Introduction

Despite over six decades of intensive immunization campaigns, tuberculosis (TB) has defied all human efforts at eradicating the disease. Instead, new drug resistant variants of the causative agent, *Mycobacterium tuberculosis* (MTB), have emerged, making it difficult to control the contagious disease. The disease kills largest number of adults than any other disease caused by an infectious organism. One third of humanity is infected with this deadly pathogen. WHO has estimated 1.45 million deaths from TB in 2010. In the same year, 8.8 million incident cases and 12 million prevalent cases of TB were also reported worldwide. Out of total TB cases, 85% have occurred only on Asian (59%) and African (26%) continents (WHO, 2011). India has the dubious distinction of having highest TB burden in the world with 2 million new cases every year, accounting for one fifth of total TB burden of the world (RNTCP, 2011).

Tuberculosis is a communicable disease. It passes from diseased person to a healthy one through tiny aerosol particles created by sneezing, coughing, laughing or like activities of infected person (Knechel, 2009). A person who inhales the TB bacterium gets infected and only a small fraction (~10%) of the totally infected population develop clinical symptoms of TB in their life time (RNTCP, 2011). The question of why only select few individuals get the disease and some even succumb to this ailment remains unanswered. Only in very few instances risk factors to TB, like HIV infection, diabetes, alcohol abuse, advanced age or corticosteroid usage have been identified. Thus, discovering the underpinnings of susceptibility to TB could provide insights into pathogenesis of this disease (Bellamy, 1998).

The interplay between the human host and its bacterial pathogen represents the interaction between two very complex systems. The virulence and pathogenicity of an infectious disease historically centers on microbial harmfulness. Koch’s postulates:

1. Pathogen must be isolated from every diseased case and not healthy one
2. Pathogen must be isolated and propagate in pure culture *in vitro*
3. Pathogen must reproduce disease by injecting into a suitable recipient
4. Pathogen be re-isolated from the host and must be identical to the original organism
have helped in the identification of various etiologic agents (Grimes, 2006). The limitations of these postulates were soon recognized by scientists as they found that presence of pathogen may not always lead to the development of disease. Additionally, isolation and propagation of pathogen from the host in vitro might not be always possible, like in *M. leprae* (Bhattacharya *et al.*, 2002). There could also be non availability of animal models for causing disease. More importantly, the postulates grossly underestimated the contribution of host’s factors in resisting the development of disease.

Prior to the discovery of MTB, the tuberculosis was considered to be hereditary disease as it occurred in many members of families (Bellamy, 1998). It has been known for a very long time that individuals differ in their susceptibility to TB. Part of the variation was attributed to environmental factors like poor hygiene, overcrowding or unhealthy living conditions and malnutrition (Kaulagekar and Radkar, 2007). Now it is believed that although the disease is not hereditary, but the host’s genetic factors do influence the outcome of this disease. This notion is also supported by twin studies which showed the incidence of TB among monozygotic twins was almost double than the dizygotic twins (Comstock, 1978).

Search for genetic determinants of susceptibility to TB got a big help with the completion of human genome project in 2001 and the availability of complete human genome sequence (Venter *et al.*, 2001). It is fairly interesting to learn that the variations between individuals at genotypic level are limited to 0.1% of their total sequence. The most common form of genetic variation exists as a single base change in the DNA sequence of an organism and is called a point mutation or single nucleotide polymorphism (SNP). Going by conservative estimates, there are around 3 million SNPs in the human genome and it is difficult to predict their precise frequency as they vary in different populations and genomic regions (Kwok and Chen, 2003). The availability of this class of genetic markers has instilled a new hope amongst researchers to ascertain the role of genetic determinants in infectious diseases.

Several attempts have been made worldwide to identify genes responsible for susceptibility to TB employing various approaches, like Genome Wide Association
Studies (GWAS), siRNA and candidate gene approach. The first GWAS study from western Africa identified a TB susceptibility gene, which have been mapped on chromosome 18q in a Gambian population (Thye et al., 2010). The siRNA based approach used the silencing of the targeted gene, leading to the non availability of its product, with the growth of the pathogen without the knowledge of mechanism or underlying pathways (Kumar et al., 2010). Using this approach 275 targets were identified that impacted the growth of *M. tuberculosis* in cultured cells. However, the most common method so far has been the candidate gene approach and is based on the identification of variations in a known gene whose function is relevant to the pathophysiology of TB. A gene has to meet the following criteria to be considered a candidate gene in a population study:

- The gene product must be functionally relevant to TB.
- Mutation within the gene must alter its function.
- TB has to show association with different alleles of this candidate gene.

According to the RNTCP 40% of human adult living in India are infected with *M. tuberculosis*. However, 90% of the infected individuals could protect themselves from the causative pathogen due to their normal immune function and only ~10% develop the TB disease in their life time (RNTCP, 2011). Evidently immunological factors are strongly implicated in the susceptibility to TB. Given that cytokines are key players in mounting immune response in a person, monitoring them could be of great help in studying the pathophysiology of disease. Additionally, the presence of SNPs in cytokine genes have accounted for inter-individual variation for synthesizing these proteins (Pravica et al., 2000; Turner et al., 1997). These findings suggest that the ability of individuals to genetically secrete higher or lower amount of these cytokines could make them differentially susceptible to TB disease. The present thesis is an extension of this concept.

In host tissue, the MTB lives in macrophages and there seems to be a constant struggle between the bacterium and the host for survival. The symptoms of the disease appear when MTB could escape immune surveillance of the host and is able to down regulate the immune system. However, if the host dependent immunological
factors predominate then the bacterium is either killed or its growth is inhibited thus putting it in latent form.

Available data on IL-10 and IFN-γ cytokines reflects that these two molecules represent two faces of immune system wherein they affect the TB pathogen in diametrically opposite ways. The former helps MTB to grow inside the host and the latter assists in killing the bacterium (Trajkovic et al., 2004). For its survival MTB will tend to evade the protective limb of the immune system of host and simultaneously will try to manipulate levels of cytokine(s) which can help it grow inside the host. The reason for targeting these two selected cytokines, in this study, is the overwhelming data supporting their role in susceptibility to TB, although a consensus on their role in different populations is still elusive.

A homodimeric cytokine, IL-10 is a 36 kDa protein whose gene is mapped to chromosome number 1 (Kim et al., 1992). It is produced by variety of cells like Th1, Th2, mast, dendritic, and macrophages. It inhibits synthesis of proinflammatory cytokines (IL-2, TNF-α, IFN-γ), chemokines, inducible nitric oxide synthase and cyclooxygenase in macrophages (Cunha et al., 1992). These anti-inflammatory properties of IL-10 could provide an affable environment for growth of MTB. This hypothesis also gets support from the finding that transgenic mice producing high amount of IL-10 developed larger mycobacterial load than control mice (Murray et al., 1997). IL-10 gene knockout mice demonstrated increased antimycobacterial immunity (Murray and Young, 1999).

In tuberculous pleurisy patients, IL-10 protein levels were significantly higher when compared with pleural fluid from control individuals (Liang et al., 2011). Over expression of IL-10 cytokine has also been observed in MTB antigen stimulated peripheral blood mononuclear cells (PBMCs) of TB patients when compared with tuberculin skin test (TST) positive normal healthy control (NHC) individuals.

Another cytokine of interest chosen for this study, IFN-γ, is a key pro-inflammatory cytokine that antagonizes the functions of IL-10. It is a dimeric protein of 146 amino acid subunits with a molecular weight ranging from 20-25 kDa. The difference in the molecular weight is due to variable degree of glycosylation of the
protein. The gene for this cytokine is mapped to chromosome 12q (Karupiah, 1997; Naylor et al., 1983). This cytokine is produced by CD4, CD8 and NK cells and plays a crucial role in controlling the growth of TB bacterium in macrophages. It augments the antigen presentation leading to recruitment of T cells essential for mycobacterial killing (van Crevel et al., 2002). Additionally, it is a major activator of macrophage resulting in growth inhibition of MTB in mice (Raja, 2004). IFN-γ gene knock out mice failed to; develop granuloma, produce reactive nitrogen intermediates and stop the growth of MTB and supplementation with exogenous recombinant IFN-γ in these mice could only delay the fatality (Flynn et al., 1993).

Reduction in IFN-γ levels in PBMCs of tuberculosis patients has been reported, which increased significantly at successful completion of anti tuberculosis therapy (ATT) (Jo et al., 2003; Hirsch et al., 1999). However, there are studies showing that IFN-γ is produced mainly during active TB disease and its serum levels decreased with therapy (Ameglio et al., 2005; Verbon et al., 1999). It has also been reported that there is significant decrease in the expression of intracellular IFN-γ in pulmonary TB patients when compared to NHCs (Anand et al., 2010). People lacking components of IFN-γ signaling pathway are highly susceptible to TB (Sahiratmadja et al., 2007). Also, children lacking either chain of IFN-γ receptor are more prone to mycobacterial diseases and fail to upregulate in vitro monocyte function in response to IFN-γ (Levin et al., 1995).

For every phenotype, there is an underlying genotype. The genes ultimately influence the way in which their products will be formed and behave in an organism. Similarly, various genotypes implicated in differential production of IL-10 and IFN-γ cytokines can make a person vulnerable or resistant to tuberculosis based on their ability to synthesize variable amounts of these cytokines. Three, most studied, single nucleotide polymorphisms (SNP) rs1800896, rs1800871, rs1800872 in the promoter region of IL-10, which are commonly known as G-1082A, C-819T and C-592A, respectively (all subsequent references to these polymorphisms will be made in accordance with their common names). These three polymorphisms can theoretically make eight different haplotypes, but in majority of populations three haplotypes GCC,
ACC and ATA are found. The GCC haplotypes is associated with high levels of IL-10 in PBMC culture, whereas ATA haplotype present with low levels of this cytokine (Thye et al., 2009; Turner et al., 1997). Likewise, IFN-γ cytokine has a rs2430561 (Commonly known as T+874A - a term which will be used in subsequent use) SNP in first intron of its gene, whose various genotypes are associated with variable circulating plasma IFN-γ levels. Presence of T allele in homozygous state is correlated with high levels of this cytokine in blood, while AT heterozygote and AA homozygotes produce this cytokines in descending order (Cardoso et al., 2010; Pravica et al., 2000).

The three genetic polymorphisms in the promoter of IL-10 gene (G-1082A, C-819T, C-592A) have been widely studied for their association with susceptibility/resistance to TB in different populations of the world. Resistance to TB has been attributed to ACC haplotype and C allele occurring at -592 site of this gene in Korean population (Shin et al., 2005). Association of G allele at the IL-10 G-1082A site with susceptibility to TB disease has been suggested by some research groups (Scola et al., 2003; Delgado et al., 2002). These observations have been contested by other investigators who failed to find any association between IL-10 G-1082A site and susceptibility to TB (Mosaad et al., 2010; Ansari et al., 2009; Tso et al., 2005; Lopez-Maderuelo et al., 2003). A meta-analysis study by Pacheco et al. (2008) also indicated that G-1082A SNP of IL-10 is not an important factor for studying susceptibility to TB.

The genetic determinants dictating the levels of IFN-γ, have also been evaluated in many populations of world. The IFN-γ gene is highly conserved during the course of evolution and very few biallelic polymorphisms are found in its intragenic region, and the one in its first intron, T+874A is widely studied (Pacheco et al., 2008). This polymorphism is significantly associated with acquiring TB disease in various populations of the world (Hashemi et al., 2011; Mosaad et al., 2010; Tso et al., 2005; Lopez-Maderuelo et al., 2003), while many investigators could not replicate these findings (Akgunes et al., 2011; Anand et al., 2010; Onay et al., 2010; Selvaraj et al., 2008).
Meta-analysis study of Pacheco et al. (2008), also concluded that T allele present in its homozygous form provides protection to the carrier of that genotype. In Pakistani population, the reverse is true where the association of TT homozygotes have been found with pulmonary TB, which was also differentially associated with severity of disease (Ansari et al., 2009). Deviating from these observations, there are reports from Indian and Turkish populations, which suggests that there is no association of tuberculosis disease with T+874A polymorphism of IFN-γ gene (Akgunes et al., 2011; Anand et al., 2010; Onay et al., 2010; Selvaraj et al., 2008).

To assess the role of IL-10 and IFN-γ cytokines in TB, there is need to address these molecules at protein and genomic level. By doing so, both phenotypic and genotypic limbs can be studied together and an apt correlation between the two can be made to understand the immunity behind this killer disease. Studies ascertaining susceptibility to TB in different populations of the world are available. Every population is unique and has its own history of evolution, infection with microorganisms, and natural selection of particular alleles for its adaptation to specific environment; therefore each one of them has to be evaluated exclusively for studying its susceptibility or resistance to a disease (Bellamy et al., 1998). Most of the studies on TB have been performed on western populations, whereas 85% of TB cases are found only in Asia and Africa. Only the smaller proportions of cases occurred in the Eastern Mediterranean Region (7%), the European Region (4%) and the Region of the Americas (3%) (WHO, 2011). Clearly, the reports available on European and American populations will be hard to replicate in Africans and Asian populations due to their very diverse geographical and ethnic background.

Rapid progress in technological developments in SNP analyses, include high throughput techniques like microarrays, automated DNA sequencing and real time polymerase chain reaction (RT-PCR). These approaches represent the modern cutting edge technologies that require sophisticated infrastructure, expensive reagents and deft technical expertise. Clearly, it is difficult to muster resources and have such infrastructure in an average Indian molecular biology laboratory. Such facilities are
often present in the national institutes or advanced centers for higher learning, which are often housed in metropolitan cities. In India a sizable population lives in the rural areas and it very likely that these populations might be neglected for the assessment of their genetic diversity due to the non availability of adequate technology in their neighborhood. Microarray is a good tool that can screen a large number of SNPs and provide a list of attractive candidate genes that can be pursued in various disease related studies. Such information can be gathered from literature. However, it will be necessary to develop rapid, reliable and financially viable approaches for studying SNP variations in routine molecular analysis. Hopefully such initiative will motivate researchers in small labs to generate much needed human genomic diversity data in different populations, highlighting susceptibility to human diseases, such as tuberculosis.

Owing to above referred findings on IL-10 and IFN-γ at their protein level studies as well as genetic association based studies it can be said that, no conclusive statement at population level can be made regarding role of these key cytokines in pathophysiology of TB. Rather, there is differential association of these cytokines at protein or genomic level within various populations. Although clinching evidence is there, which approves that host genetic factors are important in determining outcome of this disease, they only affect some populations and not all ethnic groups. There is need for more studies in determining host factors that regulate expression of immunity in MTB infection, so that a holistic approach can be made to judge the immunity behind TB disease and population based bio-markers can be identified to manage this dreaded disease. Most of the studies which have been done are either on genomic level, or protein level. If both variables are taken, then in most studies protein levels have been studied in vitro in MTB antigen stimulated PBMCs, which could not provide give a clear picture of cytokines role inside the body of host. So, there is a need of combined approach of investigating crucial immunoregulatory cytokines, IL-10 and IFN-γ at their genomic and protein levels for assessing their association with TB. As there is no such data available from North Indian Punjabi
population, the aim of present study is to evaluate role of IL-10 and IFN-γ at protein and genomic levels in the said population. The study was designed with following objectives:

- Analysis of G-1082A, C-819T, C-592A SNPs of *IL-10* and T+874A of *IFN-γ* for their association with TB.
- Determination of circulating plasma levels of IL-10 and IFN-γ proteins in TB patients at different time intervals of anti tuberculosis therapy (ATT).
- To evaluate the possible association of circulating levels of IL-10 and IFN-γ cytokines with respective genetic polymorphism in their genes.