of inducible nitric oxide synthase (iNOS) using L-arginine as a substrate is an important member of RNI (intermediates) (Schon, T., et al., 2003). NO radical is a free reactive species, created from the amino acid L-arginine by the enzymatic action of nitric oxide synthase (NOS). This endogenous NO is stabilized by carrier molecule like reduced thiol species that conserves its biological activity. This molecule readily reacts in the presence of NO to yield biologically active S-nitrosothiols that is more stable and effective than NO itself. Higher level of NO is an indication of oxidative stress. Data from murine TB provide evidence that iNOS/NO represents an important innate effector molecule that can provide immune protection in TB (Scanga, C.A., et al., 2001).
**NANOPARTICLES:**

Nanotechnology refers to the research and technological developments at atomic, molecular, and macromolecular scales, that create objects such as nanoparticles, that have at least one dimension in the range of 1-100 nm. Nanomedicine is concerned with applying nanotechnology to the treatment, diagnosis, monitoring and control of biological systems. Nanoparticles designed for drug or gene delivery are capable of entrapping the desired drug or gene in the particle or adsorbing the drug on the particle surface thereby protecting the drug from enzymatic degradation in a biological system. The nanoparticles fit into colloidal drug delivery systems, which offer advantages of drug targeting by modified body distribution.

The subcellular size of nanoparticles enables higher intracellular uptake, which benefits from reduction of undesired toxic side effects of the free drugs. With their easy accessibility in the body, nanoparticles can be transported via the circulation to different body sites, thus aiding in systemic treatments. Nanoparticles can be prepared from a variety of materials such as protein, polysaccharides and synthetic polymers. The choice of materials depends on several factors including (i) size and morphology of the nanoparticles; (ii) surface charge and permeability of the nanoparticle; (iii) degree of biodegradability, biocompatibility and cytotoxicity; (iv) drug loading and release profile desired. Further, the properties of nanoparticles can vary depending on the process of preparation, size, zeta potential, temperature, pH, or even morphology.

**Chitosan:**

Chitosan is made of randomly distributed (1-4)-linked D-glucosamine (deacetylated unit) and N-acetyl-D-glucosamine (acetylated unit). It consists of repeating units of glucosamine and N-acetyl-glucosamine, the proportions of which determine the degree of deacetylation of the polymer (Bowman, K. and Leong, K.W., 2006). The two most important factors that determine the physicochemical properties, and consequently the specific applications of chitosan, are chitosan’s degree of deacetylation (DD) and molecular weight (MW). The percentage of the deacetylated units (glucosamine monomers) in chitosan’s chains, affects the chemical, physical and
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biological properties of chitosan, such as the tensile strength of the films, the ability to chelate metal ions and the immuno-adjuvant activity. The higher the percentage of deacetylation of chitosan, the higher are the amount of free amino groups (-NH₂), which in turn, can become protonated to form cationic amine groups (-NH³⁺) producing positively charged surfaces. This polycationic nature of chitosan is expected to enhance the interactions between the chitosan surface and the negatively charged cells.

Chitosan has wide applications in medical fields, such as wound dressing, hypo-cholesterolemic agents, blood anticoagulant, anti-thrombogenic and drug delivery wound-healing materials, cosmetic preparations and textile, paper and film technologies. Owing to its excellent biocompatibility, biodegradability and its ability to get modified without changing the skeleton of the molecule has made it interesting to exploit for its therapeutic potential for delivery of proteins (Illum, L., 1998).

Since the early 1990s, efforts to develop a subunit vaccine against tuberculosis have focused on proteins released from the growing mycobacteria into the extracellular medium. Many are unique to M. tb., and, to date, only a few have been evaluated for their immunological properties and protective potential in various animal models. Several culture filtrate proteins secreted by M. tb. contribute to immunology of tuberculosis and to possess enzymatic activities associated with pathogenicity. CFP-10 is an antigen that contributes to the virulence in M. tb. CFP-10 forms a tight 1:1 heterodimeric complex with 6 kDa early secreted antigen target (ESAT-6). In the mycobacterial cell, these two proteins are interdependent on each other for stability. CFP-10, is essential for binding and attaching to the surface of host white blood cells; such as macrophages and monocytes. CFP-21 is also a major secreted protein of M. tb. that maybe considered as a promising antigen for immunotherapy. Both CFPs are hydrophobic in nature as well as have a high content of α-helical structures. 10 kDa M. tb. Culture FiltrateProtein-10 (CFP-10, also known as MTSA-10) and many other M. tb. antigens like ESAT-6, Ag85B and MPT64 induce the differentiation and maturation of dendritic cells (Natarajan, K., 2003). Since the immune system recognizes particulate antigens much better than the soluble
form, nanoparticles can be used as a delivery system for less immunogenic proteins in order to make them more immunogenic.

Delivery of anti-tuberculosis drugs or secretory proteins by nanoparticles offers potential advantages over free drug, including the potential to target specifically the tissues and cells that are infected by *M. tb.* thereby simultaneously increasing therapeutic efficacy and decreasing systemic toxicity, and the capacity for prolonged release of drug, thereby allowing less frequent dosing.

The aim of this thesis was to develop biopolymeric nanoparticulate delivery system to treat *M. tb.* infection. The problems associated with an improved delivery system for treating *M. tb.* have been discussed herein and include reducing systemic toxicity, increasing local concentrations and ultimately improving reduction of treatment of duration. Although chitosan nanoparticles have been extensively worked upon there are no reports on their interaction with secretory proteins. The area of modulation of immune response using nanoparticles encapsulating secretory proteins is yet unexplored. This study seeks to identify the optimal characteristics of chitosan nanoparticles, quantification of cytokine levels and oxidative stress induced by nanoparticles and to further ascertain whether the immune activating properties can be co-related with the generation of oxidative stress? Finally, proof-of-concept studies were carried out in an *in vivo* model to illustrate the potential for these secretory proteins encapsulated nanoparticles for protection study through CFU assay against *M. tb.* infection in a murine model.

**Chapter I** gives a comprehensive review of literature on the immune responses in *M. tb.* of various drug delivery systems. This chapter presents the role of Glutathione and NO in the tuberculosis.

Various methodologies adopted for conducting different experiments have been presented in **chapter II.** Techniques were developed to investigate the oxidative stress and immunomodulation caused by chitosan nanoparticles, secretory proteins CFP-10 and CFP-21. Methods to determine the preparation and characterisation of chitosan nanoparticles, encapsulation of the secretory proteins and their release, their
pharmacokinetics and biodistribution in animal models, as well as the *M. tb.* protection study in murine models has been presented in this chapter.

Results obtained from various experiments conducted have been discussed in the light of other published reports in the chapter *Discussion.* The production studies that were carried out in an *in vivo* model to illustrate the potential for these secretory proteins encapsulated nanoparticles for protection study through CFU assay against *M. tb.* infection in a murine model have been discussed. Also, the implication of present finding in the development of a novel drug delivery system has been elaborated in this chapter.