CHAPTER 1

REVIEW OF LITERATURE

"If you can't explain it simply, you don't understand it well enough"

Albert Einstein
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Throughout history, it had always been there, a familiar evil, yet forever changing, formless, unknowable. Where other epidemics might last weeks or months, where even the bubonic plague would be marked forever afterwards by the year it reigned, the epidemics of tuberculosis would last whole centuries and even multiples of centuries. Tuberculosis rose slowly, silently, seeping into homes of millions, like an ageless miasma. And once arrived, it never went away again. Year after year, century after century, it tightened its relentless hold, worsening whenever war or famine reduced the peoples' resistance, infecting virtually everybody, inexplicably sparing some while destroying others, bringing the young down onto their sickbeds, where the flesh slowly fell from their bones and they were consumed in the years long fever, their minds brilliantly alert until, in apocalyptic numbers, they died, like the fallen leaves of a dreadful and premature autumn.

The Forgotten Plague: How the War against Tuberculosis was Won - and Lost

Frank Ryan, 1992

Tuberculosis is a chronic granulomatous disease caused by *Mycobacterium tuberculosis*. It is believed to have occurred even in the era before the beginning of recorded history. Its causative agent, *M. tuberculosis* probably has killed more people than any other microbial pathogen, thereby earning the sobriquet, “Captain Among these Men of Death.” The genus *Mycobacterium* originated 150 million years ago (Daniel, 2006). Rene Laënnec (1781–1826) a French physician who invented the stethoscope along with his mentor Gaspard Bayle pioneered in the anatomo-clinical conceptualisation of pulmonary tuberculosis. Laënnec studied the auscultatory findings to diagnose tuberculosis. He also observed that tuberculous matter turned from grey to yellow, which
then liquified (caseation) and expelled through the airways, leaving a cavity (often calcified) during autopsy (Duffin, 1998). Unfortunately Laënnec himself became a victim and died of tuberculosis in 1826. Subsequently, Robert Koch, in 1882, identified tubercle bacillus as the etiologic agent of tuberculosis. The only method of diagnosing the organism at that time was by stethoscopic examination. The development of tuberculin skin test in 1907 and demonstration of latent tuberculous infection in asymptomatic children was therefore, a major leap in the diagnosis of the disease.

Infection of *M. tuberculosis* in a previously unsensitized, unexposed individual is known as primary tuberculosis. In 95% cases the infection is contained at this stage and the person may become latently infected. A latent tuberculosis infected individual can either remain asymptomatic for a long period of time or the disease can get reactivated following immune surveillance. This type of infection is known as secondary tuberculosis. However, in 5% of the cases the primary infection develops into an active disease. The primary infection usually occurs in the lungs. However it can also localize in pharynx (through tonsils), intestine (terminal ileum) or skin. At this stage it can either be converted into an active disease form or the individual may become latently infected depending upon the immune status.

A major breakthrough in the fight against tuberculosis came in 1908 with the discovery of the BCG (Bacillus Calmette-Guérin) vaccine. Albert Calmette and Camille Guérin subcultured a pathogenic strain of *M. bovis* in a glycerin-bile-potato mixture while studying the effect on its virulence. Eventually after a period of 13 years and having been subcultured more than 230 times, the resulting attenuated strain called Bacille Calmette-Guérin, was found to be avirulent when tested on target animals. Till date BCG remains the only tuberculosis vaccine which is being administered to 100 million children each year. BCG protects children from severe forms of tuberculosis (TB meningitis), for which reason it is still extensively used, although its efficacy varies from 0-77% in protecting adults (Bishai, 2013). Various hypotheses have been forwarded to explain the reasons for the variable efficacy of BCG and include the following:
The masking hypothesis is thought to be one of the reasons for variable efficacy. According to this hypothesis, in case the individual is already exposed to tuberculosis in areas with high background exposure, the natural immunizing effect of background tuberculosis duplicates any benefit of BCG due to a high amount of antigenic similarity between strains of mycobacteria.

Another reason, which is attributed to the genetic variation in BCG strains is the differences between BCG daughter strains. Comparative genomic and transcriptomic analysis by Brosch et al. (2007) revealed extensive variation in gene expression both between early and late BCG daughter strains and with respect to virulent tubercle bacilli. These variations lead to differences in gene expression levels, immunogenicity, and possibly, protection against tuberculosis. The antioxidant production also increased as BCG evolved (Brosch et al., 2007). The findings in these studies revealed that early BCG vaccines may be even more superior to the later ones that are widely used.

Besides this, genetic variation in the population owing to allele distribution like in case of HLA-DR (Brahmajothi et al., 1991), HLA-DQ (Goldfeld et al., 1998) and other gene polymorphisms like Vitamin D receptor (Bellamy et al., 1998) and IFN-γ receptor and natural-resistance-associated macrophage protein 1 (Nramp 1) (Bellamy et al., 1998) have been associated with the susceptibility to tuberculosis in various studies and is postulated to affect the efficacy of BCG.

Exposure of non tuberculous environmental mycobacteria like *M. avium*, *M. intracellulare* and *M. marinum* is also proposed to be one of the reasons for variable efficacy of BCG as it leads to generation of nonspecific immune response to mycobacteria. Administering BCG to someone already exposed to environmental mycobacteria fails to augment the immune response that is already present by preventing the initial multiplication of BCG in host (Brandt et al., 2002). Concurrent parasitic infection is also one of the factors hypothesized to dampen the immune response generated by BCG. This effect may be attributed to a dominant Th2 response during the parasitic infection which might mask the Th1 protective response generated by BCG.
Due to all these reasons affecting the efficacy of BCG and lack of other effective vaccines available, tuberculosis continues to be one of the major health problems of the world. The World Health Organization (WHO) has estimated 8.7 million new cases and 1.4 million deaths from tuberculosis annually. At least, 1.1 million of these TB cases are HIV (Human immunodeficiency virus) infected. To make matters worse, 3.7% of the new TB cases are MDR-TB (multidrug-resistant TB) and of these MDR-TB cases, 9% are XDR-TB (extensively drug-resistant TB) cases. MDR-TB is defined as tuberculosis that is resistant to the first-line treatment anti-TB drug groups i.e. isoniazid (INH) and rifampicin (RMP) whereas XDR-TB refers to MDR-TB resistant to the major second-line drugs groups i.e. fluoroquinolones and injectable drugs (WHO factsheet, 2012). India alone accounts for 25% of the world’s TB cases and almost 60% of MDR-TB cases are from India, China and the Russian Federation. These statistics and the failure of BCG to confer complete protection against TB necessitate the need for true correlates of tuberculosis immunity (WHO factsheet, 2012).

1.1 PATHOGENESIS OF TUBERCULOSIS

The standard test used for determining whether a person is infected with tuberculosis is the Mantoux tuberculin skin test (TST). It is a test that determines whether a person is infected with *M. tuberculosis*. Tuberculin purified protein derivative (tuberculin PPD) is obtained by precipitation of the heated products of the culture, consisting of *M. bovis* and/or *M. tuberculosis* lysate and is capable of generating delayed type of hypersensitivity response. Standard dose of 5 tuberculin units (TU) is injected intradermally in the forearm and read 48-72 hours later. The reaction is interpreted by measuring the diameter of induration caused due to delayed type of hypersensitivity reaction.

An induration of 5 mm is considered positive in individuals infected with HIV, recent contact with TB patient, persons having fibrotic changes in chest X-ray with old healed TB, or patients who have undergone organ transplants and other immunosuppressed patients. An induration of more than or equal to 10 mm is considered positive in case of children less than four years of age or if the individual is a recent immigrant from high
prevalence country, injection drug user, healthcare worker, Mycobacteriology laboratory personnel, person with high risk like diabetes, prolonged corticosteroid therapy, leukemia etc. that place them at high risk. An induration of 15 mm or more is considered positive in individuals with no known risk factors for TB (CDC factsheet 2011). The asymptomatic latent tuberculosis infected subjects can also be diagnosed by a positive TST.

The course of infection of tuberculosis initiates from the inhalation of respiratory droplet nuclei (1 to 2 mm or less in size) facilitating the entry of tubercle bacilli into the body via the respiratory tract. Larger droplets are however expectorated form the lower respiratory tract due to the physical barriers of nasopharynx and upper respiratory tract. Once the organism manages to reach the lungs they have four potential fates. In an immunocompetent person, the host can spontaneously kill the tubercle bacilli effectively and such individuals have no chance of developing the infection in future. In some cases however, the organisms begin to grow and multiply leading to primary tuberculosis. Containment of infection at this stage is known as latent infection. Latent tuberculosis infected individual may remain asymptomatic throughout life with the bacteria remaining in a dormant state or when the immune response is compromised it can cause reactivation tuberculosis. Such individuals give positive tuberculin skin test. However, infection in an immunocompromised individual can directly develop in to active disease (Schluger and Rom, 1997; Kaufmann, 2001) which may progress along several different pathways as follows:

**Progressive pulmonary tuberculosis:** In this case the enlargement of the apical region of lungs occurs with the expansion of the area of caseation. Erosion into the bronchus evacuating the caseous center leads to formation of ragged irregular cavity lined by caseous material. Hemoptysis may also occur following the evasion of blood vessels. The infection may spread to the uninfected tissue by direct expansion of the lesion, through
Figure 1.1: Etiology of tuberculosis. (A) Following inhalation of droplet nuclei containing *M. tuberculosis*, the bacilli may be spontaneously killed by the alveolar macrophages (B) or it may survive thus forming a ‘primary complex’ (C) constituting small infiltrate and draining into lymph nodes. Calcified lesions may be observed on radiographic examination and the PPD-skin test which is a marker for *M. tuberculosis*-specific T-cell response, becomes positive. In most of the cases the infection stabilizes at this stage and the bacilli may remain dormant (D). However, in few cases it may develop into active disease which is either localized (E) or disseminated to various organs/tissue of the body (F). The disseminated infection may also either get stabilized (G) or develop into active form of disease (H). Failing immune surveillance the dormant bacilli may reactivate later in life (I).
airways and lymphatics or hematogenous spread may lead to miliary pulmonary tuberculosis.

*Endobronchial, endotracheal and laryngeal tuberculosis:* In this type, the infective material spreads either through lymphatic channels or from expectorated infectious material. Several minute granulomatous lesions are formed along the mucosal lining, which are sometimes only apparent on microscopic examination.

*Systemic miliary tuberculosis:* Dissemination of infection in this case occurs when the pulmonary venous return to the heart is seeded by infective foci, which leads through the systemic arterial system. The systemic miliary spread can occur in almost any part of the body e.g. liver, bone marrow, spleen, adrenals, meninges, kidney, fallopian tube and epididymis.

*Isolated-organ tuberculosis:* Unlike systemic miliary tuberculosis in this type of infection, miliary dissemination takes place and appears in any one of the organs/tissue. The organs that are typically involved in this type of infection are meninges (tuberculous meningitis), kidney (renal tuberculosis), adrenals (important cause of Addison's disease), bones (osteomyelitis), fallopian tubes (salpingitis) and vertebrate (Pott's disease).

*Intestinal tuberculosis:* In the past drinking of contaminated milk was one of the most common reasons of infection with *M. tuberculosis*. It involves oropharyngeal lymphoid tissue along with spread to the lymph nodes in the neck. Today, most often intestinal tuberculosis is either a complication of advanced tuberculosis or consequent to swallowing coughed-up infective material. The organisms are typically trapped in the mucosa of the small and large intestine, particularly in the ileum.

### 1.2 IMMUNE RESPONSE TO TUBERCULOSIS

The vertebrate immune system protects against any infection by means of employing either the innate immune system or the adaptive immune system, depending on the situation that the host finds itself in. Furthermore, in case of an adaptive immune
response, the host has the additional choice of utilizing either the humoral or cell mediated arm of the adaptive immune response. We know that within the cell mediated arm of the adaptive immune response there are different paths that the immune system can resort to, such as either that involving the T helper system or the cytotoxic T cell system. Both these are mediated through soluble mediators such as cytokine and chemokines, and direct contact in the case of CTLs.

1.2.1 Innate immunity

Innate immunity is the first defense of the body against any infection. Many studies based on rabbit, mice and human models have emphasized on the role of innate immunity in tuberculosis. In studies on mice, it was observed that the mycobacterium susceptible mice had 20-30 folds more viable mycobacteria than the mycobacterium-resistant mice, seven days after the primary infection through inhalation of *M. tuberculosis*. This difference in the immune response was attributed to the innate immunity, as the T-cell immunity develops only two to three weeks after infection (Dannenberg, 1994). Recently, Subbian et al. (2013) attempted to understand the difference in outcome when virulent *M. tuberculosis* strain and vaccine strain were used in a rabbit model. They observed that the outcome of infection and the progression of the disease depended on the initial events taking place in the infected lung. In study on mice, It was seen that the outcome is influenced by the differential regulation of inflammation associated innate immune cells and related gene expression pattern. For e.g. in case of virulent strains, along with the increase in gene expression for inflammatory markers like STAT1, there was also recruitment and activation of macrophages, PMN, and fMLP (N-formyl-Methionyl-Leucyl-Phenylalanine)-stimulation.

Human studies also reiterate the importance of innate immunity in tuberculosis infection. Naturally acquired T-cell immunity fails to counter the exogenous reinfection of the lung (Henderson et al., 1997). Recent study on thymocytes of neonates reported a novel population of innate T cells (αβTCR+ Thymocytes) that would provide early source of IFN-γ and facilitate generation of adaptive immune response (Gold et al., 2008).
Studies have demonstrated that endocytosis of *M. tuberculosis* can either occur through non-opsonized or opsonised manner. In the latter case, complement protein C3 can act as an opsonin and complement receptor 1 (CR1), CR3 and CR4 can then facilitate the binding and uptake of the bacteria by the host macrophage (Hirsch et al., 1994). *M. tuberculosis* also utilizes the classical pathway for activation of complement by directly binding to C2a even without the presence of C4b. Subsequently C3b is generated which acts as an opsonin and binds to CR1 (Schorey et al., 1997).

Alternatively *M. tuberculosis* can directly bind to CR3 and CR4 (Zaffran and Ellner, 1997) and get endocytosed in a non-opsonized manner. The non-opsonin-mediated phagocytosis of *M. tuberculosis* by macrophages is best characterized through mannose receptor (MR) (Schlesinger, 1996). A structurally related group of proteins called collectins also facilitate the binding of *M. tuberculosis* to macrophages. They include surfactant proteins, mannose-binding lectins and C1q (Ernst, 1998). Besides phagocytosis, an effective host response also requires specific recognition of *M. tuberculosis* or mycobacterial products. Different antigens of *M. tuberculosis* are recognized by various recently identified pattern recognition receptors present on the surface of phagocytic cells. In this regard, lipoarabinomannan (LAM) acts similar to gram-negative lipopolysaccharide (LPS) and promotes immune recognition (Underhill, 1999). This recognition is facilitated by Toll-like receptors which are a group of phylogenetically conserved mediators and are crucial for microbial recognition by macrophages and DCs (Visintin et al., 2001). The TLRs are transmembrane proteins having leucine-rich motifs in extracellular domains like the other pattern-recognizing proteins of immune system. TLR is similar to IL-1 receptor (IL-1R) signaling domain and links IL-1R-associated kinase (IRAK) which is a serine kinase that activates NF-kB like transcription factor to signal the production of cytokines. In context of CD14, TLR2 and TLR6 heterodimer binds to 19-kDa *M. tuberculosis* lipoprotein, TLR4 to heat-labile cell-associated factor and TLR9 to *M. tuberculosis* DNA (Akira et al., 2003).

Innate immunity also facilitates the initiation of adaptive immunity. Antigen presentation of mycobacterial antigens by macrophages and dendritic cells is one of the very
important aspects in that regard. MHC class II which is present on antigen presenting cells present the mycobacterial antigens to CD4+ T cells after processing of these antigens in the phagolysosomal compartment. MHC class I on the other hand is present on all the nucleated cells and present mycobacterial peptides to CD8+ T cells. Studies have confirmed the importance of MHC class I mediated antigen presentation both in animal (Sousa et al., 2000) and human models (Geluk et al., 2000). Nonpolymorphic MHC class I mediated antigen presentation of mycobacterial lipoproteins by molecules like type I CD1 (-a, -b and -c) expressed on macrophages and DCs is also seen.

Type I cytokines like IL-12, IL-18 and IL-23 produced by macrophages and DCs are important mechanisms to stimulate T lymphocytes (Oppmann et al., 2000). Mutations in the gene coding for IL-12p40 (Altare et al., 1998), IL-12RB1 (De Jong et al., 1998), IFN-γ receptor 1 (Holland et al., 1998) and IFN-γ receptor 2 (Dorman and Holland, 1998) have been known to make the patient susceptible to recurrent or fatal nontuberculous mycobacterial infections. In addition to these IL-1 (Dinarello, 1996) and TNF-α (Tsenova et al., 1999) also released by activated macrophages and DCs are known to regulate T cell stimulation.

1.2.2 Acquired immunity

Acquired immunity constitutes both cell mediated (T cell mediated) as well as humoral immunity (B cell mediated). Both T cells and B cells collaborate to fight against any infection. The T cells act by promoting the killing of pathogen infected cells by apoptosis or by cytokine activation of other immune cells while B cells make antibodies to neutralize the pathogen and also target them for destruction (Moore et al., 2001).

Humoral immune response: Role of antibodies in defense against *M. tuberculosis*, an intracellular pathogen!

Since *M. tuberculosis* is an intracellular pathogen, humoral immunity was postulated not to provide any significant protective immune response to the disease. However, immunologists such as Albert Calmette and Elie Mechnikoff emphasized on elevated
cellular immunity as a major defense to tuberculosis in the twentieth century. Since then studies carried out were predominantly focused on cell mediated immune response to tuberculosis. Contrary to the preconceived notion of humoral immunity being ineffective in rendering any significant protection against tuberculosis recent studies have now highlighted the role of B cells in tuberculosis immunity.

B-cells have also been known to modulate the immune response to tuberculosis by antigen-presentation. Inspite of the fact that B-cell mediated antigen presentation is dependent on antigen and immunological conditions, studies have been carried out to utilize B lymphocytes to present antigen to T cells by specific vaccination strategies. In fact, Andersen et al., (2007) in their study demonstrated an effective boost in the BCG primed immunity against *M. tuberculosis* using one such B-cell-targeting vaccine. B cells have also been known to act on the co-stimulatory molecules (B7 and CD40) of other antigen-presenting cells and influence the stimulation of T cell response in a more indirect manner (Radhakrishnan et al., 2003). This is also supported by the study of Radhakrishnan et al. (2003) where they observed that naturally occurring human IgM antibody binds to costimulatory B7 molecule on DCs and potentiate the stimulation of T cells by DCs. They pulsed the DC with Ag and treated them with human IgM Ab in vitro and observed a potent T cell response upon the adoptive transfer of these Ab-treated Ag-pulsed DC.

The question that still lingers is whether the antibodies are protective against *M. tuberculosis* and how can B cells modulate immune response against *M. tuberculosis*? Studies using mAb against mycobacterial arabinomannan, heparin-binding hemagglutinin and 16kDa α crystalline in mouse model have been shown to be efficacious (Reljic et al, 2006). These antibodies were known to act in various manners like diminishing mycobacterial burden or decreasing inflammatory progression and thereby enhancing animal survival (Glatman-Freedman, 2006). However, in order to use humoral immunity for developing effective vaccines studies have to be carried out to correlate it with protection in human and animal models.
It was also observed that in immunosuppressive phenotypes absence of B cells diminishes the optimal containment of infection in initial stages and the inflammatory progression of TB to chronic stages is delayed. This phenomenon was explained by phase specific function of B-cells. In case of acute infection, B cells are required for effective immunity against pulmonary infection with *M. tuberculosis* and also for generating granulomatous response. However, during chronic phase of infection when the bacilli are dormant and contained, the immunologically active B-cells can act as APC and stimulate T cells, thereby prevent reactivation of disease (Tsai et al., 2006). Abraham et al. (2013) demonstrated that the strong B cell immune response generated by Rv0265 (tuberculosis PPE protein) could be used not only to differentiate tuberculosis patients from BCG vaccinated controls but also detect extrapulmonary and smear-negative pulmonary cases efficiently. Studies have to be conducted in future to further optimize and take advantage of humoral immunity in tuberculosis protection and diagnostics.

**Cell mediated immunity: Role in protection and pathogenesis**

The fate of tuberculosis infection is therefore mainly governed by the interaction of macrophages with T cells (Kaufmann, 1998). The crucial role of T lymphocytes in immunity to tuberculosis came into light with the emergence of HIV/AIDS epidemic. Tuberculosis became the most common infection following HIV infection. The T cell mediated immunity is mostly governed by CD4+ T helper cells and CD8+ T cytotoxic cells.

**1.2.2.1 CD4+ T helper cells**

*M. tuberculosis* replicates within the macrophages, hence MHC class II recognition of pathogen by CD4+ (helper T cells) cells is a crucial step in generation of immune response. Helper T cells can be divided into two phenotypes (Th1 & Th2) on the basis of cytokines they secrete (Mosmann and Coffman, 1989). The Th1 phenotype secrete pro-inflammatory cytokine IFNγ and IL-2 whereas Th2 phenotype secrete anti-inflammatory cytokine IL-4, IL-5 and IL-10. Th1 and Th2 are mutually inhibitory to each other. Condos et al. (1998) in their study made an important observation of the dominance of...
IFN-γ Th1 response in bronchoalveolar lavage of patients with milder disease showing lack of cavity formation and low bacterial burden. In contrast, in sputum smear positive patients having cavitary lesions was marked absence of Th1 response. The role of Th1 response in protective immune response to tuberculosis was therefore highlighted.

T helper cells mediate the activation of macrophages and other cell mediated reactions including cytotoxic and delayed type of hypersensitive response (Mortaz et al., 2012). The CD4+ T DTH mediates the activation of macrophages and contributes to the killing of the bacilli within the tubercle (Gideon and Flynn et al., 2011). Studies have emphasized the importance of CD4+ T cells in early response to M. tuberculosis. This observation was supported by a study carried out by Caruso et al. (1999) on the immune response of CD4+ T cell-deficient aerosol infected mice. They observed that these mice have transient deficiency of IFN-γ in the lungs. Although this deficiency was compensated by CD8+ T cells within four weeks, the mice still succumbed to infection.

1.2.2.2 CD8+ cytotoxic T cells

Another T cell subset, CD8+ T cells, contributes significantly to protection against the disease by lysing the infected cell and inducing apoptosis of the target cells (Schluger and Rom, 1998; Lazarevic and Flynn, 2002). In addition to production of Th1 cytokine IFN-γ, CD8+ T cells restrict the tuberculosis infection by cytotoxicity due to Granule-dependent exocytosis pathway. CD8+ T cells, on recognition of the infected cells release perforin-containing granules. These granules polymerizes the cell membrane of the target cells, thus allowing the entry of effector molecules like granzyme A and granzyme B (serine proteases) and lysing the target cell. CD8+ T cells also exert cytotoxic effect through Fas/ Fas ligand-mediated cytotoxicity. The Fas ligand expressed on the surface of activated CD8+ T cells is cross-linked to Fas receptor expressed on the target cell leading to recruitment of Fas-associated death domain and activation of caspase 8 and
Figure 2 The Th1/Th2 paradigm. The homeostasis between Th1 and Th2 cytokines is maintained in the body. Following tuberculosis infection, generation of early Th1 cytokine response helps to contain the infection leading to protective immunity. In case the individual is immunocompromised the Th2 response predominates over the inadequate Th1 response leading to impaired immunity and immunopathology.
finally leading to apoptosis of target cells. Due to lysis of macrophages that are infected with the tubercle bacilli the pathogen is released into the extracellular environment where it can be taken up by active macrophages that are better equipped for bactericidal action.

CD8+ T cells granules also contain granulysin which is a molecule that has direct microbicidal effect on the intracellular bacteria (Lazarevic and Flynn, 2002). This antimicrobial peptide however, is not found in mouse but is present in humans and mediates the process of killing intracellular mycobacteria. The studies on CD4+ T cell KO mice indicate that the cytotoxic potential of CD8+ T cells is dependent on CD4+ T cells and that the susceptibility of these mice to tuberculosis infection might be partially due to impaired CTL response. However, cytokine production remains a crucial mechanism of both CD4+ and CD8+ T cells to generate immune response to tuberculosis (Lazarevic and Flynn, 2002). Scanga et al. (2000) in their study on reactivation of *M. tuberculosis* infection in mice via antibody depletion of CD4+ T cells observed a marked increase in the IFN-γ and CD8+ T cells numbers indicating that CD8+ T cells can efficiently compensate for the IFN-γ levels in such mice.

While it is evident that CD4+ T cells are essential for protective immunity to tuberculosis in the early stages of infection, the critical contribution of CD8+ T cells in the later stages of infection has also been reported. Although there is little evidence in vivo, in vitro studies have shown that *M. tuberculosis* might take shelter in epithelial cells which are class II MHC negative as a survival strategy to escape antigen presentation to CD4+ T cells. In such cases the immune response by CD8+ T cells can come into play during this stage of latency (Tully et al., 2005). Besides providing protection in the above mentioned manner researchers have also proposed the possibility of unique functions of CD8+ T cells in immune response to tuberculosis infection. The endogenous antigen presentation to CD8+ T cells via MHC-I restricted manner ensures that CD8+ T cells preferentially recognize and lyse the infected cells, unlike CD4+ T cells that may recognize the infected T cells as well as those cells that have phagocytosed the dead bacteria and their antigen (Lewinsohn et al., 2003).
Figure 3 Control of *M. tuberculosis* infection. **A) Cytokine Production:** Both CD8+ and CD4+ T cells are capable of producing IFN-γ and TNF-α which in turn activates the macrophages to produce reactive oxygen and nitrogen intermediates that mediate the killing of the bacilli. **B) Cytotoxicity:** The CD8+ T cells can directly act on the infected macrophages via *i*) **perforin-containing granules** which polymerize on the cell membrane and facilitates the entry of granzyme A and granzyme B which lyse the target cells.  
*ii*) **Fas/ Fas L apoptotic pathway** mediated by Fas receptor on the macrophage that bind to Fas L on the CD8+ T cells leading to apoptosis by activation of caspase 8.  
*iii*) **Granulysin** is an antimicrobial peptide that act directly on the intracellular bacteria.
1.3 ROLE OF CYTOKINES IN TUBERCULOSIS: A BALANCING ACT

While the effectors in case of a B cell response is the soluble immunoglobulin molecules, which in case of a T cell response are primarily cytokines and chemokines. Cytokines play a key role in orchestrating the immune response. Cytokines may have a protective role, or exacerbate any disease pathology, depending on the type of cytokine involved.

Listeria monocytogenes and Salmonella manage to escape the macrophages and infect hepatocytes. The activation of these hepatocytes by cytokines, IFN-γ and TNF-α along with the infiltration of granulocytes is essential host defense against such infections (Langermans et al., 1994). During Toxoplasmosis it has been observed that Th1/Th17 response can aggravate the hypersensitivity whereas IL-10, TGF-β and IL-27 can counteract the inflammation and prevent immunopathology (Jung et al., 2012). IFN-γ/GM-CSF are being explored as a treatment which can help to improve antigen presentation and immune response against invasive fungal infections like that of Candida and Aspergillus (Mueller-Loebnitz et al, 2013). Similarly in tuberculosis also, cytokines play a major role since the disease primarily involves the T cells of the immune system.

Studies have suggested that the dynamic changes in pro- and anti-inflammatory cytokines governs the outcome of tuberculosis infection (Sahiratmadja et al., 2007; Bose and Jha, 2012). Though studies have emphasized the protective nature of proinflammatory cytokines against the disease, the anti-inflammatory cytokines prevents excessive tissue damage due to inflammation.

1.3.1 Proinflammatory cytokines

1.3.1.1 Interleukin 2 (IL-2)

T cell activation through its antigen receptor is immediately followed by de novo synthesis of IL-2 and IL-2 receptor expression which ensures selective expansion of antigen-specific effector T cell population (Lenardo et al, 1999). The proliferation of CD4+ and CD8+ T cells is thereby the major function of IL-2. The proliferation occurs via proto-oncogenes c-my and c-fos in addition to anti-apoptotic protein bcl-2. Bcl-2 is
involved in glycolysis and cellular metabolism which enhance long-term cell survival (Frauwirth and Thompson, 2004).

IL-2 is also reported to be an important cytokine for cell mediated immune response and granuloma formation (Borish et al., 2003). Studies have suggested that LTBI and active tuberculosis patients can be differentiated by combining IL-2 ELISPOT assay with IGRA. The LTBI secrete IL-2 more than active tuberculosis patients following antigen stimulation (Biselli et al., 2010). This implies that the low bacterial/antigen load may be responsible for increased number of IL-2 secreting and IL-2/IFN-γ secreting central memory T cells and decreased IFN-γ producing effector cells in LTBI individuals (Millington et al., 2011). Suter-Riniker et al. (2011) in their study demonstrated that increase in IL-2/IFN-γ ratios may be considered as a biomarker for elimination of *M. tuberculosis* infection. Recently Lindenstøm et al., (2013) emphasised on the importance of the presence of CD4+ KLRG1- non-terminally differentiated IL-2-secreting central memory T cells at the infection site to maintain the population of TNF-α or TNF-α/IFN-γ coexpressing population of T cells to control bacterial growth. These IL-2+ CD4+ T cells therefore have the potential to replenish T cells and prevent their attrition and functional exhaustion. Zhang et al. (2012) in their study suggested the use of IL-2 and GM-CSF in treatment of multidrug-resistant *M. tuberculosis*.

1.3.1.2 Interferon-γ (IFN-γ)

IFN-γ is a very important component of innate and acquired immunity to tuberculosis and is a potent activator of macrophages. The tubercle bacilli mainly reside in the macrophages but studies reveal that they are also phagocytosed by dendritic cells (Bodnar et al., 2001; Gonzalez-Juarrero and Orme 2001). It was also observed that macrophages preferentially secrete IL-18 whereas dendritic cells secrete IL-12 to stimulate the T cells to produce IFN-γ (Giacomini et al., 2001). A positive feedback loop is created as IFN-γ augments IL-12 production, which in turn induces IFN-γ production.

The protective role of IFN-γ in cell-mediated immunity to tuberculosis has been well established (Flynn et al., 1993). IFN-γ also stimulates the expression of many proteins
involved in antigen presentation including MHC class I and II (Zhou et al., 2009; Giroux et al., 2003). It diverts the differentiation of CD4+ T cells to Th1 lineage and also inhibits Th2 cell proliferation (Smeltz et al., 2002).

IFN-γ, besides being a potent activator of macrophages, enhances the microbicidal activities. It induces the production of reactive oxygen intermediates (ROIs) and nitric oxide (NOIs) (Borish and Steinke, 2003). Studies based on genetic variation in immune response affecting mycobacterial infection suggest that children with mutations in IFN-gammaR gene lead to absence of receptors on macrophage cell surface. These macrophages are unresponsive to IFN-γ stimulation and thereby leaving a defect in TNF-α production. Children having such mutations are therefore more prone to tuberculosis infection (Newport et al., 1996). It was also observed that IFN-γ gene-disrupted mice are unable to control sublethal dose of *M. tuberculosis* administered intravenously or in aerosol from leading to progressive and widespread tissue destruction, necrosis and ultimately disseminated tuberculosis infection (Cooper et al., 1993).

Hence many studies have been focused on *M. tuberculosis* specific IFN-γ production for vaccine. It was observed that PPD and *M. tuberculosis*-culture filtrate could induce IFN-γ production in healthy skin test positive and not in skin test negative healthy individual (van Crevel et al., 1999). However it was observed that the *M. tuberculosis* infected monocytes were capable of stimulating the lymphocytes of both PPD-positive and PPD-negative individuals to produce IFN-γ (Johnson and McMurray, 1994). IFN-γ production can therefore also be used as a marker for identifying latent tuberculosis infected subjects (Dyrhol-Riise et al., 2010). The test most commonly used to identify LTBI is TST skin test. However, the interpretation of TST test is subjective and can lead to incorrect diagnosis. Also false positive results are frequent in individuals who had BCG vaccination in the past or exposure to environmental mycobacteria which share common antigens with *M. tuberculosis*. Also false negative results can be obtained in case the individual is immunocompromised for e.g. HIV (Diel et al., 2010). While interferon gamma release assay (IGRA) has now been developed to overcome these limitations of TST skin test however; the utility of IGRA in countries with high incidence of TB and/or
HIV is still not known (Dyrhol-Riise, 2010; WHO report 2012). It is therefore of utmost importance to screen for immunodominant antigens/epitopes that would differentiate the active tuberculosis patients from LTBI and healthy controls.

1.3.1.3 Tumor necrosis factor – α (TNF-α)

Another Th1 cytokine which contributes significantly to immune response against tuberculosis is tumor necrosis factor – alpha (TNF-α). Mycobacteria or mycobacterial products induce the production of TNF-α by monocytes, macrophages, dendritic cells as well as T cells. In a recent study done by Allie et al. (2013), it was observed that TNF-α inactivation from both myeloid and T-cell sources rendered the mice susceptible to tuberculosis infection. They also reported in their study that while TNF-α production from myeloid cells was crucial in early stages of infection, the T-cell derived TNF-α imparts protection during chronic tuberculosis infection.

The role of TNF-α in maintenance of granuloma was studied by Jacobs et al. (2000). TNF-α is known to increase NFκB expression and thereby increasing the expression of chemokines like IL-8, GROalpha and ENA-78 (Ciesielski et al., 2002). It interacts with the extracellular matrix to direct T cell migration (Czermak et al., 1999). TNF-α neutralization led to decreased chemokine expression (Algood et al., 2004) which could in turn be associated with decreased ability of cells to remain in close proximity or migrate to or aggregate to form granulomas. TNF-α upregulates the expression of adhesion molecules like ICAM-1, VCAM-1 and E-selectin thus facilitating the tethering and diapedesis of leucocytes through the endothelium into the infected tissue. (Madge et al., 2001). Murine studies have shown that in TNF-/- mice the cell recruitment to the site of infection is delayed although it reached similar levels to those in the wild type infected mice eventually (Roach et al., 2002). This indicates that early cell migration depends on TNF-α. It also synergises with IFN-γ to activate antimycobacterial properties of macrophages thereby up-regulating reactive oxygen and nitrogen intermediates, increases NFκB expression and contributes to the recruitment, migration and retention of inflammatory cells at the site of infection (Roach et al., 2002).
Nevertheless, it was also observed that very high levels of TNF-α can lead to tissue damage and increase immunopathology of tuberculosis. TNF-α can be toxic to epithelial cells as it reduces the amount of surfactant protein produced by type II alveolar cells. Further, it also enhances fibroblast activity and increases the production of fibroblast collagenase (Brenner et al, 1989; Solis-Herruzo et al., 1988). The enhanced production of ROIs also contributes to tissue damage (Solis-Herruzo et al., 1988). TNF-α may attribute to pathological signs and symptoms of \textit{M. tuberculosis} infection like fever, weight loss, anorexia and tissue damage (Mootoo et al., 2009). \textit{M. tuberculosis} makes the infected cells sensitive to both protective effects of TNF-α as well as its toxicity. TNF-α also enhances the cellular toxicity of \textit{M. tuberculosis} (Filley et al, 1991; Filley et al, 1992; Rook et al, 1996). Hence TNF-α, is rightly labeled as ‘A cytokine with a Split Personality’ by Mootoo et al., owing to its pro- as well as anti-inflammatory attributes.

Several studies have acknowledged the presence of TNF-α production at disease site (Law et al, 1996; Casarini et al., 1999; Dlugovitzky et al., 1999). However, the systemic spill over of TNF-α may lead to unwanted inflammatory effects like fever and wasting. It was observed that clinical deterioration of the disease can be correlated with selective increase (Bekker et al., 1998) and effective anti-tuberculosis treatment with the rapid decrease in plasma levels of TNF-α (Hsieh et al.,1999).

1.3.2 Anti inflammatory cytokine

1.3.2.1 IL-10

IL-10 is a potent Th2 cytokine that inhibits activated macrophages and dendritic cells. It is produced by macrophages after binding of Lipoarabinomannan (LAM) (Shaw et al., 2000) and also shown to be produced following interaction of PPE proteins with TLR2 (Nair et al., 2009). IL-10 is also produced by T lymphocytes especially \textit{M. tuberculosis} reactive T cells (Boussiotis et al., 2000).

IL-10 downregulates IL-12 production by \textit{M. tuberculosis} infected macrophages. Hickman et al. (2002) in their study performed the cytokine profiling of infected macrophages and observed downregulation of cytokine IL-12. However when the effects of IL-10 were
neutralized by IFN-γ priming, IL-12 production was resumed. Furthermore, it antagonizes the proinflammatory cytokine response by not only downregulating the production of IFN-γ and TNF-α but also blocks the production of ROIs and NOIs which are essential for *M. tuberculosis* control by interfering with the intracellular signaling cascade, involving suppressor of cytokine signaling-3 (SOCS3; Cassatella et al., 1999). Giacomini et al. (2006) in their study on *M. tuberculosis* extracytoplasmic factor σE mutants (sigE), demonstrated the IL-10 impaired CXCL10 production in sigE mutant-infected DC. However neutralization of IL-10 restored CXCL10 secretion. IL-10 is also known to block antigen processing and presentation by various antigen presenting cells (APC).

Murine studies have demonstrated that IL-10 has a crucial role in TB reactivation from latent stage as well as in chronic pulmonary tuberculosis (Turner et al., 2002; Beamer et al., 2008). However the anti-inflammatory actions of IL-10 in chronic tuberculosis may in fact be contributing to prevention of further damage to the infected tissue. Studies based on transgenic mice models have demonstrated the role of IL-10 in preventing tissue damage and facilitation of *M. tuberculosis* growth at the same time (Murray et al., 1997). However, in case of IL-10 deficient mice no effect was observed (North, 1998) but when IL-10 receptor blocking antibody was used it as observed that there was protective effect due to absence of IL-10 (Moore et al., 2001). Studies on human population have also correlated the presence of IL-10 with the susceptibility to tuberculosis infection (Boussiotis et al., 2000; de la Barrera et al., 2004). Recent study shows that stalling of phagosome maturation due to IL-10 in human macrophage facilitates *M. tuberculosis* persistence (O’Leary et al., 2010). Thus, the benefits of anti-inflammatory effect of IL-10 on tissue damage are outweighed by its role in reactivation and persistence of *M. tuberculosis*.

**1.3.2.2 Transforming growth factor – β (TGF-β)**

Transforming growth factor β is mainly secreted by monocytes and macrophages and other cell types like dendritic cells, CD4+ T regulatory cells (Aung et al, 2005). It synergises with the effects of IL-10 and thereby modulates the immune response by down-regulating acquired immunity and de-activating macrophages. It is a pluripotent cytokine
capable of generating anti-inflammatory and some proinflammatory effects. The proinflammatory effects include enhancement of chemotaxis of monocytes and increased expression of Fc receptors (Schluger and Rom, 1998).

However, the anti-inflammatory actions of TGF-β are predominant which include promoting immune tolerance and limiting the pathological inflammation in synergy with IL-10 (Chen et al., 2003). TGF-β also suppresses the expression of costimulatory molecules in APCs and down-regulates production of IFN-γ and TNF-α thereby reducing the generation of ROIs and NOIs (Ruscetti et al., 1993) Decreased production of cytokines like IL-12 further contribute to down-regulation of T cell function and proliferation (Gorham bet al., 1998). TGF-β levels have been shown to be up-regulated in granuloma of active tuberculosis patients (Toossi et al., 1995) and high levels of TGF-β have been correlated with severe forms of the disease. Like IL-10, TGF-β also reduces the harmful inflammatory effects by its anti-inflammatory properties.

1.3.2.3 Interleukin-4 (IL-4)

IL-4 stimulates the development of Th2 cells from naïve CD4+ T cells and also induces production of IgE antibodies. IL-4 is in turn secreted by Th2 cells thereby further differentiating and proliferation Th2 cells in an autocrine fashion. It is also produced by activated mast cells and basophils (Borish and Steinke, 2003).

IL-4 exerts its immunosuppressive effect by reducing IFN-γ production by down regulating IL-12 and IL-12 receptor and also antagonizes the effects of IFN-γ leading to deactivation of macrophages (Nakamura et al, 1997). The downregulation of IL-2 by IL-4 further contributes to the Th1 to Th2 switch. Studies have also demonstrated the correlation of increased production of IL-4 in human tuberculosis patients with excessive tissue damage and cavitary disease (van Crevel, 2000). Therefore, cytokines of both pro- and anti-inflammatory groups are crucial in maintaining a balance between inflammation and bacterial killing to minimize tissue damage.
1.4 GRANULOMA IN TUBERCULOSIS: BATTLE FIELD OR SHELTER FOR MYCOBACTERIA

"On the basis of my numerous observations I consider it established that, in all tuberculous affections of man and animals, there occur constantly those bacilli which I have designated tubercle bacilli and which are distinguishable from all other microorganisms by characteristic properties." — Robert Koch, 1882.

With these words Koch announced the discovery of the etiologic agent of tuberculosis. The term “tubercle” was given by Sylvius in the year 1650 and referred to apparent lung nodules which was a common feature of “consumption” disease. Today, these tubercles are known as granulomas.

Granuloma is an organized microscopic aggregation of immune cells. It consists of infected macrophages in the center, an inner cuff of mainly resting and activated macrophages which are transformed into epithelioid cells (epithelioid cells), multinucleate giant cells and foamy cells surrounded by an outer cuff of predominantly lymphocytes and other leukocytes like neutrophils, dendritic cells and sometimes fibroblasts. The granuloma often has a necrotic center. (Ramakrishnan et al., 2012)

1.4.1 Types of Granuloma

Granuloma can be divided into two types based on their etiology:

a) Epithelioid granuloma are characterised by the presence of epithelioid cells (modified macrophages) and sharp circumscribed module such as in tuberculous granuloma or sarcoidal granuloma. The tuberculous granuloma most often presents with caseous necrosis whereas sarcoidal granuloma has non caseating center.

b) Histiocytic granuloma characterized by ill defined nodule containing phagocytic histiocytes (tissue macrophages) e.g. Foreign-body granuloma, Rheumatoid granuloma and Rheumatic granuloma
1.4.2 Immunopathology of granuloma:

Once *M. tuberculosis* enters the airways through aerosols, it first encounters macrophages and dendritic cells. Unless killed by alveolar macrophages, the surviving bacilli migrate to the lung parenchyma initiating the inflammatory response. The DC phagocytose the bacilli and drain to the thoracic lymph nodes (LN) thus priming the T cell response (Bhatt et al., 2004). When tubercle bacilli invade and replicate in the alveolar macrophage, the site of primary infection is known as Ghon focus. The draining lymphatics, regional lymph node involved along with Ghon’s focus is known as Ghon complex (Vijayan, 2002). This site is primarily and transiently infiltrated by polymorphonuclear neutrophils (PMN) which are later replaced by activated macrophages. The recruitment of inflammatory cells like PMN, macrophages and T and B lymphocytes occur under the influence of chemokines. These chemokines are in turn regulated by cytokines like TNF-α and IFN-γ. The dendritic cells then drain into the lymph nodes and sensitise the CD4+ T helper cells and CD8+ T cytotoxic T cells and initialize the adaptive immune response. The migration of these sensitized T lymphocytes along with other leukocytes like monocytes, dendritic cells and neutrophils give rise to the structure known at granuloma. The activated macrophages in the necrotic center and the dense surrounding of CD4+ and CD8+ T cells forms a cellular wall that restrict the spread of the bacteria (Saunders et al. 2002).

Granuloma therefore appears to be a prerequisite for limiting the infection. It provides a framework whereby the T cells, which produce cytokines, remain in close apposition with the macrophages that become activated and achieve a mycobacteriostatic state. It also provides a toxic environment for the mycobacteria in addition to protecting the delicate alveolar tissue in the process (Saunders et al. 2002).

After two to four weeks of infection, additional host responses to *M. tuberculosis* develop in the form of the tissue damaging response and macrophage activating response. The tissue damaging response occurs due to delayed type of hypersensitivity (DTH) reaction to various mycobacterial antigens. Whereas, the macrophage activation response is facilitated by activation of macrophages by the cell mediated immune response (CMI)
rendering the macrophages capable of killing and digesting tubercle bacilli (Raviglione and O'Brien, 2012). A strong granulomatous reaction within the first few days is a prerequisite to contain the infection which is associated with the innate immune response. The development of acquired immune response occurs in the form of CMI and DTH. The development of caseous necrotic centre has been associated with DTH. The intense cytokine and cell-cell interaction environment also leads to necrosis of macrophages and epithelioid cells. The material released due to necrosis has 'cheese like' appearance hence it is known as caseous necrosis (Fayyazi et al., 2000). This is a host defence strategy to destroy its own tissue in order to control the uninhibited intracellular multiplication of tubercle bacilli which would otherwise be detrimental. Majority of bacteria are killed in this process; however those that survive are unable to multiply in the external milieu. Within the caseous tissue, the acidic pH, low availability of oxygen and the presence of toxic fatty acid make the environment hostile for the tubercle bacilli. These anoxic conditions developed by macrophages and T lymphocytes in the granuloma results in inhibition of replication and even killing of mycobacteria. This leads to the formation of typical granulomas in tuberculosis characterised by central caseation known as soft tubercle. Although sometimes there is no caseation, and such granulomas are known as hard tubercle.

At this stage in the presence of a good CMI, the granuloma are surrounded by large number of lymphocytes particularly IFN-γ secreting CD4+ T cells causing the caseating granuloma to shrink as they become fibrotic and then calcified. (Vijayan, 2002). In immunodeficient individuals however, the M. tuberculosis infection leads to formation of granulomas containing large rich inactivated macrophages, with very few lymphocytes at the periphery (Ulrichs et al., 2005). The absence of protective CMI response and the uncontrolled caseating destructive response leads to tissue destruction. As a result the surrounding tissue is progressively damaged, causing the lesion to enlarge further. The caseous necrotic center of the granuloma cavitates and the bacilli is released into the bronchi. The spread of the bacilli from such cavitary lesions can occur through airways, lymphatics or hematogenous transmission.
1.4.3 Cell death in granuloma

Macrophages in tuberculous granuloma can undergo both necrosis and apoptosis. (Fayyazi et al., 2000). Both mycobacterial and host factors contribute in this process. Caseating granulomas formed from the breakdown of immune cells especially macrophages are characteristic feature of tuberculosis. Histological studies from the pre-chemotherapy era correlated with the presence of caseum in early granuloma with increased bacterial numbers as opposed to non-caseating lesions. The macrophage rich areas shown enhanced bacterial growth and the cellular debris contained bacterium-rich exudates indicating the release of bacteria from necrotic macrophages. These mycobacteria thereby grow more exuberantly following their release into an extracellular milieu due to macrophage necrosis (Algood et al., 2005). The proliferation of bacteria in the extracellular environment following necrosis exceeds much more than that due to phagocytosis of apoptotic macrophages (Ramakrishnan, 2012). The extent to which these extracellular bacteria are phagocytosed by new macrophages is unclear.

Apoptotic macrophages in contrast to necrosis form apoptotic bodies of intact membranes in which the bacteria remain encased. The phagocytosis of apoptotic macrophages containing bacteria by multiple uninfected macrophages provides new niches for bacterial replication and nullifying any bactericidal effects of apoptosis (Ramakrishnan, 2012). However, another notion is that when under the effect of CD8+ T cells the infected inactive macrophages undergo apoptosis and they are taken up by other activated macrophages that are better equipped to kill the tubercle bacilli (Lazarevic and Flynn, 2002).

In comparison to apoptosis, the magnitude of bacterial proliferation promoted by necrosis is much greater. The bacterial proliferation is extremely high in external milieu as compared to apoptotic death and phagocytosis. It is also proposed that by shifting the balance from apoptosis to necrosis *M. tuberculosis* reduces the DC-mediated cross-priming of T cells. Apoptosis is also assumed to facilitate bacterial killing and stimulation of T cells by promoting antigen presentation, whereas necrosis leads to release of bacteria thus enhancing inflammation and thereby tissue damage (Philips and Ernst, 2012).
Granuloma is therefore considered to be the structure to ‘wall off’ the bacteria and therefore preventing the bacilli from dissemination to other sites of the lung or other parts of the body. It facilitates the formation of local milieu for immune cells to communicate with each other by physical contact or via production of cytokines and chemokines. (Aggarwal, 2003). The presence of healed fibrotic and calcified granulomas in healthy individuals signifies that the granuloma tried to restrict the growth of bacteria but failed. It can therefore be implied that in the absence of granuloma, there will be uncontrolled proliferation and dissemination of tubercle bacilli (Ramakrishnan 2012).

1.4.4 Cell population involved in Granulomatous response

1.4.4.1 Neutrophils

Following aerosol infection polymorphonuclear neutrophils (PMN) are among the first cells to arrive within few hours at the site of infection. However, the contribution of these cells in immune response to tuberculosis is unclear. PMN are considered as first line of defense against tuberculosis and rapid migration of PMN to the site of infection is considered as a pre-requisite for focal organization of pulmonary granulomas in tuberculosis (Seiler et al., 2003). Some studies have indicated that PMN exhibit bactericidal effects though NADPH oxidase-dependent mechanisms (Kisich et al., 2002) however other studies have reported that the tubercle bacilli escapes this oxidative killing (Corleis et al., 2012). In fact, other studies suggest that limited ability of neutrophils to restrict mycobacterial growth as compared to macrophages leads to enhanced pathology and that neutrophils may favour the mycobacteria by acting as a “Trojan horse”.

They are activated by lipoarabinomannan of M. tuberculosis (Riedel et al., 1997). They contribute to the killing of bacteria and facilitate the recruitment of leukocytes through chemokines like MCP-1 and IL-8 leading to initiation of inflammatory response (Riedel et al., 1997; Seiler et al., 2003). Neutrophils do not appear to have a significant role once the granuloma matures however their presence is observed when the granuloma starts to become necrotic. Their role in granuloma necrosis is still not clear.
Figure 4: Granuloma: Microscopic aggregate of infected macrophage in the center having caseous necrosis along with other forms of macrophages including epithelioid cells, foamy cells and multinucleate giant cells. The center is surrounded by a collar of leukocytes, mainly, B and T lymphocytes, neutrophils and dendritic cells.
A. Immunocompetent host
Alveolar macrophage infection
Granuloma initiation
M. tuberculosis replication
Granuloma - M. tuberculosis control
Non-caseating granuloma - inflammation resolution
M. tuberculosis presence (low bacterial load)
Recruitment of AMs and lymphocytes
Activation of inflammation mediators

B. Immunodeficient host
Persisting M. tuberculosis
Stimulation and influx of fibroblasts
Alveolar macrophage infection
M. tuberculosis replication
Granuloma - M. tuberculosis control
Non-caseating granuloma - inflammation resolution
M. tuberculosis presence (high bacterial load)
Recruitment of AMs and lymphocytes
Activation of inflammation mediators

C. TNF-α
Disorganized granuloma
Influx lymphocytes
Influx neutrophils

D. IFN-γ
Disorganized granuloma
Influx lymphocytes
Influx neutrophils

E. IL-12
IL-18
Disorganized granuloma
Influx lymphocytes
Influx neutrophils

F. IL-10
TGF-β
Disorganized granuloma
Influx lymphocytes
Influx inactivated macrophages

1.4.4.2 Monocyte

The infected macrophages attract immune cells including other monocytes to the site of infection by chemokine production. The monocytes can be recruited from the circulation or tissue reservoirs. The role of these newly recruited monocytes in controlling *M. tuberculosis* infection remains undefined. They are either exposed to mycobacterial soluble products or get infected with *M. tuberculosis*. The local production of IFN-γ and TNF-α drives the differentiation and activation of monocytes within the granuloma.

The matrix metalloproteinases (MMPs) secreted by monocytes are known as granuloma creators and tissue destroyers (Salgame, 2011). MMP-9, secreted by monocytes following *M. tuberculosis* stimulation, degrades type IV collagen and facilitates leukocyte extravasation to the site of infection (Goetzl et al., 1996). Studies have demonstrated the role of MMP-9 in recruitment of leukocytes and formation of granuloma to restrict bacterial growth, however excessive MMP-9 may lead to host tissue injury in TB (Price et al., 2003). Volkman et al. (2010) demonstrated that the MMP-9 upregulation in epithelial cells due to secreted 6-kDa early secreted antigenic target (ESAT-6) protein leads to recruitment of newer monocytes and macrophages which is a prerequisite for granuloma maturation.

1.4.4.3 Monocyte-Derived Cells

1.4.4.3.1 Macrophages

Macrophages play a central role in immunity to *M. tuberculosis* but at the same time act as their shelter. The macrophages resort to various inflammatory and non-inflammatory mediators like cytokines and fibrinolytic enzymes to combat the infection. In case of nonpathogenic mycobacterial infection the bacteria are killed by acidification of the phagosomal compartment. *M. tuberculosis* however, prevents the incorporation of ATP/proton pump into the phagosome and restricts the fusion of this vacuole with lysosome.

In case of resting macrophages, *M. tuberculosis* can prevent phagolysosomal fusion. However, the macrophages activated by IFN-γ can stimulate autophagy and overcome...
The activated macrophages can also exert the bactericidal effects through antimicrobial peptides, reactive oxygena or nitrogen intermediates. The infected macrophages also respond to TNF-α mediated apoptosis thereby exposing the bacilli to direct antimicrobial effects (Lee et al., 2006). The apoptotic body so formed can be engulfed by newly recruited macrophages and dendritic cells to accelerate the elimination of the bacilli by inducing adaptive immune response (Hinchey et al., 2007).

### 1.4.4.3.2 Multinucleated cells

Multinucleated Langhans giant cells (MGC) are characteristic feature of tubercular granuloma. These are formed from macrophages that fuse together to form giant cells. Mycobacterial lipoarabinomannan is known to trigger the TLR2-dependent cell activation which eventually leads to fusion process (Puissegur et al., 2007). The coculture of macrophages with activated T cells also induces MGC formation through CD40/CD40L interaction and IFN-γ secretion (Sakai et al., 2011). It was observed that the the poorly virulent strains of mycobacteria could only induce small multinucleate cells (MCs) formation which have limited ability to take up mycobacteria but fail to differentiate into MGC stage. MGCs lose their ability to phagocytose bacteria as they no longer express mannose receptor/CD11b (phagocytic receptors) while they retain their antigen presentation ability. MGCs also display NADPH oxidase activity. This implies that MGCs seem to devote themselves in the elimination of bacilli ingested in the previous stages of differentiation i.e. macrophages and multinucleate cells (Lay et al., 2007).

Since the presence of MGC is considered to be hallmark of tuberculosis infection, it would be interesting to know how patient monocytes differ from healthy control monocytes in multinucleate giant cell formation and the cytokines that influence this process.

### 1.4.4.3.3 Monocyte-Derived Dendritic Cells (mDCs)

mDCs present in granulomas at early stages drain the lymph nodes and educate the T cells. It was observed that large exchange of DCs in the late stages of infection is
indicative of immune surveillance (Schreiber et al., 2011). DCs do not have very efficient microbicidal activity, however, they limit the replication of *M. tuberculosis*. DCs infected with mycobacteria stimulate the T cells but at the same time may also serve as a vehicle for the pathogen to spread in other parts of the body (Tailleux et al., 2003).

1.4.4.3.4 Foamy Macrophages

Human tuberculous granulomas classically contain foamy macrophages. Their foamy appearance is due to lipid bodies formed as a result of accumulation of intracellular lipids. The lipids present in these macrophages are predicted to serve as a source of nutrients for the bacteria. Foamy macrophages are also instrumental in maintaining *M. tuberculosis* in a dormant state. (Peyron et al., 2008). Moreover since they lose their phagocytic and bactericidal activities they have been used as model of dormancy to test the drugs active at this stage.

1.4.4.4 T lymphocytes

1.4.4.4.1 CD4 T cells

The crucial role of CD4+ T cells in combating the mycobacterial infection and granuloma formation was demonstrated in knockout mice studies. It was observed that in MHC II /" and CD4 /" mice, not only the granuloma formation was delayed by a week but the granuloma that was formed was also disorganized and failed to contain the infection despite the macrophages displaying normal NO synthesis (Caruso et al., 1999). Ordway et al. (2007) in their study on mice models demonstrated strong Th1 response (characterized by IFN-γ and TNF-α production) by the CD4+ T cells and that the removal of CD8+ T cells at this stage did not affect bacterial growth. However, the Th1 response decreased in the later stage of the disease and concomitant removal of CD8+ T cells lead to reactivation and increased bacterial growth. However, it was also observed that CD8+ T cells were unable to compensate for the reduced Th1 expression in CD4+ T cells which implies that CD4+ T cells are curial at all stages of the infection. The presence of CD4+ T cells is also essential for the expression of Th1 like response by CD8+ T cells.

1.4.4.4.2 Th17
In addition to CD4+ and CD8+ T cells Th17 type of cells are also shown to play a role in *M. tuberculosis* infection, IL-17 secreted by Th17 cells helps in recruitment of neutrophils and activating macrophages. It also recruits effector Th1 cells to the site of infection. Most often it is the γ/δ T lymphocytes that predominantly secrete IL-17; however in some cases IL-17 secreting CD4+ T cells are also present in the granuloma.

### 1.4.4.3 Regulatory T cells

FoxP3+ regulatory T cells are recruited to granuloma to function as gate keepers of excessive immune response. CD4+CD25+Foxp3+ T regs were also found in the granulomatous lesions of active tuberculosis patient. They are located not only in the primary granuloma but also at the draining mediastinal lymph node where secondary granulomas are found. Studies have suggested the role of Treg cells in establishment of persistant infection. Shafiani et al. (2010) in their study on mice observed that Treg proliferation takes place when *M. tuberculosis* is transported to the pulmonary lymph node (pLN). These *M tuberculosis* specific Tregs were responsible for delaying the priming of effector CD4+ and CD8+ T cells in the pTN and thereby their recruitment to the granuloma. This delay results in high bacterial load in these mice. This implies that *M. tuberculosis*-specific T cells specifically and significantly restrict the protective immune response during tuberculosis infection.

### 1.4.4.4 Cytotoxic T cells

Studies on CD8+ T cells deficient mice (B2m−/− MHC-I-deficient mice) revealed that these mice were more susceptible to mycobacterial infection than the wild type animals, although the severity of the disease was not more than that of the CD4+ T cell-deficient mice. The CD8+ deficient mice showed increased susceptibility to *M. tuberculosis* irrespective of the inoculum size. (Ladel et al., 1995; Flynn et al., 1992). The CD8+ T cells were found to be localized in the periphery in the early stages of infection but migrate towards the center as the disease progresses (Gonzalez-Juarrero et al., 2001). The outer mantel of activated CD8+ T cells appears to be playing a role in retaining the infection; however it reduces the possibility of contact dependent killing of infected cells.
and eradication of infection. CD8+ T cell deficient mice showed granuloma formation, however, these granulomas were functionally impaired and had marked central necrotic zone which was absent in case of wild-type mice. This pathology could be the result of lack of apoptotic induction in infected cells leading to generation of these cells and enhanced neutrophil recruitment to the site of infection (Flynn et al., 1992).

Within the granuloma, the granule mediated killing of the tubercle bacilli is the major defense mechanism used by CD8+ T cells. Rahman et al. (2009) showed that the presence of CD8+ T cells expressing low perforin and granulysin correlated with the elevated levels of Tregs. Another important role of CD8+ T cells was highlighted by Ordway et al. (2007) in which they demonstrated the release of CXCL1 chemokine by CD8+ T cells following aerosol infection with M. tuberculosis. CXCL1 facilitates the chemotaxis of CD4 and CD8 T cells to the infection site.

Hence the studies based on various T cell subsets present in tubercle granuloma indicate that with the establishment of clinical tuberculosis the immune response is skewed towards Th2/Treg suppressive response. This in turn antagonizes the Th1/Th17 protective response and the functionality of CTLs is also compromised. Therefore, strategies for designing new immunotherapies can be directed towards enhancing the cell-mediated immune response by targeting Th2/Treg cells (Rahman et al., 2009).

1.4.4.5 B lymphocytes

The role B cells play in immunity to tuberculosis and granuloma is still unclear. Maglione et al. (2007) in their study on B cell -/- mice observed exacerbated immunopathology following aerosol infection with 100 CFU (colony forming units) of M. tuberculosis along with increased influx of neutrophils to the infection site. These mice also showed increased production of IL-10 in the lungs in addition to increased susceptibility to lung infection. Phuah et al. (2012) demonstrated the presence of B cell clusters in granuloma and peripheral node in cynomologus macaques monkey model. They suggested that the presence of M. tuberculosis specific B cells secreting antibodies
and expressing elevated CXCR5 chemokine and HLA-DR expression within the granuloma might have a role in modulating local control of infection.

1.4.5 The key players of Granuloma: Cytokines

Interaction of *M. tuberculosis* with alveolar macrophages and DC leads to the production of IL-12 and IL-23 by these cells. These cytokines in turn prime the Th1 cells that play a crucial role in granuloma assembly and immune response. The activated Th1 cells release IFN-γ and IL-2. In an immunocompetent individual the granuloma is characterised by the presence of IFN-γ producing CD4+ T cells. Sugawara et al. (1998) in their study based on IFN-γ-gene- disrupted mice showed granuloma formation only by avirulent strains but not by virulent strains of *M. tuberculosis*. They observed that when these mice were subjected to infection by BCG Pasteur and H37Ra strains they induced granuloma in spleen liver and lungs. These granulomas were infiltrated by macrophages and multinucleate giant cells but lacked necrosis. However when the mice were infected with H37Rv strains (virulent strain), disseminated abscesses were formed due to failure to induce granuloma in various organs. This study therefore substantiated the importance of IFN-γ in macrophage activation and granuloma formation mechanism.

Another proinflammatory cytokine that is particularly important in this regard is TNF-α. TNF-α is known to be crucial in maintenance of granuloma. Bekker et al. (2001) in their TNF-α knockout (KO) mice demonstrated the logarithmic growth of intracellular bacilli following BCG infection. However infection with BCG-secreting murine TNF lead to bacterial killing which was in turn associated with high inducible NO synthase (iNOS) production. Also in the same study they observed that iNOS-KO macrophages could only kill the bacteria after BCG-TNF infection. These results suggest that the bacterial killing by the macrophages takes place in two ways: 1) TNF-α- dependent- iNOS dependent manner and 2) TNF-α- independent- iNOS dependent manner. Another TNF-α gene disruption study in TNF−/− mice demonstrated widespread dissemination of *M. tuberculosis* following aerosol infection and poorly formed granulomas with extensive necrosis and neutrophilic infiltration of the alveoli (Bean et al., 1999). These studies
therefore highlight the role of TNF-α in organized granuloma formation and killing of the tubercle bacilli.

In case of chronic infection apart from TNF-α and IFN-γ, IL-2 also plays a crucial role. Lindenstrøm et al. (2013) in their study observed that *M. tuberculosis* infection of BCG immunized mice was controlled up to 7 weeks of infection following which, the protection loss was correlated with the disappearance of IL-2+CD4+T cells. However, a booster dose of subunit vaccine (Ag 85B-ESAT-6 + CAF01) was able to induce the expansion of IL-2+CD4+T cells coexpressing TNF-α or TNF-α/IFN-γ which was maintained and thus controlled the bacterial growth in the late stage of infection. It was observed that the central memory cell so produced could replenish the T cells at the site of infection and prevent functional exhaustion even when the animals were challenged two weeks post infection. However, variable results have been obtained regarding the role of IL-2 in multinucleate giant cell formation (Birkness et al., 2007; Gasser and Most, 1999).

The other group of cytokines that regulates the immune response in granuloma is the anti-inflammatory cytokines e.g. IL-10. Murray et al. (1997) in their study on transgenic mice observed that although on one hand IL-10 prevents excessive tissue damage by reducing inflammation, on the other hand it contributes to the mycobacterial growth. De la Barrera et al. (2004) demonstrated increased IL-10 production in tuberculosis patient than healthy controls. The IL-10 production also affected the lytic property of T cells. However when IL-10 was neutralized with antibody or by adding exogenous IFN-γ, it led to increased lytic activity of both CD8+ and CD4+ T cells. Thus, it appears that the beneficial effect of IL-10 is overridden by its contribution to mycobacterial persistence inside host macrophages and reactivation of tuberculosis. IL-10 depletion decreases bacterial burdens and increases the influx of IFNγ-producing T cells to the site of lung infection (Redford et al., 2010).

The functions of IL-10 are further synergized by the action of TGF-β. Like IL-10, it also reduces the tissue damage as a result of pathological inflammation but at the same time suppress APCs costimulatory molecules, iNOS production and downregulates T cell
proliferation and cytokine production. Toossi et al. (1995) in their study demonstrated upregulated TGF-β in monocytes and macrophages in granuloma of active tuberculosis patients and high levels of TGF-β correlated with severe stages of infection.

Another molecule with which IL-10 synergizes is IL-4 and inhibits macrophage cytotoxic activity (Oswald et al., 1992). IL-4 is not only predominantly produced by Th2 lymphocytes but also induces Th2 differentiation. Both IL-4 and IL-10 Th2 cytokines contribute significantly in inhibiting Th1 development and activation in addition dampening macrophage activation and bactericidal activity. Animal model studies have associated the increased production of IL-4 with cavitary tuberculosis (van Crevel et al., 2000). IL-4 has also been known to contribute significantly to TNF-α toxicity (Hernandez-Pando et al., 2004). In this study it was observed that IL-4 deficient mice not only have significantly low bacterial load but also reduced TNF-α toxicity following TNF-α challenge.

1.5 SURVIVAL OF MYCOBACTERIA: DITCHING THE HOST IMMUNE RESPONSE

Despite various immune cells and responses, mycobacteria manage to escape the host immunity, survive, proliferate and spread infection. It was observed that mycobacteria resist acidification of the macrophage phagosome to pH 6.4 thus preventing the environment from being hostile for its survival and fail to fuse with lysosome (Deretic et al., 2006; Russel et al 2011). Cell-wall lipids and other mycobacterial effectors bring about the modulation of phagosome. It was observed that the mutants were partially defective in phagosome modulation which resulted in pH5.8 of the residing vacule of mycobacteria thus arresting its growth. Additionally, on activation the macrophages overcome the effect of mycobacteria on phagosome maturation and make it more acidic at pH5.2, which is a bactericidal environment.

As described earlier the development of granuloma is to facilitate the interaction of immune cells and generation of robust cellular immune response against the bacterial antigens. However, the mycobacteria manage to manipulate the response in its favour. M.
*tuberculosis* releases cell wall components lipoarabinomannan and arabinomannan inside the infected cells and accumulate in multilamellar bodies which contain both bacterial and host components. The multilamellar bodies then unite with microvesicle lysosome known as MHC class II-enriched compartment. This vesicle then escapes the infected macrophage by exocytosis and released into external milieu as exosome. The exosomes are then internalized by the neighboring cells. The mycobacterial cell wall proteins in the exosomes released from infected macrophages were identified as belonging to Ag85 family.

1.6 ROLE OF HLA IN CELL MEDIATED IMMUNITY

The two main reasons responsible for the failure of granuloma to contain infection may be: first, the failure to generate efficient T cell response to *M. tuberculosis* in immunocompromised subjects and secondly, genetic factors. Studies based on racial variation in the susceptibility to TB and twin studies emphasized the importance of genetic factors in reactivation of TB. Fernando and Britton (2006) enumerated at least 14 genes that have been associated with disease reactivation and among them HLA has been studied extensively.

Both CD4+ and CD8+ T cells recognize *M. tuberculosis* peptides presented by major histocompatibility complex (MHC) class I and class II molecules. In humans, MHC is known as human leukocyte antigen (HLA). In the same report it has been emphasised that HLA has an association with susceptibility to tuberculosis in different populations. Attempts have therefore been made to identify immunodominant epitopes of *M. tuberculosis* antigens for vaccine purpose based on their recognition of HLA.

1.6.1 Immunodominant T-cell antigens: Answer for new vaccine?

The protective T-cell response to tuberculosis is usually antigen-specific. Hence, studies have been carried out to identify immunodominant antigens for development of effective vaccines. Since T cell antigen recognition is dependent on major histocompatibility
complex (MHC), the search for immunodominant antigens have been diverted to HLA binding epitopes.

1.6.1.1 Antigen 85 complex

Secretory proteins are potential targets for vaccine design as they are the first to interact with immune cells and generate the immune response. A number of culture filtrate proteins have been identified that not only contribute to pathology of the disease but can also generate an immune response. The proteins of antigen 85 complex are a group of secreted antigens which include Ag85A, Ag85B, Ag85C that code for fbpA, fbp B and fbpC respectively. In addition a fourth gene, named fbp D and encoding MPT51 who’s primary structure is similar to other members of the Ag 85 complex was also identified. These are a major constituent of proteins secreted in the culture of mycobacteria on the synthetic, liquid Sauton medium. They are mycolyl transferase enzymes and transfer mycolyl residue from one molecule of TMM to another TMM (forming TDM or cord factor which enhances virulence) and arabinogalactan. They also have fibronectin binding property that enhances the virulence by helping in adherence and dissemination of organism in tissue (Wiker and Harboe, 1992).

1.6.1.2 Region of difference (RD region)

The studies based on the comparison of genomes revealed certain regions of the genome that are present in M. tuberculosis strain but were found to be absent in M. bovis BCG substrains and several non-tuberculosis mycobacteria (NTM), including M. avium (Mahairas et al., 1996; Gordon et al., 1999; Behr et al., 1999). These gene segments are known as regions of difference (RD). The protein encoded by these RD regions are called RD proteins. Several studies have now been carried out to study the potential of these RD antigens in generating cell mediated and/or humoral immune response.
Table 1.1 Characteristics of regions of differences (RD) present in Mycobacterium tuberculosis.

<table>
<thead>
<tr>
<th>RD</th>
<th>Size of ORF (kb)</th>
<th>ORF (number)</th>
<th>ORF (range)</th>
<th>Gene Segment(s)</th>
<th>ORF (bp range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9.5</td>
<td>9</td>
<td>Rv3871-Rv3879c</td>
<td>160</td>
<td>7534–16989</td>
</tr>
<tr>
<td>2</td>
<td>5.6</td>
<td>11</td>
<td>Rv1978-Rv1988</td>
<td>88–89</td>
<td>14211–8598</td>
</tr>
<tr>
<td>3</td>
<td>9.3</td>
<td>14</td>
<td>Rv1573-Rv1586c</td>
<td>70</td>
<td>7677–16923</td>
</tr>
<tr>
<td>4</td>
<td>1.9</td>
<td>3</td>
<td>Rv0221-Rv0223c</td>
<td>12</td>
<td>17432–19335</td>
</tr>
<tr>
<td>5</td>
<td>2.8</td>
<td>5</td>
<td>Rv3117-Rv3121</td>
<td>135</td>
<td>27437–30212</td>
</tr>
<tr>
<td>6</td>
<td>12.8</td>
<td>11</td>
<td>Rv1506c-Rv1516c</td>
<td>65</td>
<td>23614–36437</td>
</tr>
<tr>
<td>7</td>
<td>~9.0</td>
<td>8</td>
<td>Rv2346c-Rv2353c</td>
<td>103</td>
<td>17622–26584</td>
</tr>
<tr>
<td>8</td>
<td>3.4</td>
<td>4</td>
<td>Rv0309-Rv0312</td>
<td>16</td>
<td>17018–20446</td>
</tr>
<tr>
<td>9</td>
<td>18.3</td>
<td>7</td>
<td>Rv3617-Rv3623</td>
<td>153–154</td>
<td>21131–2832</td>
</tr>
<tr>
<td>10</td>
<td>3.0</td>
<td>3</td>
<td>Rv1255c-Rv1257c</td>
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<tr>
<td>11</td>
<td>28.8</td>
<td>5</td>
<td>Rv3425-Rv3429</td>
<td>145–146</td>
<td>30303–1475</td>
</tr>
<tr>
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<td>2.0</td>
<td>4</td>
<td>Rv2072c-Rv2075c</td>
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<tr>
<td>13</td>
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<td>16</td>
<td>Rv2645-Rv2660c</td>
<td>118</td>
<td>12475–23455</td>
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<tr>
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<td>8</td>
<td>Rv1766c-Rv1773c</td>
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<tr>
<td>15</td>
<td>12.7</td>
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<tr>
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<td>6</td>
<td>Rv3400-Rv3405c</td>
<td>145</td>
<td>5012–12621</td>
</tr>
</tbody>
</table>

ORF : Open reading frame  
Source: Based on reference, Behr et al., 1999.

1.6.1.3 Region of Difference 1

The RD 1 region codes nine open reading frames (ORF) including Rv3871-Rv3875 of size 9.5kb (Table 1). This region is of particular importance not only because it is consistently absent in all the BCG strains but also because the deletion of the RD-1 fragment from *M. tuberculosis* causes loss of its virulence, whereas the inclusion of this region into *M. bovis* BCG or *M. microti* resulted in increased virulence and survival properties (Behr, 2002; Lewis et al., 2003) Two ORFs included in this region are Rv3874 which encode 10-kDa culture filtrate protein (CFP-10) and Rv3875 which encodes the 6-kDa early secreted antigenic target (ESAT-6) protein. Both these proteins induce T cell mediated response (Weldingh et al., 1999). Kamath et al. (2004) in their study on mice model observed that T cells were activated by CFP-10 epitopes and were recruited in large number in early stage of infection in lung which resulted in large amounts of IFN-γ.
This property of CFP-10 makes it a potential candidate for diagnostic and vaccine purpose. Both ESAT-6 and CFP-10 play crucial role in the persistence of mycobacteria in macrophages by downregulating the production of reactive oxygen species (ROS) inside the macrophages; which in turn damps the NF-κB transactivation property.

1.6.1.4 Phospholipase C
Phospholipase C (PLC) is known to be an important virulence factor and its role in pathogenesis of organisms like *C. perfringens* (Titball, 1998; Flores-Diaz and Alape-Giron, 2003) and *P. aeruginosa* (Titball, 1998; Songer, 1997) has been established. In mycobacterium tuberculosis, phospholipase family consists of four closely related genes encoding PLCs. The plc-A, plc-B and plc-C form an operon and have been found to be relevant in virulence of *M. tuberculosis*. However the fourth gene plc-D which is located in a different region than the other three genes did not seem to have very significant role in virulence. Phospholipase C has recently been identified by potential drug target by Bakala N'goma et al. (2010). They expressed all the genes of phospholipase C family and expressed them using Mycobacterium smegmatis as expression system. These enzymes exhibited cytotoxic activity towards macrophages via direct and indirect enzymatic hydrolysis of cell membrane.

1.6.1.5 Region of Difference 12
Mustafa et al. (2011) in their study analyzed the immune response to various peptide pools of the RD region and observed three type of responses: 1) Response with Th1 bias using peptides of RD1, RD5, RD7, RD9 and RD10; 2) Response with Th2-bias in case of peptides from RD12, RD13 and RD15); and 3) Without any Th1/Th2-bias response against peptides derived from RD4, RD6 and RD11. The study emphasizes on the possible role of RD proteins with Th1 and Th2 biases in protection or pathology of the disease respectively. In another study the same group revealed that the peptide pools of RD1 region induced the production of IFN-γ whereas RD12pool and RD13 pool induced IL-10 production. The study therefore highlights the importance of antigens of RD1
region in generating a protective immune response and that of RD12 and RD13 region in inducing pathogenesis.

1.7 DEFINITION OF THE PROBLEM
In the light of what is already known in the literature, vaccine development and improved diagnostics for tuberculosis still remains a major challenge. Despite extensive research in the field of tuberculosis immunology several aspects pertaining to immune response and pathogenesis remain undeciphered. Unraveling the key aspects of the biology of the disease and protective immune response against the disease would help in designing new therapeutic approaches. The rationale of the present study was based on the following observations made from the literature that:

a) Monocytes and granuloma being important factors in disease progression and treatment, it is important to understand their role in tuberculosis. Specifically, it would be interesting to study the differences in expression of various proteins in monocytes of patients and compare them with their respective household contacts and controls.

b) Furthermore, how do the cytokines that are produced in patients, household contacts and controls influence the ability of monocytes in granuloma formation.

Based on the above rationale the objectives of the present study were the following:

1) Study of the immune response in tuberculosis patients, their corresponding household contacts and healthy controls at the basal level and following stimulation with antigen/peptide.

2) Study of in vitro granuloma formation from monocytes of tuberculosis patients and healthy controls

3) Study of proteomic profile of tuberculosis patients and household contacts.

4) Study of antigen/peptide specific CTL (ELISPOT) assay of tuberculosis patients and household contacts.