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Tuberculosis is the world’s longest running catastrophe killing more than two hundred people every hour and more than five thousand everyday. According to WHO, someone is infected with TB every second (Dye et al., 1999). The causative organism, *Mycobacterium tuberculosis*, has been estimated to infect one in every three people or nearly 2 billion individuals worldwide. Every year, approximately 8 million of these infected people will develop active TB and almost 2 million of them will die from the disease. In India alone, one person dies of TB every minute (Dye et al., 1999). Although the vast majority of individuals infected with *M. tuberculosis* are latent carriers of the organism and exhibit no overt signs of disease symptoms, these individuals represent slow-release reservoirs. The features that enable *M. tuberculosis* to persist within the tissues of its host have also allowed TB to remain one of the world’s great killers into the 21st century. This problem was anticipated by Ehrlich in (1913) who declared in an historic address at the dawn of the chemotherapy era: “Now that the liability to, and danger of, disease are to a great extent circumscribed ... the efforts of chemotherapeutics are directed as far as possible to fill up the gaps left in this ring, more especially to bring healing to diseases in which the natural powers of the organism are insufficient”. Ninety years later, the ring has not yet been closed in the case of TB, where the “natural powers” of the human immune system are clearly “insufficient” to resolve infection. Latent carriers harbour a 2-23% life time risk of developing reactivation tuberculosis. More significantly, this risk increases to 10% annually should the immune system become suppressed, as is frequently observed in individuals coinfected with the human immunodeficiency virus (HIV). As a result, the combination of *M. tuberculosis* reactivation in latently infected individuals, and the subsequent primary infection of
immunocompromised hosts unable to control infection, results in more than 3 million deaths annually. Unless alternative measures are introduced to reduce the global burden of tuberculosis, more than 90 million people are expected to die from tuberculosis within the next 30 years. Although the front-line antibiotics (isoniazid, ethambutol, pyrazinamide, and rifampicin) are reasonably effective in treating individuals suffering from active tuberculosis, an infection state characterized by active growth of the tubercle bacilli in the host, these antibiotics are by and large ineffective in eliminating *M. tuberculosis* during latent stages of infection. The reasons for this discrepancy are currently unclear, but it could be a result of the ability of *M. tuberculosis* to enter a quiescent state during periods of persistence. Regardless, this insusceptibility, coupled with the rapid emergence of multidrug-resistant isolates, the lack of a universally effective vaccine (Fine, 1995), and the continuing increase in HIV-infected individuals in many regions of the world, has made it increasingly difficult to effectively treat infected individuals and eliminate tuberculosis in humans. Thus, the development of novel anti-tuberculosis therapeutics equally effective to persistent bacilli is urgently needed if the vicious circle of reactivation from latent stages is ever to be effectively interrupted in the near future.

1.1. The bacterium: *Mycobacterium tuberculosis*

*M. tuberculosis*, the causative agent of TB, is an acid fast, weakly gram positive, rod shaped bacillus (1-4 μm in length and around 0.3-0.6 μm in breadth), and a slow growing, facultative intracellular pathogen that can survive and multiply inside macrophages (Mφs) and other mammalian cells. Phylogenetic studies among
mycobacteria by 16S rRNA sequencing (Rogall et al., 1990) showed that \( M.\ tuberculosi \)s\) belongs to a group of ‘slow growers’, also known as ‘\( M.\ tuberculosi \) complex’ requiring 3-4 weeks to form colonies, with generation time of typically ~24 hours in solid medium. The \( M.\ tuberculosi \) complex include six members: \( M.\ tuberculosi \) the causative agent in the vast majority of human tuberculosis cases; \( M.\ africanum \), an agent of human TB in sub-Saharan Africa; \( M.\ microti \), the agent of TB in voles; \( M.\ bovis \), which infects a very wide variety of mammalian species including humans; BCG, an attenuated variant of \( M.\ bovis \); and \( M.\ canetti \), a smooth variant that is very rarely encountered but causes human disease. The important features shared by other members of \( M.\ tuberculosi \) complex include a cell wall of unique composition composed by a complex outer cell wall consisting of large amount of lipid. It consists of several unique components such as lipoarabinomannan (LAM), lipomannan (LM), pthiocerol dimycocerostate (PDIM), mycocerostate, mycolic acid, trehalose dimycolate (TDM) and sulpholipids (Bernan et al., 1990; Bersa & Chatterjee, 1994). These components are suggested to be responsible for mycobacterial hydrophobicity, ability to form clumps or cords, ability to survive intracellularly and it is the cell wall that gives \( M.\ tuberculosi \) its acid-fastness, enabling it to retain basic dyes in the presence of acid alcohol. The metabolic activity of mycobacteria, including assimilation of nutrients, energy production, metabolism and biosynthesis of macromolecules, are similar to those of other bacteria (Ratledge, 1982; Wheeler & Ratledge, 1994).
1.2. Pathogenesis of *M. tuberculosis*: stages of infection

The pathogenesis of tuberculosis is complex and its manifestations diverse, reflecting a lifetime of dynamic interactions between mycobacterial virulence factors and the human immune system (Dannenberg & Rook, 1994; Ellner, 1997; Russell, 2001). Tuberculosis can be experimentally modeled in mice, guinea pigs, rabbits and rats depending on the requirements of study. The disease has many manifestations, affecting bone, the central nervous system, and other organ systems, but it is primarily a pulmonary disease that is initiated by the deposition of *M. tuberculosis*, contained in aerosol droplets, onto lung alveolar surfaces (Wiegeshaus et al., 1989; Smith, 2003). The interactions of *M. tuberculosis* with human macrophages are central to all aspects of the pathogenesis of tuberculosis (Russell, 2001; Toosi et al., 2004; Ernst, 1998; Fenton, 1998; Dannenberg & Collins, 2001). Infection by *M. tuberculosis* can be divided into three separate interrelated stages (Fig. I).

**Stage 1** - In the first stage, individuals become infected by the inhalation of air-borne droplet nuclei containing *M. tuberculosis*. Within the lungs, *M. tuberculosis* primarily infect and reside in resident phagocytic cells such as alveolar macrophages and dendritic cells, or alternatively, in monocytes recruited from peripheral blood. Although macrophages are generally thought to provide an effective initial barrier against infection by bacterial pathogens, *M. tuberculosis* has evolved numerous strategies that allow it to survive and set up initial residence within these cells. These survival strategies include mechanisms that reduce acidification of *M. tuberculosis*-containing phagosomes, modify the normal phagosomal trafficking pathway, and alter the recruitment or association of various proteins to the phagosomal membrane. Collectively, these alterations allow the
organism to avoid elimination and multiply to moderate levels during the initial stages of infection.

**Stage 2** - In contrast, the course of infection during the second stage is primarily dictated by the host's immune response. For example, infection of an immunocompromised host by *M. tuberculosis* will typically result in the establishment of an acute infection characterized by uncontrolled bacillary proliferation and dissemination of the organism to distal sites. Symptomatically, individuals suffering from acute disease exhibit persistent fatigue, anorexia, progressive weight loss, low-grade fever, and production of a chronic, often contagious cough. Alternatively, if the infected host is immunocompetent, the host's immune system will typically resolve the initial infection, or alternatively, hold the infection in check using mechanisms that prevent further bacillary proliferation, limit the dissemination of the organism, and concentrate the immune response directly to sites of infection. Although these individuals continue to be persistently infected by *M. tuberculosis* and remain latent carriers of the organism, they do not exhibit overt signs of disease symptoms and are not infectious; however, they do test positive for a delayed-type hypersensitivity response.

**Stage 3** - The third and final stage of infection is characterized by reactivation of the tubercle bacilli from latency, and subsequent initiation of secondary acute infection in the host. The mechanism(s) responsible for the transition from persistence to resumed growth following reactivation is presently unclear; however, it is likely to be influenced by factors associated with the host's immune status. Conditions known to suppress the immune system including HIV infection, steroid therapy, age, and malnutrition, increase the likelihood of *M. tuberculosis* reactivation from latency.
1.3. Latency and the evolving climate within the host

The primary mechanism utilized by the host to control *M. tuberculosis* growth during persistent infection, and limit bacillary growth and dissemination to additional sites of infection, is the formation of granulomas. Granulomas are organized aggregates of immune cells that surround foci of infected tissues. Newly formed granulomas are composed of immature mononuclear phagocytes surrounded by lymphocytic effector cells including CD4+ and CD8+ T cells. During maturation into productive granulomas, mononuclear phagocytes differentiate into macrophages and become highly activated, aggregating into multinucleated giant cells, and larger epithelioid-like cells that contain tightly interdigitated cell membranes. This barricade-like structure walls off the organism and limits further dissemination to additional sites of infection. Within the granuloma, it is generally thought that *M. tuberculosis* must adapt to a highly dynamic
growth environment. First, much of the surrounding tissue is either calcified or necrotic. Second, the interior of the granuloma is thought to be devoid of, or alternatively, contain low levels of oxygen (Wayne & Sohaskey, 2001). However, this topic is controversial and remains an area of debate. The denseness of the tissue supports this contention and the bacteria sustaining infection during this latent stage of disease appear to have a markedly reduced rate of replication. Although it is unclear in what state *M. tuberculosis* survives the latent period of infection. Persistent *M. tuberculosis* might adopt an altered physiological state, which could account for its tolerance to drugs as well as its ability to survive in the host for many years. Third, granulomas are thought to contain high carbon dioxide concentrations, and possess increased levels of aliphatic organic acids and hydrolytic enzymes. Finally, the activation of macrophages and other immune effector cells surrounding the granuloma results in the release of numerous anti-microbial compounds. While the significance of this environment on *M. tuberculosis* growth and survival during persistent infection remains relatively unclear, tubercle bacilli are frequently observed extracellularly in infected human tissues and thus are likely to encounter such conditions during the course of the disease (Fig. II and III).
Fig. II: Pathogenesis of tuberculosis (adapted from Stewart et al., 2003).

Fig. III: Model of a granuloma (adapted from Bentrup & Russell, 2001).
1.3.1. Cytokines and bactericidal products

The consistent production of key inflammatory cytokines including IFN-γ and TNF-α, as well as other bactericidal products including reactive nitrogen intermediates and/or reactive oxygen intermediates generated by surrounding macrophages and immune effector cells is thought to be a key factor keeping *M. tuberculosis* in a state of persistence within the granuloma.

1.3.1.a. Proinflammatory cytokines

Recognition of *M. tuberculosis* by phagocytic cells leads to cell activation and production of cytokines, which in itself induces further activation and cytokine production in a complex process of regulation and cross-regulation. This cytokine network plays a crucial role in the inflammatory response and the outcome of mycobacterial infections. Several proinflammatory cytokines are discussed here. TNF-α may have multiple roles in immune and pathologic responses in tuberculosis, and is required for the control of the infection. *M. tuberculosis* induces TNF-α secretion by macrophages, dendritic cells and T cells (Henderson *et al.*, 1997; Serbina & Flynn, 1999). Mice deficient in TNF-α or the 55 kDa TNF receptor succumbed quickly to *M. tuberculosis* infection, with substantially higher bacterial burdens compared to control mice (Bean *et al.*, 1999). The requirement for TNF-α in control of *M. tuberculosis* infection is complex, but it clearly is an important component for macrophage activation. TNF-α in synergy with IFN-γ induces NOS2 expression (Liew & Millott, 1990). During chronic infection, NOS2 expression in the lungs was reduced following TNF-α neutralization (Mohan *et al.*, 2001). It has been demonstrated that TNF-α (Chensue *et al.*, 1999).
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1994) and IFN-γ are involved in granuloma formation (Enelow et al., 1992). In the absence of TNF-α or the 55 kDa TNF receptor, the granulomatous response is deficient following acute *M. tuberculosis* infection in murine models. The granulomas that do form are disorganized, with fewer activated or epithelioid macrophages (Flynn et al., 1995), and lymphocyte colocalization with macrophages is impaired (Bean et al., 1999). Clearly, TNF-α affects cell migration to and localization within tissues in *M. tuberculosis* infection. TNF-α influences the expression of adhesion molecules as well as chemokines and chemokine receptors, and this is certain to affect the formation of functional granulomas in infected tissues. Thalidomide treatment in *M. tuberculosis* infected mice, which down regulated the expression of inflammatory cytokines including TNF-α, IL-6, and IL-10, reduced the size of granulomas in the lungs without a change in bacterial numbers (Moreira et al., 1997). However, the multiple mechanisms by which TNF-α promotes effective granuloma formation, maintenance, and function remain to be determined.

A second proinflammatory cytokine involved in the host response to *M. tuberculosis* is IL-1β. Like TNF-α, IL-1β is mainly produced by monocytes, macrophages, and dendritic cells, expressed in excess at the site of disease (Law et al., 1996). Studies with mice suggest an important role of IL-1β in tuberculosis: IL-1α and -1β double-KO mice (Yamada et al., 2000) and IL-1R type I-deficient mice (which do not respond to IL-1) display an increased mycobacterial outgrowth and also defective granuloma formation after infection with *M. tuberculosis* (Juffermans et al., 2000).

IL-6, which has both pro- and anti-inflammatory properties (van Heyningen et al., 1997), is produced early during mycobacterial infection and at the site of infection (Law
IL-6 may be harmful in mycobacterial infections, as it inhibits the production of TNF-α and IL-1β. IL-6-deficient mice display increased 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 adaptive T-cell immunity has fully developed (Saunders *et al.*, 2000). IL-12 is a key player in host defense against *M. tuberculosis*. IL-12 is produced mainly by phagocytic cells (Fulton *et al.*, 1996). IL-12 has a crucial role in the induction of IFN-γ production (O’Neill & Greene, 1998). The protective role of IL-12 can be inferred from the observation that IL-12 KO mice are highly susceptible to mycobacterial infections (Cooper *et al.*, 1997; Wakeham *et al.*, 1998). Apparently, IL-12 is a regulatory cytokine which connects the innate and adaptive host response to mycobacteria (Sieling *et al.*, 1994; Trinchieri, 1995) and which exerts its protective effects mainly through the induction of IFN-γ (Cooper *et al.*, 1997).

The protective role of IFN-γ in tuberculosis is well established, primarily in the context of antigen-specific T-cell immunity (Andersen, 1997). Mycobacterial antigen-specific IFN-γ production *in vitro* can be used as a surrogate marker of infection with *M. tuberculosis* (van Crevel *et al.*, 1999). This cytokine is produced by both CD4 and CD8 T cells in tuberculosis (Lalvani *et al.*, 1998; Serbina *et al.*, 1999) and is important in macrophage activation and perhaps other functions. Individuals defective in genes for IFN-γ or the IFN-γ receptor are susceptible to serious mycobacterial infections; including *M. tuberculosis* (Ottenhoff *et al.*, 1998). IFN-γ knockout (GKO) mice are the most susceptible mouse strain to virulent *M. tuberculosis* (Cooper *et al.*, 1993). Although IFN-γ production alone is insufficient to control *M. tuberculosis* infection, it is required for the protective response to this pathogen.
1.3.1.b. Anti-Inflammatory cytokines

The proinflammatory response which is initiated 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 (TGFβ), may inhibit the production or the effects of proinflammatory cytokines in tuberculosis.

IL-10 is produced by macrophages after phagocytosis of *M. tuberculosis* (Shaw et al., 2000) and after binding of mycobacterial LAM (Dahl et al., 1996). T lymphocytes, including *M. tuberculosis*-reactive T cells, are also capable of producing IL-10. In patients with tuberculosis, expression 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 antagonizes the proinflammatory cytokine response by down regulation of production of IFN-γ, TNF-α, and IL-12 (Fulton et al., 1998; Hirsch et al., 1999). Indeed, IL-10 transgenic mice with mycobacterial infection develop a larger bacterial burden (Murray et al., 1997). In line with this, IL-10-deficient mice showed a lower bacterial burden early after infection in one report (Murray & Young, 1999), albeit normal resistance in two other reports (Erb et al., 1998; North, 1998). In human tuberculosis, IL-10 production was higher in anergic patients, both before and after successful treatment, suggesting that *M. tuberculosis*-induced IL-10 production suppresses an effective immune response (Boussiotis et al., 2000). The deleterious effects of IL-4 in intracellular infections (including tuberculosis) have been ascribed to
this cytokine’s suppression of IFN-γ production (Powrie & Coffman, 1993) and macrophage activation (Appelberg et al., 1992). In mice infected with M. tuberculosis, progressive disease and reactivation of latent infection are both associated with increased production of IL-4. Similarly, overexpression of IL-4 intensified tissue damage in experimental infection (Lukacs et al., 1997). Conversely, inhibition of IL-4 production did not seem to promote cellular immunity. IL-4/- mice displayed normal instead of increased susceptibility to mycobacteria in two studies, suggesting that IL-4 may be a consequence rather than the cause of tuberculosis development (Erb et al., 1998; North et al., 1998).

TGFβ also seems to counteract protective immunity in tuberculosis. Mycobacterial products induce production of TGFβ by monocytes and dendritic cells (Toossi et al., 1995). Interestingly, LAM from virulent mycobacteria selectively induces TGFβ production (Dahl et al., 1996). TGFβ suppresses cell-mediated immunity: in T cells, TGFβ inhibits proliferation and IFN-γ production and by antagonizing antigen presentation, proinflammatory cytokine production, and cellular activation by macrophages (Toossi et al., 1998).

1.3.1.c. Chemokines

Chemotactic cytokines (chemokines) are largely responsible for recruitment of inflammatory cells to the site of infection. About 40 chemokines and 16 chemokine receptors have now been identified (Zlotnik & Yoshie, 2000). A number of chemokines have been investigated in tuberculosis. First, several reports have addressed the role of IL-8, which 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). 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). A second major chemokine is monocyte chemoattractant protein 1 (MCP-1), which is produced by and acts on monocytes and macrophages. *M. tuberculosis* preferentially induces production of MCP-1 by monocytes (Kasahara *et al.*, 1994). In murine models, deficiency of MCP-1 inhibited granuloma formation (Lu *et al.*, 1998). A third chemokine is RANTES, which is produced by a wide variety of cells and which shows promiscuous binding to multiple chemokine receptors. In murine models, expression of RANTES was associated with development of *M. bovis* induced pulmonary granulomas (Chensue *et al.*, 1999). Some other chemokines may also be involved in cell trafficking in tuberculosis (Ragno *et al.*, 2001). Inhibition of chemokine production may lead to an insufficient local tissue response.

1.3.1.d. Effector mechanisms for killing of *M. tuberculosis*

Macrophages are the main effector cells involved in killing of *M. tuberculosis*. To become active against mycobacteria, macrophages need to be activated. *In vitro* models of macrophage activation for the killing of *M. tuberculosis* seem rather artificial, and therefore the exact conditions for optimal activation remain unknown. However, it is clear that lymphocyte products, mainly IFN-γ, and proinflammatory cytokines like TNF-α are important. Putative mechanisms involved in killing of *M. tuberculosis* within the phagolysosomes of activated macrophages include the production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). The study of these
mechanisms has been hampered by differences between mice and humans. *In vitro*, mycobacteria seem resistant to killing by ROI such as superoxide and hydrogen peroxide (Chan *et al.*, 1992). A possible explanation lies in the fact that several mycobacterial products, including sulfatides and LAM, are able to scavenge ROI (Chan *et al.*, 1991; Pabst *et al.*, 1988). The role of RNI in tuberculosis also remains a matter of debate. *In vitro*, human alveolar macrophages infected with *M. bovis* BCG display increased inducible nitric oxide synthase (iNOS) mRNA, and inhibition of iNOS is followed by increased bacterial outgrowth (Nozaki *et al.*, 1997). In tuberculosis patients, alveolar macrophages show increased production of iNOS (Nicholson *et al.*, 1996). However, whether iNOS gene expression leads to *in vivo* NO production remains uncertain, as in humans posttranslational modification of iNOS may be necessary for functional activity (Salh *et al.*, 1998). Therefore, the exact contribution of RNI in human tuberculosis remains to be elucidated. Sustained intracellular growth of *M. tuberculosis* may depend on its ability to avoid destruction by lysosomal enzymes, ROI, and RNI. When phagocytosed by macrophages, bacteria typically enter specialized phagosomes that undergo progressive acidification followed by fusion with lysosomes. However, *M. tuberculosis* delays or inhibits fusion of phagosomes and lysosomes (Russell *et al.*, 1996). In addition, *M. tuberculosis* prevents phagosomal maturation and acidification of phagosomes, thereby blocking the digestive activity of acidic hydrolases (Sturgill-Koszycki *et al.*, 1994; Clemens *et al.*, 1995).
1.4. Control of persistent infection

Immune mechanisms that are involved in the control of persistent infection are unclear, although there are some indications that these differ from the initial acute response. There is evidence that CD8+ T cells take over a substantial portion of IFN-γ production during the persistent phase of infection in the mouse model (Caruso et al., 1999; Pinxteren et al., 2000) although CD4+ T cells continue to play a vital, though undefined, IFN-γ-independent role (Scanga et al., 2000). Work with other intracellular pathogens, such as *Listeria monocytogenes*, shows that CD8+ T cells have an important role in controlling persistent infection, not only by secreting cytokines such as IFN-γ, but also by lysing infected cells (Wong & Pamer, 2003). The regulatory cytokine IL-10 has little or no effect on containment of the acute infection, but overexpression of IL-10 during the persistent phase triggers reactivation (Turner et al., 2002). Supporting this notion have been the observations that (i) viable bacilli can still be recovered from granulomas of individuals that are clinically asymptomatic, (ii) activated macrophages but not resting macrophages are able to kill *M. tuberculosis*, (iii) depletion of CD4+ and/or CD8+ T cells in mice persistently infected with *M. tuberculosis* results in disease reactivation, increased bacillary growth, and a significant decline in the mean time-to-death, (iv) depletion of IFN-γ and TNF-α during persistent stages of *M. tuberculosis* infection in mice results in rapid disease reactivation, (v) chemical inhibition of the inducible nitric oxide synthase encoded by nos2, a generator of reactive nitrogen intermediates, or infection of *M. tuberculosis* into nos2−/− mice, results in greatly increased bacillary burden and decreased survival time in tissue culture and model systems of infection, and (vi) infection of *M. tuberculosis* in phox−/− mice, lacking the
superoxide-generating NADPH phagocyte oxidase, results in increased neutrophilic infiltrate and tissue damage within the resulting granulomatous lesions. Thus, the ability of \textit{M. tuberculosis} to establish infection, maintain adequate bacterial numbers during periods of long-term persistence, and reactivate under conditions of immunosuppression, are the results of highly dynamic processes characterized by initiation of host defence mechanisms on one hand, and bacteria-mediated adaptation mechanisms on the other.

1.5. Evidence for persistent infection

Prior to the antibiotic era, TB was considered a life long infection: "Once tuberculous, always tuberculous". Despite the common feature of persistence, in the pre-antibiotic era the clinical outcome of infection was clearly variable in different individuals. While some succumbed relatively quickly to a steadily progressive primary infection, others were able to contain the disease. This containment was often temporary, and post-primary TB developing after an extended incubation period was common place. The reemergence of TB in those who had previously been diagnosed with TB was generally assumed to be due to reactivation of the earlier infection. Surgical sections of tuberculous lesions was uncommon in the pre-antibiotic era, so early attempts to assess the viability of tubercle bacilli in human lesions were largely limited to necropsy specimens. Lesions obtained from cadavers were frequently shown to contain acid-fast bacilli, sometimes in great numbers. In the mid-1920s, Opie & Aronson (1927) collected several hundred necropsy specimens and inoculated this material into guinea pigs. They found that homogenates of fibrocaseous lesions of the apex of the lung typically caused
TB in guinea pigs, whereas homogenates of caseous encapsulated or calcified lesions seldom did.

Autopsy and surgical specimen investigations conducted in the early part of this century have shown that tissues from humans who are asymptomatic but who show evidence of latent TB harbour infectious *M. tuberculosis* which was demonstrated by passage of the disease to animals (Feldman & Baggenstoss, 1939; Robertson, 1933). Microscopic examinations of these tissues sometimes fail to reveal acid fast bacilli, the characteristic bacterial forms associated with active infectious TB.

A new twist was recently added to this story. More than 70 years after Opie and Aronson's original studies were published, the presence of IS6110 DNA, an insertion element found in multiple copies in the *M. tuberculosis* chromosome, was demonstrated by using in situ PCR in the superficially normal lung tissue of diseased TB patients (Hernández-Pando et al., 2000). They found bacterial DNA not only in old granulomatous lesions but also in non-granulomatous tissue, which could indicate that persistent bacteria can survive not only in calcified lesions but also in apparently normal human lung. However, this method can not distinguish between live and dead bacteria because it only identifies bacterial DNA rather than viable organisms.

Pai et al. (2000) with the help of an animal model of latency (Cornell model) used RT-PCR directed at antigen (Ag) 85B mRNA to distinguish between viable and non-viable *M. tuberculosis* in mouse organs. The selection of Ag85B as a target was based on the fact that this protein is produced in abundance by *M. tuberculosis* both in broth cultures under a variety of stress conditions and in human mononuclear phagocytes (Garbe et al., 1996; Lee & Horwitz, 1999; Wilkinson et al., 2001). The positive mRNA
signal indicates the presence of a recently viable organism because bacterial mRNA generally has an extremely short half-life and is more vulnerable to destruction than rRNA or genomic DNA, and the method differentiated between viable and non-viable as well as to a large extent replicating and non replicating organisms in mouse tissue.

The first compelling molecular evidence for the existence of extraordinarily long periods of latency in untreated humans was provided recently by a molecular epidemiology study in Denmark (Lillebaek et al., 2002). This study examined the case of a Danish man who first developed TB in 1990. When the IS6110 fingerprint of the *M. tuberculosis* strain isolated from this patient was compared to fingerprints from the National Strain Collection, which only matched to an isolate dating 1958- isolated from his father.

1.6. Latency, dormancy, and persistence

Three terms, latency, persistence, and dormancy, are commonly used in literature describing *M. tuberculosis* and TB pathogenesis. Latency was defined by Amberson (1938) as "the presence of any tuberculous lesion which fails to produce symptoms of its presence". Latency can be achieved through either the early restriction of *M. tuberculosis* growth in the lungs prior to the onset of TB 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 (the so-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 subclinical 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. (Cunningham & Spreadbury, 1998; Gangadharam, 1995; Wayne, 1994). In bacterial physiology, the term dormancy is used to define “a reversible state of low metabolic activity, in which cells can persist for extended periods without division” (Kell et al., 1995). 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 populations 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 & 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 a combined effect of both the immune system and bacterial physiology (Bloom & Mckinney, 1999).
As a pathogen, *M. tuberculosis* manifests its unusual capacity to persist in many ways. On the cellular level, mycobacteria reside within macrophages, cells that typically function to eliminate pathogens and other foreign material 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 this bacterium is cleared by anti-TB drugs. The six months or more of chemotherapy required to cure TB makes the treatment of this disease an especially formidable challenge to global public health infrastructure, particularly in the developing world.

1.7. Model systems of persistent infection

Although a good deal of information is known about host factors limiting *M. tuberculosis* growth during latent stages, surprisingly little is known about the bacterial determinants required for persistent infection. A substantial barrier in our understanding of these factors has been an inability to experimentally recreate conditions encountered by *M. tuberculosis* during periods of latency in the host. In addition, the inability to define and recapitulate the physiological state of the tubercle bacilli during this stage has also made the identification of relevant genetic determinants required for persistence difficult to define. Since processes regulating latency and *M. tuberculosis* persistence can take years to manifest in the human host, several *in vitro* and *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, they provide a useful
platform from which to initiate studies addressing specific aspects of the infection process. Furthermore, analyses using infection of surrogate *Mycobacterium* species in their respective host(s) have provided additional insights into processes regulating latent tuberculosis (Chan *et al.*, 2002; Ramakrishnan *et al.*, 2000).

1.7.1. Bacteriological models of *M. tuberculosis* persistence

The ability of the tubercle bacilli to survive environmental stress in culture was well documented by Corper & Cohn (1933), they inoculated a culture bottle with *M. tuberculosis*, incubated it at 37°C for 12 years, and demonstrated a survival of 0.01%.

1.7.1.a. The Wayne non-replicating persistence model

Lawrence Wayne has conducted pioneering studies of the bacteriological persistence of *M. tuberculosis*. The "non-replicating persistence" model (Wayne & Sohaskey, 2001) is based on the assumption that *M. tuberculosis* encounters hypoxic or anaerobic conditions during its residence within granulomatous lesions in the host. In this model, cultures of *in vitro* grown bacteria are subjected to gradual oxygen depletion by incubation in sealed cultured tubes. Oxygen depletion using this method 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, termed NRP-1, occurs when the concentration of dissolved oxygen in the medium reaches 1%. *M. tuberculosis* in this stage exhibits an abrupt discontinuation in DNA, and to some extent, RNA synthesis. In addition, the bacilli curtail protein synthesis, become resistant to several anti-mycobacterial drugs including isoniazid and rifampicin, and induce the expression of numerous gene products that (i) allow utilization of alternative energy sources, (ii) stabilize and protect essential cellular products, and (iii) regulate downstream effector genes mediating adaptive responses. Although bacilli in NRP-1 stop DNA synthesis, culture turbidity increases due to thickening of the bacterial cell wall. This stage may recapitulate the physiological growth state of *M. tuberculosis* in vivo following the emergence of acquired immunity. 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 and exhibit no further increase in cell turbidity. Furthermore, they exhibit sensitivity to metronidazol, a drug active against anaerobically growing organisms (Wayne & Sramek, 1994) and remain competent for growth reactivation following transfer into an oxygen-rich medium. The NRP-2 growth stage may recapitulate the environment that *M. tuberculosis* encounters in vivo during periods of extended persistence within a mature granuloma. While the NRP model cannot recapitulate the influence of the host's immune system, it may explain some characteristics observed during persistent infection of *M. tuberculosis* in humans. For example, a major protein induced during the transition of *M. tuberculosis* into NRP-1 is Acr, a homolog of the α-crystalline protein and a member of the heat shock family of proteins that acts as ATP-
independent chaperones. In vivo, Acr is induced in *M. tuberculosis* following infection in macrophages (Yuan *et al.*, 1998), and is recognized by the sera of individuals afflicted with pulmonary tuberculosis (Lee *et al.*, 1992). The expression of Acr during NRP-1 also correlates with pronounced thickening of the *M. tuberculosis* cell wall (Cunningham & Spreadbury, 1998).

### 1.7.1.b. The nutrient starvation model

A second *in vitro* growth model, the nutrient starvation model (Betts *et al.*, 2002), is based on the assumption that *M. tuberculosis* resides in tissues where nutrients and other essential cofactors are likely to be limiting. This model was developed to support the previous observations that *M. tuberculosis* isolated from lung lesions frequently displays altered colony morphology (cell wall thickening) and uncharacteristic staining properties (no longer acid-fast) (Nyka, 1974). Nutrient starvation model is initiated by the growth of *M. tuberculosis* in a nutrient-rich medium, transfer of the culture to a nutrient-limiting medium such as phosphate-buffered saline, and prolonged incubation under these conditions. *M. tuberculosis* cultures grown in this fashion (i) exhibit no loss in viability over a 6-week incubation period, (ii) significantly decrease their rate of respiration, (iii) show increased resistance to the anti-tubercular drugs, isoniazid and rifampicin, (iv) remain resistant to metronidazole, and (v) exhibit an unusual colony morphology and staining property that can be reversed following a transfer back to the nutrient-rich medium. At the genetic level, nutrient-starved cultures exhibit a global downshift in gene expression. For example, genes involved in amino acid biosynthesis, biosynthesis of cofactors, prosthetic groups and carriers, DNA replication, repair,
recombination and restriction/modification, energy metabolism, lipid biosynthesis, translational and post-translational modification, and virulence, are all expressed at lower levels as determined by microarray analyses. Furthermore, two dimensional gel analysis of cell extracts further confirmed that many alterations noted by gene expression result in reduced protein levels. While the effect of limited nutrient availability on the growth of *M. tuberculosis* in vivo is currently unclear, the ability to switch to secondary metabolism systems may be essential for prolonged survival in the host (Mckinney *et al.*, 2000).

1.7.2. Animal models for latent TB

1.7.2.a. Cornell mouse model

First animal model system for latent tuberculosis was developed at Cornell University in the 1950s (McCune *et al.*, 1956). In this model, mice are infected intravenously with a high dose of virulent *M. tuberculosis* and immediately treated with anti-mycobacterial drugs, such as isoniazid and pyrazinamide, for a defined period (usually 12 weeks) to reduce bacterial numbers to low levels. Antibiotic treatment results in animal tissues that are "clinically sterile" (McCune *et al.*, 1966) a state characterized by an inability to observe acid-fast bacilli in infected tissues, lack of viable bacilli as measured by plating organ homogenates, and inability to retransmit the disease to susceptible mice or guinea pigs. Although clinically sterile, spontaneous reactivation of persistent *M. tuberculosis* can be observed as early as 4 weeks following the cessation of antibiotic treatment. While this model has the advantage of achieving and maintaining low or undetectable bacterial burdens in infected tissues, as is observed during human latent infection, it is not indicative of human latency in that the administration of
antibiotics is required to induce latent infection. Furthermore, infected mice exhibit higher rates of spontaneous reactivation following cessation of antibiotic treatment compared to reactivation rates observed during human latency.

1.7.2.b. Low-dose murine model

In contrast to the Cornell model, the low-dose murine model utilizes an alternative method to recapitulate latency processes in a surrogate animal host (Orme, 1988). In this model, low-dose bacterial inocula are administered to "genetically resistant" strains of mice such as C57BL/6 or BALB/c. These mice generate an effective immune response upon infection, and are able to control bacillary proliferation. Consequently, *M. tuberculosis* enters a persistent state of infection. The low-dose model also recapitulates many characteristics of *M. tuberculosis* during infection. For example, *M. tuberculosis* exhibits relatively unimpeded growth in infected tissues during the initial stages of infection, and the growth reaches a plateau following the emergence of an adaptive immune response. In addition, although the bacterial burden in persistently infected tissues remains higher than that typically observed during latent infection in humans, mice undergo chronic infection without disease symptoms, a state that can be maintained for extended periods with little variation in bacterial burden. Furthermore, latently infected animals undergo artificially induced disease reactivation, and exhibit rates of spontaneous reactivation similar to those observed during human latency.
1.8. *M. tuberculosis* genes required for persistent infection

The ability of *M. tuberculosis* to persist in a host requires the coordinated expression of numerous bacterial virulence determinants at specific times during infection. The identification of genes utilized by *M. tuberculosis*, specifically during periods of persistence, is of particular interest because they may (i) define host conditions encountered by *M. tuberculosis* during latent infection, (ii) allow the development of novel antibiotics that could target bacilli during latent stages, and (iii) provide alternative strategies for the development of rationally attenuated *M. tuberculosis* vaccines that are safer and more efficacious than the current *Mycobacterium bovis* BCG vaccine. To date, a handful of genes have been implicated in persistent infection by *M. tuberculosis*. However, only a few of these genes have been shown to be required by *M. tuberculosis* during persistent stages of infection in an animal model system of infection.

1.8.1. *icl* and the glyoxylate shunt

Isocitrate lyase, encoded by the *icl* gene, was one of the first *M. tuberculosis* genes shown to be required for persistent infection (McKinney, 2000). Isocitrate lyase is the initial enzyme in the glyoxylate shunt, a secondary metabolic pathway that allows bacteria to utilize fatty acids as carbon and energy sources when the availability of primary carbon sources is limiting. This enzyme, in combination with malate synthase, catalyzes the conversion of isocitrate to malate, a reaction that allows maintenance of the TCA cycle and assimilation of carbon by gluconeogenesis during growth on C2 substrates. Several studies have provided compelling evidence that components of the glyoxylate shunt, in particular, isocitrate lyase, are essential for the transition of *M.
*tuberculosis* from acute to persistent infection in the host, which may be listed as: (i) *M. tuberculosis* obtained from tissues of chronically infected individuals preferentially use fatty acids over glucose as a carbon source; (ii) *icl* expression is induced in *M. tuberculosis* during growth in activated but not resting macrophages *in vitro*; (iii) an Δicl mutant of *M. tuberculosis* exhibits significantly reduced bacterial counts following infection of activated but not resting macrophages *in vitro*; (iv) Δicl mutants of *M. tuberculosis* are progressively eliminated from the lungs and extrapulmonary sites of infection in mice following the emergence of adaptive immunity, but remain unaltered in growth during acute stages of infection. The requirement for *icl* expression during *M. tuberculosis* persistence suggests that primary carbon sources may be limiting and that alternative carbon sources may be preferentially utilized in the host. Furthermore, the observation that *M. tuberculosis* may show a transition to fatty acid metabolism following the emergence of adaptive immunity, provides compelling support for the role of activated macrophages in the control of *M. tuberculosis* during periods of persistence in the host, and further underscores the dynamic nature of the interaction between the host and pathogen. While the role of *icl* in *M. tuberculosis* persistence is well demonstrated, the requirement for malate synthase in the transition of *M. tuberculosis* to fatty acid metabolism has not yet been demonstrated. For example, the gene encoding malate synthase, *glcB*, is not induced for expression in *M. tuberculosis* under conditions known to stimulate the expression of *icl* (Wayne & Sohaskey, 2001). Thus, the requirement for *icl* expression for bacterial persistence may simply be to replenish NAD in its oxidized form during the transition of *M. tuberculosis* from an aerobic to microaerophilic environment (Wayne & Sohaskey, 2001).
1.8.2. **acr** (α-crystallin protein homolog, *hspX*)

The *acr* gene, which encodes an α-crystallin homolog, is a major *M. tuberculosis* antigen recognized by the sera of a high proportion of TB patients (Verbon *et al.*, 1992) and is induced under anoxic conditions (Wayne, 1994). A *M. tuberculosis* mutant in which the *acr* gene was inactivated was severely attenuated for growth in the macrophages (Yuan *et al.*, 1998). 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). It is postulated that the chaperone-like *acr* is an important controlling element in *M. tuberculosis* latency or persistence, since overexpression of the protein inhibits *M. tuberculosis* growth (Yuan *et al.*, 1996).

1.8.3. **Mycolic acids and PcaA**

Cell wall components are also important factors regulating persistent infection by *M. tuberculosis*. In contrast to other Gram-positive organisms, the cell wall of *Mycobacterium* spp. contains a complex mixture of unique glycolipids and mycolic acids, long-chain branched fatty acids that are covalently or non-covalently attached to the cell surface. The cell wall of *M. tuberculosis* has long been implicated as a virulence factor in *M. tuberculosis* pathogenesis. For example, the extent of cell wall serpentine cording (braided microscopic bundles) is known to correlate to the virulence of *M. tuberculosis* in an animal model. Recently, a gene involved in the cording phenotype was identified and shown to be required for persistent infection by *M. tuberculosis in vivo* (Glickman *et al.*, 2000). This gene, *pcaA*, encodes a cyclopropane synthase, an enzyme that incorporates a single proximal cyclopropane ring on the α-mycolic acids present in the cell wall.
Deletion of the pcaA gene in *M. tuberculosis* results in an alteration in the cording phenotype of the strain. More importantly, deletion of pcaA reduces the ability of *M. tuberculosis* to undergo long-term persistence in the host. For example, an *M. tuberculosis* pcaA deletion mutant exhibits reduced virulence during long-term infection in the murine model of tuberculosis, although it does grow similar to the wild-type parent strain during acute stages of infection. In addition, mice infected with the pcaA mutant exhibit a significant increase in time-to-death compared to mice infected with wild-type *M. tuberculosis* in lethality experiments, a consequence resulting from a reduction in host-mediated pulmonary damage. Although the exact mechanism(s) by which pcaA expression contributes to *M. tuberculosis* persistence remains unclear, the inability to attach cyclopropane rings to cell wall mycolic acids may alter antigen presentation by the host’s immune system.

1.8.4. 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 effector gene expression in response to changes in the local environment. So far, three putative transcription factors have been implicated in *M. tuberculosis* persistence: two-component signal transduction systems, such as mprA–
mprB and devS-devR, the sigH sigma factor, and whiB3, a transcription factor of unknown function.

1.8.5. Two-component signal transduction systems

Two-component signal transduction systems are ubiquitous regulatory networks that mediate adaptive processes in response to physical or chemical environmental stimuli. These systems utilize phospho-transfer mechanisms to transmit information between a membrane-localized, histidine kinase “sensor” protein and a cytoplasmic “response regulator” transcription factor. Detection of the appropriate stimulus by the histidine kinase results in autophosphorylation and subsequent transfer of phosphate to the cognate response regulator, initiating a transcriptional regulatory cascade that results in the activation or repression of downstream effector determinants. Eleven complete two-component systems have been annotated in the M. tuberculosis genome (Cole et al., 1998), and one of these systems, mprA–mprB, has been shown to participate in persistence processes (Zahrt et al., 2001). In contrast to other “persistence” genes, the requirement for mprA–mprB by M. tuberculosis is tissue- and time course-specific. Mice infected with an mprA mutant strain of M. tuberculosis using the low-dose murine model of 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. In contrast, growth of the M. tuberculosis mprA mutant is unaltered in the liver during these stages. The importance of mprA for M. tuberculosis persistence is also suggested by the observation that the expression of this gene is differentially regulated between virulent and avirulent M.
tuberculosis strains. For example, mprA expression is silenced in *M. tuberculosis* H37Rv during intracellular growth in human and murine macrophages, whereas *mprA* expression is induced to high levels under similar conditions in the vaccine strain *M. bovis* BCG, an attenuated derivative unable to establish persistent infection. Several other *M. tuberculosis* two-component systems have also been implicated in various aspects of virulence, although their role in persistent infection remains unclear. Although the environmental signal(s) and downstream effector determinant(s) recognized by *mprA*-mprB, or any of these two-component systems, have yet to be defined, the study of two-component systems offers a multifaceted approach for understanding latency-related processes because (i) identification of the signals recognized by sensor kinase proteins, such as MprB, will ultimately define host conditions experienced by *M. tuberculosis* in vivo, and (ii) determination of the downstream effector genes regulated by response regulator proteins, such as MprA, will help define virulence determinants required for the establishment and maintenance of persistent infection.

*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).

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

Finally, the MtrA response regulator is a major antigen found in sera of *M. tuberculosis* infected individuals during acute stages of infection and immediately prior to the emergence of clinical tuberculosis (Singh et al., 2001).
A novel two component system devS/devR was reported which is expressed at higher levels in *M. tuberculosis* H37Rv as compared to H37Ra (DasGupta *et al.*, 2000) and may have role in bacillary persistence.

*M. tuberculosis* H37RvΔrelMt exhibits normal initial bacterial growth and containment in mice, but chronic infection is severely impaired, thereby implicating the importance of RelMt in long term survival of non replicating *M. tuberculosis*. (Dahl *et al.*, 2003).

1.8.6. Sigma factors

A second group of transcription factors, the sigma factors, are also required by *M. tuberculosis* during persistent stages of infection. 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 thought to direct extracytoplasmic functions and various other stress responses. For example, several extracytoplasmic function sigma factors are induced following the exposure of *M. tuberculosis* to various environmental stresses *in vitro*, including temperature, oxidative stress, pH, and infection of macrophages (Manganelli *et al.*, 1999).

One of these 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 *per se* for the growth and survival of *M. tuberculosis* in a mouse model of tuberculosis, infection with an *M. tuberculosis ΔsigH* mutant results in a significant reduction in overall lung histopathology during persistent stages of infection,
concomitant with a reduction in the recruitment of CD4+ and CD8+ T cells to infection sites. Consequently, mice infected with a $\Delta\text{sigH}$ mutant exhibit a significant increase in time-to-death compared to mice infected with the parental strain. Microarray analyses of the $\Delta\text{sigH}$ mutant suggest that more than 180 genes are regulated by this sigma factor, including genes involved in resistance to oxidative and other denaturing stresses (Kaushal et al., 2002). Thus, $\text{sigH}$ is likely to play an important role in modulating gene expression in tubercle bacilli in response to in vivo conditions, including those involved in host immunity.

1.8.7. WhiB3

A third putative transcription factor required for persistent infection has also been recently reported in $\text{M. tuberculosis}$. This determinant, $\text{whiB3}$ (Steyn, 2002) encodes a homolog of the $\text{Streptomyces coelicolor whiB}$ gene, a transcription factor whose function is required for sporulation processes. In $\text{M. tuberculosis}$, 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 $\text{rpoV}$ in $\text{Mycobacterium}$ virulence has previously been suggested, because $\text{rpoV}$ mutation in $\text{M. bovis}$ reduces virulence of this strain in a guinea pig model of tuberculosis (Collins, 1995). In $\text{M. tuberculosis}$, mutations in $\text{whiB3}$ affect bacterial persistence in vivo, but in a manner independent of overall bacterial burden. For example, while an $\text{M. tuberculosis}$ $\text{whiB3}$ 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 $\text{M. tuberculosis}$. As with the $\text{M. tuberculosis}$
rpoH mutant, this increase in survival time is likely result of changes in bacterial gene expression in the whiB3 mutant that reduce the cell-mediated immune response.

1.8.8. PE/PE-PGRS proteins

The PE/PE-PGRS proteins represent a novel class of highly related genetic determinants found in the genomes of several pathogenic *Mycobacterium* spp. including *M. tuberculosis* and *M. marinum*. The PE and PE-PGRS family members are named for their conserved Pro–Glu motif near their N-terminal, and in the case of PE-PGRS proteins, their C-terminal extension of tandem repetitions of Gly–Gly–Ala or Gly–Gly–Asn (Cole *et al.*, 1998). In the tubercle bacilli, genes from this family account for nearly 5% of the organism's coding capacity, and several lines of evidence suggest that these genes are required for various aspects of virulence, including persistent infection. First, several proteins from this family localize on 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 (Rv3367) is a dominant antigen recognized by sera of asymptomatic latent carriers (Singh *et al.*, 2001). Support for the role of PE and PE-PGRS proteins in *M. tuberculosis* persistence is also suggested by the observations that several genes from this family are important for persistent infection by *M. marinum* (Ramakrishnan *et al.*, 2000). For example, several PE and PE-PGRS genes are (i) expressed following *M. marinum* infection in macrophages, (ii) induced during the growth of *M. marinum* in granulomatous lesions *in vivo*, and (iii) required for long-term
persistence in the 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 exhibits reduced bacterial burdens in the spleen and liver of infected frogs. Frogs infected with this mutant also exhibit a markedly 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.