Chapter 2

Review of Literature
2. Review of Literature

2.1 Tuberculosis: The Global Epidemic

Tuberculosis is the cause of 1.7 million deaths annually (WHO, 2010) which is the second highest cause of mortality from a single infectious disease worldwide, after HIV/AIDS (Corbett et al., 2003). One third of the global population is latently infected with \textit{M. tb}, and new infections occur at a rate of one per second (WHO, 2004) but it remains asymptomatic for a long duration (Barnes and Cave, 2003). Probability of developing active disease in those infected is approximately 10 percent (Bloom and Small, 1998), and the possibility increases dramatically in the events of perturbations of the immune response, as happens due to the HIV infections (Girardi et al., 2000; Selwyn et al., 1989). Thus, the huge prevalence of the TB has alarmingly caused a global public health emergency.

![Map of global TB incidence rates](image)

\textit{Figure 1. Estimated TB incidence rates, by country, 2009 (Adapted from WHO Report 2010, Global Tuberculosis Control).}

What is more alarming about this pandemic is its association with poverty and its skewed occurrence. The registered number of new cases of TB worldwide roughly correlates with economic conditions, maintaining its tightest grip on the populations
of poor and under-developed countries of Africa, Asia, and Latin America (Corbett et al., 2003; Frieden et al., 2003). The incidence of TB ranges from less than 25 per 100,000 in North America to 100 to 299 per 100,000 in Asia and western Russia to over 300 per 100,000 in Southern and Central Africa (Figure I) (WHO, 2010). Furthermore, 95 per cent of all new cases and 99 percent of all deaths due to TB occur in developing countries (Dye, 2006). In industrialized countries, the steady drop in TB incidence began to level off in the mid-1980s and then stagnated or even began to increase. Re-emergence of TB as a serious public health threat worldwide has largely been because of significant increase in Multiple-Drug-Resistant TB (MDR TB) as well as synergism between HIV and M. tb infection. On an average, out of ten immuno-competent people who are infected with M. tb one will have a chance of developing active TB in their lifetimes, while among those with HIV, this rate is one out of ten in a single year. In tuberculin test positive AIDS patients one in two or three has a chance of developing active TB (Corbett et al., 2003). In industrialized countries, these cases make up only a small minority of TB cases. However in developing countries, the impact of HIV infection on the TB situation is a grave concern.

2.2 Mycobacterium tuberculosis

2.2.1 General History

The genus Mycobacterium is presumed to be originated more than 150 million years ago (Daniel, 2006). An early progenitor of M. tuberculosis probably contemporaneously co-evolved with early hominids in East Africa around 3 million years ago. The modern members of M. tuberculosis complex seem to have originated from a common progenitor about 15,000 - 35,000 years ago (Gutierrez et al., 2005). The presence of tuberculosis has been documented in the prehistoric remains of humans (4000 BC) as well as in Egyptian mummies (3000-2400 BC) (Zink et al., 2003). In earlier times tuberculosis was known as “phthisis” - a Greek term that means ‘consumption’ indicating extensive weight loss due this disease. It was Hippocrates who first identified tuberculosis as a fatal disease around 460 BC and named it phthisis. J.L. Schoenlein gave the disease its present name “tuberculosis” in 1839.

Mycobacterium tuberculosis, the bacillus causing tuberculosis, was described for the first time in 1882 by Robert Koch, who later in 1905 received the Nobel Prize
for medicine or physiology for this discovery. Albert Calmette and Camille Guerin introduced the *Mycobacterium bovis* BCG vaccine in 1921, which is still the only available vaccine against TB; however its efficacy against adult pulmonary TB remains questionable. In 1946, the antibiotic streptomycin was utilized as a treatment drug for TB (MRC, 1948). Notwithstanding, such advancements in the field of TB treatment, in 1980s and early 1990s multi-drug resistant tuberculosis (MDR-TB) and extensively-drug resistant tuberculosis (XDR TB) strains emerged as a serious threat to TB control (CDC, 1990; CDC, 1993; CDC, 2007; Crawford, 1994; Dheda et al., 2010; Neville et al., 1994). With the advent of MDR and XDR TB, the hope that TB will be completely wiped out was shattered. Prevalence of HIV/AIDS accompanied by resurgence of tuberculosis has aggravated the scenario which led the World Health Organization to declare TB a global health emergency in 1993 (WHO, Frequently asked questions about TB and HIV).

**2.2.2 Taxonomic Position of *Mycobacterium tuberculosis***

*Superkingdom*: Bacteria  
*Phylum*: Actinobacteria  
*Class*: Actinobacteria  
*Subclass*: Actinobacteridae  
*Order*: Actinomycetales  
*Suborder*: Corynebacterineae  
*Family*: Mycobacteriaceae  
*Genus*: *Mycobacterium*  
*Species group*: *Mycobacterium tuberculosis* complex  
*Species*: *Mycobacterium tuberculosis*

Major characteristics of Tuberculosis complex organisms are:

- *M. tb is an* obligate aerobe and grows most efficiently in tissues with high oxygen content, for example, in the lungs.

- It is a facultative intracellular pathogen and usually infects mononuclear phagocytes (*e.g.* macrophages, monocytes or dendritic cells).
• It grows very slowly (generation time is 12 to 18 hours) compared to other bacteria (for example, the generation time of *Escherichia coli* is only about 20-30 minutes), This physiological characteristic may contribute to its virulence.

• Its cell wall is rich in lipids and extremely hydrophobic in nature. Since the cells are hydrophobic and tend to cluster together, they are impermeable to most of the regular stains like Gram’s stain.

• They are known as “acid-fast bacilli”. Due to the presence of lipid-rich cell walls, these bacteria are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resist decolourization with acidified organic solvents and are therefore called “acid-fast”.

2.2.3 Morphology of *Mycobacterium tuberculosis*

*Mycobacterium tuberculosis* bacteria appear as long thin rods, usually straight or slightly curved (*Figure 2*) that frequently show irregular beading due to vacuoles and polyphosphate granules. The bacillus is 1-10μm (usually 3-5 μm) long and 0.2-0.6 μm wide. It often appears singly and occasionally in threads. The bacterium is non-motile, non-spore forming, and non-capsule forming. It has very high lipid content in the wall, probably the highest among all bacteria. This waxy coat confers the acid fastness, extreme hydrophobicity, and low permeability to many antibiotics (Bhatt et al., 2007; Chambers et al., 1995; Glickman and Jacobs, 2001). In the envelope structure *Mycobacteria* contain mycolic acids and complex long-chain fatty acids that are found otherwise only in *Nocardia* and *Corynebacterium*. Owing to the presence of N-glycolyl muramic acid in the place of N-acetyl muramic acid in the peptidoglycan cell wall, *M. tuberculosis* is an acid-fast bacterium, which means the resistance to decolourization with acid-alcohol solutions after staining with carbol fuchsin. This feature is of great practical importance since it is used to identify mycobacteria in pathological specimens.
Figure 2. Scanning Electron Micrograph of *Mycobacterium tuberculosis* (Image courtesy- Dr. Ray Butlar / CDC)

2.3 Pathogenesis

The respiratory tract is the main route of infection for the tubercle bacillus. The bacteria are inhaled into the respiratory tract as airborne droplets that proceed distally to the lungs to establish an infection (Gupta and Chatterji, 2005). Although tuberculosis can manifest itself at any tissue site, the lung represents both the main portal of entry and an important site of disease manifestation. Extrapulmonary tuberculosis develops in 10 to 25 percent of all the reported cases (Bloom and Small, 1998; Golden and Vikram, 2005; Mehta et al., 1991; Yoon et al., 2004). Droplets containing minute number of bacilli are expelled by individuals suffering from tuberculosis. Alveolar macrophages engulf these droplets but do not kill the pathogen. Specific T cells are stimulated in the draining lymph nodes and induce bacterial containment in small granulomatous lesions of the lung, but fail to achieve complete microbial eradication (Shoenfeld and Rose, 2005). Thus the bacteria replicate within the macrophages and induce cytokines that initiate the inflammatory response in the lungs (Algood et al., 2003; Algood et al., 2005; Flynn and Chan, 2005; Ulrichs and Kaufmann, 2006). Macrophages and lymphocytes migrate to the site of infection and form a granuloma (Gonzalez-Juarrero et al., 2001). The function of the granuloma is to prevent spread to the remainder of the lung and to other organs as well as to concentrate the immune response directly at the site of infection. The granuloma is
maintained in a persistently infected host, probably due to chronic stimulation of the immune cells, and forms a basis for a tuberculous lesion (Flynn and Chan, 2001). Live bacilli have been isolated from granulomas or tubercles in the lungs of patients with clinically inactive tuberculosis, indicating that the organism can persist in granulomatous lesions for many years (Opie and Aronson., 1928; Robertson, 1933). Less than 10 percent of the infected individuals will develop clinical disease during their lifetime (Bloom and Small, 1998), but once disease does develop and remains untreated, it is fatal within 5 years in more than half of the cases (Tiemersma et al., 2011). Disease outbreak is delayed because the progress of infection is very slow. In the adult, tuberculosis occurs most commonly as a result of the reactivation of the existing foci, rather than as a direct outcome of primary infection (Manabe and Bishai, 2000; Russell, 2007). Usually in immunologically incompetent individuals, such as newborns, the aged, and the HIV infected patients, does the primary infection habitually transforms into disease (Russell, 2007). If the infection is successfully contained, the granuloma shrinks and may eventually calcify (Doherty and Andersen, 2005). If however, the immune response does not successfully control the bacterial replication, the granulomas increase in size and cellularity. Eventually, cell death in the hypoxic centre of the granuloma leads to necrosis. In individuals whom infection converts into disease, cavitary lesions develop and bacteria increase in number in the caseous detritus. As a result of cellular disintegration and destruction, the central material of the granuloma becomes caseous (Dannenberg, 1993). In tuberculosis, this lipid rich material provides a nutrient rich source for the pathogen. Further destruction might lead to liquefaction, thereby allowing microbial dissemination. If the granuloma is close to the surface of the lung, the tissue destruction caused by necrosis can breach the mucosal surface, giving rise to the prototypic symptom of TB, a persistent cough with blood in the sputum, a process referred to as cavitation (Abebe et al., 2010; Doherty and Andersen, 2005). At this point the patient is highly infectious, spreading the bacteria by aerosol. A person with active disease infects up to 15 people annually (Kaufmann, 2000). Thus the vicious circle then continues.

### 2.3.1 Granuloma

Granuloma is a mass of immune cells, consisting of a central area of activated macrophages surrounded by activated lymphocytes, which is formed to restrict or contain the foreign substance or pathogen that can not be eliminated. The centre of the
granuloma often contains multinucleated giant cells (known as Langhans cell) formed by the fusion of activated macrophages (Lay et al., 2007; Murphy et al., 2008; Pritchard et al., 2003). These giant cells typically are surrounded by large modified macrophages that resemble epithelial cells and therefore are called epithelioid cells (Goldsby et al., 2003).

The tuberculosis granuloma is the product of a robust cellular immune response to bacterial components. Alveolar macrophages in the airways, following internalization of inhaled bacteria, are stimulated to invade the lung epithelium (Algood et al., 2005; Flynn and Chan, 2005; Ulrichs and Kaufmann, 2006). Production of TNF-α and inflammatory chemokines from the infected macrophages drives the recruitment of successive waves of neutrophils, natural killer (NK) T cells, CD4+ T cells and CD8+ T cells, each of which produce their own complement of chemokines and cytokines that amplify cellular recruitment and remodelling of the infection site (Algood et al., 2003). This inflammatory cascade is regulated and superseded by a specific cellular immune response that is linked to the production of interferon (IFN)-γ. At this stage, formation of the ‘stable’ granuloma that is responsible for immune containment during the latent, or subclinical, period of the infection becomes recognizable and the stratification of the structure emerges (Kaplan et al., 2003; Ulrichs et al., 2005). More mature-phase granulomas show marked neovascularization and develop an extensive fibrotic capsule that delineates margin between the macrophages, granulocytes, foamy macrophages and giant cells, and the lymphocytic infiltrate (Dheda et al., 2005a; Kaplan et al., 2003; Ulrichs and Kaufmann, 2006). If the immune response is strong, latent infection results in asymptomatic and non transmissible state and finally granulomas may be reduced to small fibrous and calcified lesions (Doherty and Andersen, 2005). On the contrary, following a change in the immune status of host (due to old age, malnutrition or co-infection with HIV), the granuloma caseates (decays into a structureless mass of cellular debris that resembles cheese – hence the term caseation), ruptures and spills thousands of viable, infectious bacilli into the airways. This results in the development of a productive cough that facilitates aerosol spread of infectious bacilli (Figure 3).

Granuloma has long been considered to be necessary for the containment of infection but a recent study suggested that granulomas might promote infection, rather than simply containing it (Davis and Ramakrishnan, 2009). This finding indicates that
TB granuloma may not only be considered as a crucial part of the protective immune response to disease, but also as a facilitator in the development of latent infection, which is hard for the immune system to tackle and is notoriously difficult to treat by conventional methods.

Figure 3. The pathology of granuloma. The granuloma consists of a kernel of infected macrophages surrounded by foamy macrophages and other mononuclear phagocytes, with a mantle of lymphocytes in association with a fibrous cuff of collagen and other extracellular matrix components that delineates the periphery of the structure. (Adapted from Russel, 2007)
2.4 Innate immune responses to *Mycobacterium tuberculosis*

*M. tb* infects, survives, and replicates within phagocytes (macrophages, monocytes, and immature dendritic cells) of susceptible hosts. These phagocytes provide a first line of defence, and in most cases, degrade the harboured organisms shortly after engulfment. Disintegration of the ingested organisms is initiated after phagolysosome fusion. The acidic environment of the lysosomal contents and the hydrolases that are present in this compartment destroy the microorganisms. However, *M. tb* counters this host immune defence by several mechanisms. It has been shown that mycobacterial sulfatides and lysosomotropic polyanionic glycolipids can inhibit phagolysosome fusion (Goren et al., 1976a; Goren et al., 1976b; Middlebrook et al., 1959). Furthermore, *M. tb* produces copious amounts of ammonia, which effectively neutralizes the acidic environment of the phagolysosome, and successfully inhibits phagolysosome maturation (Gordon et al., 1980).

Enzymatically cleaved antigens intersect with the cellular antigen processing machinery and are eventually presented on the cell surface in the context of Major Histocompatibility Complex (MHC) molecules. The antigenic peptides are recognized by T cells, which lead to the generation of antigen-specific T cell responses. In addition to the MHC-bound peptide, optimal activation and polarization of T cells require a microenvironment consisting of co-stimulatory molecules on the same APCs, appropriate cytokines and chemokines, and lipid mediators secreted at the inflammatory site. The micro-environment at the inflammatory site is predominantly generated by innate immune components upon recognition of Pathogen-Associated Molecular Patterns (PAMPs). These PAMPs are recognized by Pattern Recognition Receptors (PRRs) expressed by innate immune cells.

*M. tb* produces several PAMPs, including lipoarabinomannan, phenolic glycolipids, phosphatidylinositol mannosidase, and lipoproteins. These molecular patterns are recognized by innate receptors, called TLRs, on macrophages and DCs (Brightbill et al., 1999). Interestingly, ligation of these PAMPs triggers both protective and pathogenic immune responses (Hawn et al., 2006). However, it is not clear whether the balance by which these receptors are engaged during *M. tb* infection contributes to susceptibility and resistance. It has been shown that signalling through TLR2 and TLR9 provides the strongest host resistance in TB (Bafica et al., 2005). TLR2 recognizes a variety of microbial products and also forms a heterodimer with either TLR1 or TLR6 (Takeda and Akira, 2005). TLR2 binds early secreted antigenic
target protein 6 (ESAT-6) of M. tb (Pathak et al., 2007) which results in extensive modulation of host immune response. TLR2/1 recognizes mycobacterial phosphatidylinositol mannosidase and the 19-kDa lipoprotein in the cell wall, leading to the production of pro-inflammatory cytokines (Brightbill et al., 1999; Hawn et al., 2006; Underhill et al., 1999). In contrast, recognition of lipoarabinomannan by TLR2 induces production of IL-10, an attenuator of protective immune responses (Quesniaux et al., 2004). TLR9 recognizes unmethylated CpG motifs of bacterial DNA, which results in the production of pro-inflammatory cytokines (Hemmi et al., 2000). There are two types of CpGs, namely B/K-type CpG and A/D-type CPG, both of which are recognized by TLR9 (Hemmi et al., 2003). B/K-type CpG induces the production of proinflammatory cytokines IL-12 and TNF-α, which facilitate Th1 responses (Takeda and Akira, 2005). On the other hand, A/D-type CpG triggers interferon (IFN)-β (a type-I IFN) production by plasmacytoid DCs (Krug et al., 2001; Verthelyi et al., 2001). It has been shown that infection of macrophages by M. tb induces production of type-I IFNs. Type-I IFNs also assist in mounting Th1 responses. Although some studies have suggested the involvement of TLR-4 in mounting inflammatory responses, the role of this TLR in tuberculosis infections remains controversial. Upon recognition of ligands, TLRs form dimers, which then recruit the TIR domain-containing adapter molecule MyD88, which activates its downstream signals (Takeda and Akira, 2005). Except for TLR3, all TLRs signal through MyD88. Nonetheless, individual TLR signalling pathways are divergent. For example, activation of TLR3 and TLR4 signalling pathways induces type-I IFN, but activation of TLR2 and TLR5 pathways does not induce this cytokine (Takeda and Akira, 2005). However, it is clear that the MyD88 pathway is essential for production of immunostimulatory and immunoinhibitory cytokines as well as accessory molecules by APCs. MyD88-deficient mice are clearly more susceptible to M. tb infection than wild type littermates (Ryffel et al., 2005).

2.5 Dendritic Cells and Mycobacterium tuberculosis

Dendritic cells (DC) are a system of cells that are specialized for the presentation of antigen to T cells. They are the most potent of antigen presenting cells and are central to the initiation of immune responses in naïve animals (Banchereau and Steinman, 1998; Steinman, 1991). They originate in bone marrow but recent
investigations suggest that they may also be derived from either myeloid or lymphoid precursors. DC are a trace population in most tissues and form networks underlying major body surfaces such as skin, trachea and intestine, where their function is the uptake of antigens and, after migration to the draining lymph nodes, the presentation of the processed antigen. A number of properties have been established that are critical to the function of DC as the ultimate antigen presenting cell population. These include the ability to effectively take up antigen by a number of routes, which may include endocytosis by clathrin-coated pits or caveolae, macropinocytosis or phagocytosis depending on the maturation stage of the cell. High levels of expression of MHC class II and a number of costimulatory molecules that include CD80, CD86 and CD40 have been considered to contribute to the efficiency of DC as antigen presenting cells (Banchereau and Steinman, 1998).

A lot of studies have been carried out on the interaction of mycobacteria with DCs. For example, it has been shown that the infection of DCs with mycobacteria causes their activation as reflected by increase in the surface densities of various costimulatory and MHC molecules (Henderson et al., 1997). Additionally, it has been reported that infected DCs secreted increased levels of inflammatory cytokines including TNF-α, IL-1 and IL-12. DCs were also shown to phagocytose mycobacteria. Furthermore, it has been established that mycobacteria could replicate inside murine bone marrow derived DCs and although DCs were able to restrict their growth they were nevertheless less efficient than infected macrophages at eliminating the infection (Bodnar et al., 2001). A number of microbial lipopeptides and proteins have also been shown to activate and mature DCs (Hertz et al., 2001). In addition, stimulation of M. tb infected DCs via CD40 increased the ability of DCs to mount T cell responses and this was later shown to be primarily attributed towards increased expression of costimulatory and MHC molecules on their cell surface (Demangel et al., 2001). DCs have also shown to induce protective immunity in a murine model of M. tb and also against aerosol mediated infection (Demangel et al., 1999). These results further suggested the importance of DCs in priming immune responses to mycobacteria.
2.6 The Th1/Th2 Paradigm in TB

Although, one third of the global population is infected with *M. tb* (WHO, 2006), only less than 10% of infected individuals develop tuberculosis disease in their lifetime (Bloom and Small, 1998). Thus, the host has evolved the resistance mechanism(s) for controlling tuberculosis. Studies from patients and animal models indicate that T cells are indispensable for anti-tuberculosis immunity. Historically, it is accepted that CD4⁺ T cells play the central role in the resistance to TB infection (Caruso et al., 1999; Mogues et al., 2001; Scanga et al., 2000). However, recently CD8⁺ T cells have been receiving considerable attention for this disease. Individual that are resistant to tuberculosis generally develop antigen-specific Th1 responses, as determined by the production of IFN-γ, lymphotoxin (LT), and TNF-α by these cells (Kutlu et al., 2007; Salgame, 2005). On the other hand, susceptible strains of animals mount progressive Th2 responses, predominated by the production of IL-4, IL-5, and IL-13 (Kutlu et al., 2007; Rook, 2007; Rook et al., 2004). Animal models of tuberculosis have confirmed that *M. tb*-specific Th1 cells are indispensable for expulsion of the harboured tuberculoid organisms (Cooper et al., 1993; Flynn et al., 1993). Similarly, individuals defective in the genes encoding IFN-γ or the IFN-γ receptor are highly susceptible to TB (Ottenhoff et al., 1998). However, several studies have indicated that Th1 responses alone are not sufficient for protection against tuberculosis (Bhattacharyya et al., 1999; Elias et al., 2005a; Leal et al., 2001; Majlessi et al., 2006). In fact, tuberculosis disease is often characterized by delayed-type hypersensitivity (DTH) induced by purified protein derivative (PPD), which is the sign of IFN-γ mediated immune response. Therefore, elevation of Th2 responses might be responsible for enhanced susceptibility to TB. This hypothesis was strengthened by the fact that IL-4-deficient mice show accelerated resistance to *M. tb* (North, 1998; Saunders et al., 2000). Similarly, studies investigating the expression of cytokines in human granulomas in advanced TB have detected an enhanced IL-4 transcription (Fenhalls et al., 2000). Several other studies have indicated that production of IL-4 correlates well with the immunopathology and is predictive of disease progression in animal models and patients (Dheda et al., 2005b; Ordway et al., 2005; Seah et al., 2000; van Crevel et al., 2000). Furthermore, strong Th2 responses have been noted in patients who were not protected by BCG (Dlugovitzky et al., 1999; Rook et al., 2005). However, a few studies reported that peripheral blood
mononuclear cells (PBMCs) from some patients showed depressed IFN-γ production, while Th2 responses were unaltered in other patients (Lin et al., 1996; Zhang et al., 1995). Nevertheless it is clear that Th2 cells are not the only cell type conferring disease susceptibility. Thus, it is very likely that promoting Th1 responses and simultaneously inhibiting Th2 responses holds the key to effective resistance against TB. This hypothesis is strengthened by the fact that latently infected individuals, in which M. tb is effectively controlled, produce a large amount of IL-4β2, an endogenous antagonist of IL-4 generated by alternative splicing from the primary IL-4 transcript (Rook, 2007).

2.7 Th17 cells in TB

The role of T cell responses in TB is not as straightforward. During the last few years, several other T cell subsets have been discovered, and the list is still growing. Contribution of these cells in the outcome of tuberculosis pathogenesis has not been studied extensively. A third Th cell lineage, Th17 cells, has been recently described. These cells are inflammatory, are responsible for the pathogenesis of several autoimmune disorders, and provide resistance to certain bacterial and fungal infections (Jin et al., 2008). It appears that IL-17 does not play a protective role in primary immune responses during TB (Cruz et al., 2006; Khader et al., 2005). It has also been shown that BCG-infected IFN-γ-deficient mice develop enhanced numbers of IL-17-producing cells, but the susceptibility of these animals to BCG remained unaltered (Cruz et al., 2006). Another study indicated that IL-17 produced during the primary immune response inhibits the generation of an effective secondary immune response against TB (Romano et al., 2006). In contrast, several other studies have demonstrated a protective role of IL-17-producing cells for the development of secondary immune response against TB (Khader et al., 2007; Wozniak et al., 2006). It has also been documented that, in the absence of IFN-γ, a strong IL-17-dependent memory response is produced in BCG-infected animals. This memory response successfully protected animals upon subsequent challenge with M. tb (Wozniak et al., 2006).

Differentiation of Th17 cells requires simultaneous presence of IL-6 and TGF-β (Bettelli et al., 2007). M. tb infections in macrophages have been shown to produce large amounts of both of these cytokines (Toossi et al., 1995; VanHeyningen et al.,
Thus, it is expected that *M. tb* infections facilitate Th17 differentiation. However, it is evident that both the Th1 and Th2 subsets inhibit the differentiation of Th17 cells (Stockinger and Veldhoen, 2007). It is worth to re-emphasize here that the Th1/Th2 paradigm in patients and animal models of TB has been well established. Furthermore, recently it has been reported that TGF-β is dispensable for the molecular orchestration of Th17 differentiation (Das et al., 2009) instead, it strongly inhibits Th1 and Th2 differentiation mechanisms. Thus, the Th17 response is enriched by default. Consistent with this idea, IL-6-deficient mice showed a marginally increased bacterial burden in the initial phase of infection, suggesting a minor protective role of IL-6 confined within the innate immune response (Saunders et al., 2000). Taken together these data suggest that Th1 and Th17 cells are protective whereas Th2 cells assist the TB disease progression.

### 2.8 MicroRNAs and *Mycobacterium tuberculosis*

MicroRNAs (miRNAs) represent an abundant class of highly conserved endogenous small (18–25 nucleotides long) noncoding RNAs and function as a critical regulator of gene expression (Taganov et al., 2006). miRNAs bind to the 3’ untranslated region (UTR) of target mRNAs and exert their function in two ways—mainly by blocking the translation and also by inducing their cleavage (Stefani and Slack, 2008). miRNAs have been shown to play cardinal roles in biological processes including embryonic development, organogenesis, tissue development, stem cell differentiation, innate and adaptive immune responses (Hou et al., 2009; Houbaviy et al., 2003; Judson et al., 2009; Lee et al., 1993; Lu and Liston, 2009; O’Connell et al., 2010; Taganov et al., 2007; Wang et al., 2008).

The involvement of miRNAs in the fine tuning of innate immunity has been a field of intensive research. Direct role of miRNAs in the innate immunity was investigated in reports that identified miR146a performances as a negative feedback regulator in TLR signalling by targeting IRAK1 and TRAF6. miR146a can inhibit the expression of IRAK1 and TRAF6, impair NF-κB activity and suppress the expression of IL-6, IL-8, IL-1β and TNF-α (Larner-Svensson et al., 2010; Lu and Liston, 2009; Nahid et al., 2009; Starczynowski et al., 2010; Taganov et al., 2006). This miR146a is also up-regulated in *Helicobacter pylori* infection and in Tregs (Liu et al., 2010; Lu et al., 2010). The other miRNAs such as miR125a-5p was found to mediate lipid uptake.
and to decrease the secretion of some inflammatory cytokines (IL-2, IL-6, TNF-α, TGF-β) in oxidized low density lipoprotein (oxLDL)-stimulated monocyte-derived macrophages (Chen et al., 2009). miRNAs has been identified as important regulators of monocyte differentiation and maturation, granulocyte proliferation and activation, pathogen sensing, inflammatory responses and antiviral immunity (Fontana et al., 2007). In macrophages it has been shown that activation of TIRs (Toll-IL1 receptors) and TNFα receptor results in rapid expression of host miRNAs such as miR9, miR146a and miR155 (Bazzoni et al., 2009). As revealed by target gene analysis, these impair the expression levels of proteins involved in the pro-inflammatory signalling pathway including TRAF6 (miR146a), IRAK1 (miR146a), NF-κB1 (miR9), Inhibitor-κB kinase epsilon (IKKε) (miR155) and TGF-b-activated kinase 1-binding protein 2 (TAB2) (miR155) (Bazzoni et al., 2009). miRNA-mediated down-regulation of these genes allows cells to control their activation status by dampening the signalling pathways that govern the expression of pro-inflammatory cytokines.

Despite of these findings regarding the involvement of miRNAs in the regulation of innate immune responses, not much is known about the role of miRNAs in innate immune response during M. tb infection. It has been hypothesized that M. tb infection in dendritic cells or macrophages can lead to modulation of a group of miRNAs, resulting in attenuation of immune response so as to favour M. tb virulence and intracellular survival. Furthermore, miRNA profiles induced could be strain-dependent and host-dependent, which may partially explain the virulence difference among M. tb strains and host predisposition factors.

2.9 The BCG vaccine – Success, Failures and Reasons

Bacillus Calmette-Guerin (BCG), the current live vaccine against TB was developed by attenuation of M. bovis, which is closely related to M. tb (>90% DNA homology) and is a part of the M. tb complex. French scientist Albert Calmette and Camille Guerin of Pasteur institute developed BCG at the beginning of the 20th century by growing it on culture medium and monitoring its decrease in virulence in animals through this period (Calmette and Plotz, 1929). In 1921, the newly developed vaccine was administered to infants in France, where it proved a resounding success, reducing mortality by approximately 90%. Since then, it remains the only official and commercially available vaccine against TB (Lugosi, 1992). It is estimated that more than 3 billion individuals have been immunized with BCG and over 100 million doses
of BCG are administered annually, making it the most widely used vaccine in humans (WHO, 2004). Although, BCG vaccine does not confer a total and permanent immunity, it is generally accepted that BCG induces a certain degree of protection, particularly in children (Kaufmann, 2000). Meta-analysis studies have confirmed that BCG protects children, providing >80% efficacy against severe forms of TB, including tuberculous meningitis and miliary TB (Colditz et al., 1995; Trunz et al., 2006) but it has limited efficacy against adult pulmonary disease in endemic areas (Fine, 1995).

It has been found that the efficacy of BCG in imparting protection against tuberculosis varies from 0-80% (Brewer, 2000; Colditz et al., 1994). Trials conducted in 1940s and 1950s in developed countries such as UK, Denmark and North America demonstrated the vaccine to be highly efficient (70-80%), whereas more recent trials in Chingleput district of India, demonstrated no detectable protection against pulmonary tuberculosis in adults (ICMR, 1999), and some studies performed in the US showed even “negative” efficacy (Bannon, 1999; Fine, 1995). The reasons for the variable protective efficacy are unknown but several hypotheses have been proposed, including differences among the vaccine strains used in clinical studies, exposure of trial populations to environmental mycobacteria, nutritional or genetic differences in human populations, differences in trial methods, and variations among clinical \( M. tb \) strains (Behr, 2002; Brandt et al., 2002; Comstock, 1994; Demangel et al., 2005; Fine, 1995; Tsenova et al., 2007). Deletion analyses of the genome of different strains of BCG have shown that various BCG strains have lost some genes now thought to be important for establishment of protective immunity. 16 deletions encoding 129 Open Reading Frames (ORFs) have been reported, encoding several important T cell antigens such as the immunodominant molecules ESAT-6 (Early Secreted Antigenic Target -6), CFP-10 (Culture Filtrate Protein-10), CFP-21 (Culture Filtrate Protein-21) etc (Mahairas et al., 1996; Skjot et al., 2000; Weldingh and Andersen, 1999). It has been postulated that absence of such immunodominant antigens in BCG may be the cause that BCG is unable to prime a potent immune response that can protect against a subsequent \( M. tb \) infection (Andersen, 2001; Behr et al., 1999; Skjot et al., 2000). The failure to produce important T cell antigens appears to betray the purpose of a live attenuated vaccine. Support for the importance of these antigenic proteins in protection comes from novel vaccine that performs better than BCG, which is BCG strain engineered to overproduce ESAT-6 protein (Pym et al., 2003).
The loss of some of these genes might have occurred during the original attenuation process and/or during further propagation, before the lyophilization of seed lots was introduced in 1960s (Behr et al., 1999). Major antigenic proteins were found to be present in the parental strain but either absent or not expressed in several BCG vaccines. However, it is uncertain that to what extent these strain variations can account for the observed variability in the BCG vaccine efficacy (Oettinger et al., 1999). Also, persistent helminth infestation has been reported to interfere with the establishment of protective anti-TB responses. Helminths shift the immune response towards a Th2 type, thereby significantly reducing the protective efficacy of BCG (Elias et al., 2005b; Malhotra et al., 1999). It has been suggested that in developing countries failure of BCG vaccine is not due to low Th1 responses but rather because the vaccine is rendered ineffective and immuno-pathological in individuals exposed to the environmental immunological stimuli abundant in such countries (Rook et al., 1981). These explanations are not mutually exclusive and may all contribute to the heterogeneity in vaccine efficacy.

Besides variable efficacy, there are number of other limitations and major drawbacks of BCG. BCG boosters have been found to be ineffective (von Reyn and Vuola, 2002), possibly because the vaccine strain may not replicate in persons with previous immunization. PPD conversion after BCG vaccination has been considered a disadvantage in countries where tuberculin skin testing is used to identify infection with _M. tb_ (von Reyn and Vuola, 2002). Severe and life-threatening complications may occur, including severe disseminated disease in immunocompromised individuals including AIDS patients (Quinn, 1989).

Thus, in view of variable efficacy and various limitations of BCG, development of new, efficacious and safe vaccine appears to be the only option left. We believe that modification of BCG will be the key for the future effective vaccine. Therefore, determination of immune responses modulated by H37Rv and the immune response mounted by BCG is necessary.
2.10 RD1 region of *Mycobacterium tuberculosis* genome

A comparative evaluation of the genomes of the virulent mycobacteria, *M. tb* H37Rv and *M. bovis* with the vaccine strain *M. bovis* BCG revealed that many regions were missing in BCG when compared to *M. tb*. These were grouped into what are commonly known as Regions of Difference (RDs) (Mahairas et al., 1996). It has been reported that a large number of mutations have taken place during long *in vitro* propagations of this strain and these mutations have resulted in the deletion of many open reading frames (Andersen, 2001; Behr et al., 1999; Kaufmann, 2001). Furthermore, 16 deletions encoding 129 ORFs have been reported, encoding several important T cell antigens such as the immunodominant molecules ESAT-6, CFP-10, CFP-21 etc (Mahairas et al., 1996; Skjot et al., 2000; Weldingh and Andersen, 1999) and 11 of these 16 deletions were unique to *M. bovis* whereas the remaining 5 deletions were unique to BCG. One of these 5 deletions, designated RD1 (9,454 bp long segment), was deleted from all BCG strains but present in the *M. tb* complex (Harboe et al., 1996). RD1 region is a genetic locus encoding 9 proteins from Rv3871 through Rv3879 (Cole et al., 1998). Two open reading frames of RD1 region, Rv3874 and Rv3875 encode 10-kDa culture filtrate protein (CFP-10) and 6-kDa early secreted antigenic target (ESAT-6) respectively (Cole et al., 1998) and are cotranscribed and when coexpressed they form a tight 1:1 complex (Renshaw et al., 2002).

RD1 region encodes the T-cell antigen ESAT-6, which was originally isolated from a highly stimulatory low-molecular-mass fraction of *M. tb* culture filtrate (Sorensen et al., 1995). With both humans and cattle, *in vitro* studies measuring either soluble IFN-γ or IFN-γ-secreting T cells have indicated that ESAT-6 is a potential diagnostic reagent which is highly specific for active TB and is frequently recognized during disease (Ulrichs et al., 1998). Another antigen CFP10 was identified in the low-molecular-mass fraction of culture filtrate, and the gene which encodes this antigen is located in the same operon as ESAT-6 (Berthet et al., 1998). Southern blotting of genomic DNA has shown the presence of both the esat-6 and cfp10 genes in *M. tuberculosis*, *M. africanum*, and virulent *M. bovis*, whereas these two genes could not be demonstrated in any BCG vaccine strains and in NTM (nontuberculous mycobacteria), with a few exceptions (*M. kansasii*, *M. szulgai*, and *M. marinum*) (Harboe et al., 1996; Skjot et al., 2000). In agreement with this distribution, it was demonstrated that ESAT-6 was able to discriminate TB patients from both BCG-vaccinated individuals and *M. avium* patients (Lein et al., 1999). ESAT-6 elicits
strong, TB specific DTH responses in Guinea pigs, cattle and humans while CFP-10 elicits strong *M. tb* specific DTH responses in guinea pigs.

Though none of the nine open reading frames (ORFs) that comprise RD1 have a biochemically assigned function, this region has been carefully scrutinized *in silico*. The predicted functions of several RD1 region genes suggest they may have roles in protein translocation. Further, as CFP-10 and ESAT-6 lack clear secretion signals, they may require a novel secretion machinery for export (Braunstein and Belisle, 2000), and components of RD1 may form that machinery (Cole et al., 1998; Gey Van Pittius et al., 2001; Lewis et al., 2003; Pallen, 2002; Tekaia et al., 1999). This notion was proved true when disruption of individual genes (*Rv3870, Rv3871* and *Rv3877*) within this locus prevented secretion of ESAT-6 and CFP-10, providing the first genetic evidence that this region encodes for a secretion system. Further, Cox and others showed that disruption of individual RD1-region genes did not prevent production of ESAT-6 or CFP-10. However, an intact RD1 region was required to ensure that these proteins were secreted by the bacterium (Guinn et al., 2004; Pym et al., 2003; Stanley et al., 2003).

BCG into which the RD1 locus was reintroduced (allowing it to secrete the ESAT-6-CFP10 complex) was reported to be both more virulent but also more protective than the parental BCG strain (Pym et al., 2003). Interestingly, this vaccine strain (designated BCG::RD1) has been shown to trigger an immune response that is qualitatively more similar to that of *M. tb* than BCG. Specifically, vaccination with BCG::RD1 induced the recruitment of activated/effector T cells and dendritic cells to the lungs of subsequently infected mice far more efficiently than the parental BCG strain (Majlessi et al., 2005). This suggests that antigens encoded by RD1 and lost during the attenuation of BCG can significantly modify the type of immune response generated by a mycobacterial vaccine.

RD1 region seems to be a major determinant of mycobacterial pathogenesis but the mechanism by which this system affects the biology of the host cell is unknown. Many groups have suggested that this system functions to modulate early events during *M. tb* infection. RD1 region therefore represents one of the most interesting genomic regions of *M. tb* since it seems to be simultaneously involved in enhanced virulence in immuno-compromised hosts and increased protection in immuno-competent hosts and presents a challenge to researchers to identify the tactics employed by *M. tb* to ensure its survival in the intracellular environment.
2.11 The Present Study

Numerous studies have shown that Th1 cell responses are indispensable for protective immunity against TB. However, while the vaccine strain BCG induces sufficient Th1 cell response, this response does not appear to be sufficient for immune protection in many individuals. Here, we provide evidence for the first time that Th17 cell responses in the lung play a critical role for enhanced protection against TB. Surprisingly, the virulent *M. tb* strain H37Rv induced Th17 cell responses in the lung. Consequently, antibiotic-treated animals that were previously infected with H37Rv, as compared with similarly treated BCG-infected mice, generated improved protective immune responses against infection with virulent *M. tb*. We also provide evidence that the ESAT-6 protein, which is absent in BCG but present in H37Rv, induces IL-6 and TGF-β in dendritic cells in a TLR-2- and MyD88-dependent manner, which generates an environment that is conducive for the differentiation of Th17 cells in the lung. Our findings indicate that, in addition to Th1 cells, Th17 cells play a critical role in conferring optimal protection against TB.