Host-pathogen interactions of different strains of Mycobacterium tuberculosis

Chapter 1: General Introduction

1.1. Tuberculosis Epidemic

Tuberculosis (TB) has long been a major scourge for humanity, claiming millions of lives worldwide. While TB is preventable and curable, it has remained a significant cause of morbidity and mortality in resource poor nations like India. On the basis of tuberculin reactivity or Mantoux test it has been calculated that one-third of the world's population has been infected with Mycobacterium tuberculosis bacillus (MTB), the causative agent of the disease (Dye et al, 1999). Infecting bacilli are either killed or remain 'viable but latent' inside the host macrophage for decades. In the infected individuals who develop active disease, bacilli appear to evade or subvert the host’s protective cellular immune responses. However, infection with immunodeficiency virus, treatment with corticosteroids, aging and consumption of alcohol increase the potential for reactivation of latent tuberculosis. Therefore, pathogenesis depends on bacterial factors and host factors which determine the disease outcome.

The term tubercle was coined by Franciseus Sylvius (1650) of Leyden. Jean-Antoine Villemin (1865) first established the infectious nature of tuberculosis, though he was unable to isolate the organism. In 1882 Robert Koch, a country doctor from East Prussia, announced to the Physiological Society of Berlin that he had identified and cultured the tubercle bacillus. He also described a staining technique for this bacillus and proposed a perfect experimental model for the infectious nature of the bacilli. In 1895 Roentgen discovered X-rays and exposure to X-rays was used in 1904 for the detection of this disease. Ever since, radiology and bacteriology have remained the main pillars of diagnosis of tuberculosis.

Tuberculosis is also an ancient disease. Bones of old pre-historic men dating back to 8000 BC found in Germany have shown a rare tuberculosis manifestation. Spines from
skeletons excavated in Egypt (2500 to 1000 BC) have also shown changes suggestive of tuberculosis. MTB was known as King’s evil in England during the 11th and 12th centuries. Various other historical terms used for the description of tuberculosis are phthisis and consumption.

**Global burden of Tuberculosis**

In 2007, an estimated 9.27 million incident cases and approximately 1.75 million deaths due to TB occurred worldwide (WHO, 2009). After human immunodeficiency virus (HIV)/AIDS, TB is the second most common cause of death due to an infectious disease, and current trends suggest that TB will still be among the 10 leading causes of global disease burden in the year 2020 (WHO Fact Sheet, 2000). The global distribution of TB cases is skewed heavily toward low-income and emerging economies. The highest prevalence of cases is in Asia, where China, India, Bangladesh, Indonesia, and Pakistan collectively make up over 50% of the global burden. Africa, and more specifically sub-Saharan Africa, has the highest incidence rate of TB, with approximately 83 and 290 per 100,000, respectively (Figure 1). TB cases occur predominantly (approximately 6 million of the 8 million) in the economically most productive 15- to 49-year-old age group (WHO Fact Sheet, 2000). Our understanding of TB epidemiology and the efficacy of control activities have been complicated by the emergence of drug-resistant bacilli and by the synergism of TB with HIV coinfection. In 1993, the World Health Organization (WHO) took an unprecedented step and declared tuberculosis a global emergency. It is estimated that between 2000 and 2020 nearly one billion people will be newly infected, 200 million will get sick and 35 million will die from TB if control is not strengthened further (WHO Fact Sheet 2000).

**Tuberculosis in India**

One fourth of the world’s TB burden is borne by India. More than 2 million people develop active tuberculosis and more than 500,000 people die each year due to tuberculosis
in India. This means more than 1000 die each day or one every minute due to TB. TB causes more deaths per year than malaria, hepatitis, meningitis, nutritional deficiencies, sexually transmitted diseases, leprosy, and tropical diseases (WHO, 1997) put together. Prevalence of the disease is more than twice the incidence, indicating a failure of current treatment programmes. According to WHO estimates, India loses about Rs.1700 crores in economic output every year due to TB.

"Figure 1: The global incidence of TB: The number of new TB cases per 100,000 population for the year 2007 according to WHO estimates"


1.2. The Disease – Tuberculosis:

The principal risk of acquiring TB infection is from breathing small droplet nuclei of the size 10 µm carrying 3 to 10 bacteria exhaled by a sputum positive pulmonary TB patient. The size of the droplet nuclei is crucial for tubercle bacilli to reach beyond the respiratory tract and penetrate into the terminal air passage, multiply and establish an infection, (Nardell, 1993). Droplet nuclei are stable and remain suspended in air for a long time. Good ventilation can remove droplet nuclei and exposure to sunlight kills tubercle bacilli and hence these can
prevent transmission of the disease. Two important factors, which determine an individual’s risk of infection are, the concentration of droplet nuclei in contaminated air and the amount of time he/she is exposed to such air (Vijayan, 2002). The risk of infection progressing into disease varies with age, the risk being greatest in children below 3 years followed by elderly people and young adults.

**Primary Tuberculosis**

The development of pulmonary tuberculosis from its onset to the various clinical manifestations depends largely on the immune reactions of the host to the pathogen. There are two distinct effective immune responses for inhibiting the progression of the disease. First, a T-cell mediated macrophage-activating response, enhances the ability of the macrophage to kill or inhibit *Mycobacteria* (Orme et al., 1993) and the second, the tissue damaging response, is often produced during delayed-type hypersensitivity (DTH) reactions to tuberculin like products of the bacillus. The latter is used during the course of the disease to destroy macrophages within which the bacterium is multiplying (Dannenberg, 1994).

Using animal models various steps in the development of the primary disease have been understood. Among the animal models of the disease, tuberculosis in rabbits closely resembles the human disease. It has four major stages (Lurie M.B., 1928). The first stage that lasts for one week begins following the inhalation of the tubercle bacilli into an alveolus. The inhaled particle (one unit) should be small enough to reach the alveolar spaces and contain no more than three bacilli. Ten to 50 such units are required to establish an infection. It is believed that fewer than 10 live bacilli even can cause an infection (Nicas et al, 2005). The primary site of infection in the lungs, known as the "Ghon focus", is generally located in either the upper part of the lower lobe, or the lower part of the upper lobe (Kumar et al, 2007). The infected bacilli may persist or may be destroyed by the alveolar macrophages. The
outcome depends on the inherent microbicidal activity of the alveolar macrophages and virulence of the ingested bacillus.

Stage-2, a symbiotic stage (7 to 21 days) is one in which logarithmic bacillary growth and the early tuberculous lesion formation takes place. In favorable conditions, the bacilli grow logarithmically and simultaneously in infected macrophages. Monocytes derived from circulation are attracted to the infection site by various cytokines, initiating granuloma formation. The granuloma prevents dissemination of the *Mycobacteria* and provides a local environment for interaction of cells of the immune system. The initial granuloma formation is called primary tuberculosis.

Stage-3 starts after 3 weeks, lasts up to 8 weeks and is controlled by T cell immunity and DTH. This stage begins when logarithmic bacillary growth stops and the caseous necrosis develops at the centre of the granuloma due to DTH reaction produced by cytotoxic T cells. The killing of macrophages in the interior of the granuloma results in relatively large areas of necrosis, each surrounded by a layer of epitheloid leukocytes and multinucleated giant cells. These tubercules are surrounded by a cellular zone of fibroblasts, lymphocytes and blood derived monocytes. At the same time immunocompetent individuals develop a strong T cell immunity that activates macrophages and render them capable of destroying the bacilli. The caseous foci may calcify or ossify. The extent of macrophage activation determines the subsequent course of disease i.e. the strength of the host’s T cell response determines whether an infection is arrested here or progresses to the next stage. With good T cell response, the infection may be arrested permanently at this point. The granulomas subsequently heal, leaving a small fibrous and calcified lesion. If T cell response is insufficient, dissemination of the organism occurs via intrapulmonary lymphatic route with extensive involvement of the hilar lymphnodes. Spillover from lymphatics to the bloodstream
enables the bacilli to reach almost all the organs of the body especially liver, spleen and kidney.

In Stage-4 progression of the disease occurs even in an immunocompetent host and this is caused by liquefaction and cavity formation. The factors that cause liquefaction are high levels of tuberculin reactivity and elevated hydrolytic enzymes like proteases, nucleases and lipases (Converse et al, 1996; Fink & Cookson, 2005). The liquefied material is an excellent growth medium for tubercle bacilli and the bacillus multiplies extracellularly, often reaching high numbers ($>10^8$). As the host is highly sensitive to large antigenic load of the bacillus which is quite toxic to the tissues and leading to necrosis, rupture of the walls of nearby bronchi forming a cavity. The walls of most cavities consist of an external zone of collagen, the cavity’s capsule and a caseous liquefied internal zone where the oxygen content is high and nurtures the growth of the bacilli. By coughing, the patient aerosolizes this infectious material disseminating bacilli to other parts of the lung and to the outside world.

**Post-primary tuberculosis (reactivation)**

Even in a person who successfully fights his battle against TB but has bacilli in a dormant state inside granuloma, reactivation can take place that can lead to post-primary tuberculosis. The lesions in the granulomas are with necrosis and frequently occur at the apices of the upper lobes of lungs. These lesions (Assman’s foci) undergo enlargement and liquefaction of caseous centre resulting in cavitation.

**Clinical tuberculosis**

The clinical expression of infection with *M.tuberculosis* largely depends on the site of involvement and is the most important factor influencing the clinical features of tuberculosis. In an immunocompetent host approximately 85% of the reported cases of tuberculosis are pulmonary and remaining 15% include extrapulmonary or both pulmonary
and extrapulmonary cases (Gangadharam et al, 1988). But in HIV infected patients, it was reported that 38% had only pulmonary TB, 30% had extrapulmonary TB and 32% had both pulmonary and extrapulmonary TB (De Viedma et al, 2002).

**Pulmonary tuberculosis**

The lungs are the most favored site of infection of MTB and the pathogen comes by respiratory route as described earlier. Lung infection may also occur via bloodstream. This is known as a ‘Simon focus’ and is typically found in the top of the lung (Khan & Rahman, 2000). This hematogenous transmission can also spread infection to more distant sites, such as peripheral lymph nodes, the kidneys, the brain, and the bones (Herrmann & Lagrange, 2005). Cough is the most common symptom of pulmonary tuberculosis and hemoptysis (coughing blood) may result from rupture of a dilated vessel in the wall of an old cavity. In primary pulmonary TB occurring as a result of infection showing zonal infiltration of lymphocytes whereas progressive TB shows cavitation.

**Extrapulmonary tuberculosis**

Extrapulmonary tuberculosis (EPTB) includes a very wide range of conditions of diverse pathology and prognosis. The non-specific symptoms and results of investigations make diagnosis of EPTB difficult, delayed or missed, which may result in death or serious disability. Airway is almost invariably the portal of entry. If TB bacteria gain entry to the bloodstream from an area of damaged tissue, they can spread throughout the body and set up many foci of infection, all appearing as tiny, white tubercles in the tissues (Crowley & Crowley, 2010). This severe form of TB disease, most common in young children and those with HIV, is called miliary tuberculosis (TB/HIV Clinical manual, 2004). People with this disseminated TB have a high fatality rate even with treatment (about 30%) (Jacob et al, 2009). Metastatic foci thus established, may manifest into EPTB. Early forms of EPTB are cerebral and skeletal disease (via bloodstream) or lymph node, vertebral and pericardial
disease (via lymphatic). All parts of the body can be affected by the disease, though for unknown reasons it rarely affects the heart, skeletal muscles, pancreas and thyroid (Agarwal et al, 2005).

**TB Diagnosis**

The most powerful tool in any TB control program is prompt diagnosis and successful treatment of patients. There are several popular techniques used for this purpose.

**Sputum smear microscopy.** The use of stained-sputum microscopy for acid-fast bacilli still remains the most available, easy, inexpensive, and rapid diagnostic test for TB (Kent et al, 1985) specially in resource poor country like India (Steingart et al, 2006). But, the test is not totally specific and sensitive (Tuberculosis Prevention Trial, 1980). Further, diagnosis of TB by microscopy is difficult especially in children who rarely produce adequate sputum. Currently, the sensitivity of this test has improved considerably by the use of auramine-rhodamine/ fluorochrome method instead of the classic Ziehl-Neelsen stain which uses carbol-fuchsin (Wright & Wallace, 1998).

**Cultivation of Bacteria.** *Mycobacterial* culture is the ultimate proof of *Mycobacterial* infection and is often used as a reference method due to its high sensitivity and specificity (Schirm et al, 1995; Walker, 2001). However, it takes 4-6 weeks for MTB to grow on solid culture medium (e.g. agar based Middlebrook 7H10 or 7H11 or the egg-based Lowenstein-Jensson medium), and 3 weeks to grow in liquid 7H9 medium (Morgan et al, 1983). To increase the sensitivity and reduce the detection time some modifications are incorporated and new techniques have been developed.

**BACTEC 460.** The BACTEC 460 (Becton Dickinson, Sparks, Maryland) relies on radiometric detection of $^{14}\text{CO}_2$ as an indicator of bacterial growth. The Bactec vials contain Middlebrook 7H12 medium and fatty acid substrates labelled with 14C. Growing *Mycobacteria* release $^{14}\text{CO}_2$ as a metabolic end product. The gas is removed, analyzed and
the amount of radioactive $^{14}\text{C}$ released is expressed as a numerical value called the Growth Index (GI).

**Mycobacterial Growth Indicator Tube.** The Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson, Sparks, Maryland, USA) introduced 15 years ago, is a rapid (4-13 days), high throughput method. It is based on fluorescence detection of Mycobacterial growth in a tube containing a modified Middlebrook 7H9 medium together with fluorescence quenching-based oxygen sensor (a ruthenium pentahydrate substance embedded in silicone rubber) at the bottom of the tube (Reisner et al, 1995; Rusch-gerdes et al, 1999). As the bacteria grow and consume oxygen, the indicator fluoresces under ultraviolet light.

**Biomarkers**

TB diagnostic tests that rely on detection of host immunological markers currently in use include the tuberculin skin test (TST) (Huebner et al, 1993) and interferon gamma release assays (IGRAs) (Andersen et al, 2000; Pai et al, 2008).

**Tuberculin skin test.** The TST or Mantoux test or purified protein derivative (PPD) test has been used for almost a century as the standard test for the diagnosis TB infection and disease (Mendel, 1908). The TST test is based upon the type IV hypersensitivity reaction, in which a standard dose of 5 tuberculin units is injected intradermally into the forearm and read 48 to 72 hours later which is modified by Linnikova in 1939. The TST is based on the principle that T cells of individuals sensitized with Mycobacterial antigens, produce IFN-$\gamma$ and a variety of other cytokines that recruit and activate macrophages and other nonspecific inflammatory cells producing induration after an average 48 hours. The reaction is read by measuring the diameter of induration across the forearm, perpendicular to the long axis in millimetres. No induration is recorded as "0 mm", whereas reactions over 10 mm in size are considered positive in non-immunocompromised persons. The main drawback with the
clinical use of the TST is the lack of specificity due to cross-reactivity with proteins present in other *Mycobacteria*, such as BCG or mycobacterium other than tuberculosis (MOTT) (Farhat et al, 2006). Moreover, several factors such as age, poor nutrition, acute illness or immunosuppression induced by medication or HIV infection may contribute to false-negative results.

**Interferon gamma release assays.** As a replacement for the Mantoux test, several other tests are being developed. IGRAs are based on the same principle as the TST, that T cells of individuals sensitized with *M.tuberculosis* produce IFN-γ when they re-encounter *Mycobacterial* antigens. IGRAs quantify the amounts of antigen-specific IFN-γ in blood culture supernatants (Quanti FERONTB Gold, Australia) or determine the frequency of IFN-γ producing blood leukocytes (SPOT-TB assay, Oxford Immunotec, Oxford, UK) in response to specific MTB peptides such as early secretory antigenic target 6 (ESAT-6) and culture filtrate protein (CFP) 10 (Ravn et al, 2005). The only disadvantage of this assay is that immunocompromised patients show false negative due to lack of T-cells.

**Molecular methods**

Polymerase chain reaction has contributed to a more rapid and reliable diagnosis of pulmonary TB. This technique allows the amplification of specific target sequences of nucleic acids such as *hsp65*, *16SrDNA*, *38KDa*, *IS6110* etc. that can be detected through the use of nucleic acid probes; both RNA and DNA amplification systems are commercially available (Daley et al, 2007, Ling et al, 2008, Kulkarni et al, 2012).

**Tuberculosis control**

**Vaccination.** Albert Calmette and Camille Guerin produced an attenuated (BCG) strain by subculturing a strain from bovine tuberculosis for 13 years, about 230 times. This was used as a vaccine first in 1921. Though BCG is generally considered a vaccine
against Tuberculosis; it has also provided protection against leprosy in four major trials (Setia et al, 2006; Merle et al, 2010).

According to reports, BCG is ineffective in preventing infectious form of TB in adults but it gives protection against lethal form of tuberculosis like meningitis and disseminated disease (Rodrigues et al, 1993) especially in children. However, high coverage (>80%) of BCG vaccination does not contribute significantly to reduction in transmission. In view of the failure of the present BCG vaccine, a number of laboratories have attempted to develop better vaccines for TB.

Broadly, two approaches have been used to improve the TB vaccine. The first involves subunit vaccines and the other one is live vaccines. Recent results from several researchers have indicated that non-viable subunit vaccines based on soluble proteins such as ESAT-6, 85 KDa etc. with adjuvant can induce demonstrable level of specific immunity to challenge with \textit{M.tuberculosis} (Brandt et al, 2000; McShane et al, 2005). DNA vaccination with naked DNA constructs for antigen 85 and \textit{hsp60}, have been engineered. Both protein and DNA vaccine induce partial protection against experimental TB infection in mice but their efficacy has generally not been better than BCG (Kamath et al, 1999).

For the development of live TB vaccine, many of the groups preferred using BCG as their vector. BCG is good adjuvant and gives long lasting immunity without any side effects. So, modifying BCG vaccine is considered as a better approach in recombinant vaccine development. Several attempts have been made to improve immunogenicity of BCG either by enhancing its CD8+ T cell stimulating capacity or by endowing it with Th1 cell-inducing cytokines (Freidag et al, 2000). Among them, MVA85A (modified vaccinia Ankara 85A), has shown most promising result in Phase-I clinical trial, produces higher levels of long-lasting cellular immunity when used together with BCG (McShane et al, 2004). But, very recently the vaccine failed in a Phase-II clinical trial in South Africa that involved 2,794
healthy children (aged four to six months) (Tameris et al, 2013). All the other candidate vaccines are being evaluated only in animal experiments so far and hence it may take a long time to get a better vaccine in the market.

**Chemotherapy.** *M.tuberculosis* is a slow growing organism which grows intermittently or remains dormant for a prolonged period. The main goals of anti-TB chemotherapy are (1) to convert the sputum AFB positive individuals to negatives in the shortest time thereby reducing the transmission (2) to prevent the emergence of drug resistance and (3) to assure a complete cure without relapse. The World Health Organization and the International Union against Tuberculosis and Lung disease (IUATLD) recommended standardised TB treatment regimens, called directly observed treatment short course (DOTS) program in 1994 (Raviglione and Pio, 2002). A combination of four drugs referred to as first line drugs (Isoniazid, rifampicin, pyrazinamide and ethambutol) is used together in initial treatment for 6 months under close supervision. The combination of four or more drugs is required to kill several subpopulations of *M.tuberculosis* each of which has a distinctive metabolic status and therefore, varying susceptibility to anti-TB drugs. The subpopulation growing rapidly at the wall of the cavity due to favourable growth conditions of high oxygen content in a neutral pH which is particularly vulnerable to isoniazid. The second slow growing population in intracellular acidic environment is effectively killed by pyrazinamide. The third subpopulation present in caseous material when pH is neutral but the oxygenation is poor is killed efficiently by rifampicin (Davidson, 1992). There are several different possible regimens recommended which vary depending on patient category. This short course regimen can cure 95-99% of susceptible TB cases among patients not infected with HIV as compared to 60-65% of cases with HIV.

**Obstacles in TB control**

**Multi-drug resistant (MDR) tuberculosis:**
Drug resistant tuberculosis is a form of TB in which MTB is resistant to one or more anti-tuberculosis drugs. The initial resistance is a mixture of primary resistance and unknown acquired resistance (7.5% in all new TB cases). Multi-drug resistance in MTB is defined as resistance to at least rifampicin and isoniazid with or without resistance to other anti-TB drugs. MDR tuberculosis usually occurs in chronic cases where the rate of acquired resistance is around 20%, in which resistance to both rifampicin and isoniazid occurs in 4-10% of cases. The prevalence rates of MDR, particularly in developing countries, are as high as 48%. Treatment of patients with MDR tuberculosis involves treatment with second line, reserve drugs like kanamycin, cycloserine, capreomycin, norfloxacin, which are toxic as well as very expensive. In India 1-3.3% of new TB patients have MDR TB and this will account for an estimated 20,000 new infectious cases of MDR-TB every year.

Factors that contribute to increased incidence of MDR-TB include the AIDS pandemic, populations with easy access to antituberculosis medications, deterioration of public health infrastructure, inadequate training of health care providers and above all non-compliance with medication. It has been reported that there is a close correlation between intravenous drug abuse and active TB, and between HIV infection and MDR-TB. A person suffering from HIV has decreased immunity which causes lesser effective anti-TB treatment and hence leading to a high mortality rate.

**TB/HIV co-infection**

Tuberculosis is one of the most common opportunistic infections in HIV patients. Globally, 36.1 million people suffering from HIV/AIDS and 70% of them live in sub-Saharan Africa. HIV fuels the TB epidemic as it profoundly reduces T cell immune response and effectively increases the chance of reactivation of a latent TB infection. In HIV infected patients, TB often presents with an atypical picture that confounds diagnosis. Approximately 4 million people are infected with HIV in India of which approximately half are infected with...
M. tuberculosis. The increasing prevalence of HIV leading to AIDS epidemic in India represents a serious threat to TB control efforts. The proportion of tuberculosis patients among HIV seropositive individuals was shown to be 14.6%. One of the most worrisome aspects of the HIV/TB coinfection is the rapid spread of MDR-TB among HIV infected persons. Nosocomial transmission of TB is of grave concern especially where MDR-TB and HIV infection are prevalent (Pearson et al, 1992).

1.3. Mycobacterium tuberculosis: The Pathogen

Mycobacterium tuberculosis, an obligate aerobe is 1-4 µm long and 0.3-0.6 µm in diameter, nonmotile, rod-shaped bacterium distantly related to the Actinomycetes. Many nonpathogenic Mycobacteria are components of the normal flora of humans, found most often in dry and oily locales. The bacterium is a facultative intracellular parasite, usually of macrophages, and has a slow generation time, 15-20 hours, and a physiological characteristic that may contribute to its virulence. It is not classified as either Gram-positive or Gram-negative because it does not have the chemical characteristics of either, although the bacteria do contain peptidoglycan (murein) in their cell wall. They are closer to Gram-positive organisms based on the phylogenetic analysis of 16S rRNA sequences (Pitulle et al, 1992) and it stains very weakly as positive. Mycobacterium species along with members of related genus Nocardia are classified as acid-fast bacteria. Cells of acid fast bacteria retain the fuchsin dye after acid extraction while other bacteria are decolorized. The cell wall of Mycobacteria, in addition to peptidoglycan is rich in waxes which include a group of compounds known as mycolic acid. The mycolic acid reacts with fuchsin (a basic red dye) and the mycolic acid fuchsin complex acts as a permeability barrier and impedes penetration of mineral acid. Genus Mycobacterium is broadly divided into two major categories:

Fast growers- they consist of strains of species which yield colonies on solid medium that are visible to the naked eye within less than seven days.
Slow growers- they require seven or more days to yield visible colonies.

Among the slow growers the *Mycobacteria* are grouped as:

- The **Mycobacterium tuberculosis complex** (MTC): This complex comprises *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium canettii*, *Mycobacterium pinnipedii*, *Mycobacterium africanum* and *Mycobacterium microti*. *M.tuberculosis* complex *Mycobacteria* were termed as ‘typical’ and all other species of *Mycobacteria* (except *M.leprae*) were placed in the class of ‘atypical *Mycobacteria*’. Other terms used for atypical mycobacterium were ‘non-tuberculous’ *Mycobacteria* (NTM) or *Mycobacteria* other than tubercle bacilli’ (MOTT).

- The **Mycobacterium avium complex** (MAC): The MAC consists of *Mycobacterium avium*, *Mycobacterium intracellulaire* and *Mycobacterium xenopi*. It is also referred to as *Mycobacterium avium* intracellulaire (MAI) complex.

**Phenotypic identification of the MTB**

**Colony morphology**: Colonies of primary MTB cultures invariably have a characteristic patterned texture, due to tight cording of the bacterial cells (Runyon, 1970), and this feature is often used to distinguish MTB from other *Mycobacterial* species, such as *M. avium*. While virulent *M. avium* is associated with smooth and transparent morphotype, virulent MTB is mostly associated with rough morphotype although some study observed the opposite (Schaefer et al, 1970). Colony morphology conversion of *Mycobacteria* has been shown to be associated with changes in constituents of cell wall, such as glycolipids (Belisle & Brennan, 1989), lipooligosaccharides (LOSs), and mycosides (Daffe et al, 1991). Such changes in colony morphology are not usually observed among clinical isolates MTB within a few passages.

**Biochemical tests**: Biochemical indicators for the differentiation within the MTC include nitrate reduction on modified Dubos broth, niacin accumulation, growth in the presence of
thiophen-2-carboxylic hydrazide (TCH), catalase activity at room temperature, and growth characteristics on Lebek and on bromocresol purple medium (Kent & Kubica 1985).

**Genome:**

The biggest achievement in our knowledge about TB during the last decade was the availability of the complete genome sequence of the laboratory reference strain H37Rv (Cole et al, 1998). Its size is 4 million base pairs, with 3959 genes. Forty per cent of these genes have had their function characterised, with possible function postulated for another 44%. Within the genome, there are also 6 pseudogenes excluding insertion sequence elements.

It represents the second largest bacterial genome sequence currently available after that of *Escherichia coli*. The genome is rich in repetitive DNA, particularly insertion sequences, and in new multigene families and duplicated housekeeping genes. The characteristically high guanine plus cytosine content (65.5%) was found to be uniform along most of the genome, confirming the hypothesis that horizontal gene transfer events are rarely present in modern MTB (Sreevatsan et al, 1997). Only a few regions showed exception to this rule. A conspicuous group of genes with a very high G + C content (>80%) appears to be unique in *Mycobacteria* and belong to the family of PE and PPE (full form) proteins. In turn, the few genes with particularly low (50%) G + C content are those coding for transmembrane proteins or polyketide synthases. Fifty genes were found to code for functional RNAs.

Ribosomal RNA (rRNA) gene sequences are highly conserved among prokaryotes and have been used to establish phylogenetic relationships among *Mycobacteria*. The information about 16S rRNA sequence clearly separates fast growers from slow growers among *Mycobacteria* and show high levels of similarity among the group (Cox & Katoch 1986). Pathogenic and closely related slow growing *Mycobacteria* have a single rRNA (*rrn*) operon whereas fast-growing species of mycobacterium have two operons. Most of the other bacteria have multiple copies of the operon e.g. *E.coli* has seven (Bercovier et al, 1986). The
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rrn operon is situated about 1500 kb from OriC in Mycobacteria whereas other bacteria have one or more rrn operons near to OriC. The rrn operon is located downstream from a gene thought to code the enzyme UDP-N-acetylglucosamine carboxyvinyl transferase (UNAcGCT), which is important to cell wall synthesis (Gonzalez et al, 1996). The above facts explain the slow growth rate of MTB.

**Genes involved in virulence:** MTB does not have any classical virulence factors like other pathogenic bacteria. So, bacterial load in infected animals is popularly considered, as associated with virulence with this pathogen. A serine/threonine kinase, Pkn G, is suggested to be essential for the survival of Mycobacteria inside macrophages (Walburger, 2004). PknG is secreted within the Mycobacterial phagosome and involved in blocking phagosome–lysosome fusion. However, *M.tuberculosis pknG* knockout mutants are impaired in growth both in the *in vitro* stationary phase and in mice (Sassetti et al, 2003; Cowley et al, 2004). It is also demonstrated that disruption of *M.tuberculosis hma* gene results in defective oxygenated mycolic acid synthesis causing deficiency in growth in mouse. Further, disruption in cell wall related genes often cause lower bacterial growth in infected animals, as cell wall lipid components are also considered as virulent factors for this pathogen. Glyoxylate cycle enzyme isocitrate lyase (ICL) is required for fatty acid catabolism and in the glyoxylate shunt pathway. A persistent infection of MTB requires intact *icl* gene expression, so it is considered as virulent factor of pathogen. Likewise, other virulence factors have been described: catalase-peroxidase, which protects against reactive oxygen species produced by the phagocyte; *mce*, which encodes macrophage-colonizing factor, phospholipases C, lipases, and esterases, which might attack cellular, or vacuolar membrane as well as several proteases. *M.tuberculosis* sigma factors which regulate gene expression in response to environmental stress are also shown to confer virulence (Hahn et al, 2005).

**Classification and Phylogeny of Mycobacterium**
MTB has evolved through single nucleotide substitutions, deletion and duplication events, so the population structure is strongly clonal (Gagneux and Small, 2007). The typical MTC members show more than 99.95% sequence similarity at the nucleotide level, with little or no evidence for the exchange of chromosomal DNA between strains. In spite of the close sequence similarity between members of the MTC complex, it is easy to distinguish the various members using molecular biology techniques.

The phylogeny for MTB complex was established for the first time when the strains were divided into three “major genetic” groups using mutations at katG463 and gyrA95 (Sreevatsan, et al, 1997) loci. Further, the diversity in MTB genome, especially in the human-adapted strains, was demonstrated by evaluating polymorphisms at different regions like, insertion elements, spacer elements in the direct repeat region and Mycobacteria interspersed repetitive unit (Van Embden et al, 1993; Supply et al, 2001). Six phylogenetically distinct SNP cluster groups (SCGs) and five subgroups were identified based on 212 SNP markers (Filliol et al, 2000).

Comas and Gagneux, in 2009, in a robust phylogenetic study based on genomic deletion analysis, using large sequence polymorphisms (LSPs) and their geographical distribution, demonstrated that MTB strains could be grouped in six main lineages and 15 sublineages. It is possible that such clonal lineages may evolve specific virulence characteristics (Nicol & Wilkinson, 2008).

Lineage -1, Indo-oceanic group of bacteria, consists of East-African-Indian (EAI) and MANU1 strains which branched off from a common ancestor at an early stage of evolution are referred to as evolutionarily “ancient” lineages. The Indo-Oceanic lineage is almost entirely restricted to pulmonary TB patients originating from either the Indian subcontinent or Southeast Asia. This lineage was shown to induce higher Th1 response compared to Lineage-2 in the PBMC of infected person and their household contacts (Rakotosamimanana et al,
The most widely studied strains of MTB belong to Lineage 2 (East Asia/Beijing). However, the clinical and epidemiological characteristics of Lineage 2 are not consistent. A few studies described an association of Lineage-2 with extra-pulmonary (Kong et al, 2005; Kong et al, 2007) or meningeal TB (Caws et al, 2008) whereas, another study confirmed no such association (Nicol et al, 2005). Further, a few groups also proved the HIV association with this lineage (Caws et al, 2006; Middelkoop et al, 2009); while others discard such hypothesis (De Jong et al, 2009). However, in most of the studies, it was confirmed that these strains induce lower levels of proinflammatory cytokines than H37Rv (Sohn et al, 2009; Tanveer et al, 2009), and also was associated with increased growth in human monocytes (Li et al, 2002). This lineage was also linked with higher levels of necrosis and lower levels of apoptosis, in infected macrophages (Sohn et al, 2009).

Lineage-3, mostly prevalent in India and East-Africa, consists of CAS strains induce less proinflammatory cytokines compared to H37Rv (Tanveer et al, 2009). CAS strains were shown to be associated with extrapulmonary disease (Lari et al, 2009). The Euro-American lineage or Lineage-4 was identified in patients from Europe, America, Caribbean, Middle East, and all subregions of Africa and collectively accounted for 91% of all TB cases. It was also shown that Lineage-4 was predominantly involved in pulmonary than meningeal TB (Caws et al, 2008). Among the well studied strains, H37Rv/Ra, Erdman and CDC1551 belong to Lineage 4 (Euro-American). These strains grow more rapidly in liquid culture and generally induce high levels of TNF-α and IL-12 in the host (Sarkar et al, 2012).
Figure 2: A simplified schematic diagram of host–pathogen interaction in Tuberculosis: The diagram is showing the interaction of the infected antigen-presenting cell and an antigen specific T cell after MTB infection. The key pathways in the host’s immune response are shown as solid arrows that can suppress (red) or enhance (blue) bacterial growth, together with the known bacterial products (white boxes, dotted arrows) that can interfere with the host’s response. (Source: http://www.ncbi.nlm.nih.gov/pubmed/19400867)

Lineage-5 and Lineage-6 consist of Mycobacterium africanum which is prevalent in western Africa, where it causes up to 50% of smear-positive TB cases (De Jong et al, 2007). These strains are ancient and heterogeneous, lie in between M. bovis and MTB. On the basis of geographic origin and biochemical properties, M. africanum species has been subdivided into two major subgroups; those from West Africa are subtype I (Lineage-5), closer to M. bovis, while those from East Africa are closer to MTB and are subtype II (Lineage-6). Earlier animal studies suggested that M. africanum possessed lower virulence (Meyer et al, 2008) and induced mostly higher proinflammatory response in ex vivo infection. (Portevin et al, 2011) compared to MTB.
1.4. Host immunity to *M. tuberculosis*

**Innate immunity**

It is believed that the host innate immunity provides the initial resistance to infections with intracellular pathogens, such as *Mycobacteria*, before the adaptive immunity fully develops. The major cellular components involved in innate immunity include phagocytes; macrophages, neutrophils, dendritic cells (DCs); natural killer (NK) cells; γδ T cells, and soluble mediators released by these cells serve as a links to cell-mediated immunity (Figure-2). During the initial phase of infection, *Mycobacteria* are ingested by resident alveolar macrophages. However, *Mycobacteria* can also be ingested by alveolar epithelial type II pneumocytes (Bermudez & Goodman, 1996), found in greater numbers than macrophages in alveoli. Overall, phagocytic cells play a key role in restricting the multiplication and dissemination of intracellular pathogens, as well as initiation and direction of the adaptive immune response. In addition, DCs, known to be much better antigen presenters than macrophages (Tascon et al, 2000; Marino et al, 2004), play an important role in the early stages of infection through presentation of specific *Mycobacterial* antigens to T cells (Wolf et al, 2008). A number of receptors are critical for MTB detection and uptake by phagocytes. Entry of *Mycobacteria* into phagocytic cells can occur through binding to multiple receptors. The pathogen recognition and uptake is described below:

**Pathogen associated molecular patterns**

The interaction between the MTB cell wall components and host cell surface receptors is of major importance in the pathogenesis of MTB infection (Figure-3). MTB contains a wide variety of bioactive lipids that have been implicated in the pathogenesis of the bacillus (Sartain et al, 2011). About 60% of the cell wall of MTB is composed of lipids including mycolic acids, trehalose containing lipids and several lipo-conjugates (Berg et al, 2007). In particular, the surface of MTB is dominated by a group of biosynthetically related
mannosylated lipoglycoconjugates, which mediate host cell recognition and entry through pattern recognition receptors (PRRs) (Torrelles & Schlesinger, 2010). The terminal mannose caps of ManLAMs from different MTB strains vary which determine their avidity for the mannose receptor (Schlesinger et al, 1996). ManLAM caps also bind to dendritic cell (DC)-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) on DCs (Tailleux et al, 2003a). Phosphatidyl-myo-inositol mannosides (PIM) are another major phospholipid component of the Mycobacterial cell wall. They are defined as families by their number of mannose units (1–6) and as species by their number of fatty acids (2–4) (Khoo et al, 1995). Higher-order PIMs (PIM5f and PIM6f) only can be recognized by the mannose receptor. Conversely, lower-order PIMs and lipomanan (LM) are recognized by DC-SIGN and complement receptor 3 (CR3), independent of their degree of acylation. It was observed that virulent strains have significantly more higher-order PIMs and much less lower-order PIMs as compared to avirulent ones (Torrelles et al, 2006).

**Internalization of MTB by host cells**

Endocytosis of MTB involves different receptors on the phagocytic cell (Figure 3) which either bind to non-opsonized MTB or recognize opsonins on the surface of MTB. It includes complement receptor (CR) 1,3, 4, the mannose receptor, Toll like receptor (TLRs), CD14, Fc-γ receptor, scavenger receptor and surfactant protein (SP) receptors (Ernst, 1998; Stuart et al, 2005; Philips et al, 2005). Many of them function as pattern recognition receptors (PRRs), that recognise conserved features present on microbes (Janeway, 1989) and signal downstream to activate rearrangement to allow uptake via the plasma membrane (Aderem and Underhill, 1999).

**Mannose receptors:** one of the most important receptor utilized for the entry of mycobacterium is the mannose receptor (Ezekowitz et al, 1990; Schlesinger, 1993). The best characterized receptor for non-opsonin-mediated phagocytosis of MTB is the macrophage
mannose receptor (MR), which recognizes terminal mannose residues on *Mycobacteria* (Schlesinger, 1993; Schlesinger et al, 1996). MR is a C-type lectin involved in the internalization of a variety of cargo (Ezekowitz et al, 1990) and a link between innate and adaptive immunity (Prigozy et al, 1997). The expression of mannose receptor is down regulated in activated macrophages which suggest that mannose receptor mediated uptake predominantly plays a role during the initial stages of *Mycobacterial* infection.

**Complement receptors**: There have been accumulating evidences of the role of complement receptors (CR1, CR2, CR3 and CR4) in the phagocytosis for MTB (Schlesinger et al, 1990). Expression of CRs (particularly CR4) and MR increases when monocyte differentiates into macrophages. Among the complement receptors, CR3 is the most important as absence of CR3 reduce phagocytosis of MTB by approximately 70 to 80% by human macrophages and monocytes (Schlesinger et al, 1990; Schlesinger 1993).

![Figure 3: Host cell recognition and response to MTB.](http://www.wiley-vch.de/books/sample/3527318879_c01.pdf)

**Surfactant proteins**: Surfactant protein of the lung, especially SP-A and SP-D regulates the early interaction between *Mycobacteria* and macrophage. These proteins opsonise
mycobacterium and then the bacterial complex are internalized by SP receptors. SP-A thus increases the phagocytosis of *Mycobacteria* through a direct interaction of the protein with macrophage (Gaynor et al, 1995), which upregulates mannose receptor activity (Beharka et al, 2002). Interestingly, it has been reported that human immunodeficiency virus infected individuals have increased levels of Sp-A in the lungs, and this results in a threefold-greater attachment of MTB to alveolar macrophages (Downing et al, 1995). Surfactant proteins also inhibit MTB-induced NO production in IFN-γ primed murine macrophage (Pasula et al, 1999). SP-D opsonised MTB undergoes increased phago-lysosomal fusion and hence decreased intracellular survival (Ferguson et al, 2006; Harris et al, 2000). Again, it has been found to block the uptake of pathogenic strains of MTB in macrophages (Ferguson et al, 1999). It may therefore be hypothesized that the relative concentrations of different surfactant proteins also have a role in TB infection.

**Toll like receptors (TLRs):** Toll-like receptors (TLRs) are essential for microbial recognition on macrophages and dendritic cells (Visintin et al, 2001). Members of the TLR family are phylogenetically conserved mediators of innate immunity. These are transmembrane proteins containing repeated leucine-rich motifs in their extracellular domains, similar to other pattern-recognizing proteins of the innate immune system. The cytoplasmic domain of TLR is homologous to the signalling domain of IL-1 receptor (IL-1R) and links to IRAK (IL-1R-associated kinase), a serine kinase that activates transcription factors like NF-κB to signal the production of cytokines (Oddo et al, 1998). To date, at least 10 TLRs have been identified; of those TLR2, TLR4, and TLR9 seem responsible for the cellular responses to peptidoglycan and bacterial lipopeptides (Yoshimura et al, 1999), endotoxin of gram-negative bacteria (Schlesinger et al, 1990), and bacterial DNA (Gerckcn et al, 1994), respectively. MTB lysate or soluble *Mycobacterial* cell wall associated lipoproteins induce production of IL-12, a strong proinflammatory cytokine through TLRs (Brightbill et
al, 1999). MyD88 (myeloid differentiation protein 88), is a common signalling component that links all TLRs to IRAK (Oddo et al, 1998), found to be essential for MTB-induced macrophage activation (Underhill et al, 1999). A mutation of TLR2 specifically, although incompletely, inhibited MTB induced tumour necrosis factor alpha (TNF-α) production. It suggests that besides TLR2, other TLRs may be involved (Underhill et al, 1999). TLR2 was necessary for signalling by the *Mycobacterial* LPS LAM (Means et al, 1999) and a 19-kDa lipoprotein (Noss et al, 2001; Brightbill et al, 1999). TLR-4 *Mycobacterial* ligand is not well defined. Interestingly, *Mycobacterial* infection and proinflammatory cytokines increase surface expression of TLR2 (Wang et al, 1999). Furthermore, the increased expression of CD14 and TLRs did not alter uptake of MTB in *‘in vitro’* studies. Interestingly, a study showed that TLR2 activation directly led to killing of intracellular MTB in alveolar macrophages *in vitro* (Thoma-Uszynski et al, 2001). It may be anticipated that genetic polymorphism, or perhaps mutations, in the relevant TLR or the downstream signalling proteins will affect the performance of the innate host response to *Mycobacteria*.

Thus, there are multiple mechanisms for the uptake of MTB, which may lead to differences in signal transduction, immune activation, and intracellular survival of the pathogen. Virulent strains of MTB are phagocytosed through MR, while attenuated strains use other receptors (Schlesinger, 1993). It has been shown that entry through MR triggered less ROS production as there was less NADPH oxidase activation (Astarie-Dequeker et al, 1999). Moreover, it induced an anti-inflammatory signal to the phagocyte (Nigou et al, 2001).

**Host intracellular trafficking and phago-lysosome formation**

Once taken up, the bacteria begin to disrupt the mechanisms of phagosome maturation, creating an intracellular compartment that lacks the acidic, hydrolytic environment needed to kill the bacteria which is called early endosome. However, fusion with other vesicles and membrane remodelling and trafficking still occurs, allowing MTB to
acquire necessary nutrients and export its own proteins (Desjardins et al, 2005). Phagosome maturation is the process in which phagosome remolds through a series of independent events starting from its formation, complete fusion with lysosome and presentation to the surface of the phagocyte as phagocytic cup. Phagosome after internalization also shows transient access to the rapid recycling pathway, as defined by the classic marker of this pathway, transferrin (Hao and Maxfield, 2000). During the early stage of phagosome maturation, many of the early endosomal markers like ‘early endosomal antigen 1’ (EEA1), lysosomal hydrolases such as procathepsin D and Rab5 appear. Phagosome becomes more acidic through the accumulation of v-ATPases and GTPases that pump protons into the compartment and becomes hydrolytically competent through the acquisition of lysosomal enzymes. Lysosomal hydrolases are delivered to the endosomal network by both mannose – 6 –phosphate dependent and independent manner (Schweizer et al, 1996).

MTB interferes with phagosomal maturation by as yet not fully known mechanism. MTB utilise some putative transporters, iron-scavenging molecules and lipid-synthesizing molecules in preventing normal phagosome maturation. ESAT-6/CFP10 and SecA1/2 proteins on *M. tuberculosis* are considered as virulence factors that interfere with this process (Tan et al, 2006; Hou et al, 2008). This process is also dependent, to some extent on blocking of a calmodulin dependent Ca flux by multiple pathogen derived molecules (Russell, 2001; Connolly & Kusner, 2007). Lipids such as trehalose dimycolate can interfere with membrane trafficking, preventing phagosome maturation and surface expression of MHC molecules. Some phagosome-function-inhibiting lipids, such as mannose-capped lipoarabinomannan (ManLAM), appear to be mimics of host phosphatidylinositols, whose presence on the surface of the vacuole normally indicates a maturation state (Chua et al, 2004). Other molecules such as LRG-47 (MacMicking et al, 2003; Deretic et al, 2006) also interfere with tracking and control of the phagocytic vesicle. Finally, the expression by MTB of a
eukaryotic like serine/threonine protein kinase G (Pkn G) can inhibit phagosome–lysosome fusion. The abundance of known (and presumably unknown) genes involved in altering phagosome maturation and trafficking indicates that interfering with this is a major survival strategy for MTB.

**ROI & RNI:**

Putative mechanisms involved in killing of MTB within the phagolysosomes of activated macrophages include the production of reactive oxygen intermediates (ROI) or reactive nitrogen intermediates (RNI). 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 *Mycobacteria* products, including sulfatides and LAM, are able to scavenge ROI (Pabst et al, 1988; Chan et al, 1991, Neill & Klebanoff, 1998). *In vivo*, it was found that p47phox knockout mice, which lack a functional p47 unit of NADPH-oxidase needed for superoxide production, suffer from increased early overgrowth of *Mycobacteria* in experimental infection (Cooper et al, 2000). Therefore, this supports a role for ROI in the killing of MTB. On the other hand, patients with chronic granulomatous disease, who have defective production of ROI, do not seem to display increased susceptibility to tuberculosis (Winkelstein et al, 2000). Additionally, there is a growing body of evidence suggesting that reactive oxygen species (ROS) can also act as signalling molecules and influence cytokine production (Yang et al, 2007). 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 (Nozaki et al, 1997), and inhibition of iNOS was followed by increased bacterial growth in the host (Nicholson et al, 1996). In tuberculosis patients, alveolar macrophages show increased production of iNOS as well. It is now well known that MTB lipid layers are important for ROI and RNI scavenging. As, the
composition of the cell wall and hence the lipids present vary with MTB strains, it may be possible that ROI and RNI response may vary.

**Adaptive response**

Failure of innate immune mechanisms to control the growth of MTB, is possibly related to insufficient production of NO and other immune mediators, after which adaptive immunity becomes important. The increasing immune pressure mounted by the adaptive immunity restores the immunological control. Innate and adaptive immunity are closely connected. Macrophages and dendritic cells, the primary cell types involved in the innate immune response to *Mycobacteria*, play a crucial role in the initiation of adaptive immunity.

Clearance of bacteria by macrophages is in part dependent on macrophage activation by the cytokine IFN-γ secreted by CD4+ T cells, CD8+ T cells and NK cells (Boom 1996; Flynn & Chan, 2001; Kaufmann, 2001; Wang et al, 2004; Feng et al, 2006; Ngai et al, 2007). Infected macrophages secrete pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6, as well as chemokines that lead to the migration of monocyte derived macrophages and DCs to the site of infection (Means et al, 1999; Flynn & Chan, 2001; Marino et al, 2004). The migration of cells to the site of infection results in the formation of granuloma, which functions to restrict further bacterial dissemination (Co Do et al, 2004).

DCs are central to the generation of acquired immunity after carriage of antigens to draining lymph nodes, where recognition by T cells can be maximized (Marino et al, 2004; Tian et al, 2005; Wolf et al, 2008). To optimally prime pathogen-specific Th1 responses, DCs require stimulation through TLRs (Sporri & Sousa, 2005) by the pathogen as well as host-derived factors such as type I and type II IFNs, cytokines, and chemokines (Kapsenberg, 2003). In these compartments, the stimulatory capacity of mature DCs ultimately leads to effector T cell differentiation and memory T cell expansion, which in turn, confer protection.
against MTB in the lungs (Kaufmann, 2001; Kaufmann & Schaible, 2003). In contrast to macrophages, DCs have poor mechanisms to eliminate internalized *Mycobacteria* (Bodnar et al, 2001; Tailleux et al, 2003). Rather, it has been suggested that DCs offer a niche for long-term survival of intracellular bacteria (Bodnar et al, 2001; Tailleux et al, 2003). Presentation of *Mycobacterial* antigens by macrophages and dendritic cells involves distinctive mechanisms. MHC class II molecules present *Mycobacterial* proteins to antigen specific CD4+T cells. These antigens must be processed in phagolysosomal compartments in professional antigen-presenting cells. MHC class I molecules, expressed on all nucleated cells, are able to present *Mycobacterial* proteins to antigen-specific CD8+T cells. This mechanism allows for the presentation of cytosolic antigens, which may be important as certain *Mycobacterial* antigens may somehow escape the phagosome (Mazzaccaro et al, 1996). The importance of MHC class I-mediated antigen presentation has been shown in murine models (Sousa et al, 2000) and tuberculosis patients (Cho et al, 2000; Geluk et al 2000). Other than MHCs, nonpolymorphic MHC class I molecules such as type I CD1 (-a, -b, -c and -d) molecules, which are expressed on macrophages and dendritic cells, are able to present *Mycobacterial* lipoproteins to CD1- restricted NKT cells. These cells are compelling candidates, being able to respond rapidly and subsequently to activate other cell types (Harada & Taniguchi, 2003). Their apparent self-reactivity and ability to quickly release large amounts of cytokines such as IFN-γ, is important in the initiation and regulation of various immune responses (Harada & Taniguchi, 2003). NKT cells are a subset of T cells that co-express a αβ TCR (T cell receptor), but also express a variety of molecular markers that are typically associated with NK cells, such as NK1.1. Unlike conventional αβ T cells, their TCRs are far more limited in diversity and recognize lipids and glycolipids presented by CD1d molecules. *Mycobacteria* modulate expression of antigen presenting molecules in
macrophages, most likely through the production of antiinflammatory cytokines (Pancholi et al, 1993; Gercken et al, 1994).

Thus, all these mechanisms of antigen presentation enable the activation of a larger fraction of T cells. Several studies have shown that protective immunity to TB is dependent on the adaptive Th1 immune responses (Janis et al, 1989; Flynn & Chan, 2001; Kaufmann, 2001; Holtmeier and Kabelitz, 2005) mediated by macrophages, DCs, T cells and their interactions (Flynn & Chan 2001; Berrington & Hawn, 2007).

1.5. Cytokines, chemokines, and other soluble effector molecules in Tuberculosis

**Tumour necrosis factor-α**

Tumour necrosis factor-α (TNF-α) is a prototypic proinflammatory cytokine that is produced by monocytes, macrophages (Valone et al, 1988) and dendritic cells (Henderson et al, 1997) when exposed to live bacteria or microbial products derived from MTB. TNF-α plays a key role in granuloma formation (Kindler et al, 1989; Senaldi et al, 1996), induces macrophage activation, and has immunoregulatory properties (Tsenova et al, 2005). In mice, TNF-α is also important for containment of latent infection in granuloma (Mohan et al, 2001). In tuberculosis patients, TNF-α production is present at the site of disease (Law et al, 1996) and systemic spill over of TNF-α may account for unwanted inflammatory effects like fever. TNF-α is produced as a trimeric surface molecule and is cleaved by TNF-α-converting enzyme to release the trimeric molecule from the cell. Severity of the disease associated with a selective increase of TNF-α in plasma (Bekker et al, 1998), and quick recovery is associated with a rapid decrease of this cytokine in plasma (Hsieh et al, 1999). In synergy with IFN-γ, TNF-α activates macrophages to produce nitric oxide synthase 2 (NOS2), allowing the macrophage to kill intracellularly replicating MTB (Ding et al, 1988). Mice deficient in TNF-α and TNF-α receptor (TNFR) show increased susceptibility to MTB and impaired granuloma formation following infection with MTB (Flesch et al, 1995). In humans, there is
evidence that TNF-α plays an important role in host defence against MTB as patients of Crohn’s disease and rheumatoid arthritis receiving anti TNF-α antibodies are significantly susceptible to tuberculosis reactivation (including miliary and extrapulmonary) (Keane et al, 2001). Variation in both the TNF-α and TNFR genes were associated in linkage with in vitro TNF-α production as well as the development of active TB disease (Stein et al, 2007). The main polymorphisms that have been identified are found in the promoter region at −238 and −308 in Turkish, Chinese, Thai and Colombian populations (Wilkinson et al, 1999; Mege et al, 2006; Mohan et al, 2001). Although the majority of evidence suggests that the TNF-α promoter polymorphisms are not consistently associated with TB susceptibility, many of the studies have been insufficient in size to show minor differences. So, further studies are needed before possible the association with these common polymorphisms can be disregarded.

**Interleukin-1β**

IL-1β is a proinflammatory cytokine that is produced in a myriad of infections and proinflammatory conditions (Dinarello, 1996). IL-1β is produced in the cell in an inactive precursor form (pro-IL-1β) and then is cleaved by Caspase-1 to an active form, which is secreted from the cell through an unknown mechanism. Conversion of pro-IL-1β to IL-1β can occur with numerous bacterial stimuli, including MTB, and can also occur when bacterial products are sensed within the cytoplasm by NLR proteins (i.e. NOD2), which contain a CARD domain important for Caspase-1 activation and inflammasome function. Once secreted, IL-1β acts primarily through IL-1R type I receptor, which further activates the expression of other proinflammatory cytokines. In some cases, however, IL-1β function is blocked by either binding to a membrane-bound decoy receptor (IL-1R type II) or by a soluble receptor antagonist (IL-1RA) that blocks the proinflammatory effects of IL-1β. Evidence that IL-1β plays an important role in the pathogenesis of MTB comes from studies...
on IL-1 knockout animals. Animals that have both IL-1β and IL-1α deleted are unable to clear the Mycobacteria form granulomas as efficiently as wild type mice (Yamada et al, 2000). Furthermore, animals those lack the IL-1R type I have impaired survival and are unable to contain growth of the organism in vivo (Juffermans et al, 2000). This response may be because of impaired cell-mediated immunity, as measured by IFN-γ production. There is also evidence that susceptibility to clinical disease in an African cohort with MTB is associated with IL-1 gene cluster, specifically variation in the IL-1RA gene (Juffermans et al, 2000). In this study, other polymorphisms in the IL-1β and IL-1α gene did not show significant difference in association with TB susceptibility. Polymorphisms in non-coding regions of IL-1β have been linked to human variation in cytokine production and a −511 C allele in the promoter region of the gene is associated with protection from acquiring pulmonary TB (Awomoyi et al, 2005). Furthermore, studies carried out on emigrated Indians in London showed a relationship between polymorphisms in both IL-1β and IL-1RA and the functional ratio of expression and the acquisition of tuberculous pleurisy (P = 0.028), although multiple polymorphisms in either IL-1β or IL-1RA gene when analyzed individually were not associated with differences in TB resistance (Bellamy et al, 1998). The question whether variation in genes of the IL-1 cluster confer risk to clinical TB disease continues to be premature, and further studies are needed before substantial conclusions can be made.

**IL-12/IL-12Rβ1**

IL-12 is a heterodimeric, covalently linked cytokine comprised of two subunits (p40 and p35). The p40 subunit is present in both IL-12 and IL-23, while the p35 subunit is specific for IL-12. IL-12 is mainly secreted by hematopoietic phagocytic cells (monocytes, macrophages, and neutrophils) and dendritic cells, and promotes T-cell differentiation into T-helper 1 (Th1) cells and production of IFN-γ by signalling through IL-12Rβ1 and IL-12Rβ2 (Trinchieri G, 2003). IL-12 plays an important role in stimulating IFN-γ production and
establishing a potent Th1 response to intracellular pathogens such as MTB and Salmonella. Early studies showed that mice, when given exogenous IL-12, developed increased resistance to MTB (Cooper et al, 1997). Furthermore, mice deficient in IL-12p40 and IL-12p35 showed enhanced susceptibility to MTB infection (Khader et al, 2005). The role of IL-12 in Mycobacterial disease has been firmly established by the presence of patients with uncommon polymorphisms or mutations that predispose to severe disseminated Mycobacterial infection in a Mendelian fashion (Casanova & Abel, 2002). In addition to these Mendelian phenotypes, there is conflicting evidence that common variations in the IL-12Rβ1 gene confer susceptibility to MTB. In a Japanese cohort, increase in susceptibility to MTB was reported when three missense non-synonymous polymorphisms (M365T, G378R, and Q214R) were present (Akahoshi et al, 2003). These identical missense polymorphisms were present in a Moroccan cohort at high frequency, but no susceptibility was associated with these SNPs (Remus et al, 2004).

**IL-6**

IL-6 is detected early during Mycobacterial infection at the site of infection (Law et al, 1996; Holland et al, 1998) and suppresses T cell responses (Van Heyningen et al, 1997). As it inhibits the production of TNF-α and IL-1β and promotes in vitro growth of Mycobacterium avium (Shiratsuchi et al, 1991), it can be said that IL-6 promotes infection. Other reports support a protective role for IL-6: IL-6-deficient mice display increased susceptibility to infection with MTB (Ladel et al, 1997), which seems related to a deficient production of IFN-γ early in the infection.

**IFN-γ/IFN-γR**

IFN-γ, the prototypic cytokine of the Th1 cell response, is a cytokine essential for the effective control of MTB in the host. IFN-γ is produced by CD4+ T cells, CD8+ T cells, and NK cells, and it stimulates a mycobactericidal response in macrophages characterized by the
induction of NOS (Flynn et al, 2001; Feng et al, 2006). Indeed, IFN-γ gene knockout (KO) mice are highly susceptible to M.tuberculosis (Cooper et al, 1993) and individuals lacking receptors for IFN-γ suffer from recurrent, sometimes lethal Mycobacterial infections (Flynn et al, 1993; Holland et al, 1998). Th2-type cytokines inhibit the in vitro production of IFN-γ (Powrie & Coffman, 1993; Lucey et al, 1996) and may, therefore, weaken host defence. Mice that fail to produce IFN-γ have disseminated Mycobacterial infection whether challenged by aerosolized route or intravenously (Cooper et al, 1993; Flynn et al, 1993). Furthermore, increased resistance to MTB following intravenous IL-12 administration was abrogated in IFN-γ gene-disrupted mice (Flynn et al, 1993). In humans, a series of uncommon genetic mutations in IFN-γR1 and R2 lead to Mendelian susceptibility to Mycobacterial disease (MSMD) (Casanova & Abel, 2002). There have been inconsistent findings using IFN-γR1 microsatellite markers and association studies with TB susceptibility.

Common polymorphisms in the IFN-γ gene play a role in TB susceptibility. Three polymorphisms in the IFN-γ gene have been studied in various populations (A-1616G, T+874A, C+3234T) (Lio et al, 2002; Rossouw et al, 2003; Vidyarani et al, 2006; Cooke et al, 2006). Variation in the promoter region of the IFN-γ gene disrupts an NF-κB binding site (T+874A) and is associated with an increased frequency of TB in a study comparing 314 South Africans with pulmonary and meningeal TB versus 235 healthy controls (Rossouw et al, 2003). Overall, the evidence strongly indicates that IFN-γ is significantly associated with susceptibility to active TB disease.

**Anti-Inflammatory Cytokines**

**Interleukin-10**

IL-10 is a cytokine produced by macrophages, dendritic cells, B cells and regulatory T-cell subsets after binding of Mycobacterial LAM (Dahl et al, 1996). 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; Barnes et al, 1993). *Ex vivo* production of IL-10 was shown to be upregulated in tuberculosis by some investigators (Torres et al, 1998), but this was not found by others (Lin et al, 1996). IL-10 antagonizes the proinflammatory cytokine response by down regulation of production of IFN-γ, TNF-α, and IL-12 (Gong et al, 1996), essential for protective immunity in tuberculosis. IL-10 transgenic mice with *Mycobacterium* infection develop a larger bacterial burden (Murray et al, 1997). In human tuberculosis, IL-10 production was higher in anergic patients, both before and after successful treatment, suggesting that MTB-induced IL-10 production suppressed an effective immune response.

**TGF-β**

Monocyte and dendritic cells also produce TGF-β, an anti-inflammatory cytokine in response to *Mycobacterium* products which seems to counteract protective immunity in tuberculosis. LAM from virulent *Mycobacteria* selectively induces TGF-β production (Toossi et al, 1995). Like IL-10, TGF-β is produced in excess during tuberculosis at the site of disease (Condos et al, 1998). TGF-β suppresses cell-mediated immunity: it inhibits T cell proliferation and IFN-γ production by T cells and in macrophages it antagonizes antigen presentation, proinflammatory cytokine production, and cellular activation (Epstein et al, 2000). In addition, TGF-β may be involved in tissue damage and fibrosis during tuberculosis, as it promotes the production and deposition of macrophage collagenases (Toossi et al, 1998) and collagen matrix. Naturally occurring inhibitors of TGF-β eliminate the suppressive effects of TGF-β on mononuclear cells from tuberculosis patients and in macrophages infected with MTB (Hirsch et al, 1997). TGF-β selectively induces IL-10 production, and both cytokines show synergism in the suppression of IFN-γ production.

**IL-4**
IL-4 interferes with intracellular MTB infection by the suppression of cytokine IFN-γ and macrophage activation. In mice progressive disease (Hernandez-Pando et al, 1996) and reactivation of latent infection (Howard et al, 1999) 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 double knockout 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 (North, 1998).

**Chemokines: MCP-1 and IL-8**

CCL2, also called monocyte chemoattractant protein 1, is a β chemokine that is induced by monocytes infected with MTB (Lin et al, 1998). CCL2 causes chemotaxis of memory T lymphocytes, NK cells, and macrophages to sites of inflammation. In animal models, there is evidence that CCL2 may modulate disease severity. Mice that overexpress CCL2 showed high levels of CCL2 in all tissues, and these animals were susceptible to intracellular pathogens (Rutledge et al, 1995). Mice with targeted gene deletion of CCL2 failed to show increased susceptibility to MTB (Lu et al, 1998). CCL2 may cause decreased IL-12p40 production and skew the T-cell response away from a Th1 response (Chensue et al, 1996). In humans, variation in the promoter region of CCL2 causes significant changes in chemokine expression to IL-1β (Rovin & Saxena, 1999). Furthermore, genetic analysis in Brazilian patients has indicated that susceptibility to intracellular pathogens (*Leishmania*, MTB) are linked to chromosome 17q11–12, which codes for the CCL2 protein, along with many other chemokines and NOS2A (Jamieson et al, 2004). These authors, however, failed to show significant association of CCL2 promoter polymorphisms with increased TB susceptibility. In contrast, another study with both Mexican and Korean cohorts found increased susceptibility to TB in those with the A-2518G CCL2 promoter polymorphism.
(Jamieson et al, 2004). Overall, elevated levels of CCL2 may favour Th2 cytokine response and decreased IL-12 production.

Another chemokine produced by phagocytic cells and tissue cells after simulation with MTB is IL-8 (Riedel & Kaufmann, 1997). Increased levels of IL-8 are seen in the bronchoalveolar lavage fluid of humans with TB infection, and proportionately higher levels of IL-8 may be associated with increased mortality (Friedland et al, 1995). A polymorphism in the promoter region of IL-8, T-251A, is seen in high frequency in both Caucasian and African American populations. This polymorphism not only is associated with enhanced severity to bronchiolitis in infants with respiratory syncytial virus (Hull et al, 2000) but is also significantly with MTB infection (Ma et al, 2003). Further studies need to be carried out to identify the role IL-8 plays in MTB infection.

1.6. Genetic susceptibility to TB

A series of studies over the past 50 years suggest that host genetic factors influence susceptibility to TB (Casanova & Abel, 2002; Cooke et al, 2006). Although previous studies have uncovered some of the genes involved in human predisposition to Mycobacterial infections, a comprehensive understanding of genetic susceptibility factors remains an elusive and important goal. There are four major lines of evidence to support a genetic basis for susceptibility to TB. First, studies in twins indicate that TB incidence rates among monozygotic twins are more than twice the rate of dizygotic twins (31.4 versus 14.9 TB cases per 100 twins for monozygotic and dizygotic twins, respectively, $P < 0.05$, binary variable multiple regression analysis) (Comstock, 1978). Second, several primary immunodeficiency disorders are associated with susceptibility to Mycobacteria in a Mendelian fashion attributable to rare single gene mutations with high penetrance. These disorders include severe combined immunodeficiency, hyper-immunoglobulin (Ig) E syndrome, chronic granulomatous disease, anhidrotic ectodermal dysplasia with immunodeficiency, hyper-IgM
syndrome, and Mendelian susceptibility to *Mycobacterial* disease (MSMD) (Casanova & Abel, 2002). This latter group of disorders is more selectively associated with *Mycobacterial* infection and sometimes also with *Salmonella* but not with excessive susceptibility to other pathogens. Most of the infections associated with these Mendelian disorders have been from BCG or environmental bacteria. However, some of these disorders are also associated with MTB susceptibility (Lienhardt et al, 2005). The third type of evidence for relationship host genetic makeup and TB susceptibility comes from the study of complex inheritance patterns, where the assumption is that genetic influence is polygenic and attributable to alleles that are common in the population with low penetrance for any single allele. Several genome-wide studies of susceptibility to TB have been performed with family-based linkage studies. These studies have identified several loci that include 2q35 in a Canadian population (Greenwood et al, 2000), 8q12–13 in a study from Morocco (El Baghdadi et al, 2006), 17q11.2 in Brazil (Jamieson et al, 2004), and 15q and Xq in populations from The Gambia and South Africa (Cervino et al, 2002). Fine mapping of the 15q locus in families from Africa (The Gambia, Guinea, and South Africa) suggests that Ube3a or a closely linked gene may contain the causative locus (Barreiro et al, 2006). Efforts to identify the genes underlying each of these associations are ongoing. The fourth line of evidence comes from candidate gene association studies. These studies evaluate whether common polymorphisms in candidate genes are associated with susceptibility to disease. The most common study design is a case–control format with comparison of polymorphism (single nucleotide, insertions, deletions, or microsatellite markers) frequencies between cases and controls. The strength of this study design is the capacity to enrol large cohort sizes. One disadvantage is the problem of population heterogeneity or admixture, where differences in ethnic composition of the cases and controls can lead to false associations that are not attributable to differences in disease susceptibility. A number of candidate genes have been identified in case–control studies for
their possible role in TB susceptibility. The most promising candidates that have shown consistent effects in their association with TB susceptibility in multiple studies include HLA (DRB1) and Slc11a1 (Bellamy, 2003). Other genes with strongly suggestive associations include IFN-γ, TIRAP/MAL, and CCL2. Advances in genomic technology and immunology have accelerated the candidate gene association studies in infectious diseases. Together, these studies are providing insight into human susceptibility to TB and the underlying mechanisms of pathogenesis from genetically regulated variation of macrophage function and the innate immune response.

1.7. Rationale of the study:

A hallmark of the natural history of tuberculosis has long been variability in the outcome of disease. Only 30% of exposed persons show evidence of infection and of those infected, only 10% become ill. Among those who become ill, there is variability in disease time course, severity, and anatomic distribution. MTB acquires genetic changes with time which confers the virulence property to the pathogen as well as the ability to modulate host immune response in its own favour. The precise pathogenesis of TB and the factors determining the highly variable outcome of infection are only partly understood.

It was initially believed that MTB complex constituted a genetically highly conserved group of bacteria with limited phenotypic differences, hence most of the earlier immunological studies have used a limited number of laboratory strains, such as H37Ra, H37Rv and BCG. In 1905, MTB strain H37 was cultivated from the sputa of a 19-year-old male with pulmonary tuberculosis. Later on, this isolated strain was used as standard laboratory virulent strain. In 1922, it was noted that this strain had changed the colony morphology and was not able to produce disease. This attenuated progeny came to be known as H37Ra, an avirulent standard laboratory strain. BCG strain, the world’s most widely used vaccine has also been generated analogously from a *M. bovis* isolate. The clear phenotypic
differences between H37Ra and H37Rv, BCG and *M. bovis* have inspired several groups to seek the genomic differences in them. A 25 kb DNA fragment called *ivg* (*in vivo* growth-promoting locus) has been identified in H37Rv but not in the attenuated H37Ra (Pascopella et al, 1994; Brosch et al, 1999; Gordon et al, 1999). In addition, a 7.9 kb fragment containing genes coding for a putative sugar transferase, oxidoreductase, and a membrane protein are seen in the genome of H37Ra but not H37Rv (Brosch et al, 1999; Gordon et al, 1999). Interestingly, the introduction into H37Ra of genes that are restricted to H37Rv did not correct the attenuated phenotype of H37Ra (Pascopella et al, 1994; Brosch et al, 1999). Studies comparing H37Rv, H37Ra, and BCG have identified phenotypic differences *in vivo* and *in vitro*. For instance, the number of bacteria recovered from the lungs and spleens of infected guinea pigs after inoculation with H37Ra was 100-fold less than with H37Rv at 3 weeks and declined rapidly thereafter (Alsaadi & Smith, 1973). Differences in virulence have been investigated in monocyte and macrophage models. Moreover, epidemiological data showed that differences in transmissibility and virulence among MTB strains are related to the genetic background of the organisms (Valway et al, 1998). Thus, pathogenesis in tuberculosis is driven by many components of the host immune system, pathogen and environment (Van der Spuy et al, 2009). Environmental and host factors clearly contribute to the clinical and epidemiologic behaviour of strains. But, the pathogen variability factor must also be carefully integrated into the investigative process. A coherent knowledge about the factors is still lacking.

The interaction of pathogen with its host cell is a very important area to study in order to know the different survival strategies followed by the pathogen. But, for MTB it is not very easy. First of all, accurately determining laboratory and clinically relevant phenotypes is a significant challenge. For example, early reports of the unusually high growth rate of CDC1551 in mice appear in retrospect to be largely due to the relative attenuation of the
comparison strains. One additional difficulty in trying to link genomic diversity to phenotypic diversity has been the lack of appropriate tools to index genomic diversity and classify strains. As, MTB is a genetically monomorphic organism, single genotyping tool is uninformative for this pathogen (Achtman, 2008; Comas et al, 2009). So, our limited understanding of the genetics of MTB makes it difficult to predict which genetic polymorphisms may be of consequence. Another problem is with the laboratory adaptation of the strains.

The fact that genetically distinct strains of MTB have been prevalent in different population makes it possible to surmise that the susceptibility of these populations may be linked to the immune response against those strains. Again, the genetically different MTB strains from different lineages have been shown to induce differential host responses in macrophages, cell lines and mouse models (Hoal-van Helden et al, 2001; Lopez et al, 2003) and are demonstrated to vary with respect to their virulence, pathology and bacterial load (Marquina-Castillo et al, 2009). It was shown that selected W-Beijing strains elicit less proinflammatotry and Th1 type cytokines than the non-W-Beijing strains. Further, it was reported that CAS1 and Beijing strains, belonging to lineage 2, have lower growth rate and induce lower levels of proinflammatory cytokines in THP-1 cells (Tanveer et al, 2009) as well as macrophages from human PBMNC compared to standard laboratory strain H37Rv (Portevin et al, 2011). In contrast to these observations, another group detected higher induction of TNF-α by Beijing strain in human macrophages (Chacón Salinas et al, 2005). In a mouse model, genetically different MTB strains elicited dissimilar immune responses in the lung, which determines differences in pathology and mortality. The Beijing genotype induced the highest mortality compared to H37Rv and Canetti genotype (Lopez et al, 2003). Additionally, Keane et al, 2000 showed that apoptosis contributed to innate host defence and is influenced by the virulence of the strains. Avirulent or attenuated strains induced
significantly more apoptosis than virulent strains in alveolar macrophages (Keane et al, 2000). Further, it was discernible that depending on TNF-α and IL-10 ratio induced by them, MTB strains modulated the host cell survival and apoptosis (Rojas et al, 1999). It was also noticed that the apoptosis of host cell was also governed by phagocytic index of respective strains (Rajavelu & Das, 2007). Thus, studies published so far evaluated one or two individual parameters, however, comprehensive studies to correlate different parameters induced by well typed clinical isolates have not been reported. Further, it is noticed that there is no comparative evaluation of different genotypes of MTB isolated from India for the immunological responses and other parameters induced by them.

The aim of present study therefore,

- To select and characterize clinical isolates of *Mycobacterium tuberculosis* collected from Mumbai, India by different molecular biology techniques
- To analyze the several innate host responses induced by different clinical isolates of *Mycobacterium tuberculosis* in THP-1 cells
- To assess the cytokine response induced by different clinical isolates of *Mycobacterium tuberculosis* in monocyte derived macrophages (MDM), monocyte derived dendritic cells (MDDC) and whole blood from healthy individuals
- To evaluate the host responses after infection with the same clinical isolates in BALB/c mice