Despite intensive vaccination efforts over the past six decades, Tuberculosis (TB) has defied all efforts for its eradication. It is an infectious disease caused by bacterium *Mycobacterium tuberculosis* (*M. tuberculosis*). There are over 2 billion people, nearly a third of the world population, infected with this dreaded pathogen. To this pool, 9.27 million individuals are added each year and 1.75 million lose their lives. India alone has 1/5th load of world’s TB disease with 40% of its adult population infected with *M. tuberculosis*, leading to annual death rate of about 0.4 million. These alarming figures exist when only 10% of infected individuals develop the clinical disease in their lifetime.

It is not known with certainty what types of immune or genetic factors are involved in susceptibility or resistance to TB. Remarkable progress has been made in the recent past regarding the contributions of different cytokines in the pathogenesis of this disease. The T cells and infected macrophages are essential components of the protective immune mechanism and their interaction with each other is fundamental to immunity against *M. tuberculosis*, which largely depends on the interplay of different cytokines produced during infection. New evidence suggests that variable levels of these key mediators of immune response could alter individual’s susceptibility to TB. One vital component that could induce differential expression of cytokines among individuals is the presence of alternative alleles of Single Nucleotide Polymorphisms (SNPs) in different regions of the genes of these cytokines. Thus, the SNPs responsible for the variable circulating levels of cytokines become an attractive target for association-based studies in different populations of the world.

Many pro-inflammatory and anti-inflammatory cytokines are produced during TB infection and play an important role either in elimination or aggravation of the disease. IFN-γ is a key pro-inflammatory cytokine produced during the course of the disease, which is thought to enhance protective immunity towards *M. tuberculosis*. In contrast, IL-10, an anti-inflammatory, immunosuppressive, and macrophage deactivating cytokine, is reported to aggravate the disease. It is mainly produced by T cells, B cells and macrophages. This cytokine has inhibitory effect on IFN-γ production from T cells, secretion of tumor necrosis factor, expression of costimulatory molecules and MHC class II antigens on macrophages. Different studies have defined the role of IL-10 in immune
response to Mycobacterial infections. Transgenic mice secreting increased levels of IL-10 were more susceptible to mycobacterial infection, although their IFN-\(\gamma\) levels were similar to those of wild type, whilst \textit{IL-10} knock out mice are noticed to be more immune toward TB. In human studies, the systemic levels of IL-10 in TB patients have been deliberated by a few investigators. Most studies report no statistically significant change in circulating IL-10 levels of TB patients before and after medication. However, some of them observed significantly higher IL-10 production, which normalized at the end of therapy. 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.

The production of IL-10 is genetically determined. There are three biallelic polymorphism in the \textit{IL-10} gene promoter, at -1082(G/A), -819(C/T) and -592(C/A) positions from the transcriptional start site, reported to be accountable for high or low production of this cytokine. Theoretically eight haplotypes (GCC, GCA, GTA, GTC, ATA, ATC, ACC and ACA) could be generated from these three loci. Most studies have observed only three major haplotypes viz. GCC, ATA, ACC, and are shown to be associated with differential production of this cytokine. The GCC haplotype is associated with high IL-10 production in peripheral blood mononuclear cell (PBMC) culture. Different \textit{IL-10} haplotypes and genotypes have been associated positively or otherwise with the TB infection, like C-592A SNP and ACC haplotype are significantly associated with decreased risk of clinical TB. In contrast to this, reports are also available that failed to record similar findings. The association of G-1082A SNP with increased susceptibility to TB infection has also been reported.

IFN-\(\gamma\) is required for host defense against broad range of pathogens and is especially critical for mycobacterial immunity. IFN-\(\gamma\), produced primarily by T cells and NK cells, is an important mediator of macrophage activation. It is responsible for activation of monocytes and stimulation of giant cells formation. Beside this, IFN-\(\gamma\) deficient mice have been reported to be highly susceptible to TB infection. Exogenously added IFN-\(\gamma\) reduced the bacterial load and increased survival of experimental mice, demonstrating the importance of IFN-\(\gamma\) in the immune response to \textit{M. tuberculosis}. These
findings suggest that persons producing elevated IFN-γ may have better immunity against TB infection.

The circulating levels of IFN-γ are reported to be controlled by a SNP in the first introns of its gene. A T+874A polymorphism directly influences the level of IFN-γ production with T being its high producer allele. Presence of A allele in its homozygous form at this site make the persons more prone to the risk of *M. tuberculosis* infection.

Understanding the molecular mechanism(s) underlying protective immunity is a prerequisite for the development of improved therapies to combat this dreaded disease. So, it would be of great importance if pathology behind TB and its relation to some key cytokines, like IL-10 and IFN-γ could be studied in detail. Few studies on these aspects have been conducted in the West and on Western populations; however there is a clear need to understand the contribution of the mentioned variables in Indian population. As there is virtually no data available from North Indian population on these cytokines for their association with TB, hence this study was designed to fill the existing void. We rationalize to investigate IL-10 and IFN-γ at their genomic and protein levels in Punjabi population with the 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.

This study was conducted on 665 individuals, out of which 450 were TB patients. Blood samples were collected from the patients visiting TB and Chest hospital, Government Medical College, Amritsar, and Department of TB and Chest, Sri Guru Ram Das Institute of Medical Sciences and Research, Amritsar, Punjab. Patient population was further divided into different groups based on gender, category (I, II or III based on DOTS programme) or time period of anti-tuberculosis therapy (ATT) (0 day of ATT, two months and six months after ATT). The remaining 215 individuals were normal healthy control (NHC) subjects, living in the same region.
Information about their name, age, sex, endogamous group, personal history and family history of TB was collected from these subjects. An attempt was made not to include people with a history of TB, asthma or other allergic diseases in the NHC group. Their age, sex and endogamous group was matched with that of TB patients.

Blood sample collected from each individual was divided into two aliquots – one for genomic DNA isolation and another for the estimation of circulating protein levels. WBC pellet from the first aliquot and plasma from the second was isolated and stored at -20°C. DNA was isolated from WBC pellet following inorganic method. Extracted DNA was quantified using UV-Spectrophotometry and quality was monitored by A260/A280 ratio and agarose gel electrophoresis.

Amplification Refractory Mutation System-Polymerase Chain reaction (ARMS-PCR) method was employed for genotyping SNPs in IL-10 gene promoter (G-1082A, C-819T, C-592A) and a new method was developed for construction of its haplotypes. Genotyping of T+874A polymorphism in first intron of IFN-γ gene was also accomplished using ARMS-PCR assay. To carry out ARMS-PCR, two complementary reactions were established for each allele in two different tubes, consisting of target DNA, allele specific ARMS primers, common primer and other PCR components like dNTPs, buffer and the Taq DNA polymerase. The reaction conditions were optimized in investigator’s laboratory. The obtained amplicons were run on ethidium bromide stained agarose gel. The results were seen on UV-transilluminator and saved for further analysis. Representative PCR products were also sequenced commercially for confirmation of obtained results.

Circulating plasma protein levels of IL-10 and IFN-γ were determined by using Enzyme Linked Immunosorbent Assay (ELISA), in NHCs and TB patients at different time intervals of ATT, by commercially available ELISA kits using known standards. Experiments were performed according to the instructions of manufacturer.

The genotypic data and allelic counts were subjected to standard statistical analysis, like their fit to Hardy Weinberg Equilibrium (HWE), for any difference between different groups by chi square test and Odds ratio (OR). The data of cytokine levels
obtained was analyzed by one-way analysis of variance (ANOVA) and Fisher’s protected least significant difference (Fisher’s PLSD) as post-hoc test.

Vital information has been generated in this investigation elucidating the distribution of G-1082A, C-819T, C-592A SNPs and their haplotypes in IL-10 gene, and T+874A SNP in the IFN-γ gene in North Indian Punjabi population. Additionally, the role of circulating levels of these two cytokines during the course of the disease has also been ascertained. Tremendous alteration in the levels of IFN-γ with the anti-TB treatment presents this cytokine as a probable biomarker for monitoring the effects of ATT. Clearly such a finding could go a long way in the better management of this disease. Contrary to existing reports, IFN-γ production did not differ significantly within the three genotypes of T+874A SNP of this cytokine in Punjabi population. This observation was substantiated by the finding that none of the T+874A genotypes or alleles revealed any significant association with TB patients in North Indian Punjabi population.

All the three IL-10 gene loci (-1082, -819 and -592) were found to be in linkage disequilibrium. Comparison of the distribution of alleles for -819 and -592 IL-10 loci between male and female TB patients revealed significant value of odds ratio. Furthermore, the deviation from HWE, as shown by NHC males (for -1082 locus) and both populations (for -819 and -592 loci) suggests that these loci are probably under selective pressure of evolutionary forces for their role in TB. It is important to mention here that the +874 site in IFN-γ gene did not reveal any deviation from HWE when genotyped in the same populations. This supports the notion that loci, for IL-10 and IFN-γ, are differentially acted upon by the evolutionary forces and hence deviation in HWE for one locus can not be construed for another in the same population. The strategy adopted for constructing the haplotypes for the mentioned three polymorphic sites of IL-10 gene in present study was a novel one. It is a rapid and inexpensive method of determining the haplotypes by using ARMS primers. The present study confirmed earlier observation that IL-10 cytokine levels vary between different haplotypes, with GCC being high producer in its homozygous state. However, no differences were observed in circulating levels of IL-10 cytokine between NHCs and TB patients. Interestingly, IFN-γ
was found to be associated with circulating plasma levels in TB, while IL-10 cytokine was seen to be correlating at genotypic levels.

In conclusion, the data generated in this study presents a holistic view of the role of IL-10 and IFN-γ cytokines at genomic and protein levels in containing TB disease in the host. Understanding the genetics as well as expression of these two pivotal proteins will help in formulating future strategies for the better management of this dreaded disease.