CHAPTER-1

INTRODUCTION AND REVIEW OF LITERATURE
1.1 Introduction

Tuberculosis (TB) is a slow progressive, chronic granulomatous disease caused by *Mycobacterium tuberculosis* (*M. tb.*), an intracellular pathogen that is capable of establishing and causing life-long devastating infection in humans. Over 2 million people die of TB worldwide each year, around 400,000 of them in India alone. TB represents 3.7 percent of India's total disease burden, 11 times that of malaria, and is the leading cause of death, causing about 1.7 million new cases every year in India. The increase in drug resistance for anti-TB drugs pose an increasingly serious public health hazard with a high economic burden for India for several decades to come (WHO Report, 2017).

*M. tb.* is a highly contagious, airborne, slow-growing, Gram-positive aerobic rod-shaped acid-fast bacillus. The cell wall has high lipid content and allows the bacteria to survive within macrophages. Macrophage serve as the primary cells where the Mycobacteria invade and actively replicate within maturation arrested phagosome. Initially the survival of an intracellular organism is controlled by the innate immune response at the site of infection. The macrophage plays a major role in the innate immune system, and its ability to mount an appropriate response is therefore of prime importance in the immunopathology of TB. Thus, the pathogenicity of mycobacteria lies in the fact that they are extremely well adapted to the host macrophage environment.

The primary infection is generally asymptomatic and goes unnoticed. Infection and concomitant inflammatory reactions resolve once acquired immunity develops and a few surviving bacteria become dormant. But in a few patients, especially in immunocompromised patients and young children, there may be a quick progression to primary disease. The dormant organisms may also become reactivated in some patients to produce the disease.
Hypersensitivity reactions to mycobacterial proteins may cause extensive tissue damage in lungs that can spread virtually to any organ.

When bactericidal mechanisms invoked by the macrophage (respiratory burst, NO production, immune signaling) fail to clear the intracellular bacilli, the Mycobacterium is able to establish infection within the host. The TB patient then requires treatment of the disease by the intervention of chemotherapy.

One of the popular and highly efficacious therapeutic regimens embodied in the guidelines of RNTCP (the Revised National Tuberculosis Control Program) is the Directly Observed Therapy, Short Course (DOTS), which is for a minimum duration of 6 months. The RNTCP recommends initiation of therapy with two or more of the first-line drugs [streptomycin (STR), isoniazid (INH), Rifampicin (RIF), ethambutol (ETB) and pyrazinamide (PZA)]. Conventional dosage forms used in TB employ the oral route (as for INH, RIF) or intramuscular injections (STR). These result in low drug bioavailability at the site of infection (phagosomal compartments of macrophage) and therefore have to be taken in relatively high doses to build up an effective concentration of the drugs in the blood. In addition, the DOTS treatment regimen is long and arduous, making patient compliance difficult and leading to the rapidly expanding problem of drug resistance.

The targeted delivery of anti-TB drugs via micro- or nano-particles, liposomes, etc. to macrophage, (the cells that harbor these bacteria), therefore, forms an effective therapeutic approach against TB. Recent researches by several investigators on drug-loaded particulate delivery systems has shown that these particles are rapidly phagocytosed by macrophage and lead to the development of high intracellular drug concentrations, macrophage activation and significant enhancement in the anti-microbial efficacy of the loaded drugs.
In this context, β-Glucan particles, from the cell walls of baker’s yeast, are hollow and porous 2–4 μm microspheres, and are widely used as supplements in human nutrition. These are the major yeast pathogen-associated molecular pattern (PAMP), which are mainly identified by the pattern-recognition receptor (PRR) dectin-1, present on macrophage. Glucan particles are rapidly taken up by macrophages in vitro when encountered. The hollow cavity and porous nature of the GP also allow for the high loading of payload molecules. These properties makes them suitable as carriers for targeted drug delivery of loaded molecules to macrophages. Several fungal β-glucans appear to be effective immunomodulators, and they appear to impact positively on cancers and several bacterial infections.
1.2 Review of literature

*M. tb.* is a facultative, aerobic, slow growing pathogen that enters the respiratory airways and lungs by inhalation. When inhaled, a single infectious droplet may be enough to cause the disease. Most droplets end up in the upper respiratory tract, where the microbes are killed, but a few penetrate further down.

1.2.1 Current situation of Tuberculosis

TB is one of the leading killers of young adults worldwide and the global scourge of multidrug resistant tuberculosis (MDR-TB) is reaching epidemic proportions, causing about 1.5 million deaths worldwide, which represent over 95% of the deaths in low and middle-income countries (WHO, Tuberculosis fact sheet, October 2014). India’s TB burden is the highest in world, with 10.4 million incident cases of TB reported in 2016 across the world, of which ~2 million people are reported to die every year due to this curable disease (Global TB Control 2017 Report). The only available pediatric vaccine, using *Mycobacterium bovis* BCG, is protective against severe forms of childhood TB, but is ineffective against TB in adults. TB has, therefore, become a global pandemic and is a priority concern of our country as well.

TB is the world's second most common cause of death from infectious disease, after acquired immunodeficiency syndrome (AIDS) (Frieden et. al., 2005). It is endemic in most developing countries and resurgent in developed and developing countries with high rates of human immunodeficiency virus (HIV) infection.

In 2015, there were an estimated 10.4 million new TB cases worldwide, of which 5.9 million (56%) were among men, 3.5 million (34%) among women and 1.0 million (10%) among children (Global TB report, 2015). People living with HIV accounted for 1.2 million (11%) of all new TB cases. Six countries accounted for 60% of the new cases: India,
Indonesia, China, Nigeria, Pakistan and South Africa. Thus, the global progress depends on major advances in TB prevention and care in these countries. Worldwide, the rate of decline in TB incidence remained at only 1.5% from 2014 to 2015. This needs to accelerate to a 4–5% annual decline by 2020 to reach the first turning point of the End TB Strategy. In 2015, there were an estimated 480,000 new cases of MDR-TB (resistance to Rifampicin and Isoniazid) and an additional 100,000 people with Rifampicin-resistant TB (RR-TB) who were also newly eligible for MDR-TB treatment. WHO recommends that all patients with Rifampicin-resistant TB (RR-TB) are treated with a second-line MDR-TB regimen.

1.2.1.1 Pathogenesis and immunology of TB

The first stage of TB is initiated with inhalation of droplets generated by a person with active disease. The bacteria reach the alveoli in the lungs, where the alveolar macrophages rapidly phagocytose them. The intracellular bacteria inhibits the phagosome-lysosome fusion and thus survives and replicates within macrophage, that normally serves as the first line of defense in our immune system (Sturgill-Koszycki et. al., 1996). Several receptors are involved in the uptake process including mannose receptors, Toll-like receptor 2 (TLR2) and Toll-like receptor 4 (TLR4), surfactant protein A receptors, CD14, scavenger receptors, complement receptors and immunoglobulin receptors (Bhatt and Salgame, 2007). However, the mycobacteria have evolved to evade the host defence mechanisms, enabling their intracellular survival in the host macrophage and replicate within ‘maturation arrested phagosomes’ that do not fuse with lysosomes and thus are not fully acidified (Sinai and Joiner, 1997), thereby avoiding low pH exposure and hydrolytic surroundings of phagolysosomes (Vergne et. al., 2004).
In the second stage, mycobacterium multiplies in the macrophage, eventually causing its lysis. This results in the cellular damage which attracts the inflammatory cells and blood monocytes to the area. Monocytes differentiate into macrophages and attempt to attack the microbe which is ingested by the macrophages and grow inside the phagocyte. These macrophages again lyse and die due to bacterial load (Mueller and Pieters, 2009).

Two to three weeks after infection, the third stage begins. T cell immunity develops, and lymphocytes drift to the region of infection. Presentation of mycobacterial antigens to the T cells causes their stimulation, resulting in the release of γ-interferon and other cytokines. The γ-interferon activates macrophage to secrete IL-12, TNF-α, IL-8, and other proinflammatory cytokines. Rapid growth of the M. tb stops and, at this stage, the host cells develop cell-mediated immunity. The bacteria surviving outside cells are resistant to antibody activated complement attack due to the high lipid content of mycobacterial cell wall (Todar, 2009).

Cell-mediated immunity is also responsible for much of the TB pathology. Tissue damage can also take place when activated macrophage releases lytic enzymes, reactive intermediates and various cytokines. At this stage the immune cells, specifically the macrophages, enclose the mycobacteria inside tubercles. In between these structures, the atmosphere is anoxic and acidic and prevents the growth of mycobacteria. This balance between host and mycobacterium is called ‘latency’ which is one of the hallmarks of TB.

In the fifth and final stage, the tubercles may dissolve by many factors such as malnutrition, immunosuppression, steroid use, or HIV infection. For unknown reasons, the centers of tubercles may liquefy, providing an outstanding growth medium for the microbe which now begins to grow rapidly in the extracellular fluid. The large number of bacteria and the immune
response against them eventually cause the lung tissue near the tubercles to become necrotic and form a cavity (Sherman et. al., 1980).

Most TB infections stop at the third stage. The cell-mediated immune response involves CD4+ (helper) and CD8+ (cytotoxic) T cells, and both play significant role in protection against TB. Antibacterial activity of macrophages is enhanced by the CD4+ (helper) T cells by releasing cytokines like interferon-γ (IFN-γ) and TNF-α, whereas CD8+ cells destroy infected macrophages and possibly M. tb. by releasing different cytotoxic mediators like perforins, granzymes and granulysin (Cooper, 2009). In spite of our enhanced information of immune response to M. tb., the type of immune response required for the effective immunity is not fully understood.

1.2.2 Different forms of TB

There are two different types of TB, latent TB and active TB. In the case of latent TB, bacteria remain dormant in body and can last for much longer time treated by taking single anti-TB medicine for 9 months duration (Debjit et. al., 2009). While, in case of active TB, bacteria multiply and spread in the body, thereby causing damage to the infected tissues.

1.2.2.1 Role of appropriate host immune response

The first step in immune response to M. tb. is its recognition as invading pathogen, followed by activation of innate host defense responses, and the subsequent initiation of adaptive immune responses. The development of an appropriate host immune response mediated by CD4+ T cells, as well as cytokines IL-12, IFN-γ and TNF-α, play a critical role in the control of M.tb infection (O'Garra et. al., 2013; Bozzano et. al., 2014) through mechanisms that involve altered production of reactive nitrogen intermediates (RNI) and prevention of
phagosome maturation, *M. tb* are able to survive within the host cells. Mycobacterial persistence leads to granuloma formation, which limits the spread of infection and masks bacteria from immune response, resulting in a latent infection that does not progress to symptomatic TB (Flynn and Chan, 2001).

The generation of appropriate innate and Th1 adaptive response is critical for macrophage activation, formation and maintenance of the granulomas so as to prevent spread of infection (Saunders and Britton, 2007) and control TB. Studies have demonstrated that the control of TB is dependent on IL-12 and Th1 cell responses (Altare *et. al.*, 1998, Urdahl *et. al.*, 2011). Th1 cell responses are critical in macrophage activation, and the formation and maintenance of the granuloma (Saunders and Britton, 2007).

The data from acute, chronic murine and human TB indicate that TNF-α plays an important role in *M. tb*. Constraining granuloma formation and limiting pathology in TB (Hasan *et. al.*, 2003). In contrast, Th2 response is abundant during infection with *M. tb*. (Flynn and Chan, 2001, Collins and Kaufmann, 2001). A small percentage of infected individuals develop symptoms, as fever, cough, chest pain, and night sweats (Redford *et. al.*, 2011), that are linked to breakdown in Th1 responses meant to isolate the infection. One of the common findings in individuals with symptomatic TB, (either from primary exposure or from reactivation of latent infection) is a high concentration of IL-10 in the blood (Boussiotis *et. al.*, 2000, Redford *et. al.*, 2011). IL-10 production has been associated with the reactivation of TB in a mouse model (Turner *et. al.*, 2002), thereby supporting a critical role for IL-10 production in TB pathogenesis. IL-10 dampens macrophage activation, impairs pro-inflammatory Th1 responses and stimulates development of Th2 cell responses and alternatively activated macrophage (AAM) (Kahnert *et. al.*, 2006). This leads to breakdown of granulomas that are
critical in limiting the spread of infection and disease severity. When the immune system of the patient is unable to clear the bacteria, the patient then needs an intervention of chemotherapy.

1.2.3 Chemotherapy for TB

1.2.3.1 First-line anti-tuberculosis drugs

TB is treated with first-line drugs as a combination therapy with INH, RIF, PYZ and ETB for 4 to 6 months. These drugs are administered orally and have outstanding potency against *M. tb.* (Grange and Zumla, 2002).

1.2.3.2 Second-line anti-tuberculosis drugs

When the *M. tb.* strain is resistant to INH and RIF, two of the most powerful first-line drugs; it develops into more complex form of TB known as MDR-TB. A combination of second-line drugs used to cure MDR-TB is aminoglycosides such as amikacin and kanamycin, polypeptides such as capreomycin, viomycin, and enviomycin, fluoroquinolones such as ciprofloxacin, levofloxacine and moxifloxacine, and thioamides such as ethionamide, prothionamide and cycloserine (Grange and Zumla, 2002). Second-line drugs are more toxic and are more expensive than first-line drugs, and treatment may last for much longer periods of time (Debit et al., 2009).

1.2.3.3 Third-line anti-tuberculosis drugs

To overcome the problems of first and second line drugs, WHO included the third-line drugs for treating TB include Rifabutin (RB), linezolid, thioridazine, arginine, vitamin D and macrolides such as clarithromycin and thioacetazone (Grange and Zumla, 2002). Like other available drugs for the treatment of TB, third-line drugs are not as effective or their efficacy has not been proven (Lalloo and Ambaram, 2010).
1.2.4 Drug regimens

Although the ‘Directly Observed Treatment, Short Course’ chemotherapy (DOTS) is an internationally recommended approach for TB, the treatment regimen is long and arduous, making patient compliance difficult. According to the ‘Tuberculosis Control Project, World Bank’, with older treatment regimens, the completion rates have traditionally been about 30 percent. As a result, some strains of the bacteria have become resistant to many of the available antibiotics and the problem is still growing. The emergence of drug resistant strains of Mycobacteria and the millions of deaths of TB patients imply that the drug concentrations established in blood and in cells are not sufficient to clear bacteria. Clearly, there is a need for providing a shorter alternative to 6 month for TB treatment which patients don’t complete.

1.2.5 TB burden in India

Each year about 2.2 million people develop TB in India and an estimated 220,000 die from the disease. Some estimates calculate the deaths as being twice as high (TB India RNTCP, 2016). TB can affect any age, caste or class but cases are mainly poor people including slum dwellers, tribal populations, prisoners and immune-compromised patients. The financial load of TB in India is very high and from 2006 to 2014, it effects the cost the Indian economy a huge USD 340 billion (TB India RNTCP, 2016).

TB treatment and care in India is provided by the government bodies as the RNTCP as well as through private sector health providers. In 2015 the RNTCP investigated a population of 1.28 billion and a total of 9,132,306 new cases of suspected TB were examined through sputum smear microscopy from which 1,423,181 people were diagnosed and registered for further TB treatment (TB India RNTCP, 2016).
1.2.5.1 Multidrug-resistant tuberculosis (MDR-TB)

This is an arduous form of TB, defined by resistance to at least two of the standard first-line anti-tuberculosis drugs such as Isoniazid, Rifampicin, Ethambutol and Streptomycin (WHO, 2016).

Inadequate or inconsistent treatment has allowed MDR-TB to emerge and spread rapidly. Nowadays, the treatment for drug-resistant TB consists of second-line drugs and takes almost two years and, in addition, the treatment is very complex, expensive and toxic (http://www.tballiance.org/why/the-tb-pandemic.php). Many second-line drugs are lethal and have severe side effects. Treatment for MDR-TB is administered for a minimum of 2 years and involves daily injections (Nasiruddin et. al., 2017). All these components hold a significant challenge to government and health care departments. The World Health Organization (WHO) aims to treat 80% of the MDR-TB cases by 2015. Without unique, simple, and inexpensive treatments for MDR-TB, this is next to impossible. WHO has predicted that more than 2 million people have developed MDR-TB between 2011 and 2015 (WHO, 2016).

1.2.5.2 Extensively drug-resistant TB (XDR-TB)

Extensively drug resistant (XDR-TB) poses a major risk to public health. This is more brutal form of MDR-TB and is characterized by resistance to any fluoroquinolone and at least one of the three injectable second-line drugs (CDC, 2006). In the year 2006, XDR-TB outbroke in KwaZulu-Natal, South Africa; 52 out of 53 people who contracted the disease died within few months (WHO, 2016). 70% of XDR-TB patients were estimated to die within a month of diagnosis. WHO has suggested that roughly 5% of MRD-TB cases are XDR-TB.
1.2.6 Various drug delivery systems

Although current anti-TB drugs are effective, novel strategies must be urgently developed in order to accomplish targeted delivery of these drugs and avert the development or increase in resistance. If managed properly, combinations of first- (and also second-) generation anti-TB drugs can still show better efficacy. New advanced technologies like the design of nano- or micro- carrier based targeted drug delivery system are thus being further investigated for TB treatment.

1.2.6.1 Microemulsions

The concept of microemulsions was first introduced by Hoar and Schulman in 1943 (Hoar and Schulman, 1943). Microemulsion is a system of water, oil and an amphiphile (surfactant and co-surfactant) which is a single optically isotropic and thermodynamically stable liquid solution (Danielsson and Lindman, 1981). Microemulsions have gained a lot of attention for the development and design of new drug delivery systems in recent years due to their thermodynamic stability, high diffusion and absorption rates, ease of preparation and high solubility (Ritschel et. al., 1991, Sarciaux et. al., 1995). They aid in the improvement of drug bioavailability (Pouton et. al., 1997), resistance against enzymatic hydrolysis and reduced toxicity (Bhargava et. al., 1987). Stable microemulsions usually have droplet diameter within the range of 10–100 nm, and hence these systems are also termed as Nano-emulsions. Microemulsions have broad applicability in the development of colloidal drug delivery systems for the purpose of drug targeting and sustained release behaviour of the encapsulated molecules. There are three different types of microemulsions depending on composition: (a) oil-in-water (o/w), (b) bi-continuous, and (c) water-in-oil (w/o) microemulsions (Lawrence and Rees, 2000). Mehta et. al., reported the encapsulation of one or more anti-TB drugs with
the use of tween-based microemulsion systems (Mehta et. al., 2007; 2010). They successfully formulated microemulsions composed of oleic acid, phosphate buffer, Tween 80 and ethanol, and examined its potential as a delivery system for anti-TB drugs (RIF, INH, and PZA).

In another study, Kumar et. al., (2011) performed the inclusion of INH in o/w microemulsion or w/o microemulsion comprising of Triton X-100 and Acetic acid in the ratio of 1:1, followed by addition of cetyltrimethylammonium dichromate, chloroform and water; this microemulsion system presents the opportunity of sustained release, increasing drug solubility and bioavailability. Talegaonkar et. al., (2008) studied a means of concentrating RIF in order to make this drug more efficient for oral drug delivery by microemulsion, which was composed of a reaction product of a castor oil and ethylene oxide. This study established that RIF was effective and may likely prevail over the problem, since lowering the dose lessens the toxicity.

1.2.6.2 Micro-and nano-particles

Inhaled microparticles containing anti-TB drugs are rapidly phagocytosed by alveolar macrophage and are targeted thereby to macrophage cytosol, which is primarily the site where the Mycobacteria reside and actively multiply (Sharma et. al., 2001). The phagocytic uptake of microparticles not only delivers a payload of drug intracellularly, but also activates the phagocyte, upregulating the anti-microbial defense mechanisms (Sharma et. al., 2007, Yadav et. al., 2009, Sharma et. al., 2011). This suggests that administration of microparticles containing anti-TB drugs may be useful for treating TB, by targeting macrophage-resident persistent mycobacteria, improving drug efficacy and thereby offering promises of dose and dosing-frequency reduction as well as toxicity alleviation.
1.2.6.3 Targeted drug delivery against TB

Biodegradable polymers, liposomes, and microspheres have been developed in order to reduce the dosing frequency and days of the treatment (Hari et. al., 2010). Attempts have also been made to enhance the delivery of loaded drug to specific lung tissue as an overall requirement of formulations meant for targeting lung macrophage in an efficient manner. Targeted drug delivery allows controlled release of drugs and cause minimum toxicity as compared to the conventional oral dosage. Therefore, while free antibiotics need to be administered daily, new formulations such as nano- or microparticles have been seen to effective even when administered after a few days.

Injectable INH loaded PLGA microparticles have been prepared by Dutt and Khuller (2001) and successfully evaluated for treatment of TB in M.tb. H37Ra infected mice model. Similarly, RIF loaded biodegradable microparticles have been evaluated for anti-TB activity following H37Rv infection in mice model for targeted delivery to host macrophage. These were also shown to significantly decrease the number of viable mycobacteria at 7 days following initial infection within H37Rv infected murine macrophage cells J774 and human Mono Mac 6 cell lines (Barrow et. al., 1998) and in M.bovis BCG infected rat alveolar macrophage NR8383 (Hirota et. al., 2010). A study on the comparison of the anti-TB efficacy of RIF loaded microparticles (RIF-PLGA) with pure drug administered to respiratory tract of M.tb. H37Rv infected guinea pigs, exhibited that the guinea pigs treated with RIF-PLGA had a significantly smaller number of viable bacteria, reduced inflammation and lung damage than lactose or saline control, PLGA or RIF treated animals (Suarez et. al., 2001b). RIF–PLGA microspheres have been reported to be safe due to their “silent” nature when taken into rat
macrophage cells and that they are promising for the treatment of tuberculosis (Hirota et al., 2010).

INH or RIF stealth liposomes with enhanced affinity towards lung tissue were prepared by modifying the surface of stealth liposomes by tagging O-stearylmylopectin so as to enhance the drug targeting to lungs, and were seen to have increased affinity towards the lung tissue of mice (Deol and Khuller, 1997). Multiple emulsions of RIF also gave a controlled release profile, and coating of these emulsions with polysaccharide was found to reduce toxicity compared to the free soluble drug (Khopade et al., 2000).

Various researchers have proposed the development and use of dry powder inhalations (DPI) for pulmonary delivery of drugs in the lungs (Patton, 1998). Particles delivered to lungs are rapidly phagocytosed by alveolar macrophages (Evora et al., 1998). Inhalable or respirable delivery systems (microparticles) containing anti-TB drug combinations have been proposed by several researchers (Patton, 1998, O’Hara and Hickey, 2000, Suarez et al., 2001a,b, Hino et al., 2005).

Lipid microsphere formulation containing RIF were shown to be a promising preparation for delivery via the respiratory tract to TB patients (Takenaga et al., 2008). The concept of using submicron carrier systems by incorporation of one or more anti-TB drugs for the delivery of antibiotics (Gursoy et al., 2004) at the site of infection has gained increasing interest in recent years. The group of Gopal Khuller (Chandigarh, India) administered antibiotics enclosed within biodegradable poly lactide-co-glycolide (PLGA) nanoparticles to different animals infected with M. tb. via the lung-aerosol or oral routes (Pandey et al., 2003, Sharma et al., 2004). The study showed that the administration of two-third of the recommended dose (for first line anti-TB drugs) every 10th day sterilized lungs and spleens
(Sharma et al., 2004). Similarly, alginate nanoparticles incorporating anti-TB drugs at 70-90% encapsulation efficiency were also standardized as carriers for pulmonary delivery systems, and sterilized the lungs in three doses administered at 15-day intervals (Ahmad et al., 2006, Ahmad et al., 2007, Zahoor et al., 2005).

Studies reported by the Gupta and Khuller group also demonstrate very high efficacy and sterilizing activity of econazole with moxifloxacin, if RIF is added to the combination (Ahmad et al., 2008). Similarly, Sterling et al., (2011) has shown that a new combination drug regimen, comprising of 3 months of isoniazid and Rifapentine was as effective as a 9-month isoniazid regimen for treatment of latent TB. The short-course, 3-month regimen had higher treatment completion rates and was also well tolerated in human patients.

1.2.6.3.1 Immune response modulation by delivery systems

An important reason for the current failure to control TB is that even when the best chemotherapy is used the treatment must be continued for at least 6 months. Apart from the existing of ‘persisters’, the other reason for prolonged treatment is that an inadequate pattern of immune response does not correct itself during treatment.

Treatment with drug containing microparticles has been shown to induce several biochemical and cellular events, as respiratory burst, production of NO and Th1 cytokines, particularly TNF-α and apoptosis (Prior et al., 2002, Sharma et al., 2007, Yadav et al., 2009). Thus, some of the microparticles (containing anti-TB drugs) have been shown to target macrophage, the antigen presenting cells (APCs) and also rescue at least some elements of ‘classical’ activation in targeted cells (Sharma et al., 2007, Yadav et al., 2010), instead of inducing ‘alternate’ activation or immuno-suppression (Kahnert et al., 2006). With such knowledge, it is possible to reverse the balance towards Th1 response with protective efficacy
and at last to achieve the aim of immunotherapy.

1.2.6.4 Glucan Particles as a novel drug delivery vehicle

The development of efficient drug delivery systems represents an important challenge to deal with issues such as drug solubility, targeting, *in vivo* stability, clearance, and cytotoxicity (Kang and Im, 2014, Crielaard *et. al.*, 2012, Park *et. al.*, 2014, Vanniasinghe *et. al.*, 2009, Tzakos *et. al.*, 2013, Gerwin *et. al.*, 2006).

1.2.6.4.1 Chemical Structure of β-glucan

Glucan particles (GP) are glucose polymers (fig. 1.1), consisting of a backbone of β-(1→3)-linked β-D-glucopyranosyl units with β-(1→6) - linked side chains (Brown and Gordon, 2003), which are hollow, 1–4 µm porous particles prepared from *Saccharomyces cerevisiae* (Baker’s yeast) and contain additional trace amounts of chitin. Natural products like β-glucans have been consumed for probably thousands of years especially in China and Japan, and have long been considered to improve general health (Chen and Seviour, 2007, Lindequist *et. al.*, 2005). However, only recently their importance in health and disease has been recognized. GP are FDA approved as generally recognized as safe (GRAS) (U.S. Food and Drug Administration, 2011).

They are mainly found as cell wall components in fungi, yeast and bacteria and also in cereals as barley and oat. Recently, methods to encapsulate macromolecular drugs inside GP based on the “in situ layer-by-layer synthesis of electrostatically bound complexes caged within hollow yeast cell wall particles” have been developed (Soto and Ostroff, 2008, Soto *et. al.*, 2011). The hollow cavity of GP allows efficient encapsulation of payload molecules and
targeted drug delivery into phagocytic cells with surface β-glucan receptors (dectin-1 and Complement Receptor 3 or CR3) such as macrophage, neutrophils and dendritic cells (Brown and Gordon, 2001). Such particles are therefore, being used for macrophage targeted delivery of macromolecules [i.e., proteins (Huang et. al., 2009), DNA (Soto, Ostroff, 2008), and siRNA (Aouadi et. al., 2009, Tesz et. al., 2011)]. Neutral, small drug molecules, such as antibiotic RIF have been encapsulated (precipitated) inside the GP using a hydrogel matrix composed of calcium alginate or chitosan that seals the GP pores, thereby slowing down drug release (Soto et. al., 2010). Such particles contained precipitated nanoparticles of RIF caged within hollow GP. Recently, Glucan particles have been used to encapsulate gallium nanoparticles that were shown to inhibit HIV in human macrophage (Soto et. al., 2016).

β-Glucans exists in single or triple helical conformation in solution, of which the triple helical configuration glucans have been reported as powerful immunomodulators (Falch et. al., 2000). β-Glucans also act as “natural polysaccharide immunomodulators,” and are known for their ability to activate the immune system. These characteristics make GP an effective drug delivery carrier to target phagocytic cells in the immune system.

The size of GP also favors their uptake by M cells in Peyer's patches and subsequent dissemination to the mesenteric lymph nodes, blood circulation and spleen (Eldridge et. al., 1990, Hong et. al., 2004).
1.2.6.4.2 Protective Effects of β-Glucans on immune system

In numerous animal models, β-glucans have been shown to have broad anti-infective activities, with the predominant immuno-pharmacological effects being the activation of neutrophils, macrophage and NK cells (Ross et al., 1999, Tzianabos, 2000). Large molecular weight and/or particulate glucans appear to be able to activate leukocytes directly, stimulating their phagocytic, cytotoxic and anti-microbial activities, including reactive oxygen intermediates (ROS), as well as stimulating the production of proinflammatory mediators, cytokines and chemokines [IL-1, IL-6, TNF-α, IFN-γ and IL-8] (Vetvicka et al., 2004, Berner et al., 2005, Brown, 2006). The GP-enhanced immunotherapy of mouse mammary carcinoma
has been shown to convert non protective Th2 response to protective Th1 cellular immune response (Baran et al., 2007). It has been shown that murine macrophages produced IL-12 after in vivo stimulation with other β-1,3 glucan-WGP from baker's yeast. IL-12 derived from macrophages can stimulate IFN-γ production from T cells, thereby favoring a Th1-pattern of response (Baran et al., 2007). They also stimulate macrophage phagocytosis of apoptotic cells through upregulation of phosphatidylserine receptor (Fadok et al., 2000).

1.2.6.4.2.1 Anti-infective activity of β-glucans

Zymosan, a cell wall extract from S. cerevisiae, has been reported to enhance the effectiveness of vaccine against HIV by stimulating cell-mediated immunity through activation of complement system as well as through production of interferon gamma (Ara et al., 2001).

Other β-glucans such as SCG, a 1,3-β-D glucan from Sparassis crispa, Poly-[1-6]-D-glucopyranosyl-[1-3]-D-glucopyranose glucan (PGG) and lentinan, are effective against several bacterial infections. For example, lentinan reduced M. tb. infection in rat model in vivo by increasing macrophage numbers and in vitro by increasing the killing ability of macrophage towards M. tb. (Markova et al., 2003, Markova et al., 2005). Overall, these and many other studies suggest that β-glucan treatment, when used as prophylactics, may boost the immune system to fight against subsequent infections.

1.2.6.4.3 Receptor mediated signaling for β-Glucan recognition

1.2.6.4.3.1 Dectin-1: signaling pattern recognition receptor for β-Glucans

GP uptake has been demonstrated to be dectin-1 dependent in vitro (Huang et al., 2009). Dectin-1 is a pattern-recognition receptor which expressed on the surface of all
phagocytes that detect β-glucans in fungal cell walls and stimulate direct cellular antimicrobial activity.

A great deal of work on macrophages and dendritic cells using zymosan as a ligand has established that Dectin-1 is required for inflammatory (cytokine and chemokine production, including TNF-α, CXC-chemokine ligand 2 (CXCL2), IL-2, IL-10 and IL-12) innate immune responses (Brown and Gordon, 2003, Rogers et al., 2005), respiratory burst (Underhill et al., 2005) and phagocytosis (Herre et al., 2004a, Underhill et al., 2005). The induction of cytokines like TNF-α and IL-12 (Brown et al., 2003) required collaboration with TLR2. A significant role for dectin-1 has been shown, in cooperation with TLR2, to activate the macrophage’s proinflammatory response to a mycobacterial infection (Yadav et al., 2006).

Studies by Bose et al., (2013) reported the immunomodulatory and immunostimulatory properties of baker’s yeast β-1, 3/1, 6 glucans, mediated through their ability to be recognized by human innate immune cells.

1.2.6.5 Natural Mucoadhesive polymer, Alginate

Sodium alginate, a salt of alginic acid (brown algae), a linear copolymer of α-guluronic acid and α-mannuronic acid, has the ability to form a gel/meshwork in the presence of divalent cations such as CaCl₂. This gel is mucoadhesive and is likely to adhere to mucosa for prolonged periods of time and should have the potential for releasing the drug in sustained and controlled manner. Alginate particles as Anti-TB drug carriers have been prepared and studied for oral drug delivery systems for TB treatment (Ahmad et al., 2006, Ahmad et al., 2007).
Alginate (ALG) is an aqueous soluble linear bio-degradable polysaccharide extracted from the brown sea weed, comprising of alternating chains of 1-4 linked α-L-guluronic and β-D-mannuronic acid residues. Previous research reported that ALG is mucoadhesive, biodegradable, and biocompatible and has potential role for various pharmaceutical and biomedical applications in drug delivery and cell encapsulation (Gombotz and Wee, 2012, Smidsrod and Skja, 1990). ALG micro- and nano-particles can be obtained easily by inducing gelation with calcium ions (Pan et. al., 2002, Mladenovska et. al., 2007). Such easy-gelling properties of ALG can be used to produce a pre-gel consisting of very small aggregates of gel particles, followed by the addition of an aqueous polycationic solution to make a polyelectrolyte complex coating (De and Robinson, 2003).

Recently, chitosan (CS) has been selected as an alternative cationic polymer. Chitosan, a linear polysaccharide consisting of glucosamine and N-acetylglucosamine units, is biocompatible, biodegradable, and nontoxic in the application of peroral delivery of drugs (Murata et. al., 2007). The addition of CS can not only provide nanoparticles a positive surface charge, but also prolong the time that the active ingredients contact with the epithelium and enhance absorption via the para-cellular transport pathway through the tight junctions (Kotze et. al., 1999, Sarmento et. al., 2007).

Microspheres prepared with bioadhesive and biodegradable polymers undergo selective uptake by the macrophage in lung mucosa and by the M cells of Peyer patches in gastrointestinal (GI) mucosa. Bioadhesive microspheres based formulations offer application as an ideal carrier system that can be tailored to adhere to any mucosal tissue, including those found in oral cavity and throughout the respiratory and gastrointestinal tract.
ALG is a negatively charged polymer which may interact with positively charged polymers such as chitosan. Recently, ALG beads containing active ingredients have been prepared by the gelation of alginate with calcium cations (Tonnesen and Karlsen, 2002). From a regulatory point of view, the U.S. Food and Drug Administration (US-FDA) recognizes ALG as a “Generally Referred As Safe” (GRAS) material, a designation that applies to substances accepted as safe for alimentary use by qualified experts (Chang and Chang, 2007). The like bioadhesive, mucoadhesive, biocompatible and nonirritant properties of ALG permit its makes potential role in the manufacturing of adhesive tablets for buccal drug delivery (Choi and Kim, 2000) and wound dressings with different features such as exudates absorption, moisture conservation, and wound healing (Qin, 2008, Boateng et. al., 2008, Thomas et. al., 2000, Timmons, 2008).
1.3 Hypothesis

Yeast derived GP may act as a potential carrier for targeted delivery of payload molecules of a new and efficient drug, Rifabutin to macrophage cells, with minimum wastage of drug and minimum toxicity. The recognition and internalization of drug-loaded glucan particles by PRRs on macrophage would allow targeted delivery of a payload of encapsulated drug. Therefore, in addition to drug delivery, the GP-based system might also activate the host cells to overcome the M. tb. induced immunosuppression, and promote appropriate innate and adaptive immunity required to effectively eliminate intracellular mycobacteria.

1.4 Aim of the study

The aim of this study was to prepare and characterize yeast-derived β-glucan particles containing a large payload of Rifabutin (RB). A novel, particle-based drug delivery technology based on the in situ layer-by-layer synthesis of an effective Anti-TB drug, within the hollow yeast cell wall particles (YCWP), enmeshed within alginate matrix was thus developed. We also aimed to attain a slow and sustained release profile of the drug (thereby, prolong drug release) from the alginate matrix, enhanced drug bioavailability at the site of infection and promotion of immune potentiation so as to kill the intracellular M. tb. more efficiently than the soluble drug at an equivalent concentration.
1.5 Objectives

- Preparation of yeast derived Glucan Microparticles (GP) containing Anti-Tuberculosis Drug.
- Evaluation of the immune responses to drugs administered as GP.
- Evaluation of the therapeutic potential of candidate formulation(s)
1.6 Significance of the study

We report the synthesis and characterization of GP-based formulation that is biodegradable and biocompatible, thermostable and has the capability to encapsulate a large payload of a hydrophobic anti-TB drug (RB). This is significant because low drug loading implies a need for repeated administration and inability to address the non-compliance problem and thus a risk for development of drug resistance.

To the best of my knowledge, there is no report of research on targeted drug delivery by yeast derived β-glucan microparticles incorporating Anti-TB drug(s) from any of the laboratories in India. Such a targeted delivery would help in achieving higher intracellular drug concentrations into target cell (host macrophage), the abode of replicating bacteria and therefore, would more effective against *M. tb.* than conventional antibiotic treatment regimens.

This technology has substantial potential to reduce antibiotic resistance and adverse effects associated with conventional TB therapy, such as drug wastage, severe hepatotoxicity, etc. The main advantage of this targeted delivery system lies in the ability to reduce the minimum time required, antibiotic dosages and also the adverse side effects associated with these drugs. This technology therefore, may be of clinical significance in treating chronic bacterial infection in TB patients who need prolonged administration of antibiotics. This study might facilitate translation of this drug delivery system into clinics to improve the treatment of TB.