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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 *M. tuberculosis*, and new infections occur at a rate of one per second (WHO) but it remains asymptomatic for a long duration (Barnes and Cave et al., 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.

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 underdeveloped 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 2.1)(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. tuberculosis* infection.
On an average, out of ten immuno-competent people who are infected with *M. tuberculosis* 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 Causative agent of Tuberculosis: *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 to 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 : Mycobacterium tuberculosis

Major characteristics of Tuberculosis complex organisms are:

- *M. tuberculosis* 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 2.2) that frequently show irregular beading due to vacuoles and polyphosphate granules. The bacillus is 1-10µm (usually 3-5µm) long and 0.2- 0.6µm 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 2.2. Scanning Electron Micrograph of Mycobacterium tuberculosis (Image courtesy- Dr. Ray Butlar / CDC)
2.3 Pathogenesis of *Mycobacterium tuberculosis*

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., 2011; 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 Events in the pathogenesis of TB

There are different stages that define the progression of a disease after inhalation of bacilli.

**First stage:** It begins with the inhalation of tubercle bacilli. Alveolar macrophages ingest the bacilli and often destroy them. The destruction of mycobacteria depends on the intrinsic microbicidal capacity of host phagocytes and virulence factors of the ingested mycobacteria.
Second stage (Symbiotic stage): Mycobacteria, which escape the initial intracellular destruction, multiply in the macrophages. This leads to the disruption of macrophages. Blood monocytes and other inflammatory cells are recruited to the lung. These monocytes differentiate into macrophages that again readily ingest but do not destroy the mycobacteria. Bacteria grow logarithmically, and blood-derived macrophages accumulate, but little tissue damage occurs.

Third stage: T-cell immunity develops with antigen-specific T lymphocytes that arrive and proliferate within the early lesions or tubercles and, then, activate macrophages to kill the intracellular mycobacteria. The early logarithmic bacillary growth stops. Central solid necrosis in these primary lesions inhibits extracellular growth of mycobacteria. As a result, infection may become stationary or dormant.

Fourth stage (Post-primary tuberculosis): Disease may progress, and hematogenous dissemination may take place after primary infection, as well as months or years afterwards, under the conditions of failing immune surveillance. Liquefied caseous foci provide excellent conditions for extracellular growth of *M. tuberculosis*. Cavity formation may lead to rupture of nearby bronchi, allowing the bacilli to spread through the airways to other parts of the lung and the outside environment.

2.3.2 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 cannot 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 coinfection 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 2.3). 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.
2.3 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. tuberculosis* 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
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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. tuberculosis* 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. tuberculosis* 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. tuberculosis* 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. tuberculosis* infection contributes to susceptibility and resistance. It has been shown that signaling 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. tuberculosis* (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. tuberculosis 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 signaling pathways are divergent. For example, activation of TLR3 and TLR4 signaling 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. tuberculosis infection than wild type littermates (Ryffel et al., 2005).

2.4.1 Recognition of M. tuberculosis by Toll-Like Receptors (TLRs)

Toll-like receptors (TLRs) are phylogenetically conserved mediators of innate immunity that are essential for microbial recognition by macrophages and dendritic cells. Engagement of TLRs by M. tuberculosis ligands is an early event in the interaction of M.
tuberculosis with its host cell (Figure 2.4). M. tuberculosis expresses a large repertoire of TLR2 ligands. The 19-kDa lipoprotein (LpqH), a secretory antigen of M. tuberculosis, was the first M. tuberculosis ligand shown to interact specifically with TLR2 to induce TNF-α and nitric oxide production from both murine and human macrophages (Brightbill et al., 1999). LprA (Rv1270) (Pecora et al., 2006) and LprG (Rv1411c) (Gehring et al., 2004) are two other mycobacterial lipoproteins that are TLR2 agonists. In addition to lipoproteins, lipomannan (Quesniaux et al., 2004) and phosphatidylinositol-myo-mannoside (PIM) (Gilleron et al., 1997) also interact with TLR2 to initiate cellular activation (Jones et al., 2001). However, with regards to PIMs, Abel et al. (Abel et al., 2002) demonstrated that PIM structures could also elicit cellular activation via TLR4.

![Figure 2.4. Phagocytosis and immune recognition of M. tuberculosis.](image)

**Figure 2.4. Phagocytosis and immune recognition of M. tuberculosis.**

Various receptors have been identified for phagocytosis of M. tuberculosis by macrophages and dendritic cells: complement receptors are primarily responsible for uptake of opsonized M. tuberculosis; MRs and scavenger receptors for uptake of nonopsonized M. tuberculosis. TLRs play a central role in immune recognition of M. tuberculosis. In the context of CD14, TLR2 binds lipoarabinomannan, a heterodimer of
TLR2 and TLR6 binds a 19-kDa M. tuberculosis lipoprotein, TLR4 binds to an undefined heat labile cell-associated factor, and (possibly) TLR9 binds to M. tuberculosis DNA. After binding to TLRs, common signaling pathways lead to cell activation and cytokine production. TLRs are expressed not only at the cell surface but also in phagosomes; therefore, immune activation may occur with or without phagocytosis. On the other hand, phagocytosis alone probably does not lead to immune activation without the involvement of TLRs. (Adapted from van Crevel et al., 2002)

Immune recognition of the major mycobacterial cell wall component, lipoarabinomannan (LAM), resembles that of gram-negative bacterial lipopolysaccharide (LPS) (Zhang et al., 1993). Several circulating factors and receptors are involved. Plasma LPS-binding protein enhances macrophage responses to LPS and LAM by transferring these microbial products to the cell surface receptor CD14 (Fenton and Golenbock 1998). Similarly, soluble CD14 confers responsiveness to both LAM and LPS in CD14 negative cells (Yu et al., 1998). Besides TLR2 and TLR4, other TLRs are also involved in immune recognition of M. tuberculosis: heterodimerization of TLR2 with TLR6 or TLR1 is necessary for signal transduction (Bulut et al., 2001), and TLR9 binds CpG dinucleotides present in bacterial DNA (Hajjar et al., 2001; Hemmi et al., 2000).

2.4.2 Recognition of M. tuberculosis by DC-Specific ICAM-3 Grabbing Non-integrin (DCSIGN)

DC-Specific ICAM-3 Grabbing Non-integrin, DC-SIGN is a calcium-dependent (C-type) lectin, containing a carbohydrate recognition domain (CRD) at its extracellular COOH-terminal end that recognizes mannose-rich molecules (Geijtenbeek et al, 2000). DC-SIGN was initially described as a receptor for ICAM-3 at the surface of T cells, triggering the formation of the immunological synapse between DCs and naive T lymphocytes. Interestingly, DC-SIGN binds to HIV and simian immunodeficiency viruses, and is involved in the trans-infection of CD4 T lymphocytes by HIV- or SIV-infected DCs (Geijtenbeek et al, 2000a). DC-SIGN has also been recently involved in Leishmania amastigotes binding to DCs (Colmenares et al, 2002). Tailleux et al. showed that M. tuberculosis infects DCs via ligation of DC-SIGN by the mycobacterial surface-
exposed lipoglycan lipoarabinomannan (LAM). Freshly isolated LDCs were found to express DC-SIGN, and *M. tuberculosis* – derived material was detected in DC-SIGN+ CD14+ HLA-DR+ cells in Lymph nodes from patients with tuberculosis (Tailleux *et al.*, 2003). This C-type lectin receptors extensively characterized with respect to mycobacteria. DC-SIGN/CD209 has been reported to be the predominant *M. tuberculosis* receptor on human DCs, whereas the mycobacterial macrophage receptors, CR3 and MR, appear to play a minor role, if any, in this binding (Buettner *et al.*, 2005). It is noteworthy that DC-SIGN is expressed by human pulmonary DCs and mycobacteria-derived antigens have been detected in DC-SIGN+ DCs in lymph nodes from patients with TB, suggesting that interactions between the lectin and the bacillus may occur during the natural course of infection. Study by Tailleux *et al.*, (2005), reveals that during the natural course of TB in human lungs, soluble host and/or mycobacterial factor(s) induce DC-SIGN expression by alveolar macrophages, which renders the cells highly prone to infection by the tubercle bacillus. DC-SIGN induction in alveolar M/s may have important consequences on lung colonization by *M. tuberculosis*, as well as on host immune and inflammatory responses, which will require further investigation in cell and animal models, as well as in patients with TB.

In the mouse, the ‘DC-SIGN’ loci is much more complex than in humans and encompasses seven genes, namely, SIGNR1-5, SIGNR7-8 and one pseudogene SIGNR6. Subsequently, Caminschi *et al* (2006) reported the functional comparison of mouse DC-SIGN also known as CIRE with human DC-SIGN and showed that like human DC-SIGN, CIRE or mouse-DC-SIGN bound mannosylated residues.

Mycobacteria interact with DC-SIGN to affect TLR4-mediated immune responses by DCs, suggesting that this C-type lectin modulates TLR4 signaling (Geijtenbeek *et al.*, 2003). The ligand responsible for this DC-SIGN-mediated immune modulation is ManLAM, a cell-wall component abundantly expressed by *Mycobacterium tuberculosis*. Binding of ManLAM to DC-SIGN impairs lipopolysaccharide (LPS)-induced maturation of DCs and increases the production of the immunosuppressive cytokine interleukin-10.
(IL-10) (Geijtenbeek et al., 2003). The intracellular signaling triggered upon DC-SIGN engagement (ERK and PI3K activation, transient rise in intracellular calcium concentration) impairs IL-12 and enhances IL-10 release and, this might explain why DC-SIGN ligation favors a pro-Th2/protolerogenic dendritic cell maturation (Caparrós et al., 2006).

Interestingly, the interactions of *M. tuberculosis* vis-à-vis TLR and DC-SIGN have shown interesting correlates. While interactions with TLR result in activation of DCs characterized by high IL-12 secretion, interactions with DC-SIGN prevent DC activation by blocking NF-κB activation. For example, TLR9 has been shown to regulate Th1 responses to *M. tuberculosis* in cooperation with TLR2 (Bafica et al., 2005; von Meyenn et al., 2006).

The mechanism has been identified by which DC-SIGN modulates TLR-dependent responses in human DCs (Gringhuis et al., 2007). Triggering of DC-SIGN by ManLAM activated the serine and threonine kinase Raf-1, independently of TLR signaling. Activation of Raf-1 led to acetylation of p65, one of the key activating subunits of NF-κB, but only after TLR signaling had activated NF-κB. Strikingly, acetylation of p65 prolonged transcriptional activity of NF-κB and enhanced the transcription rate from the IL10 gene. They further demonstrated that this Raf-1-acetylation-dependent pathway is central to modulation of TLR-specific immune responses elicited by DCs in response to mycobacteria, fungi, and viruses. The above mechanistic studies were done with TLR3, TLR4 and TLR5 signaling. Thus, DC-SIGN signaling was found to modulate not only TLR4- but also TLR3- and TLR5-induced cytokine responses. The endosome-resident TLR3 thus signals through TRIF, while TLR5 on the outer membrane depends on the binding of MyD88, whereas TLR4 induces both MyD88- and TRIF-dependent pathways, all culminating in the activation of the transcription factor NF-κB (Kawai and Akira, 2006). However, similar studies comparing the activities of TLR2 and DC-SIGNR1 in the mouse system have not been investigated as yet.
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. tuberculosis* infected DCs via CD40 increased the ability of DCs to mount T cell responses and this was later shown to be
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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. tuberculosis* and also against aerosol mediated infection (Demangel et al., 1999). These results further suggested the importance of DCs in priming immune responses to mycobacteria.

2.5.1 Dendritic Cells as immunotherapeutic agents

*M. bovis* BCG or *M. tuberculosis* antigen(s) pulsed DCs have been found to impart significant protection against experimental mycobacterial infection. In a study by Demangel et al., *in vitro* derived DC were infected with BCG, which induced their maturation, as shown by the increased expression of MHC class II antigens, CD80 and CD86 co-stimulatory molecules as well as the enhanced levels of IL-1, IL-6, IL-12, IL-10 and IL-1 receptor antagonist as indicated by the increased mRNA levels. When administered intratracheally in mice, these infected DCs induced a potent T cell response and an increased production of IFN-γ to mycobacterial antigens in the mediastinal lymph nodes, leading to a significant protection against aerosol *M. tuberculosis* infection. Intriguingly, although the vaccination schedule for BCG-infected DCs was much shorter than subcutaneous BCG vaccination (7 days as compared to 100 days), both types of vaccination showed similar levels of protection. These data suggest that DCs can be the potent inducers of a cellular immune response against mycobacteria and support the concept of combining DC strategies with mycobacterial vaccines for protective immunity against tuberculosis (Demangel et al., 1999).

Another strategy to use DC-based vaccines have focused on pulsing *ex vivo* derived DCs with whole proteins or immunodominant peptides (McShane et al., 2002; Gonzalez-Juarrero et al., 2002). DCs pulsed with whole Ag85A protein delivered intranasally gave rise to increased numbers of IFN-γ-secreting CD4+ and CD8+ T-cells in the lungs (Gonzalez-Juarrero et al., 2002). However, these cells were not able to confer protection against pulmonary *M. tuberculosis* challenge. A study has shown that DCs pulsed with
Ag85A immunodominant CD4+ and CD8+ peptides induced slightly better immune responses than DCs pulsed with whole protein when delivered intramuscularly or intravenously (Malowany et al., 2006). This enhanced immune activation is probably due to the increased antigen presentation, by the infected DCs on MHC I as well as antigen presentation of secreted Ag85A by endogenous DCs on MHC II. While ex vivo-derived DCs pulsed with antigenic peptides or proteins have resulted in mediocre induction of protective immunity, virally transduced DCs were capable of much greater protective immunity. Such virally transduced DC vaccines may, thus, serve as therapeutic vaccines to treat intracellular infections without causing tissue damage and virus-neutralizing antibodies. This emphasizes the fact that DCs can act as natural adjuvants for effective priming of antimycobacterial adaptive immunity and in subsequent development of an efficacious vaccine (Demangel et al., 1999; Tascon et al., 2000).

2.5.2 Dendritic Cells as an important target for *M. tuberculosis* mediated immune evasion

As in the case of macrophages, mycobacteria also manipulate important functions of DCs, which is not surprising considering their importance in the anti-mycobacterial immune response. In mice infected with GFP-expressing *M. tuberculosis*, it has been demonstrated that the DC subpopulation that is most efficacious in stimulating CD4+ T cells in the lymph node is the one that carries a lower burden of mycobacteria (Wolf et al., 2007). There is also evidence that live *M. tuberculosis* can inhibit MHC class II antigen presentation without a decrease in the surface expression of MHC class II and costimulatory molecules (Hava et al., 2008). The poor antigen presentation observed in *M. tuberculosis*-infected DCs could be explained by a mis-timing of antigen availability and the maturation process, as proposed by Hava et al. (2008). DCs are extremely responsive and can undergo the process of maturation rapidly following stimulation. Particularly in the case of slow-growing intracellular pathogens, or the arrest of phagosome maturation, it may be that the activation program proceeds ahead, before the
cell can acquire abundant mycobacterial antigens (Mortellaro et al., 2009). This apparently simple case of poor timing may represent a novel way for pathogens to manipulate DCs and thus the immune response to their own advantage.

*M. tuberculosis* has several ligands for different TLRs as well as for C-type lectins such as DCSIGN. Mycobacteria-DC-SIGN interactions at the molecular level have revealed that DC-SIGN discriminates between *Mycobacterium* spp. through selective recognition of the mannose caps on LAM molecules, expressed by slow-growing virulent *M. tuberculosis* and *M. avium* but not by the fast-growing mycobacteria such as *M. smegmatis* that express arabinose on their LAM molecules (Maeda et al., 2003). ManLAM inhibits lipopolysaccharide (LPS)-induced DC maturation by interacting with DC-SIGN. Neutralization of DC-SIGN restores LPS-induced DC maturation in the presence of ManLAM (Turville et al., 2001). Functional characterization of DC-SIGN-*M. tuberculosis* interactions has shown that specific targeting of DC-SIGN by *M. tuberculosis* through ManLAM is a mechanism to impair DC maturation and to induce production of the anti-inflammatory cytokine, IL-10 (Geijtenbeek et al., 2003). This suggests that DC-SIGN, following binding to ManLAM, delivers a signal that interferes with mycobacteria-induced DC maturation signals. These results indicate that *M. tuberculosis* targets DC-SIGN to suppress cellular immune responses since immature DCs that secrete IL-10 would not only inefficiently stimulate T cells, but also induce a state of antigen-specific tolerance. Recently, Srivastava *et al*, have also shown that DC-SIGNR1 induces suppressor responses during *M. tuberculosis* infection via enhanced expression of SOCS1. The study has shown that increased expression of SOCS1 could be one of the suppressor molecules that contribute towards induction of suppressive responses by ManLAM via regulation of IL-12 and IL-12 receptor levels.

Many *M. tuberculosis* secretory antigens including culture filtrate protein (CFP)-10, early secreted antigenic target (ESAT)-6, MPT64, antigen 85B (Ag85B), whole cell lysate and total CFPs (when used collectively) induce the differentiation of mouse bone marrow leukocyte precursors into DCs (Latchumanan *et al.*, 2002). These secretory antigens
differentiated DCs show morphological and phenotypic similarities to DCs differentiated conventionally with granulocyte macrophage-colony stimulating factor (GM-CSF). However, functional characterization of these DCs shows that a challenge with mycobacterial components induce suppressor responses in an IL-10 and TGF-β dependent mechanism (Balkhi et al., 2004). These results indicate that secretion of some of these antigens at the sites of infection is a strategy employed by mycobacteria to subvert protective pro-inflammatory responses and induce suppressor responses at sites of infection. Some *M. tuberculosis* genes have been studied that were expressed inside infected macrophages at different times post-infection and characterized the immune responses mediated by these genes. The modulations in DC and macrophage functions by these genes as they are expressed during the course of infection were studied. Further, the ability of these genes to alter T cell priming and functions was also investigated. In parallel, the intra-cellular mechanisms employed by the antigens in the modulation of immune responses were also studied (Gupta et al., 2010).

Further, these strategies and mechanisms employed by the bacteria to subvert immune responses have been described extensively in the following section on description of the problem that follows the current chapter.

### 2.6 Role of Macrophages in tuberculosis infection

*M. tuberculosis* usually enters the alveolar passages of exposed humans in an aerosol droplet, where its first contact is thought to be with the resident macrophages. Macrophages are the primary habitat of *M. tuberculosis*; unlike other microbial pathogens that depend on the avoidance of phagocytosis to survive, *M. tuberculosis* preferentially targets the macrophage vacuoles. *M. tuberculosis* resides within macrophage and persists in a granuloma which consists of macrophages and giant cells, T cells, B cells and fibroblasts. Macrophages are the phagocytes at the front line of the host immune defense against microbial pathogens, which constitutes a potent antimicrobial component of both innate and cell mediated immunity. However, the precise mechanisms by which these
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cells mediate killing or inhibition of bacterial pathogens are not clearly understood. The effector functions by which macrophages mediate antimicrobial activity include phagolysosome fusion, generation of ROI by the oxidative burst, production of RNI via the NOS2-dependent cytotoxic pathway and mechanisms mediated by cytokines.

2.6.1 Phagolysosome fusion

Phagosomes formed by phagocytosis of large particulate materials including microbes, fuse with lysosomes containing potent hydrolytic enzymes capable of degrading a whole range of macromolecules including microbes at acidic pH of 4.5-5. Phagocytosed microorganisms are subject to degradation by intralysosomal acidic hydrolases upon phagolysosome fusion (Cohn, 1963). Hart et al., in 1972 first hypothesized that M. tuberculosis survived inside macrophages by preventing this phagolysosomal fusion, a phenomenon observed only with phagosomes containing live bacilli. These phagosomes do not undergo further acidification, due to the absence of proton-ATPase molecules from the vacuolar membrane, and this reduced level of acidification allows the intracellular survival and growth of mycobacteria (Sturgill-Koszycki et al., 1994). These observations have been supported by many subsequent studies that included identifying specific markers for phagosome and lysosome and observing their distribution in various endocytic compartments (Russell, 1995; Deretic and Fratti, 1999). It has been reported that mycobacterial sulfatides (Goren et al., 1976), derivatives of multi-acylated trehalose 2-sulfate (Goren et al., 1976a), a lysosomotropic polyanionic glycolipid (Middlebrook et al., 1959), have the ability to inhibit phagolysosomal fusion. In vitro studies have demonstrated that ammonia generated by M. tuberculosis inhibits phagolysosomal fusion by affecting salutatory movement of lysosomes and by alkalizing the intralysosomal compartment (Gordon 1980; Hart et al., 1983). However, the precise mechanism by which ammonia prevents phagolysosomal fusion is not yet known. Also, the GTPases of the Ras family, known to play a role in the interaction between various endocytic compartments (Desjardins et al., 1994; Pfeffer, 1992; Zerial et al., 1993), have been a target of investigation aimed at understanding the inability of mycobacterial phagosomes
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to mature to phagolysosomes (Via et al., 1997). Thus, mycobacterial phagosomes retain
Rab5, which directs the interaction between early endocytic compartments and
phagosomes (Desjardins et al., 1994; Desjardins et al., 1995), and exclude Rab7 (Press et
al., 1998), a GTPase that regulates late endosomal membrane trafficking. Presence of
TACO (tryptophan aspartate-containing coat) protein on the phagosomal membrane
containing live mycobacteria provides a direct explanation for the inability of
mycobacteria-containing phagosomes to fuse with lysosomes (Ferrari et al., 1999). The
50-kDa host cell protein specific for phagosomes containing live bacilli is recruited and is
retained on to the mycobacterial phagosomal membrane of BCG-infected macrophages
there but not to the phagosomes containing dead BCG bacteria and endosomal vesicles of
uninfected cells. Thus, viability of phagocytosed bacilli is the prerequisite to the retention
of TACO by which mycobacteria inhibit phagolysosomal fusion and evade potent
lysosomal antimicrobial functions of macrophages. Elucidation of the mechanisms by
which *M. tuberculosis* contains phagosomes and retains TACO will provide insight into
the pathogenesis of the tubercle bacillus.

2.7 Role of Cytokines against *M. tuberculosis*

2.7.1 Interferon-γ (IFN-γ)

IFN-γ is produced by both CD4 and CD8 T cells in tuberculosis (Lyadova et al., 1998;
Lalvani et al., 1998; Serbina et al., 1999), by *M. tuberculosis* infected alveolar
macrophages in IL-12 dependent manner (Wang et al., 1999; Fenton et al., 1997) and by
NK cells. The protective role of IFN-γ in tuberculosis is well established (Flynn et al.,
1993), primarily in the context of antigen-specific T-cell immunity. IFN-γ plays a key
role in control of *M. tuberculosis* infection. IFN-γ knockout (GKO) mice are the most
susceptible to virulent *M. tuberculosis* (Cooper et al., 1993; Flynn et al., 1993). IFN-γ
GKO mice, upon *M. tuberculosis* infection show uncontrolled bacillary growth, necrotic
granulomas, and defective macrophage activation with low NOS2 expression (Flynn et
al., 1993; Dalton et al., 1993). However, the mean survival time for *M. tuberculosis*-
infected NOS2-/- mice is at least twice that of GKO mice (MacMicking et al., 1997; Flynn et al., 1993), suggesting that there are IFN-γ-dependent, NOS2-independent mechanisms of protection against tuberculosis. Studies in humans have shown that individuals with mutations in genes for IFN-γ or the IFN-γ receptor are prone to serious mycobacterial infections, including *M. tuberculosis* (Ottenhof et al., 1998). Some studies have demonstrated that IFN-γ levels are depressed in the serum of patients with active tuberculosis (Lin et al., 1996; Zhang et al., 1995). Moreover, *M. tuberculosis* and its components can prevent macrophages from responding adequately to IFN-γ by inhibiting IFN-γ signaling in human macrophages by disrupting the association of the transcription activator STAT1 with the transcriptional coactivators, namely CREB-binding protein (CBP) and p300 (Ting et al., 1999).

### 2.7.2 Tumor Necrosis Factor-α (TNF-α)

TNF-α plays an important role in the host response against *Mycobacterium tuberculosis*. TNF-α is secreted by macrophages, dendritic cells and T cells upon activation with *M. tuberculosis*. TNF-α is an essential component of tuberculous granuloma and a potent mediator of macrophage activation. In case of mice deficient in TNF-α or the 55-kDa TNF receptor, *M. tuberculosis* infection resulted in a deficient granulomatous response followed by rapid death of mice with substantially higher number of bacilli in the lung compared to those observed in the control mice (Flynn et al., 1995; Fenhalls et al., 2000). TNF-α synergizes with IFN-γ to induce the iNOS expression and hence is critical for control of the acute *M. tuberculosis* infection (Flesch et al., 1990; Chan et al., 1992; Liew et al., 1990). Nitric oxide production in the granulomas of TNFRp55/-/- as well as TNF-α-/- mice was delayed in response to *M. tuberculosis* infection (Flynn et al., 1995; Bean et al., 1999). Mice inoculated with a low-dose of *M. tuberculosis* were found to maintain a stable bacterial load even six months later. However, neutralization of TNF-α with anti-TNF-α antibody resulted in increased bacillary counts in the lungs followed by 100% mortality of these animals (Flesch and Kaufmann, 1990). Anti-TNF-α antibody treatment of an arthritis patient also resulted in development of fatal disseminated tuberculosis.
(Maini et al., 1999). Intriguingly, TNF-α has also been shown to permit the multiplication of intracellular bacteria in human alveolar macrophages. Treatment of these *M. tuberculosis* infected macrophages with neutralizing anti-TNF antibody reduced the growth rate of intracellular bacteria, whereas bacterial replication was augmented by addition of exogenous TNF-α (Engele et al., 2002). Although presence of TNF-α is not an absolute requirement for necrosis of lung tissue, it is considered to be a major factor in the host-mediated destruction of lung tissues (Aung et al., 2000). In fact, recombinant BCG expressing very high levels of TNF-α has been reported to cause destructive inflammation (Bekker et al., 2000). At the same time, TNF-α is known to contribute to the inhibition of *M. tuberculosis* growth in macrophages by a mechanism that is dependent on apoptosis and independent of IFN-γ activity (Keane et al., 2002). Thus, TNF-α has an important role as a modulator of inflammation and has a complex and multifaceted role in protection and immunopathology in tuberculosis infection.

### 2.7.3 Interleukin-12 (IL-12)

IL-12, produced mainly by phagocytic cells, is a key player in the host defense against *M. tuberculosis*. IL-12 is important in controlling *M. tuberculosis* infection and is strongly induced after phagocytosis of *M. tuberculosis* bacilli by macrophages and dendritic cells (Ladel et al., 1997; Henderson, 1997). It helps in the development of TH1 response leading to production of IFN-γ. In tuberculosis, IL-12 has been detected in lung infiltrates (Casarini et al., 1999; Taha et al., 1997), in pleurisy (Zhang et al., 1994), in granulomas (Bergeron et al., 1997), and in lymphadenitis (Lin et al., 1996). The expression of IL-12 receptors is also increased at the site of disease (Zhang et al., 1999). IL-12 when administered during the early phase of *M. tuberculosis* infection in BALB/c mice, results in decreased bacterial numbers and increased mean survival time (Flynn et al., 1995a). Also, IL-12p40 gene deficient mice produce low amounts of IFN-γ and therefore, are observed to be more susceptible to *M. tuberculosis* infection with increased bacterial numbers and decreased survival time as compared to control mice, suggesting a protective role of IL-12 in *M. tuberculosis* infection (Cooper et al., 1997). In humans,
studies have shown that humans having mutations in IL-12p40 or the IL-12 receptor genes and are more susceptible to disseminated BCG and *M. avium* infections due to lower production of IFN-γ by T cells, although *M. tuberculosis* infections were not reported (Ottenhof *et al.*, 1998; Casanova, 2000). The IL-12 encoding plasmid DNA when administered to mice with a chronic *M. tuberculosis* infection, results in reduced bacterial numbers (Lowrie *et al.*, 1999), suggesting that IL-12 induction may play an important role in the design of a tuberculosis vaccine.

### 2.7.4 Interleukin-6 (IL-6)

IL-6 has an important implication in inflammation and T cell differentiation in response to *M. tuberculosis* (Saunders *et al.*, 2000). Suppressive role of IL-6 on T cell responses has been demonstrated in BCG-infected macrophages (vanHeyningen *et al.*, 1997). Spleen cells from IL-6-/- mice produced elevated levels of IL-4 and decreased levels of gamma interferon compared to the control mice (Ladel *et al.*, 1997a). However, upon development of acquired immunity, IL-6-/- mice successfully controlled the infection, retaining the memory response to subsequent aerosol challenge (Saunders *et al.*, 2000). However, if a high intravenous dose of *M. tuberculosis* inoculum was given, the IL-6-/- mice did succumb to infection compared to the IL-6-competent control mice, perhaps due to overwhelming of the defective innate response by introduction of large bacterial numbers (Ladel *et al.*, 1997a).

### 2.7.5 Interleukin-10 (IL-10)

IL-10 is an anti-inflammatory cytokine produced by macrophages after phagocytosis of *M. tuberculosis* (Shaw *et al.*, 2000) and T cells during *M. tuberculosis* infection, possesses macrophage-deactivating properties, including downregulation of IL-12 production, which in turn decreases IFN-γ production by T cells. IL-10 is known to inhibit CD4 T cell responses as well as antigen presentation by the cells infected with mycobacteria (Rojas *et al.*, 1999a, Rojas *et al.*, 1999). Although, IL-10-/- mice were not
more resistant to acute *M. tuberculosis* compared to wild type mice, transgenic mice constitutively expressing IL-10 were less capable of clearing a BCG infection (North, 1998; Murray et al., 1997). Also, transgenic mice over-expressing IL-10 showed no increase in the susceptibility to *M. tuberculosis* during the early stages of infection, but showed evidence of reactivation of tuberculosis during the chronic phase of the infection with a highly significant increase in bacterial numbers within the lungs associated with the formation of macrophage, dominated lesions, decreased mRNA production for TNF-α and IL-12p40, and a decrease in the antigen-specific IFN-γ secretion (Turner et al., 2002). Attenuation of in vitro T cell proliferation by macrophages from tuberculosis patients was partially reversed by IL-10 inhibition. Together, these data suggests that IL-10 plays a pivotal role during the chronic/latent stage of pulmonary tuberculosis.

### 2.7.6 Interleukin-1 (IL-1)

IL-1, along with TNF-α, plays an important role in the acute phase response such as fever and cachexia, prominent in TB. In addition, IL-1 facilitates T lymphocyte expression of IL-2 receptors and IL-2 release. The major antigens of mycobacteria triggering IL-1 release and TNF-α have been identified (Wallis et al., 1990). IL-1 has been implicated in immunosuppressive mechanisms, which is an important feature in tubercular immunity (Fujiwara et al., 1986).

### 2.7.7 Transforming Growth Factor-β (TGF-β)

This anti-inflammatory cytokine has been implicated in the suppression of T cell responses in tuberculosis patients (Hirsch et al., 1997). TGF-β is present in the granulomatous lesions of tuberculosis patients and is produced by human monocytes after stimulation with *M. tuberculosis* (Toossi et al., 1995) or lipoarabinomannan (Dahl et al., 1996). Reportedly it inhibits the T cell responses to *M. tuberculosis* (Hirsch et al., 1997; Rojas et al., 1999a) as well as participates in the macrophage deactivation by inhibiting the IFN-γ-induced NOS2 production (Ding et al., 1990). Regulation of this cytokine is
very complex and occurs at various levels. The in vivo role of TGF-β in protection or pathology in tuberculosis has not been directly tested.

2.8 Role of Reactive Oxygen intermediates (ROI):

Hydrogen peroxide (H₂O₂), one of the ROI generated by macrophages via the oxidative burst, was the first identified effector molecule that mediated mycobactericidal effects of mononuclear phagocytes (Walker et al., 1981). Activation of macrophages by cytokines leads to the production of reactive oxygen species (ROS), which is capable of killing intracellular mycobacteria (Nathan and Shiloh, 2000). However, the significance of toxic oxygen species in the control of tuberculosis remains controversial as many reports indicate that ROS are not crucial for controlling intracellular mycobacteria (Wayne and Sohaskey, 2001). The ability of ROI to kill *M. tuberculosis* has been demonstrated only in mice (Flesch and Kauffman, 1987) and remains to be confirmed in humans. Despite the demonstration that H₂O₂ generated by cytokine-activated macrophages was mycobacteriocidal (Walker et al., 1981), the ability of ROI to kill *M. tuberculosis* remains to be confirmed (Flesch et al., 1987; Chan et al., 1992). Indeed, mycobacteria are capable of evading the toxic effect of ROI by various means (Zahrt and Deretic, 2002). Mycobacterial components lipoarabinomannan (LAM) (Chan et al., 1991) and phenolic glycolipid I (PGL-I) are potent oxygen radical scavengers (Chan et al., 1989) and mycobacterial sulfatides interfere with the oxygen radical–dependent antimicrobial mechanism of macrophages. Despite these findings, a role of ROI in defense against the tubercle bacillus cannot be entirely excluded as reports have shown that mice deficient in the NADPH oxidase complex exhibit enhanced susceptibility to *M. tuberculosis* infection (Adams et al., 1997; Cooper et al., 2000).
2.9 Autophagy

Autophagy is an innate immune defence mechanism which forms a double membrane vesicle called autophagosome which delivers the engulfed material to lysosome for degradation (Figure 2.5) (Armstrong et al., 1975). In the context of *M. tuberculosis* infection, autophagy not only acts as an antibacterial mechanism for the clearance of intracellular pathogen, but also limiting the secretion of pro-inflammatory cytokines such as IL-1α, IL-1β, to avoid excessive inflammation. Comparing with autophagy-proficient littermates, *M. tuberculosis* infection of Atg5 deficiency mice exhibited increased bacillary burden and excessive pulmonary inflammation, which is characterized by neutrophil infiltration and IL-17 response with increased IL-1α levels. The increased IL-1α production by autophagy-deficient macrophages is independent of the inflammasome.

![Figure 2.5 Schematic representation of the ALP pathway.](image)

*The autophagosome creation and also the autophago-lysosome after the fusion with the lysosome are characteristically appeared. (Adapted from Alicia Meléndez, 2009)*

The increase of IL-1α in Atg5 deficient macrophages might be associated with elevated ROS, activation downstream of ROS, rather than the absolute levels of calpain. This might represent a novel proinflammatory pathway downstream of autophagy (Castillo et al., 2012). Increased IL-1β due to inflammasome activation enhances maturation of *M.*
*tuberculosis* phagosome which reduces intracellular bacterial burden in vivo. Additionally, the defence against *M. tuberculosis* requires a strong TH1 response with secretion of IFN-γ which has many effects including induction of autophagy (Tomioka *et al*., 2011; Shi *et al*., 2010). Autophagy induced by IFN-γ results into suppression of *M. tuberculosis* survival in human and murine macrophage cell lines (Shi *et al*., 2010). Autophagy is also modulated by ROS. Autophagy mediated killing of *M. tuberculosis* requires ROS, since autophagy induced by P2X7, TLR or vitamin D3 requires ROS (Songane *et al*., 2012).

Furthermore, autophagy might confer host protection during antibiotic therapy against *M. tuberculosis* infection through maintaining a balance between the innate antimicrobial and proinflammatory responses. Given the role of autophagy in the removal of intracellular *M. tuberculosis* in vitro, autophagy-based TB therapy is a tempting goal (Ni Cheallaigh *et al*., 2011). Currently, this strategy is largely pursued as the following. Firstly, old drug for novel use. Such options contain mTOR inhibitors, such as rapamycin (sirolimus) and everolimus, both are currently approved for clinical use to prevent transplant rejection (Ni Cheallaigh *et al*., 2011). Rapamycin is a classical inducer of autophagy by inhibiting Tor (Deretic *et al*., 2008). Tor is widely conserved from yeast to man. Unfortunately, the adverse effects of rapamycin, especially immunosuppression, preclude its use in TB treatment. Small molecule enhancers of rapamycin (SMERs) capable of boosting the growth inhibitory effects of rapamycin in the budding yeast Saccharomyces cerevisiae have been found via a novel two-step screening process. Moreover, comparing with either the SMERs or rapamycin treatment alone, these SMERs in combination with rapamycin led to higher removal rate of autophagy substrates (Floto *et al*., 2007), suggesting its potential in TB therapy.

### 2.10 The Th1/Th2 Paradigm in TB

Although, one third of the global population is infected with *M. tuberculosis* (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, lymphotixin (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. tuberculosis*-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. tuberculosis* (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. tuberculosis* is effectively controlled, produce a large amount of IL-4δ2, an endogenous antagonist of IL-4 generated by alternative splicing from the primary IL-4 transcript (Rook, 2007).

2.11 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. tuberculosis* (Wozniak et al., 2006). Differentiation of Th17 cells requires simultaneous presence of IL-6 and TGF-β (Bettelli et al., 2007). *M. tuberculosis*
infections in macrophages have been shown to produce large amounts of both of these cytokines (Toossi et al., 1995; VanHeyningen et al., 1997). Thus, it is expected that *M. tuberculosis* 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.

### 2.12 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. tuberculosis* (>90% DNA homology) and is a part of the *M. tuberculosis* 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 et al., 1999; Fine et al., 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. tuberculosis strains (Behr, 2002; Brandt et al., 2002; Comstock et al., 1994; Demangel et al., 2005; Fine et al., 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. tuberculosis infection (Andersen et al., 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 \textit{M. tuberculosis} (von Reyn and Vuola, 2002). Severe and life-threatening complications may occur, including severe disseminated disease in immuno-compromised 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.
2.13 RNAi screens

One of the most effective tools of functional genomics is the use of RNAi interference to understand gene functions. In the last few years a large number of genome scale RNAi screens have been performed to understand a plethora of biological processes in drosophila, animals, eukaryotes, humans, nematodes, viruses infection and mammalian cell based systems (Bakal et al., 2010). These processes include cell cycle, signal transduction pathways, host pathogen interactions, fat regulation and cancer biology among others (Bakal et al., 2010). Such screens have not only enabled a systems level understanding of cellular processes, they have also helped deepen the mechanistic insights by identification of novel components in these processes. RNA interference is essentially a loss-of–function technique, where the effect of gene knockdown can be assessed directly as a functional (biological) readout. It reveals the role of a gene in a specific biological context and depending on the experimental design it may be small scale or high throughput (Bakal et al., 2010). Though the use of RNAi at a small scale has also revealed a wealth of information, its most important impact has been the ability to perform high throughput RNAi based screens. Such formats allow the function of a large number of genes to be interrogated concurrently.

Several studies have demonstrated the utility of RNAi based screens using siRNA libraries to identify genes that play crucial roles in specific pathways leading to functional and phenotypic outcomes (Moffat et al., 2006) and in identifying potential drug targets (Ito et al., 2005). For example, siRNA libraries against the apoptotic pathway have been used to identify several cancer regulated genes (Ovcharenko et al., 2007). Further, siRNA libraries have been used in systematic and cost-effective genome-wide loss-of-function screens with the aim of assessing the role of specific genes in neoplastic phenotypes, and the rapid identification of novel drug targets (lorns et al., 200). The apoptotic pathway library has been used to identify genes active during apoptosis, proliferation and cell cycle (Alenzi, et al., 2004). MAPK pathway siRNA libraries have used to study cell cycle regulation (Su, et al., 1996). Similarly, the roles of
phosphatases that regulate NF-κB signaling have been studied (Li et al., 2006). Recently, genes regulating the level of ploidy in HeLa cells were profiled using genome-scale RNAi profiling and over 2000 genes were identified to regulate ploidy in cancerous cells (Kittler et al., 2007).