2.1 Tuberculosis (TB)

Tuberculosis (TB) is a major health concern and cause of deaths worldwide. Reports are flooded with information of TB explaining it as scourge (Murray, 2004), deadly disease (Algood et al., 2003), captain of all these men of death (Ducati et al., 2006), leading infectious killer (Di Pietrantonio and Schurr, 2005). These words are not to glorify the disease of TB but to explicate the havoc it is causing to the mankind. The disease is not new to the world. Approximate five thousand year old Egyptian mummies show signs of TB (Ackerknecht, 1982), and was common in ancient Greece and imperial Rome (Dubos and Dubos, 1987). Its description can be found in ancient Chinese and Egyptian manuscripts (Liu and Modlin, 2008) and sacred Indian texts of Vedas like Rig Veda and Atharva Veda, shaped around 3500-1800BC ago (Tuli, 2004). However, the causing agent, bacteria, *Mycobacterium tuberculosis* was discovered nearly 130 years before by Robert Koch (Koch, 1882). Despite highly efficacious treatments available from decades the disease of TB is still devastating. TB was declared as “global emergency” by the WHO in 1993 and it was for the first time that an infectious disease got such dubious distinction. The reason for declaring TB as emergency, lied in the fact that, it is a foremost killer disease, takes a toll on health of individuals and also has huge impact on overall social and economic development of a country (WHO, 2011; Berrington and Hawn, 2007; NIAID, 2002).

Staggering statistics of TB disease talked about in next section, portrays the ugly face of this dreaded disease.

2.1.1 Epidemiology

Human race have entered the new millennium, with towering developments in the areas of biotechnology and medicine, still the dream of an effective cure against deadly disease of TB has not been fulfilled. This havoc must be haunting the guardians of society for its eradication, as around one third of world’s population have become reservoir of TB infection. From these infectious individuals, only 5-10% develop active disease, thus 90-95% retain the infection in the form of latent TB (Cooper, 2009; Dye et al., 1999). The estimate of devastation by TB can be done by looking at the statistics of 16th annual report on global control of TB published by WHO (2011). The report says
that this disease took a heavy toll on human lives by killing almost 1.1 million HIV negative TB patients in the year 2010. There were additional 0.35 million deaths of HIV positive TB patients. In the same year estimated 8.8 million incidents cases of TB were notified. This figure of incidences is an increase from 8.3 million in year 2000 and around 6.6 million cases in 1990. Although, it appears from given figure that TB incidents are increasing from 1990 onwards, but they are due to population growth. Regarding per capita number, they are falling, but with very slow rate of less than 1% in year. The TB incidences were 142 per 100 000 persons in 2004 which came down to 139 per 100 000 persons in year 2007 (WHO, 2009). Asia and Africa contributed maximum to TB with estimated 59% and 26% cases in the year 2010. County wise data estimates India on the top of TB chart with 2-2.5 million cases, which are about one quarter (approximately 26%) of total cases on globe. Other four countries with high number of TB incidence are china with 0.9-1.2 million population of TB patients, South Africa with 0.4-0.59 million cases, Indonesia with 0.37-0.54 million patients, and Pakistan with 0.33-0.48 million TB cases (WHO, 2011).

Regarding two severe forms of TB, multi drug resistance-TB (MDR-TB) and extensively drug resistant-TB (XDR-TB), there were about 0.3 million of MDR-TB case in world (WHO, 2011). In case of MDR-TB too, India has the dubious distinction of being at first place with 131 000 case. By the July month of year 2010, atleast 58 countries have reported at least one case of XDR-TB, including India (RNTCP, 2011).

Although, India top the world with highest case of TB, it ranks 17th among 22 high burden countries in terms of TB incidence rate. Because of, continuous efforts of revised national tuberculosis control programme (RNTCP) TB mortality in the country has reduced to 23 per 100 000 population in 2010 from an estimated 42 per 100 000 population in 1990. The figures are encouraging but the disease should not be taken frivolously, as 40% of Indians are still infected with TB bacilli (RNTCP, 2011). Daily, around 5000 people develop the disease in this country, and 1000 succumb to this scourge. Unfortunately, the most of morbidity and mortality due to this disease occurs in age group of 15 to 54 which is regarded as most productive years for social and economical development (RNTCP, 2009; RNTCP, 2011).
2.1.2 Etiology

Tuberculosis was thought to be a genetic disease (Bellamy, 1998; Dubos and Dubos, 1987), which ran into families, long before its actual cause was identified. Then the researchers were successful to define it as an infectious disease. Villemin discovered that TB can be transmitted to animals (Palomino et al., 2007). After him Cohnheim, Salomonsen (Murray, 2004), Baumgarten experimented with TB and inoculated it successfully in anterior chamber of eye. Later on Tappeiner was also triumphant with inhalation trial. So, it was almost known with certainty that TB is an infectious disease but the agent for its etiology was still a mystery. Than came the year 1882 and all the perceptions and hypothesis related to cause of TB were nullified. It was the discovery of its causative agent, a bacterium, *Mycobacterium tuberculosis*, (MTB) by Robert Koch (Koch, 1882). The microbe is a slow growing acid fast bacterium which spreads through respiratory route (Flynn and Chann, 2001).

It is believed that TB existed in cattle before man became host to it. With the dawn of civilization when man started domesticating cattle TB germ transmitted from cattle to man (Sharma and Mohan, 2004).

In human beings it spreads through droplet infection. When a diseased person harboring MTB in his sputum, coughs, sneezes, exhales or even talks, tiny droplets having TB bacteria in them, are released into air. These tiny droplets known as ‘droplet nuclei’ can remain infectious for hours after expectoration (Knechel, 2009). These aerosols when inhaled by any individual present in the vicinity of patient, cause infection. However, it is important to mention here that not all the individuals who get this bacterium become diseased and can contain MTB in their body for infinite time in form of latent TB (Chandra et al., 2011).

After inhalation, the defense system of body comes into play. Most of the bacteria got trapped in upper part of respiratory tract in mucus secreted by goblet cells and the cilia on the surface of cells beat to remove this mucus outside. Bacteria, which bypass this physical defense, reach the alveoli in lungs. Here they are immediately engulfed by macrophages and they slowly go on dividing in these immune cells. Phagocytosis of MTB by macrophages starts a series of immune functions, by virtue of which, the bacterial infection can be controlled or can become an active TB disease. Initially, the macrophages secrete proteolytic enzymes and cytokines to destroy the bacteria. Secreted
cytokines attracts T cells to the site of infection, which constitutes cell mediated immunity. The accumulation of both macrophage and T cells give rise to formations of nodular like lesions called granulomas. In the center of these lesions macrophages get destroyed and a solid necrotic tissue is formed, which restricts the spread of MTB and give rise to latency of TB. In a person with strong immunity, these lesions contain the MTB successfully but in an individual with low immunity the necrotic lesions undergo liquefaction (Knechel, 2009). The person now starts building the symptoms of active TB disease, which will be discussed in types and symptoms part (2.1.3) of this review. The wall of granuloma breaks which drains MTB containing semi-liquid into bronchus, blood or lymphatic vessels. Previous condition can infect other person, when this get coughed up in sputum while later condition can spread the bacteria to other body parts through lymphatic system or bloodstream. Bacteria mainly multiply in the organs with high oxygen pressure, like upper lobes of lungs, kidneys, bone marrow and meninges (Palomino et al., 2007).

The important facts is that the real harm to body tissues and symptoms of TB are not due to the MTB alone, but also because of immune reaction that body develops against the bacteria (Doherty and Andersen, 2005). An imperative factor behind this is a cytokine, Tumor Necrosis Factor (TNF)-α. It has an important role in the formation of granuloma, which restricts the TB germ, but its excessive production cause tissue necrosis, fever and cachexia, which makes a person feeble and bed ridden (Condos and Rom, 2004).

Although, TB is caused by a bacterium, there are some risk factors which make a person more prone to this deadly disease. Some of them to be counted are genetic make up of host, HIV infection, consumption of alcohol and drugs, crowded living, regular contact with TB patients like in health care workers (Cantwell et al., 1998).

2.1.3 Types and Symptoms

Tuberculosis is classified as pulmonary TB and/or Extrapulmonary TB depending upon the site of infection. Pulmonary TB is mainly characterized by formation of lesions in lungs, which can be identified by X-ray examination. The further establishment of disease can be done by sputum smear. Patients are further divided into sputum smear positive and sputum smear negative on the basis of AFB microscopy. When the MTB disseminate to other body parts such as pleura, lymph nodes, kidneys, brain,
genitourinary system, skin, joints or bones, it is known as extra pulmonary TB (EPTB) (Knechel, 2009; Palomino et al., 2007).

The PTB is most common type of TB which accounts for almost 80% of all forms of TB disease (Iseman, 2000). The patients may have both type of TB at one time, but that is classified into Pulmonary TB (RNTCP, 2005). The principle symptom of PTB is cough with sputum that lasts for more than three weeks. The intensity of cough is high in night and early morning. The sputum may contain blood, which is called hemoptysis. The other symptoms which are normally same for both PTB and EPTB are low grade fever with rise of temperature in evening, chest pain, weight loss, loss of appetite, weakness, night sweats and dyspnea (Palomino et al., 2007; Sri, 2003).

2.1.4 Categorization of Patients and Anti-tuberculosis Therapy

After diagnosis of TB in patients, they are divided into three categories (table 2.1) according to DOTS and RNTCP guidelines and treatment is started. Treatment is done according to the guidelines of DOTS programme. In this programme it is ensured that patient swallow the drug in the presence of a dedicated health worker or a social volunteer. The drug regimen of anti-tuberculosis therapy has been divided in to intensive phase and continuation phase. Intensive phase usually continues for two months and then continuation phase for next four or five months. Sputum is tested for the presence or absence of MTB after intensive phase, in between continuation phase and at the end of the therapy. Accordingly, the precautions are taken and the drug regimen is changed (RNTCP, 2005).

Table 2.1: Category of treatment of TB patients

<table>
<thead>
<tr>
<th>Category of Treatment</th>
<th>Type of Patient</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Category I</strong></td>
<td>New sputum smear-positive</td>
</tr>
<tr>
<td></td>
<td>Seriously ill, new sputum smear-negative</td>
</tr>
<tr>
<td></td>
<td>Seriously ill, new extra-pulmonary</td>
</tr>
<tr>
<td><strong>Category II</strong></td>
<td>Sputum smear-positive Relapse</td>
</tr>
<tr>
<td></td>
<td>Sputum smear-positive Failure</td>
</tr>
<tr>
<td></td>
<td>Sputum smear-positive, Treatment After Default</td>
</tr>
<tr>
<td><strong>Category III</strong></td>
<td>New Sputum smear-negative, not seriously ill</td>
</tr>
<tr>
<td></td>
<td>New Extra-pulmonary, not seriously ill</td>
</tr>
</tbody>
</table>
2.1.5 Relentless March (MDR, XDR, Allies with HIV)

The persistence of TB regardless of the availability of cheap drugs and moreover free treatment by governments makes this disease more apprehensive. The situation further aggravates when TB turns into MDR or XDR or associates with HIV.

In MDR-TB, the strains of TB bacilli are resistant to isoniazid and rifampicin, the drugs which are regarded as best in anti TB medicines. So, the patients with MDR-TB need to have second line of drugs, which is expensive and difficult to be proceeded by financially poor patients (RNTCP, 2011). The MDR-TB is a consequence of past history of TB, severity of illness, defaulting from ATT, poor knowledge on part of patients about this disease. Even genetic factors of host, like polymorphisms in HLA class II regions are also found to be responsible for this extreme form of TB (Sharma et al., 2003). MDR-TB not only leads to extra cost of cure, but also increases chance of transmission of highly pathogenic strains responsible for this type of disease (RNTCP, 2009).

Rather rare form of TB is XDR, the patient with this type is certain to have premature death, even after having treatment. These patients are resistant to even second line medication of fluoroquinolones, with already resistant to isoniazid and rifampicin. They also do not respond to injectable drugs, amikacin, kanamycin or capreomycin or at least to one of them (Dietrich and Doherty, 2009). WHO classified XDR-TB as a serious emerging threat to global public health in 2006, especially in those countries which have high prevalence of HIV. The idea of its destructive power can be had from the story of a small town of Tugela Ferry in KwaZulu-Natal province of South Africa. Church of Scotland Hospital serves this area with high HIV rate. In 2006, out of 536 TB patients, 53 were diagnosed with XDR-TB in this hospital out of which 52 died within days of detection (RNTCP, 2009; Migliori et al., 2008).

The dual infection of TB and HIV magnify the death toll, especially in poor countries. HIV infection enhances the risk of TB and also the risk of reactivation of latent TB by 20 times compared to HIV negative individual. TB is one of the earliest infections, known to infect HIV patients. The lives of HIV patients with TB get worsen with higher viral load, and death rate in those patients is higher when compared to HIV patients without TB (Dietrich and Doherty, 2009). TB is regarded as principal cause of deaths
among HIV patients. According to RNTCP report (2009), 8% of new TB cases were HIV positive and 12% TB deaths happened in HIV positive individuals.

### 2.1.6 Social and Economic Burden of TB

Tuberculosis is not only a disease but also a great social stigma (RNTCP, 2011). People with TB can not share their sufferings openly as people around them start avoiding them. Prejudices due to TB can be counted in many ways. School dropout in children, problem in getting married, outcast at workplace, community and even in household are some of them. In India nearly 300,000 school children dropout from the schools and more than 100,000 women with TB disease are abandoned by their families every year. The death rate in women due to TB is much higher when compared to any other infectious disease or all other aggregate causes of deaths in women. The age group of 15-55 years have highest death rate which is regarded as the most productive years in social and economic scenario. In terms of economic loss, India looses approximate of 3 billion US dollars as indirect cost and 300 million US dollars in form of direct cost. Every year because of TB more than 170 million work-days are lost (RNTCP, 2009; RNTCP, 2005).

### 2.2 Immunopathology of TB

Nature has made the biological systems to be very robust. Any disturbance in their internal or external environment can be adjusted to an extent, with some modifications in their essential life supporting components and physiology. If the change happened to be an abiotic factor, all the modulations has to be made in part of host, otherwise for any biotic entities trying to accommodate each other in their environment, the regulation of their components has to be made from both sides. Host-pathogen relationship has been cited as a good example to this notion and successful infection of host by TB bacterium is an important element to be studied for well being of human race. It has now been recognized that the host cell can go from total extermination of MTB from human body to active disease of TB or their can be asymptomatic latent infection of bacteria (Chandra et al., 2011).

It is a well known fact that only 5-10% of individuals infected with TB bacteria develop clinical TB disease and remaining 90-95% escape from the clutches of this menace (RNTCP, 2011). This observation tells that the immune system of human body is
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quite efficient for the management of TB. Nonetheless, the dialogue between MTB and host immune cells is too complex to be completely understood till now. Adding to this intricacy is the existence of different strains and lineages of MTB (Basil et al., 2011; Sharma et al., 2008) and they employ their own approach of modulating human immune system for survival in host tissue (Kumar et al., 2010). It has been elucidated that TB bacilli are very successful in evading host immune system. Their secretory antigen induces the full maturation of DCs at site of infection and thus down regulating pro-inflammatory response crucial for eradication of MTB (Natarajan et al., 2003). *Mycobacterium tuberculosis* secreted antigen (MTSA)-10 was shown to interfere with lipopolysaccharide (LPS) signaling, thus reducing the generation of effective immune response against MTB. This antigen also disrupts the host cell signaling machinery so as to downregulate transcription of genes which are essential for macrophage to work against TB bacterium (Basu et al., 2009; Basu et al., 2006). Also all the host factors do not impede the growth of TB bacilli but there are some which can facilitate their survival inside host (Jayaswal et al., 2010).

TB can be controlled by the both innate and adaptive constituents of immune system (Palomino et al., 2007). Dendritic cells and macrophages are important immune entities in the body of host as they are first to encounter and harbor the TB bacteria. They help to contain the disease of TB by initiating different immunologic cascades while on the other hand MTB survives and reproduce in them by evading all of them. Macrophages along with other different types of immune cells and immune mediators take part in the process of eradication of MTB from body. So, the cross talk between the host and the pathogen is important to understand.

2.2.1 Macrophages and MTB

Phenotypes of the phagocyte in the lungs have become a controversial issue. It was demonstrated that the phagocytic cells which accumulate in the lungs are macrophages derived from monocytes (Cooper, 2009). In recent years, the observation, that dendritic cells (DCs) can be infected by mycobacteria *in vivo* have confronted the notion that only macrophage are principal host cells for MTB. Further, the cells of DC phenotype were determined to be primary host for MTB in infected lungs (Wolf et al., 2007; Jiao et al., 2002). However, the origin of these cells and function in controlling
MTB infection is still under investigation (Cooper, 2009). So, because of complex nature of cellular environment in MTB infected lungs and paucity of data on cells other than macrophage, macrophage has been discussed in this review as principle host cells for TB bacilli.

The interaction of MTB and macrophages was briefly talked about in section 2.1.2. The macrophages are the cells which engulf the bacterium in alveoli, who survives the physical defense created by goblet cells. Interaction of macrophage and MTB starts by their binding to each other. Different types of cell surface receptors like Complement receptors (CR1, CR2, CR3 and CR4) and mannose receptors (MR) bind bacteria to macrophage, which are later phagocytosed by these immune cells. Microbes are than degraded by acidic hydrolases after phagosome-lysosome fusion, by reactive oxygen intermediates (ROIs) or reactive nitrogen intermediates (RNIs) (Raja, 2004; Raupach and Kaufmann, 2001). ROIs and RNIs are generated by macrophages after they get activated by immune mediators like IFN-γ and TNF-α. TNF-α is ineffective alone but in combination with IFN-γ they strongly activate macrophage for antimycobacterial properties (Flynn and Chan, 2001). However, MTB has also evolved strategies to defend itself from these aggressive molecules and approach of host cells. To survive inside macrophage it has developed mechanism that circumvents phagosome-lysosome fusion. Mycobacterial sulphatides are reported to be important molecules for inhibition of phagolysosomal fusion. Ammonia produced in cultures by TB bacterium has also been demonstrated to be responsible for this inhibition in vitro (Raja, 2004; Natarajan et al., 2003). To escape from ROIs or RNIs, the MTB tries to evade physical contact with them. If, in case, it fails in this management, bacteria survive by detoxification mechanisms which include production of molecular scavengers, antioxidant enzymes, DNA repair processes and expression of specific antioxidant regulons (Raupach and Kaufmann, 2001).

Programmed cell death or apoptosis of MTB infected macrophage is also considered to be a mechanism against proliferation of this bacterium. Molloy et al., (1994) reported that apoptosis of macrophages results in low viability of MTB. Studies have controversies regarding role of apoptosis in mycobacterial load, as some say that load is reduced while others have conflicting views to this (Raja, 2004). Xenophagy in
infected macrophage has also been demonstrated to be an important mechanism for clearance of intracellular MTB. However the bacterium too is, quite able to attenuate the activation of xenophagic pathways for ensuring its own survival in host cells (Kumar et al., 2010).

MTB has been shown to be an organism, which is a classical example, for which the body requires cell mediated immunity as the bacterium resides in macrophages. Thus, the T cells which are important component of cell mediated immune response have been appraised to some extent in the ensuing headings.

2.2.2 The Role of T Lymphocytes

Different types of T lymphocytes like CD4, CD8, γδ and CD1 restricted are important for optimum immunity against MTB, but out of these CD4 T cells were regarded of significant importance followed by CD8 T cells (Kaufmann, 2002). Now the scenario of research has changed and lot of work is being done on CD8 T cells (Flynn and Chan, 2001). In TB infection, T cell activation can take place through both MHC class II as well as class I presentation. It is not known with certainty that when MTB reside in phagosome and its antigen are readily accessible for MHC class II processing why it also has access for MHC class I processing? The two hypotheses for this are available, one states that MTB form pore in the phagosome membrane so that it can gain access to cytoplasm nutrients, but through these pores antigen translocation can also takes place for MHC class I processing (Mazzaccaro et al., 1996). Second one state that direct antigen transport does not take place, but the apoptosis of macrophage leads to formation of extracellular vesicles having MTB antigens. These vesicles are taken up by dendritic cells, which are than presented in context of MHC class I (Flynn and Chan, 2001).

2.2.2.1 CD4 T Cells

The MHC class II presentation of MTB antigen to CD4 T cells is an evident result of TB infection. These cells hold a vital place for themselves in protective immunity against mycobacteria. Major effector function of CD4 cells is believed to be the production of IFN-γ cytokine, which activate the macrophages, which in turn control or eradicate TB bacterium (Raja, 2004). In murine studies, it has been shown that mice having depleted number of CD4 T cells fall prey to TB (Caruso et al., 1999). The HIV
infection in human beings clearly reveals that depletion of CD4 T cells, greatly enhance susceptibility to MTB infection (Raja, 2004).

2.2.2.2 CD8 T Cells

CD8 T cells are thought to be more important in later stages of TB disease (Serbina and Flynn, 2001). They also produce IFN-γ and have cytotoxic activity (Shams \textit{et al.}, 2001; Stenger, 2001). The main function is controversial but, it is suggested that they eliminate non responsive macrophage resulting in release of mycobacteria, which are then taken up by freshly recruited phagocytes with antimycobacterial capacity. This has been demonstrated that if MHC class II is deficient, there is impairment in the response to acute TB infection, whereas MHC class I deficiency have less effect on immunity. On the other hand in case of chronic TB infection less responsiveness of MHC class I have great influence on anti-MTB immunity (Doherty and Andersen, 2005). CD8 T cells are implicated in direct killing of mycobacteria. This is achieved by use of perforin, required to form pore and executed by granulysin that has cytotoxic effect on bacteria (Rom and Garay, 2004).

2.2.2.3 CD1 Restricted and γδ T Cells

The role of human γδ T cells has been shown in early phase of immune response against mycobacteria. These cells recognize non-peptidic antigens like pyrophosphate, alkylamine and nucleotide antigens (Morita \textit{et al.}, 1996). The population of γδ T cells increased during MTB infection and they also acquired memory function after TB infection (Doherty and Andersen, 2005; Ulrichs \textit{et al.}, 2003).

CD1 family consists of antigen presenting molecules, which are encoded by genes located outside to MHC. Group 1 CD1 molecules are found on professional antigen presenting cells, while group 2 CD1 are found on cells like nonprofessional APCs, certain B-cell subsets. In mouse group 2 CD1 is more widely distributed and found on T cells, B cells, dendritic cells, hepatocytes, and some epithelial cells (Kindt \textit{et al.}, 2007). Group 1 CD1 present mycobacterial lipid and glycolipid antigens and group 2 CD1 interact with natural killer (NK) T cells. The CD1 system has been reported to be concerned with cell mediated response against MTB. The actual role of CD1 restricted T cells against MTB remains to be elucidated. However, initial studies report that their effector role can be production of IFN-γ and cytotoxic activities, which may not target
macrophages. They also appear to recognize glycolipids in the wall of MTB and distinguish them from self-derived lipids (Doherty and Andersen, 2005; van Crevel et al., 2002). Group 2 CD1 restricted NKT cells get activated by MTB cell wall components and were involved in granuloma formation (Rom and Garay, 2004).

### 2.2.3 The Cytokines

For an effective immune response against TB bacterium, different cells of immune system like macrophage, dendritic cells and T cells are needed and the specific interactions between them are required (Natarajan et al., 2003). These interactions are mediated by low molecular weight (up to 30 kDa) soluble proteins, secreted by leukocytes and various other cells of body, in response to some stimulus. These soluble proteins are collectively known as cytokines and have a major effector function of cell to cell communication (Kindt et al., 2007). They have the ability to modulate the immune response to pathogens and play an important role in antimycobacterial immunity (Natarajan et al., 2001).

#### 2.2.3.1 Pro-inflammatory Cytokines

The Mycolic acids present in the cell wall of *Mycobacterium tuberculosis* stimulate the host inflammatory response leading to the formation of granuloma, NK and T cell responses, activation of macrophages and upregulation of antigen presentation. It has been demonstrated that mycolates, trehalose dimycolate, cord factors present in cell wall of MTB selectively induce production of Th1 cytokine response through signal transducer and activator of transcription-4 (STAT-4) signaling (Doherty and Andersen, 2005). Th1 cytokines, IFN-γ and TNF-α, are produced with other proinflammatory cytokines like IL-6, IL-12, IL-15 and IL-18. IL-6 deficient mice have increased susceptibility to MTB infections, additionally it was found to be harmful during mycobacterial infections as it inhibits production of TNF-α. IL-12 and IL-18 in conjunction play a vital role in the IFN-γ induction. IL-15 is found to be helpful in stimulating T-cells and NK-cells proliferation and activation (Schroder et al., 2004; van Crevel et al., 2002). The costimulatory activity of TNF-α in IFN-γ based activation of macrophage against tuberculosis bacterium has been established. It was demonstrated that IFN-γ and not TNF-α stimulates the macrophage for functioning against MTB, but blocking of TNF-α greatly reduces the activation of APCs (Flesch and Kaufmann, 1993;
Flynn et al., 1995). TNF-α is also required for granuloma formation. Impairing TNF-α function or its receptors increases the bacterial load and weakens formations of granulomatous lesions (Kaufmann, 2002). It has been established that macrophages activated by IFN-γ have high capacity of pinocytosis and receptor mediated phagocytosis, in addition to increased ability of killing microbes (Schroder et al., 2004).

2.2.3.2 Anti-inflammatory Cytokines

The response of pro-inflammatory cytokines is antagonized by anti-inflammatory factors. Three main anti-inflammatory cytokines involved are IL-10, IL-4 and TGF-β (van Crevel et al., 2002). IL-10, a potential immunosuppressive protein, has been shown in villainous role in clearance of MTB infection (Ellner, 2010; Kumar et al., 2010). It is established that MTB cell wall component, mannosylated lipoarabinomannan, inhibited dendritic cell maturation by interfering with its surface receptors and induced them to produce IL-10 (Wu et al., 2011) and TGF-β (Dahl et al., 1996). IL-10 cytokine down regulates protective immunity against TB by inhibiting the production of IFN-γ, TNF-α and IL-12 cytokines (Fulton et al., 1998; Gong et al., 1996; Hirsch et al., 1999). Thus IL-10 modulates the host defense against tuberculosis bacterium (Abhimanyu et al., 2011; Redford et al., 2011). Similarly, TGF-β also works against protective immunity in TB. It suppresses cell mediated immunity, IFN-γ production, antigen presentation in macrophage, pro-inflammatory cytokine production and cellular activation (Raja, 2004). It is also implicated in tissue damage and fibrosis during TB. TGF-β enhances the production and deposition of macrophage collagenase (Toossi and Ellner, 1998) and collagen matrix (Sporn et al., 1986). This cytokine induces the production of IL-10 and both suppress the IFN-γ production. The deleterious effects of IL-4 are also similar to IL-10 and TGF-β as it is found to be involved in suppression of IFN-γ and macrophage activation (Appelberg et al., 1992; de Waal Malefyt et al., 1993; Powrie and Coffman, 1993; Lucey et al., 1996). In mice model of TB infection, it has been seen that disease progresses and reactivates with the increased production of IL-4. Tissue damage has also been associated with over expression of IL-4. However studies have also indicated that IL-4 may be a consequence of MTB infection rather than a cause of TB, as IL-4 knockout mice presented with normal susceptibility to ailment of TB rather than enhanced vulnerability (Doherty and Andersen, 2005; van Crevel et al., 2002).
From the various studies it seems that pro-inflammatory cytokines provided protection from TB and anti-inflammatory had the opposite effect. However, this does not mean that anti-inflammatory factors are adversary to the host as uncontrolled pro-inflammatory response leads to tissue damage. Activation of macrophage by inflammatory cells promote bacterial killing and must be tightly regulated to avoid any damage to host tissue. Production of anti-inflammatory cytokines help regulate this inflammatory process, nevertheless this can give chance to MTB for its growth in host tissue. It can be speculated here that genetically determined high or low production of cytokines may render an individual vulnerable or resistant to MTB infection depending upon their nature of function (Redford et al., 2011; van Crevel et al., 2002).

2.3 Host Genetics and TB Vulnerability

In section 2.1.2, it was made apparent that for a long time TB was known as a genetic disease, before its actual cause was deciphered to be a bacterium. Now when the field of genomics is treading a fast path, it has been established that though this disease is not a genetic one, a genetic component of host can render it susceptible or resistant entity. While scanning the scientific literature describing the infection of TB, an amazing point emerged that out of infected individuals only 5-10% develops clinical signs of this ailment (RNTCP, 2011). This means that a dynamic interaction happens between the two participants that lead to the development of TB. Clearly, the genetic factors play a decisive role in protecting 90% of infected individuals from getting the disease. On the other hand, it is also possible that hereditary factors play a role in succumbing to this deadly disease. Studying the genetics behind TB can put some light into the disease pathogenesis and thus assisting in the management of this old foe.

Number of genetic elements like different cytokine genes (Ben-Selma et al., 2011a; Onay et al., 2010; Pacheco et al., 2008), autophagy genes (Kleinnijenhuis et al., 2011), HLA system (Sharma et al., 2003), Nramp (Merza et al., 2009), vitamin D receptor (Sharma et al., 2011), IFN-γ receptor (Onay et al., 2010), purinergic P2X7 receptor (Sharma et al., 2010), Toll like receptor gene (Dalgic et al., 2011) etc. has been associated with human TB. How a certain gene is regarded as a marker of association to a disease, can be evaluated in different ways. Different strategies have been evolved, some of which include animal model studies, candidate gene approach, family studies and
genome wide linkage and association studies to elucidate the role of genetic factors in susceptibility to TB (Marquet and Schurr, 2001; Selvaraj, 2004).

2.3.1 Animal Models of Study

Animal models have helped immensely in studying TB disease (Schluger and Rom, 1998; Pietrantonio and Schurr, 2005). It is easier to dictate and/or modulate the genetic makeup of the animals and to inject them with MTB. Animals like Guinea pigs, rabbits, mice are being used as model of human TB as they immunologically, histologically and in the form of clinical TB disease resemble the humans (Shanks et al., 2009).

There seems to be very less research on Guinea pigs and rabbits. Former animal was used by Robert Koch in 1882 to demonstrate that TB was caused by Mycobacterium tuberculosis (Koch, 1882). Inbred rabbits have also been shown to differentially acquire the infection of MTB. Resistant strains develop pulmonary form of TB while susceptible one developed hematogeneously spread disease. This inheritance pattern was thought to be genetically determined (Lurie, 1952). Much work has been done on mice strains to see putative genes responsible for resistance or susceptibility to MTB infection. The NRAMP1 gene was the first one to be responsible for abnormal sensitivity of mice towards BCG infection, Salmonella and Leishmania (Palomino et al., 2007). Later, this gene has been associated with susceptibility to TB in many human populations (Bellamy, 2002). Various genetically modified mice strains like C3HeB/Fej (susceptible), C57BL/6J (resistant), HcB/Dem recombinant congenic strains, mice with altered cytokine gene are now being used to study the genetics of host (Pietrantonio and Schurr, 2005; Turner et al., 2002; Murray and Young, 1999; Ladel et al., 1997; Hagenbaugh et al., 1997).

2.3.2 Candidate Gene Approach

The candidate gene approach is based on the study of polymorphism in the known genes and association of these polymorphisms with a disease (Moffatt and Cookson, 1999; Kwon and Goate, 2000). The approach is used when some hypothesis regarding disease causation exists. The basic strategy for candidate gene approach involves:

- The choice of the genes to be tested. A candidate gene is one whose product has functional properties that makes its involvement in disease causation likely. The
choice of the gene to be tested is a critical step in this strategy. Ideally candidate gene is present in the area of the chromosome that has previously been associated with disease, using the positional cloning approach.

- The next step involves identifying polymorphism in the gene which has functional significance.
- Performing allele association studies. These are case-control studies in which the frequency of the each of the alleles is compared in affected and unaffected individuals. An allele is said to be associated with the disease if it occurs at a significantly higher frequency among the affected compared to the unaffected normal healthy control subjects.

As most candidate gene studies follow case-control design, the ethnic matching of subjects is very important, otherwise false genetic association can be found for a particular disease. In one such example, it has been found that Caucasian Americans are more resistant to MTB infection than African Americans (Stead et al., 1990). It means that ethnicity has great influence on genes because of process of evolution. On the basis of case-control studies various genes with single nucleotide polymorphism, like NRAMP1, vitamin D receptor gene (VDR) (Merza et al., 2009), TNF-α (Kumar et al., 2008), IFN-γ, IL-10 (Pacheco et al., 2008) have been associated with manifestation of TB. However, their association may vary in different populations.

### 2.3.3 Family Studies

Family studies may include twins, sib pairs, parents and child (trios) or complex families which provide clue about the associations of a disease with genetic component of host. Beiguelman (1981) reported that whenever development of an infectious disease is more frequent in parents and offspring, than in between spouses, then the role of genes can not be ruled out in catching of ailment. However, there remains ambiguity in terms of household exposure to family members and thus no convincing demonstration can be found for genetic predisposition. In the example of twins, contradiction in views emerges out. Monozygotic twins are identical genetically, while dizygotic are like any other sibling. Now, if it is assumed that same environmental exposure to both type of twins then if both gets TB or both remains healthy, can be attributed to genetic components. In a study from United States approximate three fold more concordance has been found in
monozygotic twins than in dizygotic twins irrespective of history of exposure (Kallmann and Reisner, 1943). Dizygotic twins had same concordance as seen in non twin siblings. However, in contrast to this it has also been discussed that environmental factors outweigh the importance of hereditary factor in getting the infection of TB (Simonds, 1963). One more area in which family studies loose point, is that they have less power than case-control studies as they have generally fewer subjects (Palomino et al., 2007; van der Eijk et al., 2007).

2.3.4 Genome wide Linkage and Association Studies

Genome wide research has a big benefit over candidate gene studies. Unlike in candidate gene studies these do not require prior knowledge of structure or function of a gene. Number of novels genes can be identified, which previously were unknown for their association with a disease (Marquet and Schurr, 2001).

In genome wide linkage study, a large number of microsatellite markers of importance are used for linkage analysis. Study is carried out by using affected sib pairs. Those families are collected which have two diseased siblings and whose parents do not have the disease. This approach tests whether segregation of markers takes place according to the Mendelian inheritance or not (Selvaraj, 2004; Marquet and Schurr, 2001).

In case of genome wide association studies millions of SNPs can be studied in whole of genome. Because of evolving technologies like DNA sequencing, microarray and software tools for biology this has become a reality. The replication of results of genome wide association in independent populations is a necessity as they are found to have a high false discovery rate (Bellamy, 1998; Burgner et al., 2006).

2.4 Single Nucleotide Polymorphisms (SNPs): Emerging Markers of Susceptibility

Single nucleotide polymorphisms (SNPs) are variations in DNA sequence due to change in single nucleotide (A,T,C,or G) in the genome sequence. The single base change must be present in at least 1% of population to be considered as a SNP (Micklos et al., 2003). It is known that more than 99% human genome is same and less than 1% of change creates all the distinctions in human beings. In these changes about 90% is constituted by SNPs which accounts for 1 per 1200 bases in a total of 3.2 billion bases in human genome. Approximate 3.2 million SNPs, occurring in the human genome, can be
found anywhere in promoter region, intron or exon of a gene (Sadeghnejad, 2006; Kwok and Chen, 2003; Micklos et al., 2003). Any difference in genetic make up of an individual from others, dictates how that person will respond to a disease, to environmental factors, to drugs or any therapy etc. As, the highest number of variations in human genome are due to SNPs, they constitute an important part of biomedical research (Gray et al., 2000). They are also evolutionary very stable (Farkas, 2004) which makes them important markers for population studies (Sadeghnejad, 2006).

In recent times, the SNP have become workhorse of high throughput genotyping technologies like genome wide association studies. SNPmap and HAPmap projects are in place to create a database of SNPs and to look for linkage disequilibrium in them (Wild et al., 2011; Gray et al. 2000). These all modern assets could come up with new genes and markers of susceptibility to human diseases, building strategies for staving off ailments, allergies or atleast preventing them and looking for drug benefits to humans. Personalized medicine is one concept in which appropriate drug for a particular individual could be determined well in advance of treatment by going through the SNP profile of the patient (Micklos et al., 2003).

As such a disease is not caused by SNPs but they can predict, in advance, towards the genetic predisposition of a person for a particular disease. Many diseases like TB (Sharma et al., 2011; Pacheco et al., 2008), asthma (Kumar et al., 2008), rheumatoid arthritis (Sugiura et al., 2002), cancers (Silva et al., 2011), leprosy (Malhotra et al., 2005), migraine (Samaan et al., 2011), Alzheimer (Antoniades et al., 2011), Diabetes (Moon et al., 2011), End Stage Renal Disease (Mohindru et al., 2004) are being looked for SNP based vulnerability. In case of tuberculosis the SNPs in different population has been associated with susceptibility. Many genes like HLA (Sharma et al., 2003), VDR gene (Sharma et al., 2011), P2X7 (Sharma et al., 2010), IFN-γ (Ben Selma et al., 2011a), TNF-α (Kumar et al., 2008), TGF-β (Kumar et al., 2007), IL-10 (Pacheco et al., 2008) have SNPs which have been looked for their association with TB.

2.5 IL-10 and IFN-γ: Candidate Cytokines to TB Immunity

Vital implications of IL-10 and IFN-γ cytokines in TB and their antagonistic role to each other makes them interesting targets to be studies in pathophysiology of this dreaded disease. IFN-γ is an important mediator for eradication of MTB from the
host cell whilst IL-10 help in survival of TB bacilli by suppressing the immune system. It has been observed that low IFN-γ and high IL-10 production can prove to be detrimental for host, and this work can cleverly be done by MTB itself through modulation of host immune response (Trajkovic et al., 2004). The TB bacteria modulate the immune system in such a way, so as to upregulate the expression of immunosuppressive cytokines like IL-10. The function of cytokine IFN-γ with strong antimicrobial properties is disrupted by modulating its receptors on MTB infected macrophage. In such events, the presence of IFN-γ in plenty, can not be used for killing of TB bacterium (Kumar et al., 2010).

Both the mentioned cytokines have SNPs in their genes, which account for their differential production. Studies conducted in various populations of the world have shown association of these polymorphisms and of systemic levels of cytokines with TB (Anand et al., 2010; Henao et al., 2006; Lopez-Maderuelo et al., 2003; Olobo et al., 2001). It is interesting to note that similar data is lacking on these cytokines from Punjab, India.

2.5.1 Interleukin-10 (IL-10)

Intrleukin-10 is an important bio-molecule of immune system. In humans, it is an 18.5 kDa homodimeric cytokine with subunits having length of 160 amino acids (Moore et al., 1993). It inhibits the secretion of pro-inflammatory cytokines like IFN-γ, IL-2, TNF-α and suppress the antigen presentation capacity of APCs, therefore, it is known by various names like cytokine synthesis inhibitory factor (CSIF), immunosuppressive cytokine and is also described as anti-inflammatory cytokine. This cytokine is mainly produced by Th2 subsets of CD4+ helper cells, activated macrophages and some activated B cells. However, Th0 and Th1 cells are also known to produce IL-10 following activation. The gene for this cytokine has been mapped to chromosome 1q with ~4.7kb genomic DNA consisting of five exons (Lalani et al., 1997; Turner et al., 1997).

Interleukin-10 has been implicated in the pathophysiology of TB due to its immunosuppressive activity. It is regarded that the high production of this cytokine can render an individual susceptible to TB as it inhibits the production of IFN-γ, TNF-α and suppress activity of macrophages, thus resisting the elimination of MTB. On the other hand a report also suggested the probable role of IL-10 in converting DCs into
macrophage like cells with increased virulence against MTB and thus inhibiting the
growth of MTB in host (Fortsch et al., 2000)

2.5.1.1 IL-10 and Tuberculosis

2.5.1.1.1 Animal Studies

Many animals, predictive for humans, biologically and immunologically are being
used as a model for studying human diseases (Shanks et al., 2009). Guinea pigs and mice
have been utilized by many workers. Murray et al., (1997) and Murray and Young (1999)
in their studies have defined the role of IL-10 in immune response to mycobacterial
infections. They have demonstrated that transgenic mice which secrete increased levels of
IL-10 are more susceptible to mycobacterial infection, although their IFN-γ levels were
similar to those of wild type. Based on this study, they further studied IL-10 knock out
mice and noticed that the animals were more immune toward TB. Over expression of this
immunosuppressive cytokine has also been shown to reactivate TB in transgenic mice,
with significant increase in number of MTB within lungs (Turner et al., 2002).
Measurement of IL-10 mRNA from BCG vaccinated Guinea pig PBMCs, spleen and
BAL cells revealed little change in level of IL-10 expression when compared with normal
one. However, there was no increase in its mRNA in spleen and BAL cells as speculated
by Kawahara et al., (2002). It has also been reported that IL-10 modulate the induction of
apoptosis in MTB infected murine macrophage. Murine rIL-10 behaves opposite to
rTNF-α. Later reduces apoptosis while the former augments this process of cellular
destruction. Overall the balance between TNF-α and IL-10 cytokines, produced by MTB
stimuli determines the process of apoptosis, suggesting its role in the innate
antimycobacterial mechanisms (Rojas et al., 1999). As it is a known fact that macrophage
survival is essential for MTB to remain in host body and IL-10 induced reduction of
apoptosis of macrophage can be helpful to TB bacterium for its survival in host.

2.5.1.1.2 IL-10 Levels and Human TB

In human studies, number of researchers has studied levels of IL-10 cytokine in
TB patients. The studies do not converge at a point, regarding levels of this cytokine in
TB patients. Where some say the levels are high in TB patients, others say otherwise or
some of them report no change in levels of this cytokine when compared with NHCs or
cured individuals. This observation can be owed to different type of samples and different
Review of Literature

procedures used for IL-10 measurements. Various procedures included measurement of IL-10 from serum, plasma or PBMCs stimulated with attenuated MTB, Lipopolysaccharide (LPS) or purified protein derivative (PPD) etc.

Number of reports notify the difference in serum IL-10 levels in TB patient, cured individuals and NHCs. (Deveci et al., 2005; Vankayalapati et al., 2003; Verbon et al., 1999). Deveci et al., (2005), while studying TB patients at different time intervals of ATT, found that active pulmonary TB patients had higher levels of IL-10 than inactive pulmonary TB cases and NHCs. Significant difference in serum IL-10 levels was observed in patients before start of ATT, when compared with patients after 4 and 6 months of anti TB regimen. The difference was non-significant on comparison with patients after 2 months of ATT. High serum IL-10 levels in patients with TB have also been found by Vankayalapati et al., (2003), when compared to healthy TST responders. In yet another study which recruited five different groups of subjects, it was noted that significant difference in serum IL-10 levels exists among them. Active TB patients had highest levels of this protein followed by patients during therapy, patients after treatment and contacts of TB patients. Least IL-10 serum levels were found in skin test negative healthy controls (Verbon et al., 1999).

Some of the studies used ex vivo cytokine production capacity of isolated PBMCs after stimulation with MTB antigen. In such cases, about 35% elevated IL-10 cytokine have been reported in PPD stimulated PBMCs cultures of TB patients by Lopez-Maderuelo et al., (2003), when comparison was made with PBMCs from contacts or NHCs. In response to LPS stimuli IL-10 production was significantly higher in PBMCs of active TB patients, which decreased at the end of therapy. However when MTB sonicate was used as stimulus, the levels of this very cytokine remained below detection limit in both TB patients and NHCs (Sahiratmadja et al., 2007). In anergic TB patients it has been found that IL-10 production remain higher even after successful treatment, which suggest towards suppression of effective immune system because of MTB induced IL-10. Here anergy has been defined as the lack of skin reactivity to tuberculin PPD (Boussiotis et al., 2000)

Ameglio et al., (2005) reported that there is no change in the serum IL-10 levels with progression of ATT when it was measured in TB patients before the start of therapy.
and on conclusion of therapy. However, epithelial lining fluid show increased value of IL-10 in patients after six months of ATT, when compared to IL-10 level at the start of medication. It may suggest local synthesis of this anti-inflammatory cytokine indicating towards incomplete cure of disease. Similar results were shown in MTB-stimulated cultured PBMCs by Vankayalapati et al., (2003). They measured IL-10 in culture supernatants of PBMCs of TB patients and NHCs and came to conclude that there is no significant difference in the levels of this protein between two groups studied.

Study of Olobo et al., (2001) have some unique set of results, which demonstrated that pulmonary TB patients before cure have high plasma IL-10 levels, when compared to cured patients, but contacts of TB patients have even higher IL-10 levels. Controls have levels higher than TB patients before and after cure, but lower than contacts.

As IL-10 is reported to inhibit the mycobacterial elimination, the immunity of a person to TB infection may be reflected in high or low circulating IL-10 levels.

2.5.1.1.3 Different Genotypes and IL-10 Production

The genetic control for the production of IL-10 cytokine lies in the three biallelic polymorphism occurring in promoter of its gene, from transcriptional start site at -592, -819 and -1082 positions. Polymorphism on these three sites occur from C to A at -592 commonly represented as C-592A, from C to T at -819 shown as C-819T and from G to A at -1082 generally written as G-1082A. These three SNPs have been reported to be accountable for high or low production of this cytokine. Most of the studies have focused only on G-1082A SNP. In this case individuals with genotype GG are found to be high producer of IL-10 protein followed by GA and least production is seen in AA genotype. When C-819T and C-592A polymorphisms are also taken into account, the effect of genotypes on IL-10 production can be redefined. Theoretically, eight haplotypes viz. GCC, GCA, GTA, GTC, ATA, ATC, ACC and ACA can be generated from these three loci. But, these three positions are reported to be in partial linkage disequilibrium, thus affecting the transcription of IL-10 gene and as a result the production of this cytokine. Although, most studies have observed only three major haplotypes viz. GCC, ATA, ACC, all associated with differential production of this cytokine. There are also reports of occurrence of rare haplotypes such as GTA, ATC, GTC and ACA (Afzal et al., 2011; Scarel-Caminaga et al., 2004). GCC is related to high IL-10 production in peripheral
blood mononuclear cell (PBMC) culture (Turner et al., 1997). The production defined by all the three haplotypes can be summarized in table 2.2.

### Table 2.2: Relation of IL-10 gene promoter SNPs (-1082, -819 and -592) and its production

<table>
<thead>
<tr>
<th>Diploptype</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATA-ATA</td>
<td>Low IL-10 production</td>
</tr>
<tr>
<td>ACC-ACC</td>
<td>Low IL-10 production</td>
</tr>
<tr>
<td>ACC-ATA</td>
<td>Low IL-10 production</td>
</tr>
<tr>
<td>GCC-ACC</td>
<td>Intermediate IL-10 production</td>
</tr>
<tr>
<td>GCC-ATA</td>
<td>Intermediate IL-10 production</td>
</tr>
<tr>
<td>GCC-GCC</td>
<td>Highest IL-10 production</td>
</tr>
</tbody>
</table>

### 2.5.1.1.4 IL-10 SNPs, Haplotypes and Their Association with TB

Association based studies of different IL-10 polymorphisms with susceptibility to disease of TB has been done by various research groups. Evaluation of Chinese Han population for C-819T SNP of IL-10 promoter indicates that there is no association of this polymorphism with TB in that particular population (Ma et al., 2010). Mosaad et al.,(2010) on Egyptian people, Ansari et al., (2009) on Pakistani population, a meta-analysis study by Pacheco et al.,(2008) on eight different research articles, a study done by Lopez-maderuelo et al.,(2003) on Spanish white population and a report by Anand et al.,(2007) from South Indian population demonstrated that G-1082A polymorphism of IL-10 gene promoter is not associated with either susceptibility or resistance to TB disease. Nonetheless, Pacheco et al.,(2008) also reported that when only pulmonary TB patients are taken into account the G allele of G-1082A SNP had trend towards protection from TB, although non-significant.

In contrast to these reports, Ben Selma et al., (2011) observed that GA genotype of G-1082A polymorphism provides with increased risk of susceptibility to EPTB in Tunisian population, whereas AA genotype is associated with resistance to PTB. In the same study GCC haplotype (from -1082, -819, -592 sites of IL-10 gene promoter) was
reported to be a risk factor for TB. Scola et al. (2003) in Sicilian population found that carrier of A allele at -1082 site are significantly decreased in TB patients. Heterozygosity of G-1082A polymorphism has also been associated with susceptibility to TB disease in Cambodian population (Delgado et al., 2002). Shin et al., (2005) found that C allele bearing genotype of C-592A polymorphism was significantly higher in NHC than in patients with TB, while G-1082A has no association with this disease. In a study on Dravidian population, from South India it was noticed that although there was no association of TB with G-1082A SNP of IL-10, CC genotype of C-819T polymorphism was marginally decreased in TB patients compared to NHCs (Selvaraj et al., 2008). In Turkish TB patients C allele at -819 and -592 sites of IL-10 promoter was found to be more common than in NHCs (Akgunes et al., 2011).

Henao et al., (2006), studied all the three polymorphisms in Colombian patients and concluded that neither genotypes of these three SNPs nor haplotypes are associated with susceptibility to pulmonary and milliary TB. However, AA genotype of G-1082A site was found to be associated with pleural TB. Tso et al., (2005) too failed to show any association of IL-10 promoter polymorphisms with Hong Kong Chinese TB patients. Interestingly, they did not get any homozygous GCC haplotypes.

2.5.2 Interferon-Gamma (IFN-γ)

Interferons (IFNs) were initially discovered as mediators that obstructed the replication of viruses (Sen, 2001). They have been classified into type I and type II according to the receptors to which they bind and the homology of sequence. Type I interferons are IFN-α subtypes, IFN-β, IFN-ω and IFN-τ. All type-I IFNs are structurally similar and bind to common receptor (Bach et al., 1997). Almost all type of cells secretes them in very low amount in humans and IFN-τ has only been reported in ruminants. IFN-γ is only type II interferon, which is structurally different to type I forms, have different receptors to bind with and located on separate chromosome locus (Young, 1996; Bach et al., 1997). Previously, it was believed that only Th1, CD8+ and NK cells secretes IFN-γ, but now it has become clear that B cells, NK T cells and APCs also produce this cytokine. When IFN-γ is produced by NK cells and APCs, it is believed that it is being produced as early host defense against infections but T cells production of this cytokine has been linked with adaptive immune response (Frucht et al., 2001; Sen, 2001).
Cytokine secreted by APCs like IL-12 and IL-18 act as positive stimulators of IFN-γ whilst IL-4, IL-10 and TGF-β are thought to be acting as negative regulators (Schroder et al., 2004).

The gene of IFN-γ cytokine has been mapped to chromosome 12 at location 12q24.1, containing four exons with DNA size of 4.95kb. In terms of protein characteristics, it is a dimeric protein having subunits of 146 amino acids (aa). It is synthesized as a precursor protein of 166 aa containing secretory signal sequence of 23aa. Under denaturing conditions IFN-γ is observed to have two forms of 20 kDa and 25 kDa. This difference is due to differential extent of glycolyisation of two forms. 40-60 kDa forms of IFN-γ have also been reported under non-denaturing conditions (Karupiah, 1997; Fiers et al., 1982; Naylor et al., 1983).

The IFN-γ cytokine has been strongly implicated in immunopathology of TB. This protein is regarded as a key macrophage activating factor in conjunction with other cytokines (Flynn and Chan, 2001). In turn macrophages are principle cells in regulating anti-mycobacterial immunity (Natarajan et al., 2003). The importance of this cytokine in the disease of TB also lies in the fact that functional deficiency of IFN-γ due to mutation in IFN-γ receptor gene or modulation of this receptor increases the vulnerability of person towards mycobacterial infection (Singhal et al., 2007). Investigations on vaccine development against TB also look for high IFN-γ expression in host due to some MTB antigens (Kumar et al., 2010). The polymorphism in IFN-γ gene and its circulating levels has also been associated with TB in various populations (Ben-Selma et al., 2011a; Anand et al., 2010; Henao et al., 2006; Lopez-Maderuelo et al., 2003; Olobo et al., 2001). Further, this cytokine is also being promoted as having diagnostic potential for TB Villena et al., (1996), as well as in its therapy (Robak, 1994). The role of IFN-γ in TB is being discussed in detail in subsequent titles.

2.5.2.1 IFN-γ Levels in TB Patients

Plenty of studies have tried to illustrate the role of IFN-γ cytokine in TB disease by reporting its levels both in humans (Mattos et al., 2010) and animal models (de Steenwinkel et al., 2011). Studies are done either by stimulating PBMCs with live or inactivated M. tuberculosis, MTB antigens or measuring directly the systemic levels in plasma or serum. There are also reports which present both of the methods. Levels of
IFN-γ are reported to be elevated in patients with active TB disease by most of studies while others antagonize them. They vary considerably in their observations and as such do not converge to give any unified view about the role of this vary cytokine in TB (Mattos et al., 2010; Vallinoto et al., 2010; Lopez-Maderuelo et al., 2003; Vankayalapati et al., 2003; Olobo et al., 2001).

In Brazilian population, NHCs are reported to have more of IFN-γ levels in comparison to TB patients. Grouping of patients on basis of disease site depicted that PTB patients have high production of IFN-γ than EPTB patient (Vallinoto et al., 2010). Contrary to that, in an Indian study decreased expression of IFN-γ was observed in TB patients compared to NHCs (Anand et al., 2011).

There is an interesting article published in 2003 by Vankayalapati et al., from Texas, in which they measured serum cytokine concentrations in TB patients and NHCs and compared them with inactivated *M. tuberculosis* induced production of cytokines in cultured PBMCs of those individuals. The IFN-γ cytokine was considerably high in serum of TB patients when compared with NHCs. This scenario completely differed when concentration of this cytokine was measured in *M. tuberculosi*s stimulated PBMCs as cells of TB patients produced lesser amount of IFN-γ than cells of NHCs. So this study reveals that *in vivo* and *in vitro* difference do exist in cytokine production and two can not be correlated with each other.

The part of the study of Vankayalapati et al., (2003) dealing with *M. tuberculosis* induced production of cytokines in PBMCs has been well supported by Sahiratmadja et al., (2007), and Vidyarani et al., (2006). They also reported the similar results. PBMCs of newly diagnosed TB patients produced significantly lesser amount of IFN-γ in response to live or sonicate *M. tuberculosis*, Phytohemagglutinin (PHA) or culture filtrate (CF) than cells of NHCs. The level goes on increasing with the advancement of therapy. Spontaneous production of IFN-γ was also considerably higher in PBMCs of NHCs when compared to patients. Besides, IFN-γ levels correlates with severity of the TB disease. Stimulated PBMCs of patients with mild and moderate TB produced higher amount of this protein than of those with advanced TB. Instead of PBMCs when whole blood was stimulated with PHA, LPS or CF, somewhat similar trend was found regarding levels of IFN-γ. It was suppressed in EPTB1 and PTB patients when compared with TST+ control.
subjects (Jamil et al., 2007). *M. tuberculosis* induced PBMCs of tuberculous pleuritis patients also produced lesser amount of IFN-\(\gamma\) when compared to production from pleural fluid cells. Though, there was no comparison with NHCs like other studies (Barnes et al., 1992).

In contrast to above studies, Veenstra et al. (2007) demonstrated different results. They studied the IFN-\(\gamma\) response in TB patients and found that IFN-\(\gamma\) production was significantly higher in stimulated PBMCs of patients who are diagnosed with TB than NHCs and those who have completed their anti tuberculosis therapy. Percent increase in IFN-\(\gamma\) producing CD8 and CD4 T lymphocytes was also higher in TB patients which turned to normal on completion of treatment. On splitting the studied individuals as acute TB patients, contacts of TB patients (persons living in close proximity to patients) and NHCs it was observed that production of IFN-\(\gamma\) from their PPD stimulated PBMCs was in descending order, starting from patients with acute TB (Handzel et al., 2007).

After some glimpse on *ex vivo* experimentation concerning levels of IFN-\(\gamma\) in TB patients, a bit of more information on this subjects by discussing its levels in body fluid like serum, bronchoalveolar lavage fluid (BALF) or pleural fluid can be had. Serum of patients with active PTB is reported to have significantly higher levels of IFN-\(\gamma\) when compared to cured PTB patients, contacts of patients or NHCs. These levels decrease considerably after two months of treatment and further go on decreasing with the ATT. On subdividing the patients on the basis of different symptoms it was observed that patients with fever, anorexia and malaise have elevated amounts of this protein than patients without fever and later said symptoms. Difference in levels was statistically significant (Deveci et al., 2005; Verbon et al., 1999).

Apart from reports of high serum IFN-\(\gamma\) levels in initial stages of TB, Barnes et al., (1992) did not find any detectable amount of this vary cytokine in serum of patients with tuberculous pleuritis. However, pleural fluid had noticeably high measurement of IFN-\(\gamma\). Little information is also available on cytokine levels in BALF. Like in serum, BALF also contained higher levels of IFN-\(\gamma\) at start of TB therapy which reduced significantly on completion of six months of treatment (Ameglio et al., 2005). The levels of IFN-\(\gamma\) were also reported to be altering with site of disease. It was notified that patients with pleural effusion had high IFN-\(\gamma\) levels in their pleural effusions rather than in
peripheral blood. Contrary to it, persons who are suffering from miliary TB were found to contain high IFN-γ protein in blood when compared to BALF (Sharma et al., 2002).

A cohort study was conducted by Pai et al., (2006) from Sevagram India, in which they evaluated sensitivity of QuantiFERON-TB Gold in tube assay and determined longitudinal changes in IFN-γ response in confirmed TB patients. They found that there were no significant changes in IFN-γ levels with the progression of treatment, although they decreased slightly. Also the QuantiFERON-Gold (QFT-G) activity remained approximately unaltered during therapy.

2.5.2.2 IFN-γ Levels and T+874A SNP

The production of IFN-γ cytokine is reported to be genetically controlled. There are two studies published by Pravica et al., in 1999 and 2000 which put weight to this articulation. It was described that there exists a CA microsatellite sequence in the first intron of IFN-γ gene, and that it is a polymorphic sequence. Pravica et al., in 1999 showed that, five different alleles of this microsatellite sequence occur in population of United Kingdom. The alleles differ in numbers of CA repeats. Allele number 1 to 5 corresponds to 11 to 15 CA repeats, respectively. The important finding which was reported by them was that, in vitro production of IFN-γ was higher in peripheral blood leukocytes which have microsatellite region with 12 CA repeats, depicting allele number 2. Further, it was revealed that those persons who have allele 2 in homozygous state produce significantly high level of IFN-γ than individuals with any other allele of said microsatellite in first intron of IFN-γ gene.

Adding to the above investigation, this group reported a novel SNP in human IFN-γ gene. This polymorphism, from nucleotide T to A existed at 5' end of CA repeat in first intron of this gene. The position of SNP was +874 bases from transcription start site. This biallelic polymorphism has been mentioned as T+874A in all next encounters. The vital result of their study was, complete linkage disequilibrium between, high IFN-γ producing, 12 CA repeat allele of microsatellite region and occurrence of T allele at T+874A SNP. DNA sequencing confirmed the individuals homozygous for allele 2 (12 CA repeat allele) as TT homozygotes, heterozygotes for this allele as TA and negative for this vary allele as AA homozygous (Pravica et al., 2000). So as already certain by 12 CA repeat allele polymorphism, T allele can be regarded as high producer and A allele as low
producer of IFN-γ cytokine. A transcription factor NF-κB is reported to play an important role in differential production of IFN-γ (Yamada et al., 2001). This factor is known to control the expression of IFN-γ cytokine. The gene knockout mice for NF-κB produce less IFN-γ than their counterparts. This association indicates regulation of IFN-γ expression by NF-κB (Rossouw et al., 2003; Yamada et al., 2001). The low and high production of IFN-γ, because of different alleles of T+874A SNP, was attributed to binding site of this transcription factor. This site coincides with T+874A dinucleotide polymorphism of IFN-γ gene. The ‘T’ allele at +874 site creates a DNA sequence AATCTC and to this sequence NF-κB activity has been shown by electrophoretic mobility shift assay. Assay shows specific binding of NF-κB to T allele containing DNA sequence. Hence, ‘T to A’ polymorphism at +874 site in first intron of human IFN-γ gene could explain the difference in IFN-γ cytokine production.

2.5.2.3 T+874A SNP and TB

Three consecutive investigations done by Awad et al., (1999) and Pravica et al., (1999, 2000), yielded intriguing results with regard to production of IFN-γ cytokine and its management by a SNP and role of that SNP in disease. It was revealed that, allele 2 of a variable length CA repeat microsatellite region, occurring in first intron of human IFN-γ gene is associated with high production of IFN-γ in vitro. In the mean time, it was explained that, this allele 2 has significant association with allograft fibrosis in lung transplant recipient, thus confirming biological role of this polymorphism in vivo.

Based on above studies, the debate about the role or association of T+874A SNP of IFN-γ and TB can be made. Before investigations of Pravica et al., (1999, 2000), it was already known that IFN-γ is an important biomolecule for the activation of macrophage (Nathan et al., 1983), the cells phagocytizing MTB and helping in its eradication. So, it can be understood that any process which will alter IFN-γ function may eventually interfere with MTB elimination. The production of IFN-γ is under control of T+874A SNP. After studying these aspects, it can be hypothesized that allele ‘T’ being high producer can provide additional protection against MTB infection and allele ‘A’ may render an individual susceptible to TB infection.

To look into this hypothesis, different studies available on T+874A SNP and its association with TB were analyzed. The results of studies differed on this association. Lio
et al., (2002), while evaluating such association on Sicilian population concluded that the frequency of ‘T’ allele was less in TB patients, compared to NHS, but the difference was not statistically significant. When they compared genotypic frequencies of patient and control populations, there was significant difference in the distribution of ‘TT’ genotype. This genotype was significantly reduced in TB patients (p=0.024). The difference in statistical significance of allelic and genotypic comparisons among patients and controls can be attributed to fact that, effect of a given gene is because of allele pair combination received from mother and father, and not because of a single allele, and there is a dosage dependent effect on IFN-\(\gamma\) production (Pravica et al., 1999, 2000).

The association of individuals, homozygous for allele ‘A’ at +874 site in intronic region of \(IFN-\gamma\) gene, with susceptibility to TB was also significant in Brazilian population (Vallinoto et al., 2010) and Egyptian population (Mosaad et al., 2010). In Iranian population too, AA genotype of T+874A SNP was found to be increasing risk of developing TB by 3.3 times (Hashemi et al., 2011). In Spanish population (Lopez-Maderuelo et al., 2003) the ‘AA’ homozygotes were reported to be at, 3.75 times increased risk of becoming victim to TB infection (OR=3.75, CI=2.26-6.23). In contrast to study of Lio et al., (2002), who did not find any significant difference in allele frequencies of TB patients and controls, this study reported an overrepresentation of allele ‘A’ in patients with TB (OR=2.71, CI=1.54-4.75).

In a family based study in South African colored population, it was concluded that, there was very high transmission of ‘A’ allele of T+874A SNP to TB patients, by their parents (p=0.0048). In further analysis of same data, families with more than one affected siblings were deducted to remove any bias. It was then observed that ‘A’ allele transmission to patient sibling became much more significant (p=0.0008). In the same report, they too did case-control study for TB and reported the protective role of ‘T’ allele against MTB infection. It was significantly higher in controls with p value for allelic and genotypic frequencies of 0.0055 and 0.017, respectively (Rossouw et al., 2003).

The higher frequency of AA genotype of T+874A dinucleotide in \(IFN-\gamma\) gene in TB patients, was confirmed in Hong Kong Chinese population. Individual with AA genotype were found to be 3.79 (CI=1.93-7.45) times more susceptible to TB (Tso et al., 2005). Positive correlation of allele A and susceptibility to MTB infection has also been
found in Southeastern Chinese population. Individuals carrying ‘AA’ genotype were shown to be 1.98 times more vulnerable to TB disease than those carrying ‘AT’ or ‘TT’ allele pair (Ding et al., 2008). Brazilian population studied by Amim et al., (2007), reproduced the relationship of \( IFN-\gamma \) T+874A SNP with TB. The results of their study depicted that the individuals with ‘A’ allele in homozygous condition have more than two fold extra chance of vulnerability to TB (OR= 2.62, CI=1.16–5.91).

Significantly lower values of ‘T’ allele and ‘TT’ genotype frequency, in TB patients has also been stated by Sallakci et al., (2007) in Turkish population, when compared with control population of same setting. They acknowledged the ‘TT’ genotype for providing resistance to individuals by 30% against TB, while mentioning negative role of ‘AA’ genotype which increases chances of developing TB by 1.41 times.

Recently, a meta-analysis was done by Pacheco et al., (2008) to discover the role of different SNPs in \( IFN-\gamma \), \( IL-10 \) and \( TNF-\alpha \) cytokine gene, for susceptibility to TB. The \( IFN-\gamma \) T+874A SNP was studied and analyzed from the data of 13 different studies from all over world, with different ethnicities. The results of this analysis too indicated towards protective effect of ‘T’ allele of T+874A SNP. The Odds ratio for ‘T’ allele, ‘T’ carrier and ‘TT’ genotype were found to be 0.75 (CI=0.63-0.88), 0.76 (CI=0.60-0.94) and 0.59 (CI=0.45-0.78), respectively.

Till now, the situations explaining constructive effect of ‘T’ allele in immunity against TB and antagonistic effect of ‘A’ allele, occurring at +874 site of \( IFN-\gamma \) gene were probed. This status is not always true and there are reasons to explain this contradiction. There are studies, whose results depict that T allele is a risk factor for TB or there exist no association between T+874A SNP of \( IFN-\gamma \) and TB.

Ansari et al., (2009) while evaluating risk to tuberculosis in Pakistani population, observed that there was increase in frequency of T allele of T+874A SNP in PTB patients, while there was no association of this allele in EPTB.

Tuberculosis susceptibility was studied in Malawi population with \( IFN-\gamma \) as one of the candidate gene, but no association of T+874A SNP in this gene was found with this disease (Fitness et al., 2004). Similar results were found in Croatian population (Etokebe et al., 2006). However, the small difference, which existed, was in the relation of ‘TT’ and ‘AA’ genotypes of \( IFN-\gamma \) T+874A SNP with AFB negative and positive TB.
Number of ‘TT’ homozygotes was higher in AFB negative patients, thus showing some relation of high IFN-γ producing genotype with reduced mycobacterial load.

In continuation to susceptibility studies, based on relationship of IFN-γ T+874A SNP and TB disease, an investigation was conducted on populations of three West African countries Gambia, Guinea Bissau, and the Republic of Conakry by Cooke et al., (2006). The findings of the study revealed that ‘AA’ genotype of IFN-γ T+874A SNP, do not makes a person less or more vulnerable to MTB infection. A study published by Moran et al., in 2007, added to the aphorism of non association of IFN-γ T+874A polymorphism with TB. It was a study which included different populations like, African-American, Caucasian and Hispanics. No significant difference was found for T+874A genotypic frequencies of controls when compared to ethnically matched patients, with different forms of TB, thus suggesting no association of this polymorphism with TB susceptibility and severity or different forms of TB.

Contradictory, to the studies of Tso et al., (2005) and Ding et al., (2008) on Chinese populations, Wu et al., (2008) reported similar genotypic frequencies of IFN-γ T+874A SNP in TB patients and NHS belonging to Chinese population. Thus, suggesting non-association of said polymorphism with development of TB.

In Colombian and Pakistani population, a different type of association was found. Low IFN-γ producing Allele ‘A’ of T+874A SNP was not associated with TB susceptibility, but high producer allele ‘T’ was cause of some different forms of TB. Study on former population done by (Henao et al., 2006) stated that, IFN-γ T+874A SNP was not associated with pulmonary TB. Frequencies of both ‘T’ and ‘A’ allele were almost similar in controls and TB patients. But, when ‘A’ allele of TST- and TST+ NHS and PTB patients was compared with that of pleural and milliary TB patients, former group had higher frequency. The frequency of ‘T’ allele, which is thought to create resistance to TB, was high in patients with pleural and milliary TB. ‘TT’ homozygotes were less in patients with PTB. Genotypic frequencies of IFN-γ T+874A SNP polymorphism significantly differed in three forms of TB studied. This statistical significance was lost when control populations were included for comparison with TB patients of different groups.
On similar line, the results of investigation on Pakistani population indicated that ‘TT’ homozygosity of $IFN-\gamma$ T+874A SNP was positively associated with PTB. However, when PTB patients were divided into minimal, moderate and advanced type, the association of ‘T’ allele restricted only to patients with minimal and moderate PTB ($p=0.01$ and $0.02$, respectively). Odds ratio displayed 2-3 fold increase in risk development for minimal and moderate TB disease, for individuals having ‘T’ allele of $IFN-\gamma$ T+874A SNP (Ansari et al., 2009).

From India, the reports of $IFN-\gamma$ T+874A SNP and its relationship with TB are available for south Indian populations. One study, done by Vidyarani et al.,(2006) explained non-association of above said polymorphism, with TB susceptibility. The genotypic and allelic frequencies of T+874A polymorphism did not differ significantly in PTB patients and control population. Selvaraj et al.,(2008) also studied influence of $IFN-\gamma$ T+874A SNP in development of TB in similar population. The subjects under study were Dravidian descent of Tamil Nadu state of India. No variation of allelic and genotypic frequencies of mentioned dinucleotide polymorphism was found among NHS and PTB patients.

After going through some of these reports, available from different parts of the world, a combined picture can be drawn, which ensue towards assumption that a single nucleotid polymorphism T+874A occurring in the first intron of human $IFN-\gamma$ gene, may be associated with TB susceptibility in some ethnic groups, while not in others. It can also interpreted, that little work has been done on this polymorphism in Indian population with regard to TB vulnerability. The present study put an effort to fill this lacuna by studying different population from North India. Thus, better role of this polymorphism in regulation of host immunity can be described, and new strategies can be made to combat the progress of this deadly disease.

2.5.2.4 IFN-\gamma in Diagnosis and Treatment of TB

Numbers of clinical tests are available for the diagnosis of TB. Starting from very common, Ziehl-Neelsen staining and culture of TB bacilli, the new techniques have expanded the horizons, to nucleic acid amplification tests like PCR. Even the species and drug resistant strains can be identified by automatic sequence analysis (Cho, 2007). In
between these magnificent tests, the role of IFN-γ in the TB diagnosis is also gaining pace.

The diagnostic competence of IFN-γ in tuberculous pleural effusion has been well mentioned (Villena et al., 1994). The comparison of IFN-γ levels in pleural effusions, occurring from different pathologies or autoimmune diseases depicted the higher value of IFN-γ in effusion occurring from TB and it was well above the cut-off limit of 3.7 U/ml proposed by them. IFN-γ appeared to be better diagnostic tool than pleural biopsy and adenosine deaminase (ADA) for tuberculous pleurisy. It can identify patients, who are not diagnosed by biopsy. Further, unlike ADA the results do not differ for HIV positive and HIV negative individuals who are either immunocompromised or immunocompetent. On comparison of four different biological markers (ADA, IFN-γ, immunosuppressive acidic protein and soluble IL-2 receptor) for diagnosis of tuberculous pleural effusion, IFN-γ emerged as best indicator with high sensitivity and specificity (Aoe et al., 2003).

The diagnosis of latent TB through IFN-γ assay is providing new approach in the management and control of TB, even in high endemic areas of this dreaded disease. QuantiFERON (QFT) tests and T-SPOT.TB (SPOT-TB) assays are providing efficient and specific detection of latent MTB. QFT include QFT-G and QFT-GIT, measure amount of IFN-γ produced by T-cells after 16-24 hours after incubation with MTB specific antigens like ESAT-6, CFP-10 or TB7.7 proteins. SPOT-TB assay essentially count the number of effector T-cells which produce IFN-γ after stimulation with said MTB antigens (Cho, 2007). The role of IFN-γ as healing prognostic marker has also been supported by the study of Moura et al., (2004). They found that the levels of IFN-γ increased significantly at the end of treatment of TB disease. Goletti et al., (2010) proposed a novel method for detecting IFN-γ response in Indian context. It was observed that IFN-γ response to RD-1 selected peptide is associated with TB disease in India and this assay has higher specificity compared to QFT and TST tests.

The role of IFN-γ in treatment of diseases has also been discussed in early nineties. Patients with chronic granulomatous disease have been found to be benefiting from long term treatment with recombinant IFN-γ (Robak, 1994). IFN-γ aerosol therapy on MDR-TB patients have resulted in change of positive sputum smear to negative, gain in body weight and reduction in cavitary lesion size. As an adjuvant therapy also, IFN-γ
have promised a great deal. When it is administered intramuscular for 6 months with chemotherapy patients show reduced lesion size, negative sputum and culture and increased body mass index. Another trial have observed that giving IFN-γ as adjuvant therapy with normal anti-TB drugs leads to increased levels of STAT1, IRF-1, and IRF-9 in BAL cells (Condos et al., 1997). IFN-γ actively participates in enhanced signal transduction and gene expression in macrophage, thus it can act as potential therapeutic agent of TB (Miller et al., 2009).

2.6 Concluding Remarks

In overall scenario, it can be pointed out that there are numerous studies available on the role of cytokines like IL-10 and IFN-γ in the dreaded disease of TB. But, no generalized consensus can be made on the potential of these cytokines in susceptibility or resistance to TB either on genetic or on protein levels. There is a lucid picture regarding the function of these two immunological gears in disease of TB on basis of population. This observation in itself has a point to make, that every population has its own history of evolution. The process of natural selection may explain if a particular population show association to TB based on these two markers. It can not be said in general that if a genetic marker is associated with a particular disease in a said population of the world, it will too show association in another population. In North Indian Punjabi Population, there was no study regarding the role played by IL-10 or IFN-γ cytokine in rendering people susceptible or resistant to the havoc of TB. To fill this existing void of information on these two pivotal immunological markers the present study was designed, so that we can come through the truth behind role of IL-10 or IFN-γ in occurrence of TB in this particular population.