Chapter 2

Review of Literature
2.1. Tuberculosis

Tuberculosis is one of the world’s most devastating bacterial diseases, as a number one killer of humanity for several centuries (Revised, WHO Fact sheet, 2006). The earliest unambiguous detection of *Mycobacterium tuberculosis* was in the remains of bison dated 18,000 years ago (Rothschild *et al.*, 2001). The origin of *M. tuberculosis*, the causative agent of tuberculosis (TB), has been the subject of recent investigation, and it is thought that the bacteria in the genus *Mycobacterium*, like other actinomycetes, were initially found in soil and that some species evolved to live in mammals (Smith *et al.*, 2003). Skeletal remains show prehistoric humans (4000 BC) had TB, and tubercular decay has been found in the spines of mummies from 3000–2400 BC (Zink *et al.*, 2003). Phthisis is a Greek term for tuberculosis; around 460 BC, Hippocrates identified phthisis as the most widespread disease of the times involving coughing up blood and fever, which was almost always fatal (Hippocrates, 2006). Genetic studies suggest that TB was present in South America for about 2,000 years (Konomi *et al.*, 2002). In South America, the earliest evidence of tuberculosis is associated with the Paracas-Cavema culture (circa 750 BC to circa 100 AD) (prehistoric findings, 2003).

The history of TB starts with the announcement by Robert Koch that tuberculosis is caused by *M. tuberculosis*. Before that Hippocrates speculated that it is hereditary disease (4th century BC) while Aristotle stressed on its contagious nature. It was only Edward Trudeau who showed that TB could be induced in rabbits with a purified culture of virulent *M. tuberculosis*, and environmental conditions play very important role in the development and progression of disease (Angelichio *et al.*, 2002).

TB epidemic spread widely in Europe during the 16th and 17th century and peaked during 19th century (Smith *et al.*, 2003). About 32% of the world’s population or 1.86 billion people are infected with *M. tuberculosis*. Every year, approximately 9 million of these infected people develop active TB (WHO, 2006). TB is the longest running catastrophe, killing more than 200 people every hour and more than 5000 every day. However, by the mid-1950, there was a sharp decline in TB incidence due to the continued progress in understanding the immune responses to infection, an increasing armamentarium of effective anti-tuberculosis drugs as well as better health facilities and improved sanitation. The antibiotic era, started after the discovery of streptomycin by Schatz and Waksman in the late 1940s and its widespread use against TB, followed by the introduction of antibiotics like isoniazid, rifampin, and pyrazinamide has helped greatly in
reducing the morbidity and mortality due to TB (Smith et al., 2003). In addition, the widespread use of BCG, an attenuated vaccine strain produced by the sequential passage of a virulent *Mycobacterium bovis* strain by Calmette and Guerin, has lowered the incidence of TB in recent years. Nevertheless, these antibiotics are effective in treating individuals suffering from active tuberculosis, an infection state characterized by active growth of the tubercle bacilli in the host; these are by and large ineffective in eliminating *M. tuberculosis* during latent stages of infection. Regardless, insusceptibility to antibiotics, along with the failure of BCG vaccine, resurgence of TB due to HIV pandemic and emergence of multi drug resistance isolates has made it difficult to treat infected individuals and eliminate tuberculosis in humans. This has led WHO to declare tuberculosis a global emergency which indicates that the development of novel antituberculosis therapeutics and vaccine candidate equally effective to all forms (latent as well as active) of tuberculosis are the need of hour.

2.1.1. The *Mycobacterium* Complex

The causal organism of tuberculosis belongs to genus mycobacteria. It was first isolated by Robert Koch in 1882. It is an obligate aerobe but also shows anaerobic metabolism. *M. tuberculosis* is a non-motile, rod-shaped, slightly curved bacillus, usually 2-4 μm in length and 0.2-0.5 μm in width (Cox, 2004). The bacterium is a facultative intracellular parasite that can survive and multiply successfully inside macrophages and other mammalian cells. The bacterium has slow generation time of about 15-20 hours. *In vitro* grown culture shows that they are present in the form of chain and often form distinctive serpentine cords.

Phylogenetic studies using 16S rRNA by Rogall et al., 1990 showed that the genus has four groups viz; *M. tuberculosis* complex, non-tuberculous mycobacteria (NTM), *M. ulcerans* and *M. leprae*. Among these, *M. tuberculosis* belongs to a group of ‘slow growers’, also known as ‘*M. tuberculosis* complex’ requiring 3-4 weeks to form colonies, with generation time of typically ~24 hours on solid medium. This complex involves *Mycobacterium tuberculosis*, *Mycobacterium africanum*, *Mycobacterium microti*, *Mycobacterium bovis*, and *Mycobacterium bovis* BCG (Rogall et al., 1990). The other category of mycobacterium called as ‘fast growers’ with generation time of 3-4 hours. There are 17 species of rapidly growing mycobacteria comprising two pathogenic organisms *Mycobacterium fortuitum* and *Mycobacterium chelonae*. Despite 99.9%
similarity with ‘slow growers’ at genetic level, these organisms have adapted to different host and possess varying degree of pathogenicity and virulence to their host. Some of the representative species from slow and fast growers are:

*Mycobacterium tuberculosis* is the most common etiological agent causing tuberculosis in humans. Humans are the only reservoir for this bacterium.

*Mycobacterium bovis* has the widest range of hosts. In addition to its primary host cattle, it may affect several domestic and wild animals including dears, lions, seals and laboratory animals like rabbits. It can infect humans but with pasteurization of mild and other safe practices, its effect is limited.

*Mycobacterium bovis BCG* is a laboratory attenuated strain of *M. bovis* generated by several passages of *M. bovis* in a potato medium by Calmette and Guérin at the Pasteur Institute, Lille in 1882. Presently it is the only vaccine available for tuberculosis (Thoen *et al.*, 2006; Kumar *et al.*, 2007). It is used commonly as representative of *M. tuberculosis* H37Rv as slow growers in several research studies.

*Mycobacterium africanum* was originally isolated from man in equatorial Africa. It resembles *M. bovis* in being microaerophilic and susceptible to TCH but resembles *M. tuberculosis* in being susceptible to pyrazinamide.

*Mycobacterium microti* is a rarely encountered pathogen of voles and other small mammals like rodents. It was first isolated in UK in 1930. It is also found in immunocompromised patients (Niemann *et al.*, 2000).

*Mycobacterium fortuitum* is also called as ‘cold blooded tubercle bacilli’ as it was isolated from frog. It causes injection abscesses and, rarely, keratitis, pulmonary and disseminated disease.

*Mycobacterium chelonae* is also ‘cold blooded tubercle bacilli’ isolated from turtle. It causes injection abscesses, wound infections, pulmonary lesions, disseminated disease and peritonitis in patients with renal failure treated by chronic peritoneal dialysis.
2.1.2. Unique features of *M. tuberculosis*

The unique feature that enables *M. tuberculosis* to infect and persists within the tissues of its host is its cell wall. All the members of *M. tuberculosis* complex include a cell wall of unique composition, with a complex outer cell wall consisting of large amount of cell wall lipid. Because of its unique cell wall it is neither classified as Gram-positive nor Gram-negative, although the bacteria contain peptidoglycan (murein) in their cell wall. They use N-glycolylmuramic acid in place of N-acetyl muramic acid in their peptidoglycan (cell wall) (Beman *et al.*, 1990; Bersa and Chatterjee, 1994). The bacilli stain very weak with gram positive stains, are classified as acid-fast bacteria due to their impermeability of certain dyes and stains. Despite this, once stained, acid-fast bacteria will retain dyes when heated and treated with acidified organic compounds like the Ziehl-Neelson stain (Madison *et al.*, 2001).

The cell wall of mycobacteria is unique among prokaryotes and plays a major role in determining its virulence. It is the most complex cell wall in nature and its major distinguishing characteristic is the presence of very high lipid content, over 60% of the mycobacterial cell wall is lipid. The cell wall contains three distinct layers: inner and outer electron-dense layers separated by an electron transparent layer (Paul and Beveridge *et al.*, 1992). The cell wall is rich in waxes and lipids, especially mycolic acids which are covalently linked to arabinogalactans. The cell wall complex contains peptidoglycan and has a complex glycolipid structure forming the outermost layer and complex lipids. The lipid fraction of cell wall consists of three major components mycolic acid, cord factor and wax.

*Mycolic acids* are unique alpha-branched lipids found in cell walls of *Mycobacterium*. They make up 50% of the dry weight of the mycobacterial cell envelope. Mycolic acids are strong hydrophobic molecules that form a lipid shell around the organism and affect permeability properties at the cell surface. They are covalently linked to arabinogalactan which are in turn linked to peptidoglycan. Mycolic acids are a significant determinant of virulence in *M. tuberculosis*.

*Cord Factor* or Trehalose 6, 6 dimycolate is responsible for the serpentine cording in the virulent strains. Cord factor is toxic to mammalian cells and inhibitor of PMN migration,
is most abundantly produced in virulent strains of mycobacteria. It causes granuloma formation (Paul and Beveridge et al., 1992).

**Wax-D** in the cell envelope is the major component of Freund's complete adjuvant (CFA). Other important components also include lipoarabinomannan, sulfatides.

### 2.2. Pathogenesis of *M. tuberculosis*

Pathogenesis of tuberculosis is complex and diverse. The disease has many manifestations affecting central nervous system, the lymphatic system, the circulatory system, the genitourinary system, bones, and joints, but it is primarily regarded as pulmonary disease affecting lungs (WHO report, 2006). Although the disease progression depends on number of factors like prior exposure, vaccination, infectious dose and immune status of the host, the pathogenesis of TB can be summarized into different stages as under (Figure. 2.1).

**Stage1. Initial uptake of Bacteria**

Primary infection starts with the inhalation of infectious particles present in the atmosphere produced by TB patients during coughing and sneezing. When a healthy person inhales, the smaller particles which escape the first line of respiratory defense enter into alveolar passages where they are phagocytosed by resident alveolar macrophages, alveolar epithelial type II pneumocytes and dendritic cells present in the lungs. This process is initiated by bacterial contact with macrophage mannose and/or complement receptors (Schlesinger, 1993), or a macrophage cell entry protein expressed by virulent mycobacteria (Riley, 1995). The resulting "infectious focus" made up of inflammatory cells, is referred to as a primary focus. The infected macrophages produce cytokines and chemokines that attract other phagocytic cells, including monocytes, other alveolar macrophages, and neutrophils, which eventually form a nodular granulomatous structure called the 'tubercle'. The bacilli and the antigens which are liberated from tubercle are drained by the macrophages through the lymphatic system to the nearest lymph node. Inside the lymph node, the T-lymphocytes identify the *M. tuberculosis* antigens and are transformed into specific T-lymphocytes, leading to liberation of lymphokines and activation of macrophages that inhibit the growth of the phagocytosed bacilli. At this stage calcification of granuloma occurs, and these calcified granuloma and draining lymphnodes
which are remnants of primary infections are called ‘Ghon Complexes’ (Ghon, 1923; Houben et al., 2006).

The infected individual, at this stage develop humoral and cell mediated immunity (delayed type hypersensitivity). Delayed type hypersensitivity is demonstrated by tuberculin testing. The individual at this stage is symptomatic and remain unnoticed. All of these clinical and immunological phenomena in infected host constitute Primary tuberculosis infection.

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**Figure 2.1 Pathogenesis of tuberculosis (Adapted from Stewart et al., 2003).**

Stage 2. Secondary tuberculosis infection

This stage is marked by hematogenous circulation of bacteria to many organs including other parts of the lungs. This hematogenous spread results in the extrapulmonary tuberculosis, also called as ‘milliary tuberculosis’. The secondary lesions caused by milliary TB can occur at almost any anatomical location, but usually involve the genitourinary system, bones, joints, lymph nodes, and peritoneum (Agarwal et al., 2005; Herrmann et al., 2005). However, a number of bacilli may remain latent in this secondary foci for months or even years but can be reactivated depending upon immune state of the host. This reactivation is the cause of clinical disease at extra pulmonary sites and of a proportion of cases of pulmonary tuberculosis due to endogenous reactivation.
Stage 3. Dormancy

For most individuals living in endemic areas, infection with \textit{M. tuberculosis} progresses to the T-cell activation stage (conversion to skin test positivity), but in only a few individuals does the primary infection manifests as clinical disease. Following exposure to \textit{M. tuberculosis}, 30\% of individuals become infected, with only \(~10\%\) of these developing active tuberculosis, and 90\% developing latent infection. 2-23\% of the patients with latent TB reactivate into the typical adult form of pulmonary TB at some later stage in life (Parrish \textit{et al.}, 1998). Progression to clinical disease is dramatically increased to a 5-10 \% annual risk in HIV-infected individuals (WHO, 2006).

The course of infection during this stage is primarily dictated by the host’s immune response and it develops in only immunocompetent host. In such infected host, the immune system typically resolve the initial infection, or alternatively, hold the infection in check using the mechanism that prevent further bacillary multiplication, limit the dissemination of the organism, and concentrate the immune response directly to the site of infection. Such individuals continue to be persistently infected with \textit{M. tuberculosis} and remain latent carriers of the organism, they do not exhibit overt disease symptoms and are not contagious, thus, carrying a persistent or latent infection. However, latently infected individuals test positive for a delayed-type hypersensitivity response. Lesions containing latent bacilli represent an epidemiologically important reservoir for persistence of the organism in the human population. It is believed that upon liquefaction of the tubercle this ‘latent’ infection can reactivate and cause a secondary infection with clinical manifestations of tuberculosis even after decades (Grosset, 1980).

2.3. Immune response to \textit{M. tuberculosis}

The immune response to \textit{M. tuberculosis} is complex and multifaceted. Many components of the immune response are necessary or important in the protective response. These include CD4 and CD8 T cells, cytokines such as IFN-\(\gamma\) and TNF-\(\alpha\), and macrophage activation. Activated macrophages are the most effective killer of \textit{M. tuberculosis}, by aggregation of many macrophages and forming granuloma. Apart from individual macrophages, the very hostile anoxic and acidic environment in the centre of the lesion is also very important in overcoming infection (Figure. 2.2).
2.3.1. Macrophages

The macrophage is key to the control of *M. tuberculosis* infection. Macrophage-*Mycobacterium* interactions and the role of macrophage in host response can be summarized under the following headings: surface binding of *M. tuberculosis* to macrophages; phagosome-lysosome fusion; mycobacterial growth inhibition/killing; recruitment of accessory immune cells for local inflammatory response and presentation of antigens to T cells for development of acquired immunity. Phagocytosis of mycobacteria is mediated by interaction between mycobacterial surface glycoprotein lipoarabinomannan (LAM) (Schlesinger *et al.*, 1994) and Complement receptors (CR1, CR2, CR3 and CR4), mannose receptors (MR) and other cell surface receptor molecules on macrophages. The organism can multiply within resting macrophages, but can be inhibited or killed when the macrophage is activated (Suter, 1952; Mackaness, 1969). IFN-γ is the key activating agent that triggers these antimycobacterial effects (Rook *et al.*, 1986; Flesch *et al.*, 1987). Tumor necrosis factor alpha (TNF-α), although ineffective alone, synergizes with IFN-γ to induce antimycobacterial effects in macrophages (Flesch and Kaufmann, 1990). The major effector mechanism by which IFN-γ and TNF-α show its antimycobacterial activity is the induction of the production of nitric oxide and related reactive nitrogen intermediates (RNIs) by macrophages via the action of the inducible form of nitric oxide synthase (NOS2) (Ding *et al.*, 1988). Mice deficient in NOS2 activity are very susceptible to acute or chronic *M. tuberculosis* infection, compared to wild-type mice (Chan *et al.*, 1992; MacMicking *et al.*, 1997; Flynn *et al.*, 1998; Scanga., 2001). Another antimycobacterial mechanism of macrophages is phagolysosome fusion. Lysosomes, in the late endocytic pathway, contain numerous hydrolytic enzymes and are a very acidic organelle. Fusion of the lysosome with phagosome-containing ingested bacteria is a primary mechanism by which macrophages control infections. Phagolysosome fusion is increased when the macrophage is activated with IFN-γ or other cytokines (McKinney *et al.*, 2000). *M. tuberculosis* is initially within a phagosome, and has been shown to inhibit phagolysosome fusion and acidification in "non-activated" macrophages, and thereby avoid being killed by the macrophage (Armstrong, 1971; Hart *et al.*, 1972; Sturgill-Koszycki *et al.*, 1994). The mechanism by which this occurs is still unclear, although a number of possible molecules are known involved in this evasion response, including mycobacterial sulfatides, glutamine synthetase, and modulation of the phagosomal membrane.
2.3.2. Cellular response

*M. tuberculosis* is an intracellular pathogen and the cell mediated immune response primarily contributes to the control of infection and protection against tuberculosis. This response involves mainly T lymphocytes activating microbicidal functions of macrophages through the release of IFN-γ (Flesch and Kaufmann, 1990). Priming of native T lymphocytes against mycobacterial antigens occur in the proximal draining lymph nodes by Dendritic Cells (DCs). DCs do this, by capturing antigens and draining to lymph nodes, where they express high amounts of presentation molecules, such as MHC-I or II, as well as co-stimulatory molecules, such as CD80 and CD86 (Mellman et al., 2001), and soluble factors, such as IL-12, IL-18 or IL-23 (Tailleux et al., 2003). Toll-Like Receptor-2 (TLR-2) play an important role, in T cell activation as it is able to mature DC myeloid precursors into competent antigen-presenting cells expressing CD1 proteins (a, b, c). Mycobacteria were shown to provide two signals for the activation of lipid reactive T cells: lipid antigens that activate T cell receptors, and lipid adjuvant that activates antigen-presenting cells (APCs) through TLR-2 (Roura-Mir et al., 2005). Once primed, memory CD4 and CD8 T cells become central components of the acquired immune system. CD4 and CD8 T cells kill pathogens by attracting infected cells and secreting cytolytic and antimicrobial effector molecules like CCL5 etc. which efficiently attract *M. tuberculosis* infected macrophages. *In vitro*, infected macrophages trigger the expression by CD8 T cells of granulysin and perforin, two compounds which are highly active against drug sensitive and drug-resistant *M. tuberculosis* clinical isolates (Stegelmann et al., 2005). Besides, Natural killer cells (NK cells) are also bactericidal against mycobacteria. It is reported, that IFN-γ and monokines, such as IL-15 and IL-18, play a crucial role in the regulation of CD8 T cells against *M. tuberculosis* infection by NK cells. NK cells improve also the function of γδ T cells, another type of lymphocytes which play a role in the immune response against *M. tuberculosis* (Zhang et al., 2006).

2.3.3. Humoral Response

Humoral response against tuberculosis is not very effective. Although antibodies against *M. tuberculosis* may not allow the transfer of immunity against tuberculosis, they seem to have an opsonising role and thereby improve phagocytosis by macrophages or the cytotoxic actions of killer lymphocytes. BCG-induced antibodies were shown to significantly enhance the cell-mediated immune response with an increased proliferation
and IFN-γ production in mycobacterium specific CD4 and CD8 T cells. Mycobacterium specific antibodies seem capable of enhancing both innate and cell-mediated immune responses to mycobacteria (De Valliere et al., 2005). It also enhances the inhibition effects of neutrophils and monocytes/macrophages on mycobacterial growth.

Figure 2.2 Main features of Immune Response to *M. tuberculosis* (Adapted from Ulrichs and Kaufmann, 2006).

2.3.4. Cyokines and bactericidal products

The last important components of immune response against mycobacteria are cytokines chemokines and effector molecules. They play important role in hosts–bacterial interactions. This cytokine network regulates the inflammatory response and the outcome of mycobacterial infections. The pro-inflammatory response induced by *M. tuberculosis* is antagonized by anti-inflammatory mechanisms. Soluble cytokine receptors (e.g. soluble TNF-α receptors I and II) prevent binding of cytokines to cellular receptors, thereby blocking further signaling. In addition, three anti-inflammatory cytokines, IL-4, IL-10, and transforming growth factor beta (TGF-β), may inhibit the production or the effects of pro-inflammatory cytokines during tuberculosis infection. Role of several pro-inflammatory cytokines TNF-α, IFN-γ, IL-1β, IL-6, IL-12, IL-18 and IL-15 involved in host–*M. tuberculosis* interactions is discussed briefly.
2.3.5. Proinflammatory cytokines

Phagocytosis and recognition of *M. tuberculosis* leads to cell activation and production of cytokines which play a crucial role in the inflammatory response and the outcome of mycobacterial infections. The most important proinflammatory cytokines is TNF-α. Stimulation of monocytes, macrophages, and dendritic cells with mycobacteria or mycobacterial products induces the production of TNF-α at the site of disease (Barnes *et al.*, 1992; Law *et al.*, 1996; Henderson *et al.*, 1997; Serbina and Flynn, 1999).

**Tumor necrosis factor-α (TNF-α)** in synergy with IFN-γ induces NOS2 expression (Liew *et al.*, 1990). During chronic infection, NOS2 expression in the lungs was reduced following TNF-α neutralization (Mohan *et al.*, 2001) leading to reactivation of the disease. However, the multiple mechanisms by which TNF-α promotes effective granuloma formation and maintenance remain to be determined.

**Interferon-γ (IFN-γ)** is the second proinflammatory cytokine involved in the host response to mycobacteria. It plays an important role in protection in the context of antigen-specific T-cell immunity and macrophage activation (Andersen, 1997). This cytokine is produced by both CD4+ and CD8+ T cells during tuberculosis infection (Lalvani *et al.*, 1998; Serbina *et al.*, 1999). Individuals defective in genes for IFN-γ or the IFN-γ receptor are highly susceptible to *M. tuberculosis* and other mycobacterial infections (Ottenhoff *et al.*, 1998). The susceptibility to *M. tuberculosis* increases in IFN-γ knockout (GKO) mice (Cooper *et al.*, 1993) because of defective macrophage activation and low expression of NOS2 (Dalton *et al.*, 1993).

**Interleukin-1β** is another pro-inflammatory cytokine produced mainly by monocytes, macrophages and dendritic cells (Law *et al.*, 1996). This cytokine is involved in acute phase response to *M. tuberculosis* such as fever, cachexia, prominent in tuberculosis. IL-1α and IL-1β double-KO mice (Yamada *et al.*, 2000) display an increased mycobacterial outgrowth and also defective granuloma formation after infection with *M. tuberculosis* (Juffermans *et al.*, 2000).

**Interleukin-2** has a pivotal role in generating an immune response by inducing an expansion of the pool of lymphocytes specific for an antigen and also, it can influence the
course of mycobacterial infections, either alone or in combination with other cytokines (Blanchard et al., 1989).

**Interleukin-6** has both pro- and anti-inflammatory properties (Van Heyningen et al., 1997) and play multiples role in the immune response, including inflammation, hematopoiesis, and differentiation of T-cells. IL-6 deficient show increase susceptibility to infection with *M. tuberculosis* (Ladel et al., 1997), which seems related to a deficient production of IFN-γ early in the infection, before the development of T-cell immunity (Saunders et al., 2000).

**Interleukin-12**, produced mainly by phagocytic cells plays crucial role in defense against *M. tuberculosis* (Fulton et al., 1996). It is responsible for the induction of IFN-γ production (O’Neill and Greene, 1998). It is a regulatory cytokine which act mainly by inducing the secretion of IFN-γ and connects innate and adaptive host response to mycobacteria (Sieling et al., 1994; Trinchieri, 1995). The exogenous administration of IL-12 to Balb/C mice can improve survival (Flynn et al., 1995). It has been shown that administration of IL-12 DNA could substantially reduce bacterial numbers in mice with chronic *M. tuberculosis* infection (Lowrie et al., 1999), suggesting that the induction of this cytokine is an important factor in the design of a tuberculosis vaccine.

### 2.3.6. Anti-inflammatory cytokines

The proinflammatory response initiated after phagocytosis is antagonized by anti-inflammatory cytokines. IL-4, IL-10, and TGF-β are three key anti-inflammatory cytokine minimizes the effect of proinflammatory cytokines in tuberculosis.

**Interleukin-10** is the most important anti-inflammatory cytokines. It exerts effect mainly by down regulating the production of IFN-γ, TNF-α, and IL-12 (Fulton et al., 1998; Hirsch et al., 1999). It directly inhibits CD4+ T-cell response, as well as by inhibiting APC functions of cells infected with *M. tuberculosis*. Phagocytosis of *M. tuberculosis* (Shaw et al., 2000) and binding of mycobacterial LAM triggers the production of IL-10 by macrophages (Dahl et al., 1996). Besides, it is also produced by T-lymphocytes and *M. tuberculosis* reactive T-cells. In infected individual, ubiquitous presence of IL-10 mRNA has been demonstrated in circulating mononuclear cells, at the site of disease in pleural fluid, and in alveolar lavage fluid (Gerosa et al., 1999). IL-10
transgenic mice with mycobacterial infection develop a larger bacterial burden (Murray et al., 1997, Murray and young, 1999).

Transforming growth factor-beta (TGF-β) TGF-β is present in the granulomatous lesions of TB patients and is produced by human monocytes after stimulation with M. tuberculosis (Toossi et al., 1995) or lipoarabinomannan (Dahl et al., 1996). TGF-β has important anti-inflammatory effects, including deactivation of macrophage production of ROI and RNI (Ding et al., 1990), inhibition of T cell proliferation (Rojas et al., 1999), interference with NK and CTL function and downregulation of IFN-γ, TNF-α and IL-1 release (Ruscetti et al., 1993; Toossi et al., 1995) have shown that when TGF-β is added to co-cultures of mononuclear phagocytes and M. tuberculosis, both phagocytosis and growth inhibition were inhibited in a dose dependent manner. Part of the ability of macrophages to inhibit mycobacterial growth may depend on the relative influence of IFN-γ and TGF-β in any given focus of infection.

2.3.7. Chemokines

Chemokines (Chemotactic cytokines) are responsible for recruitment of inflammatory cells to the site of infection. Till now 40 chemokines and 16 chemokine receptors are identified (Zlotnik and Yoshie, 2000). IL-8 is the principal chemokine, it attracts neutrophils, T lymphocytes, and possibly monocytes. Upon phagocytosis of M. tuberculosis or stimulation with LAM, macrophages produce IL-8 (Juffermans et al., 1999; Zhang et al., 1995). It can also be expressed by fibroblasts, keratinocytes, and lymphocytes. This production is substantially blocked by neutralization of TNF-α and IL-1β, indicating that IL-8 production is largely under the control of these cytokines (Zhang et al., 1995). Other chemokines include monocyte chemoattractant protein-1 (MCP-1), and secreted RANTES. Chemokine and chemokine receptor expression contribute to the formation and maintenance of granuloma in chronic infections such as TB. In in vitro and in vivo murine models, M. tuberculosis induced production of a variety of chemokines, including RANTES, macrophage inflammatory protein MIP-1a, MIP-2, MCP-1, MCP-3, MCP-5 and IP10 (Rhoades et al., 1995). Mice over expressing MCP-1 (Rutledge et al., 1995), but not MCP-/- (Lu et al., 1998), were more susceptible to M. tuberculosis infection than were wild type mice. RANTES, MCP-1, MIP1-a and IL-8 were released by human alveolar macrophages upon infection with M. tuberculosis in vitro (Lane et al.,
and monocytes, lymph node cells and BAL fluid from pulmonary TB patients had increased levels of a subset of these chemokines compared to healthy controls (Kurashima et al., 1997; Lane et al., 1999). In human studies, CCR5, the receptor for RANTES, MIP-a and MIP-b, was increased on macrophages following in vitro M. tuberculosis infection and on alveolar macrophages in BAL from TB patients (Fraziano et al., 1999).

2.4. Models for Measuring Mycobacterial Pathogenesis

Mycobacterial virulence and pathogenesis are studied both by, tissue culture, using macrophages, dendritic cells and pneumocytes and in animal models (Bermudez and Goodman, 1996; Birkness et al., 1999, Bodnar et al., 2001; Hickman et al., 2002; Jiao et al., 2002). Although tissue culture models are easier and give faster results, they can be used to study only early stages of infection. Therefore animal models are preferred because all stages of infection can be screened. The choice of animal models for virulence studies is important, and the three major models, mice, guinea pigs, and rabbits, each have their advantages and disadvantages.

Mice: Mice are the most commonly and extensively used in vivo model because of their relatively easier handling and low cost of maintenance. Well studied genetics, existence of inbred strains, and even strains with mutations in some essential immune function genes further increase their utility as in vivo models. The vast knowledge of immunology of mice and the availability of reagents for quantitative and qualitative measurement of different aspects (such as cytokine levels) of immune system makes them the model of choice for immunological studies (Orme, 1988; Orme, 1994; Orme and McMurray, 1996). However, the progression of TB in mice is different from human in that the granuloma formed are not as distinct, but their less sensitivity to the disease as other animal model and ability to develop chronic infection like human is the biggest advantage of mice as in vivo model.

Guinea pig: Guinea pigs are much more sensitive to M. tuberculosis than mice. All the stages of the disease, including early stages of granuloma formation are similar to humans in this animal which increases it’s suitability as animal model. The disadvantages of using guinea pigs are the lack of inbred strains and reagents, as well as high maintenance cost (Orme and McMurray, 1996).
Rabbit: Rabbit as a model for tuberculosis, was used extensively by Max. B. Lurie (Lurie, 1932). The major advantage over other animal models in that the granuloma formation and stages of disease progression like caseation, liquefaction, and cavitation is very much similar to human TB. This model is used extensively for the study of initial stages of infection and mechanism of immunity (Orme and McMurray, 1996; Converse et al., 1996). The disadvantages of rabbits are similar to those of guinea pigs, but their upkeep is even more expensive.

Non-human primate model: NHPs are also used in TB research for decades. Cynomolgus macaques (Macaca fascicularis) are the most commonly used non-human primate model. This model is primarily used to study latent infection. It is an ideal model for studying granuloma formation and functions as its pathology is almost identical to humans. The histopathologic characteristics of NHP granulomas are extremely similar to human granulomas (Capuano, 2003). However, the cost of maintenance, limited resource, and genetic variability between each monkey limits its use as model.

Macrophages: Macrophages are the most ideal model to analyze mycobacterial virulence. They serve as good model for the early stages of infection and initial responses which occur during the phagocytosis of M. tuberculosis by residual macrophages in the lung alveoli. Model macrophages from mice and humans are frequently used, which are either primary culture or immortalized cell lines. Mice macrophages, isolated from bone marrow, lung alveoli, or peritoneal exudates are primary macrophages (not immortalized) and are representatives of the actual in vivo situation, but they are difficult to obtain and show greater variability. Mouse macrophage cell lines, including the widely used J774 line and MH-S cells (Melo and Stokes, 2000) are also available and are used to observe the M. tuberculosis killing activity of the cells.

Human macrophages like THP-1 and U937 are also widely used (Tsuchiya et al., 1982; Wei et al., 2000). Besides, primary cultures derived from peripheral blood monocytes (MDM), alveolar macrophages from branchiolar lavages are also used. Studies have shown that differentiated THP-1 macrophages are quite similar to human MDM in their response to M. tuberculosis infection. In addition, many reagents are available to study human host responses to M. tuberculosis infection (Stokes et al., 1999).
However, there are certain caveats in using macrophage as model for virulence study. Some *M. tuberculosis* mutants do not exhibit an attenuated phenotype in macrophages yet are defective for growth in mice and/or cause fewer histopathology changes (Stewart *et al.*, 2001; Dubnau *et al.*, 2002). Another problem is that macrophages isolated from different organs of the same animal may respond differently to *M. tuberculosis* infection.

### 2.5. Mycobacterial persistence

*M. tuberculosis* has unique ability to show persistent infection. The ability to cause persistent infection is a fundamental aspect of the interaction between many diverse viral, bacterial and eukaryotic pathogens and their mammalian hosts. Persistence may be defined as a group of organisms that, after causing an initial disease state, are kept in check by an adaptive immune response, but are not completely cleared from the host and persist in a privileged niche, perhaps inside host cell, for long periods of time. This pathogenic mycobacterium causes several long-term infections in their respective hosts.

Three terms, latency, persistence, and dormancy are frequently used in literature describing *M. tuberculosis* and pathogenesis. Latency was defined by Amberson, (1938) as “the presence of any tuberculosis lesion which fails to produce symptoms of its presence”. It’s a state in which person has proven *M. tuberculosis* infection (either by skin test or by immunological test) without clinical signs or symptoms of tuberculosis. This represents a state in which the host immune response is able to contain the infection. At the most fundamental level, latent tuberculosis can be viewed as equilibrium between host and bacillus. Latent TB occurs after an individual has been exposed to *M. tuberculosis*, infection has been established, and immune response has been generated to control the pathogen and force it into a quiescent state. Latency is achieved either by early restriction of *M. tuberculosis* growth in the lungs prior to the onset of disease, or the spontaneous resolution of primary TB. Most people exposed to *M. tuberculosis* mount a vigorous cell-mediated immune response that arrests the progress of the infection, largely limiting it to the initial site of invasion in the lung parenchyma and the local draining lymph nodes (called Ghon complex) (Ghon, 1923). The complete elimination of the pathogen, however, is slow and difficult to achieve. Without antibiotic treatment, chronic or latent infection is thought to be the typical outcome of TB infection. Latent TB can reactivate after years or
even decades of sub clinical persistence, leading to progressive disease and active transmission of the pathogen.

Dormancy has been used to describe both TB disease as well as the metabolic state of the tubercle bacillus (Wayne, 1994; Gangadharam, 1995; Cunningham & Spreadbury, 1998). In terms of bacterial physiology, dormancy is defined as “a reversible state of low metabolic activity, in which cells can persist for extended period without cell division”. TB lesions are described as active or dormant, based on whether the associated pathology is progressing or healing, respectively. Active lesions generally contain easily detectable population of acid-fast, culturable *M. tuberculosis*, but the precise bacteriological status of dormant lesions remains unclear despite nearly a century of study and debate. The term dormancy has also become strongly associated with an *in vitro* model of *M. tuberculosis* growth under limiting oxygen tension, developed by Wayne and Hayes (1996). It has been suggested that this model may approximate the state of *M. tuberculosis* surviving in closed, necrotic lesions during clinical latency. It should be emphasized that the model remains speculative, since the location and physiologic state of *M. tuberculosis* during latency have not yet been firmly established. It is suggested that an altered physiological state of persistent *M. tuberculosis* accounts for its tolerance to drugs as well as the ability to survive in the host for many years.

The word persistence literally means “continuing steadfastly or obstinately, especially in the face of opposition or adversity”. Persistence is likely to be the combined effect of both the immune system and bacterial physiology (Bloom and Mckinney, 1999). As a pathogen, *M. tuberculosis* manifests its unusual capacity to persist in many ways. On the cellular level, mycobacterium resides within macrophages, cells that typically function to eliminate pathogens and other foreign materials from the body. At a more systemic level, *M. tuberculosis* is able to avoid elimination from the human host despite the development of vigorous cell-mediated immunity. Another less obvious but profoundly important manifestation of *M. tuberculosis* persistence is the slow rate at which the bacterium is cleared by anti-TB drugs.

### 2.5.1. Characteristic features of persistent infection

A number of hypotheses have been proposed to explain the physiological status and characters of persistent bacteria. Prior to the antibiotic era TB was considered life long infections: “Once tuberculosis always tuberculosis”. It was only the autopsy and surgical
specimen investigation conducted in the early part of 20\textsuperscript{th} century have shown that tissues from humans who are asymptomatic but who show evidence of latent TB harbors infectious \textit{M. tuberculosis} which was demonstrated by the passage of the disease to animals (Feldman and Baggenstoss, 1939; Robertson, 1993). Besides, the presence of IS6110 DNA, an insertion element found in multiple copies in the \textit{M. tuberculosis}, was also demonstrated in normal lung tissue of diseased tissue patients. They found bacterial DNA in both old granulomatous lesions and non granulomatous tissues, which indicate the presence of persistent mycobacteria and their ability to survive in calcified lesions as well as normal human lung.

Although the exact location of viable latent mycobacteria during persistent infection is unknown, bacteria are often found inside macrophages within granulomas, which are formed in response to persistent intracellular mycobacteria (Adams, 1976). Granuloma formation is the sole mechanism utilized by the host to prevent bacillary growth and subsequent dissemination to additional infection sites. Tuberculous granuloma in humans and mice contain an organized collection of differentiated macrophages, T lymphocytes, some B lymphocytes, dendritic cells, neutrophils, fibroblasts and extracellular matrix components (Flynn and Chan, 2001; Peters and Ernst, 2003). Granulomas arise initially from aggregates of mononuclear phagocytes that surround individual infected macrophages. These macrophages get activated and in some cases fuse to form giant cells, T lymphocytes and other immune cells are recruited early during granuloma formation (Roach \textit{et al.}, 1999). The lesion formed is sealed off from surrounding tissue by epitheloid cells, which have tightly interdigitated cell membranes that form zipper like arrays, and adjacent cells, and which can also be fibrotic and calcified. In the centre of granulomas there is usually an area of caseous necrosis – a region of cellular debris that has a distinct appearance (Figure 3.3). Within the granuloma, much of the tissue is calcified or necrotic, making the mycobacteria to adapt to a highly versatile growth environment. Granulomas contain low levels of oxygen, high concentration of carbon dioxide concentrations, increased levels of aliphatic organic acids, hydrolytic enzymes, and numerous antimicrobial compounds.

Different approaches have been used to explain the metabolic state of persistent bacteria. Chemotherapy trials for latent tuberculosis with isoniazid (INH) showed efficacy in decreasing the risk of reactivation, which indicates that latent bacteria have some metabolic activity (Comstock \textit{et al.}, 1979). Also, detection of Ag 85 transcripts by RT-PCR in multiple tissues that were negative for cultivable bacteria, further confirmed that
the bacilli are viable and metabolically active (Pai et al., 2000). Results of these studies indicate an active, but restricted metabolism, present below the threshold level of immune detection.

The third and most peculiar characteristic of persistent state is related to its replication ability. Evidences in humans that the persisting bacteria are in a dormant, spore-like state comes from the results of culturing and staining diseased tissues from patients who have undergone chemotherapy, which might have resulted in false negative culturing results (Manabe and Bishai, 2000; McKinney, 2000; Parrish et al., 1998). Other hypothesis entails that persistent bacteria are either in a non-replicative state or have low levels of replication within the granuloma (Parrish et al., 1998), and a constant bacterial load is maintained by a balance between bacterial replication, and killing by the immune system. Mouse plateau model representing replication status of persistent bacteria has also shown that persistent bacteria either replicates very slowly or not at all (Rees and D’Arcy Hart, 1960).

Figure 3.3 Model of a granuloma. At a late stage of tuberculosis, a granuloma with a caseous necrotic centre is formed. The centre consists of a cheese-like semi solid structure that is low in oxygen and rich in lipids and proteins derived from dead cells as well as bacteria. The centre is surrounded by activated or partially activated macrophages and further by non-activated macrophages and lymphocytes.
Another fact associated with persistent bacteria is their altered colony morphology and thickening of cell wall which make these persistent bacteria resistant to different drugs. Dormant form of bacilli show uncharacteristic staining properties and are no longer acid-fast (Seiler et al., 2003). Persistent forms of mycobacterium are resistant to anti-tubercular drugs, to which they are susceptible in the proliferating state of infection (Stewart et al., 2003). Drug that act on cell division processes and cell wall synthesis have a reduced effect on non-replicating cultures. The induction of stress-response defenses and a decrease in metabolic rate also tend to increase tolerance to drug action. Reduction in bacterial replication is also associated with a reduction in drug susceptibility.

2.6. Model systems of persistent infection

There are a number of important questions that remain to be answered with respect to latency and reactivation in tuberculosis. Since processes regulating latency and \textit{M. tuberculosis} persistence can take years to manifest in the human host, several \textit{in vitro} and \textit{in vivo} systems have been developed to mimic aspects of latent infection. While these model systems are limited in their ability to fully recapitulate host and bacterial characteristics and have limitations, they provide a useful platform from which to initiate studies addressing specific aspects of the infection, dormancy and reactivation process. Furthermore, analyses using infection of surrogate Mycobacterium species in their respective host(s) have provided additional insights into processes regulating latent tuberculosis (Ramakrishnan et al., 2000; Chan et al., 2002).

2.6.1. \textit{In vitro} model of \textit{M. tuberculosis} persistence

All \textit{in vitro} models are based on the assumption that latent bacilli reside inside granuloma, and all \textit{in vitro} parameters are defined in a way to mimic the harsh environmental conditions that bacteria encounter inside granuloma.

2.6.1.1. The Wayne non-replicating persistence model

Wayne's non-replicating model (Wayne and Hayes, 1996; Wayne and Sohaskey, 2001) is based on the assumption that \textit{M. tuberculosis} encounters anaerobic conditions within granulomatous lesions inside the host. In this model, cultures of \textit{in vitro} grown bacteria are subjected to gradual oxygen depletion by incubation in sealed culture tubes.
Oxygen depletion triggers a dormancy response in the bacilli that is termed non-replicating persistence (NRP), a physiological state thought to mimic the one exhibited by *M. tuberculosis* during various stages of persistent infection. The transition of *M. tuberculosis* to a state of NRP is characterized by three distinct growth stages. In the first stage, the dissolved oxygen concentration in the medium is high and the bacilli exhibit normal logarithmic growth. This stage may recapitulate the physiological growth state of *M. tuberculosis in vivo* prior to the emergence of cell-mediated immunity. The second stage, called NRP-1, occurs when dissolved oxygen in medium reaches 1%. This stage mimics the physiological growth state of *M. tuberculosis in vivo* following the emergence of acquired immunity. During this stage, bacteria curtail protein synthesis, show discontinuation in DNA, and to some extent RNA synthesis, become resistant to several anti-mycobacterial drugs including isoniazid and rifampicin, and also show the upregulation of genes involved in glyoxylate shunt pathway which allow the utilization of alternative energy sources. During the third and final stage, NRP-2, dissolved oxygen concentrations drop below 0.06% in the culture medium, and bacilli switch from aerobic respiration to anaerobiosis. Bacilli in this state arrest growth at a uniform stage of the cell cycle. Furthermore, they exhibit sensitivity to metronidazol, a drug active against anaerobically growing organisms (Wayne and Saramek, 1994) and remain competent for growth reactivation following transfer into an oxygen-rich medium. These stages recapitulate the environment *M. tuberculosis* encounters *in vivo* during periods of extended persistence within a mature granuloma. While the NRP cannot recapitulate the influence of the host’s immune system, it may explain some characteristics observed during persistent infection of *M. tuberculosis* infection in humans (Yuan *et al.*, 1998).

2.6.1.2. The nutrient starvation model

The second *in vitro* growth model is the nutrient starvation model (Betts *et al.*, 2002). This model is based on the assumption that *M. tuberculosis* resides in tissues where nutrients and other essential cofactors are likely to be limiting. This model is initiated by the growth of *M. tuberculosis* in nutrient-rich medium, transfer of the culture in nutrient-limiting medium such as phosphate-buffered saline, and prolonged incubation under these conditions. *M. tuberculosis* cultures grown in this approach exhibited no loss in viability over an incubation periods of 6 weeks, decreased respiration rate and showed resistance to anti-tubercular drugs. As determined by microarray analysis nutrient starved cultures
exhibit a global shift down in gene expression. 2-D gel analysis of cell extracts has further confirmed reduced protein level in the starvation model.

2.6.2. *In vivo* models

2.6.2.1. Cornell mouse model

The Cornell mouse model (also known as drug induced model) was the first animal model system for latent tuberculosis. This model was developed at Cornell University in 1950s (McCune *et al*., 1956). In this model, *M. tuberculosis* infected mice are treated with antimycobacterial drugs [isoniazid and pyrazinamide], which reduces the bacterial burdens to undetectable level in mouse lungs (McCune *et al*., 1966). After antibiotic treatment reactivation of infection can either occur spontaneously or in response to immunosuppressive agents like glucocorticoids. This model mimics the low bacterial burden in mice, as in humans, but the reduced bacterial burden due to antibiotic treatment is not similar to protective immune response developed in humans. Variants of Cornell model has been reported differing in inoculating dose, antibiotic treatment and rest period before immune suppression (Scanga, *et al*., 1999).

2.6.2.2. Low-dose murine model

The low-dose murine model (also known as the chronic or plateau model) was developed by O’rme (1988). In this model, low-dose bacterial inocula are administered to “genetically resistant” strains of mice such as C5BL/6 or BALB/c intravenously or via aerosol. This results in acute phase of bacterial replication followed by a steady state of infection directed by the onset of adaptive immune response. During this state constant CFU is maintained in the lungs over many months. This chronic model resembles latency in humans in that it depends on host immune response to check the infection. However, due to large bacterial numbers and progressive pathology, it is more similar to chronic infection rather than human latent TB infection (Rhoades *et al*., 1997).

Other animal models also have been developed. One such model involves the use of *M. marinum*, which causes TB in ectothermic hosts such as frogs and fish. This model is used to study mycobacterial pathogenesis (Cosma *et al*., 2003).
2.7. Proteins implicated in mycobacterial persistence

The ability of \textit{M. tuberculosis} to persist in a host may require the coordinated expression of several bacterial virulence determinants at specific times during infection. The repertoire of genes expressed by \textit{M. tuberculosis} during period of persistence is of great importance because they may define the host conditions encountered by \textit{M. tuberculosis} during latent infection, as well as they allow the development of novel antibiotics and more efficacious vaccine that could target bacilli during latent/persistent stages. A range of genes that are expressed during, mycobacterial persistence have been identified. The products of these genes fall into three basic categories: respiratory enzymes, stress-related products and metabolic enzymes and, proteins involved in fatty acid catabolism.

2.7.1. Respiratory Proteins

During persistence organisms undergo eventual downshift in metabolism, and allow themselves to survive successfully in the host (Wayne and Hayes, 1996; Smeulders, 1999). The bacteria adapt their metabolism to anaerobiosis by switching to nitrate respiration and reductive amination of glyoxylate (Wayne and Hayes, 1996). The most commonly expressed respiratory protein during persistent infection is NarX, a putative "fused nitrate reductase". NarX might function as a respiratory nitrate reductase when oxygen is no longer available as the terminal electron acceptor. This gene is present in the form of cluster as \textit{narGHJI} in both \textit{M. tuberculosis} and BCG (Weber \textit{et al.}, 2000). It has been reported that \textit{narG} deficient mutant is unable to exploit nitrate as a source of oxygen and because of the scarcity of oxygen in the mature granuloma, the bacteria show reduced virulence as the infection progress. Wayne and Hayes (1996) have shown a marked increase in nitrite production in \textit{M. tuberculosis} cultures grown with nitrate during hypoxic shift down from aerobic growth to anaerobic persistence. Another gene whose expression is up-regulated during hypoxic response is NarK2, a putative nitrite extrusion protein. This protein helps in the extrusion of nitrite. Nitrate respiration helps the bacteria to survive in oxygen depleted areas of inflammatory or necrotic tissues where nitrate occur as degradation product. Nitrate in turn further could be used as a terminal electron acceptor under anaerobic condition, which would provide \textit{M. tuberculosis} with a distinct survival advantage under nitrite conditions (Virtanen, 1960; Hutter \textit{et al.}, 1999).
2.7.2. Stress-response and general metabolic proteins

The nature of metabolic adaptations to the anaerobic non-replicating state are largely unknown (Wayne et al., 1982). However, the *M. tuberculosis* genome revealed the presence of enzymes that are involved in anaerobic respiration and fermentation. In *M. tuberculosis*, transcription of *hmp*, increases under microaerophilic conditions when the culture enters stationary phase and under nitrosative stress. This enzyme is homologous to genes encoding flavohemoglobin in *E. coli*. Hmp protein plays a role in protecting *M. tuberculosis* from nitrosative stress, which is the main mechanism utilized by host to clear mycobacterial infections. This protein act as an NO di-oxygenase, which converts NO to NO3-(aerobically) or N2O (anaerobically), thereby functioning as an NO trap when induced by NO or nitrosative stress (Poole et al., 2000).

Another protein, whose expression increase manifold is 16 kDa α-crystalline like protein (Acr protein). This protein is associated with stationary phase (Yuan et al., 1996), is induced under RNI stress (Garbe et al., 1999) and is an immunodominant antigen that is necessary for survival in macrophages (Yuan et al., 1998). When subjected to anaerobic stress, *M. tuberculosis* and *M. bovis* BCG induce a massive up-regulation of the production of this protein, which is associated with a thickened cell envelope (Cunningham et al., 1998; Lim et al., 1999; Boon et al., 2001; Desjardin et al., 2001). This protein can stabilize cell structure during long-term survival and permits the bacilli to survive within the low-oxygen environment of the granuloma (Wilkinson et al., 1998). Its expression is controlled by the sigma factor SigF, an alternate RNA polymerase sigma factor is expressed in stationary phase and under stress conditions in vitro (Michele et al., 1999). It is nonessential both in axenic culture and for survival in macrophages in vitro. However, although loss of SigF does not prevent the mutant strain from producing a lethal infection, death of BALB/c mice infected by the mutant strains was significantly delayed (Chen et al., 2000).

2.7.3. Proteins involved in fatty acid metabolism

The most important proteins, activated during adaptation to persistence and microaerophilic conditions (Wayne et al., 1982) involve enzymes of glyoxylate bypass. There is a significant increase in a putative enzyme glyoxylate dehydrogenase and isocitrate lyase (ICL) activity. The up-regulated expression of these enzymes allows *M.
*tuberculosis* to utilize acetyl Co-A as a carbon source produced by the metabolism of fatty acid via the β-oxidation cycle and produces succinate which is the main precursor for the synthesis of sugars. Consequently, enabling *M. tuberculosis* to synthesize carbohydrate from fatty acids, as well as supply intermediates to support the TCA cycle. This is obviously of major importance when fatty acids are the main source of carbon and energy, as has been suggested to be the case for *M. tuberculosis* and *M. leprae* in chronically infected tissues (Bloch, 1956; Wheeler and Ratledge, 1988). Recently studies have revealed that ICL expression is up-regulated during infection of macrophages by mycobacterium species (Höner zu Bentrup *et al.*, 1999). It has been shown that *M. tuberculosis* in which the gene encoding ICL was deleted, impaired survival of bacilli occur in activated murine macrophages as well as in the late stage infection in the lungs of mice. The requirement for the ICL gene for bacterial survival during late-stage infection indicates a change of environment that require the bacteria to alter their diet from carbohydrate to lipid and also confirm the link between the immune status of the host and the metabolism of *M. tuberculosis* because the dependence on ICL seems to coincide with the onset of acquired immunity against the bacterium. The abundance of genes encoding enzymes involved in fatty acid degradation supports the suggestion that *M. tuberculosis* uses host lipids while growing *in vivo* (Cooper, 1999). This was further confirm by and observed that *in vitro*-grown bacteria had a preference for carbohydrates, whereas *in vivo*-grown bacteria preferred fatty acids (Nathan and Shiloh, 2000).

2.7.4. Regulatory Proteins implicated in Mycobacterial persistence

2.7.4.1. Transcription factors

Apart from the requirement of secondary metabolism systems and lipids, genes encoding transcription factors are also required by *M. tuberculosis* for long-term persistence. Transcription factors play an important role in modulating bacterial response during infection, because they provide a direct mechanism to quickly initiate adaptive responses. At a molecular level, transcription factors allow the tubercle bacilli to rapidly increase or decrease effectors gene expression in response to changes in the local environment. So far, three putative transcription factors have been implicated in *M. tuberculosis* persistence: the two-component signal transduction systems, the sigH sigma factor, and *whiB3*, a transcription factor of unknown function.
2.7.4.2. Two-component signal transduction systems

Two-component signal transduction system mediates adaptive processes in response to physical or chemical environmental stimuli. *M. tuberculosis* has eleven complete two-component systems (Cole et al., 1998), and one of these systems, mprA-mprB, participate in persistent processes (Zahrt et al., 2001). The expression of this system in bacteria is tissue- and time course-specific. Mice infected with an mprA mutant strain of *M. tuberculosis* exhibit reduced bacterial burdens in the lungs during persistent, but not acute stages of infection, and the growth of the mprA mutant is reduced in the spleen during both acute and persistent stages of infection. Several other *M. tuberculosis* two-component systems have also been implicated but their role in persistent infection remains unclear.

*phoP* (Rv0757), which codes for a putative transcription regulatory factor of the two component system PhoP/PhoR, is a response regulator required for intracellular growth of *M. tuberculosis* during acute stages of infection (Perez et al., 2001; Walter et al., 2006).

The *prrA–prrB* system is expressed in *M. tuberculosis* during growth in human macrophages *in vitro* (Graham and Clark-Curtiss, 1999), and is required during the initial stages of acute infection *in vivo* (Ewann et al., 2002).

A novel two-component system *devS/devR* was reported which is expressed at higher levels in *M. tuberculosis* H37Rv as compared to H37Ra (Das Gupta et al., 2000) and may have role in bacillary persistence.

*M. tuberculosis* H37RvΔrelMtb exhibits normal initial bacterial growth and containment in mice, but chronic infection is severely impaired, thereby implicating the importance of RelMtb in long term survival of non replicating *M. tuberculosis* (Dahl et al., 2003).

2.7.4.3. Sigma factors

A second group of transcription factors, required by *M. tuberculosis* during persistent stages of infection are sigma factors. These proteins are subunits of RNA polymerase and are responsible for directing the transcription of genes. In *M. tuberculosis*, 13 sigma factor genes have been annotated in the genome (Cole et al., 1998), nine of which belong to a special subfamily and direct extra cytoplasmic functions and various other stress responses. For example several sigma factors are activated following exposure
of *M. tuberculosis* to various environmental stresses *in vitro*, including temperature, oxidative stress, pH, and infection of macrophages (Manganelli *et al.*, 1999).

One such sigma factors, sigH is required by *M. tuberculosis in vivo* for progressive pulmonary disease during latent infection (Kaushal *et al.*, 2002). Although sigH expression is not required for the growth and survival of *M. tuberculosis* in a mouse model of tuberculosis, infection with *M. tuberculosis* sigH mutant results in a significant reduction in overall lung histopathology during persistent stages of infection, as well as reduction in the recruitment of CD4+ and CD8+ T cells to infection sites. Microarray analyses of the sigH mutant suggest the up-regulation of genes that are involved in resistance to oxidative and other denaturing stresses (Kaushal *et al.*, 2002). Thus, sigH is likely to play an important role in modulating gene expression in the tubercle bacilli in response to *in vivo* conditions, including those involved in host immunity.

### 2.7.4.4. WhiB3

A third putative transcription factor expressed in *M. tuberculosis* during persistent infection is whiB (Steyn *et al.*, 2002). This determinant, whiB3 is homologous to the whiB gene of *Streptomyces coelicolor*, a transcription factor involved in sporulation processes. In *M. tuberculosis*, WhiB3 interacts with the C-terminal region of RpoV, the principal sigma factor of strain H37Rv, to activate expression of yet-to-be-identified virulence determinants. The role of rpoV in *Mycobacterium* virulence is already known, as an rpoV mutation in *M. bovis* reduces virulence of this strain in a guinea pig model of tuberculosis (Collins, 1995). In *M. tuberculosis*, mutations in whiB3, although affect bacterial persistence but overall bacterial burden remains same. Similarly, *M. tuberculosis* whiB3 mutant grows normally in the organs of infected mice during either acute or persistent stages of infection. These mice exhibit significantly prolonged survival times compared to mice infected with wild-type *M. tuberculosis*. This increase in survival time is due to the result of changes in bacterial gene expression in the whiB3 mutant that reduce the cell-mediated immune response.

### 2.7.4.5. PE/PE-PGRS proteins

The PE/PE-PGRS proteins represent a novel class of proteins found in the genomes of several pathogenic *Mycobacterium* spp. including *M. tuberculosis* and *M. marinum*. The PE proteins have conserved Pro-Glu motif near their N-terminal, and in
case of PE-PGRS proteins, there is C-terminal extension of tandem repetition of Gly–Gly–Ala or Gly–Gly–Asn (Cole et al., 1981). In mycobacteria, genes from this family accounts for nearly 5% of the coding sequences, and suggested that these genes are required for virulence and persistent infection. First, several proteins from this family localize to the cell surface and influence cell surface interactions between mycobacteria and the macrophage (Brennan et al., 2001). Second, significant humoral and cellular immune responses are generated in vivo following the expression of several PE-PGRS and PE family members, respectively (Delogu, 2001). Finally, a PE-PGRS protein is a dominant antigen recognized by sera of asymptomatic latent carriers (Singh et al., 2001). The role of PE and PE-PGRS in mycobacterial persistence is assured by the expression of several genes from this family in persistent infection by M. marinum (Ramakrishnan et al., 2000). For example, several PE and PE-PGRS genes are expressed following M. marinum infection in macrophages. Some genes are involved during the growth of M. marinum in granulomatous lesions in vivo, while others are required for long-term persistence in the poikilothermic animal model system of infection. For example, an M. marinum strain carrying a mutation in a PE-PGRS gene, mag-24 is defective in persistent infection and exhibited reduced bacterial burdens in the spleen and liver of infected frogs. Frogs infected with this mutant exhibited attenuated granulomatous response. Taken together, these results suggest a role for PE-PGRS genes in aspects of persistent infection that include modulation of the host’s immune response.

2.8. Reactivation

 Reactivation refers to a clinical state in which a person has developed clinically apparent disease as opposed to clinically non-symptomatic state. This represents a change in the immune response in which the host is no longer able to contain the infection as compared to latent infection where host response prevents active disease from occurring, and the bacterium avoids elimination. Primary infection with M. tuberculosis leads to clinical disease in only approximately 10% of cases. Most infected individuals are able to mount an effective immune response, which limits the proliferation of the bacilli and produces a long-lasting partial immunity. This immunity is for both to new infections (also known as “exogenous reinfection”) and to the reactivation of latent bacilli (also known as “endogenous reactivation”). Although, a number of host factors responsible for reactivation/resuscitation of latent bacilli is known like immune status of the host, use of
steroids etc, a very little is known about mycobacterial factors. Recently, one such factor involved in reactivation/resuscitation of dormant bacteria has been reported. They are a family of protein named as resuscitation promoting factors (Mukamolova et al., 1998a, b). It has lysozyme type of enzymatic activity (Cohen-Gonsaud et al., 2004). But, how these proteins play role on resuscitation of dormant bacteria is yet to be known. How these proteins signal, which led mycobacterial cell to reactivate from dormancy is unclear.

2.8.1. Is the transition to and from dormancy active, programmed process?

While the survival of differentiated eukaryote is maintained by continuous provision of external signals, it is not known whether the transition to true dormancy is part of an essentially ordered developmental programme (Raff, 1992). It is yet to be known that whether the process is similar to the other developmental processes such as sporulation (Errington, 1996; Losick, 1999), stationary phase in Gram-negative organisms (Kjelleberg, 1993; Kolter et al., 1993). Alternatively progressions into a dormant state reflect a gratuitous and graceful degradation from a state of normal activity and culturability. Loss of many different functions during this process is responsible for dormancy (McDougald et al., 1998; Barer and Harwood, 1999; Kell and Young, 2000). Similarly the return from dormancy to culturability involves either a reproducible and ordered programme of gene expression or some repair/recovery processes. Studies on M. luteus system, in which there is coherent timings of the loss and gain of metabolic and biochemical functions (Kaprelyants and Kell, 1993; Mukamolova et al., 1995), suggest the process of dormancy and resuscitation are both active and programmed.

2.8.2. Reusucitating Promoting Factors

Resuscitating promoting Factors are a family of protein isolated originally from the culture supernatant of M. luteus. This family of proteins belongs to all those varieties of different autocrine chemical compounds (Stephens, 1986) produced as secondary metabolite, and responsible for eliciting and coordinating the differentiation of prokaryotes during processes such as sporulation, conjugation and expression of virulence (Ji et al., 1995; Fuqua et al., 1996). These molecules are different from nutrients and have some common properties like i) they are produced by the organism themselves ii) they are active.
at very low concentrations, and iii) unless they are generated from prohormones their metabolism is not necessary for activity. The chemical nature of these pheromonal compounds varies widely; those from Gram-negative organism are generally low molecular weight, particularly N-acyl homoserine lactone derivative, while Gram-positive organisms use proteins and polypeptides (Ji et al., 1995; Solomon et al., 1995; Solomon and Grossman., 1996; Solomon et al., 1996; Nodwell et al., 1996; Dunny and Leonard., 1997).

An Rpf protein from *M. luteus* purified to homogeneity, has the properties of cytokine, and possesses both autocrine and paracrine signaling function. It stimulates the growth of viable cells and also involved in the normal control of cell multiplication (Mukamolova et al., 1998). It has been found to resuscitate the cells rendered to stationary phase or dormant state due to prolonged incubation. Rpf from *M. luteus* show cross reactivity as it permits the resuscitation of several other high G+C Gram-positive organisms such as *M. avium, M. bovis* (BCG), *M. kansasii, M. smegmatis*, and *M. tuberculosis*.

### 2.8.3. Distribution of Resuscitating Promoting Factors

Resuscitating promoting Factors are widespread in actinobacteria. The DNA sequences databases currently contain more than 30 members of the *rpf* gene family and most organisms contain several representatives. These are identified in the genome of many members of actinobacteria such as *Mycobacterium* (*M. tuberculosis, M. bovis, M. smegmatis, M. leprae*), *Corynebacterium* (*C. glutamicum, C. efficiens, C. diphtheriae*), *Streptomyces* (*S. coelicolor, S. avermitilis*) and *Nocardia* (*N. farcinica*) showing a variable number per genome (Voloshin and Kaprelyants, 2004). Various studies both on the level of DNA and protein reveals that Rpf domain is also present in *Microbacterium lacticum, Arthrobacter globiformis, Amycolatopsis mediterranei, Brevibacterium liquefaciens, B. linens and Rhodococcus fascians* (Voloshin and Kaprelyants, 2004). All new *rpf* found in these organisms show strong homology to the known *rpf* family members. Slot blot hybridization using antibodies against the most conserved amino acid stretch of the Rpf domain shows the presence of these genes also in *A. mediterranei, B. iodinum, A. cerinus, B. liquifaciens* (Volker and Martin, 2006).
2.8.4. Resuscitating Promoting Factors of *Mycobacterium tuberculosis*

The available genome sequences of several Mycobacterium species were searched to identify gene products homologous to the *rpf* of *M. luteus*. The search identified 19 mycobacterial ORFs which shared significant homology with the *M. luteus* Rpf: 5 ORFs of *M. tuberculosis* (Rv0867c, Rv1009, Rv1884c, Rv2389c, and Rv2450c), 3 ORFs of *M. leprae* (ML0250, ML2030, ML2151), 5 ORFs of *M. bovis* similar to *M. tuberculosis*, 2 ORFs of *M. smegmatis* (unfinished genome), 2 ORFs of *M. avium* and 2 ORFs of *M. avium* subsp. *paratuberculosis* (unfinished genome) (Mukamolova et al., 2002b; Voloshin and Kaprelyants, 2004). In addition to sharing the conserved domains with the *M. luteus* Rpf, the mycobacterial ORFs share significant homology with each other. Rv0867 share 63% identity with other members of Rpf. Rv1009c share 43% homology with Rv2450c in a 147 amino acid overlap and Rv1884c shares 55.4% identity with Rv2398c in a 101 amino acid overlap.

RpfA, secretory protein (Gomez et al., 2000), is comparatively a large protein in which the Rpf-like segment is followed by an extensive series (residue 146-320) of proline + alanine rich repeats with the consensus sequences. In mycobacterial genome this gene occurs as monocistronic operons within the clusters of genes involved in molybdopterate biosynthesis.

RpfB, present in continuity with *ksgA* which is predicted to encode dimethyladenosine transferas is anchored to the outer surface of the cell membrane by an N-terminal prokaryotic membrane lipoprotein lipid attachment site. This protein share similarity with the N-terminal Mce domain which is involved in entry into and survival inside macrophages (Arruda et al., 1993).

The status of RpfC and RpfD is not very clear and are supposed to be secretory proteins. RpfD is located between HemN and NirA, which probably encodes protein used in coproporphyrinogen III decarboxylation and nitrate reduction respectively. RpfC, is present in a seven gene operon containing a mycosyltransferase (FbpB), upstream and a probable dehydrogenase, lipoprotein (Rv1881c), and cytochrome P450 (Rv1880c) downstream. RpfE comprises a monocistronic operon, has a trans-membrane helix close to its N-terminus.

All the *rpf* genes of *M. tuberculosis* are required for the growth of vegetative cells in minimal media at very low inoculum densities, as well as the resuscitation of the dormant/stationary phase cells. They are active at picomolar concentrations and involoved
in intercellular signaling. However, recently it has been reported that Rpf domain show structural similarity with both lysozymes and related bacterial peptidoglycan (PG) – degrading enzymes termed lytic transglycolases, which are implicated in metabolism of the PG layer of the bacterial cell wall (Cohen-Gonsaud et al., 2004; Cohen-Gonsaud et al., 2005). It is however, unclear that how such an enzymatic activity is associated with resuscitation effect on dormant bacteria \textit{in vitro} or \textit{in vivo}. It has been hypothesized that alteration of cell wall structure, might gives this protein the potential to overcome a block to cell growth and cell division (Keep et al., 2006). Alternatively, either by signaling proteins of released cell wall components (PG fragment) or altered cell wall permeability signals bacterium to divide \textit{in vitro} or \textit{in vivo} (Jacobs et al., 1997; Dziarski, 2003; Royet et al., 2003; Jacobs et al., 1994; McDonald et al., 2005).