Chapter 5  DISCUSSION

Tuberculosis still remains a global health problem particularly in the developing countries. It is generally accepted that one third of the world population have latent *M.tb* infection, but very few of them develop the active disease during their life time. It is a very important and also interesting to investigate the factors which make some persons susceptible to the disease while provide protection to others. The exact reason behind the development of active TB in small group of persons is still unknown. Although it is believed that tuberculosis is an outcome of a complex procedure, which includes involvement of several factors, of which socio economic conditions, sanitation, environmental factors, host and pathogen genetic factors etc are of major concern.

Another mycobacterial disease, leprosy, also has its worst impact on human being, known since ancient history. Although effective treatment is available for leprosy and most of the countries have eradicated the disease, but still, some of the regions in the world have showing slightly increased rate of new case detection during the last two years, including in India.

Host immune response plays a vital role in the development of any disease. It is well accepted that a better understanding of the protective immune response of the host and the cause of susceptibility to the disease are essential for the development of effective vaccines, diagnostic markers or generation of immune targeted drugs.

Cytokines and chemokines are the important counterparts of the host immune system to fight against infection. Cytokines and chemokines together recruit the immune cells to the site of infection, stimulate cell proliferation and also stimulate the cells to secret other cytokines and chemokines.

The monocyte chemoattractant protein-1 (MCP-1/CCL2) is a very important chemokine, which has potent chemotactic property for monocytes. It recruits monocytes to the site of infection. Besides monocytes, it also regulates the migration and infiltration of memory T lymphocytes and natural killer (NK) cells. Studies indicate that MCP-1 is also involved in the polarized Th2 responses. Single nucleotide polymorphism at -2518 position, where A changes to G, has been reported to be associated with tuberculosis in some populations, however contradictory results were obtained in different studies in several
other populations. Similarly, controversial findings were also reported for another polymorphism in MCP-1 gene at position -362 where G changes to C.

As the role of these polymorphisms are not clear and is controversial, our objective in the present study was to investigate the functional significance of these two polymorphisms in tuberculosis cases and healthy controls from north Indian population from Agra. So far no report was available for the role of these two polymorphism in leprosy cases, we made a parallele investigation in leprosy patients also. We investigated the association of MCP-1 with Th1 cytokines, such as IL-12, IFN-γ and TNF-α in PTB and leprosy both. Besides these cytokines, TGF-β was also studied and correlated with MCP-1, because TGF-β works as suppressor of T cell response.

Thus, the focus of our study was to explore the likely role of MCP-1 at protein level and MCP-1 gene polymorphisms (-2518A/G and -362G/C) in tuberculosis and leprosy.

5.1 MCP-1 -2518A/G and -362G/C polymorphism in PTB cases and healthy controls

Robin and Saxena (1999), first identified the -2518A/G polymorphism and found that the -2518 G allele was associated with increased MCP-1 production in response to different stimulating agents (Robin and Saxena1999). Flores-Villanueva et al (2005) first reported the association of -2518A/G polymorphism with tuberculosis, they observed that G/G and A/G genotypes are strongly associated with increased odds of developing active pulmonary tuberculosis after infection in Mexicans and Koreans (Flores-Villanueva et al 2005). Later, several other studies were conducted which reported contradictory results. In our study, we screened 373 PTB cases and 248 healthy controls for the -2518A/G polymorphism. Our results indicate that the frequency of G/G genotype is higher in patients as compared to controls and is significantly associated with tuberculosis (OR=1.97, P=0.02). The findings seen to correspond with the earlier reports from Mexicans, Koreans, Peruvians, Chinese Han population, Zambian, Tunisian and Canadian population where G/G genotype of -2518 A/G polymorphism was reported to be associated with pulmonary tuberculosis (Flores-Villanueva et al 2005, Ganachari et al 2010, Yang et al 2009, Buijtels et al 2008, Selam et al 2011, Larcombe et al 2008).

However our findings are contradictory to the study of Thye et al and Arji et al carried out in the population of Ghana and Morroca, respectively, where they reported that G/G
genotype was associated with the protection against pulmonary tuberculosis (Thye et al 2009, Arji et al 2012). Interestingly studies carried out in the populations of Brazil, Russia, China, South Africa, Iran, Gambia and Argentina, showed lack of association of -2518A/G polymorphism with pulmonary tuberculosis (Jamieson et al 2004, Thye et al 2009, Chu et al 2007, Naderi et al 2011, Edwards et al 2012). Our results also indicate that the frequency of A/G genotype was higher in healthy controls, suggesting its protective role in PTB (OR=0.60, P=0.002). However this finding is contradictory to observations of Flores-Villanueva et al made in the population of Mexico and Korea, where they showed that A/G genotype was also associated with pulmonary tuberculosis (Flores-Villanueva et al 2005). While the results favour the findings reported by Thye et al that A/G genotype provides protection against pulmonary tuberculosis. The frequency of A and G alleles is almost equal in cases and controls in our study, which is different from the previous studies where significant difference in the frequency of G allele was found, indicating its association with pulmonary tuberculosis.

In Indian scenario, no association of -2518A/G polymorphism was found with pulmonary tuberculosis in South Indian population and in the Sahariya tribe of Madhya Pradesh (Alagarasu et al 2009, Singh et al 2013, Mishra et al 2012). In contrast, our observations indicate a strong association of -2518A/G polymorphism with PTB cases in the population of Agra and surrounding regions of Northern India. In our study the study subjects belong to Hindi speaking Indo Aryan population of Braj Bhumi region of U.P. This region consists of mixed population of different religions and also contains very large number of migratory population from farflung districts of U.P and nearby states, so the studied population possibly have a mixture of different genetic makeup. While in the study of Alagarasu et al and Singh et al subjects were from Tamil speaking Dravidian descent of South Indian population of Chennai. Similarly Mishra et al worked on Shariya tribe which is an isolated population and restricted to a very small geographical area of central India. This difference of ethnic background of the study subjects, may be a possible reason for varying findings of our study versus the observations of studies from South India and Madhya Pradesh. As our findings are drawn from a comparatively larger sample size this information could further help to understand the role of this -2518A/G SNP and this highlights the needs of analyses of more ethnic groups.
A recent meta analysis showed that G/G genotype and G allele is significantly associated with susceptibility to pulmonary disease in Asians, Americans and Hispanics (Feng et al 2011, Gong et al 2013).

Beside overall case control analysis, PTB cases were stratified according to category (cat-I or cat-II) and bacillary load, as observed during AFB smear test. Genotype and allele frequency was estimated in each of the group and compared with that of healthy controls. The results indicate that G/G genotype is significantly associated with category I-PTB cases (OR=2.05, P=0.02), but not with category II-PTB cases.

PTB cases were grouped according to their BI status as 1+, 2+, 3+ AFB and the genotype frequencies were compared between each of these and healthy controls. Analysis indicates significant association of G/G genotype with AFB2+ and AFB3+ PTB cases, while no correlation was observed with AFB1+ PTB cases. But, the allele frequencies did not differ between cases and controls and no association of G or A allele was observed with the PTB cases.

We also screened -362G/C polymorphism in 330 PTB cases and 235 healthy controls. Our results showed that the frequency of C/C genotype was highest in PTB cases in comparison to controls and was associated with the susceptibility to pulmonary tuberculosis. In contrast the frequency of G/C genotype was found to be higher in healthy controls and could be associated with protection against pulmonary tuberculosis. However the frequencies of G and C alleles were not different between PTB cases and healthy controls, indicating lack of association either with the susceptibility or resistance.

Our results are different from those of reported by Mishra et al (2012), where they did not find any significant difference in the genotype and allele frequencies between cases and controls in sahariya tribe, a population from central India (Mishra et al 2012). The present result also differe from that of Thye et al (2009) who reported both C/C and G/C genotypes and C allele of -362 G/C polymorphism to be associated with the protection against pulmonary tuberculosis (Thye et al 2009).

Thye et al (2009) in their study, also showed that the haplotype combination -2518G/-362C was strongly associated with the resistance to pulmonary tuberculosis (Thye et al 2009). While in our study, the haplotype combination -2518A/-362C was found significantly associated with the susceptibility to pulmonary tuberculosis disease.
Stratification of PTB cases according to different categories showed that C/C genotype was associated with category I PTB cases but not with category II PTB cases. Similarly, when the stratification of PTB cases was done on the basis of bacillary load, as observed during AFB smear test, the C/C genotype was found to be associated with AFB 2+ and AFB3+ PTB cases, but no difference in C/C genotype frequency was observed between AFB1+ PTB cases and healthy controls.

Haplotype analysis showed that A-C haplotype, A allele of -2518A/G polymorphism and C allele of -362G/C polymorphism found to be highly associated with tuberculosis susceptibility.

5.2 Serum MCP-1 and other cytokines level in PTB cases and healthy controls

We measured the MCP-1, IL-12p70, IFN-γ, TNF-α and TGF-β in the serum samples of 120 PTB cases and 54 healthy controls. The concentration of MCP-1, IL-12p70 and TNF-α were found to be higher in PTB cases as compared to healthy controls. The difference was significant for MCP-1 and IL-12p70, while, for TNF-α, it was just above the significant value p>0.05. Almost equal concentration of serum IFN-γ was found in PTB cases and healthy controls, while significantly higher level of TGF-β was measured in healthy controls than in PTB cases.

Our data supports the previous findings of higher level of MCP-1 in PTB cases than in healthy controls (Flores-Villanueva et al 2005, Ganachari et al 2010, Park et al 2007, Brown et al 2007). However, there are reports which did not find any difference in plasma or serum MCP-1 level between PTB cases and healthy controls (Alessandri et al 2006, Hasan et al 2005).

We also studied the correlation between MCP-1 and other cytokines in PTB cases and healthy controls. Results showed significant positive correlation between MCP-1 and IL-12p70, which is contradictory to the previous findings of Flores-Villanueva et al, where they showed a negative correlation between MCP-1 and IL-12p40 in PTB cases (Flores-Villanueva et al 2005). Our findings also showed a positive correlation between MCP-1 and IFN-γ, TNF-α and TGF-β in PTB cases. In controls also, similar results were found between MCP-1 and IL-12p70, TNF-α and TGF-β, except IFN-γ where the p-value was not significant.
The PTB cases and healthy controls were stratified according to MCP-1 -2518A/G polymorphism and serum concentrations of MCP-1 and other cytokines were analyzed in different genotype groups. The serum MCP-1 level was found highest in PTB cases with A/A genotype, followed by the carriers of A/G and G/G genotypes. However the MCP-1 concentration was found to be lowest in the PTB cases with G/G genotype. Our observation is in concordance with the previous reports from Japanese population with diabetes and Korean population with systemic lupus erythematosus (SLE), where also the highest concentration of serum MCP-1 was observed in A/A and lowest in subjects with G/G genotypes (Kouyama et al 2008, Kim et al 2002). However, contradictory observations have also been made, reporting elevated level in G/G genotype, followed by A/G genotype and lowest in A/A genotype subjects in populations of Mexico, Korea and Peru with tuberculosis, in Korean population with Osteoarthritis and in population of USA with systemic lupus erythematosus(SLE). In all these studies the MCP-1 concentration was measured in plasma rather than serum (Flores-Villanueva et al 2005, Ganachari et al 2010, Park et al 2007, Brown et al 2007). In healthy controls also, serum MCP-1 level was highest in A/A genotype, followed by the carriers of A/G and G/G genotypes, but the mean MCP-1 level was lower in each genotype in comparision to PTB cases. Serum IL-12p70 and IFN-γ levels were also found elevated in the carrier of A/A genotype, followed by the carriers of A/G and G/G genotype PTB cases. Both IL-12p70 and IFN-γ showed significant positive correlation with MCP-1 in PTB cases.

Flores-Villanueva et al (2005) reported that IL-12p40 was highest in PTB cases with G/G genotype, followed by A/G and A/A genotypes, which is different from our findings. They concluded that -2518G allele increases the MCP-1 concentration, which downregulates the IL-12 secretion that leads to defective T cell response and makes host susceptible for the tuberculc infection. Our study, however, dose not favour the conclusion drawn by Flores-Villanueva et al.

The serum level of IL-12p70 and IFN-γ in different genotypes of healthy controls were different than the pattern observed in PTB cases. IL-12p70 level was highest in the controls with A/G genotype, followed by A/A and G/G genotypes. While IFN-γ was highest in G/G genotype, followed by A/A and A/G genotype controls.
Reverse pattern was observed in case of serum TNF-α level in PTB cases with different genotypes. The highest TNF-α level was found in the cases with G/G genotype, followed by A/G genotype, while lowest concentration was found in the carriers of G/G genotype. In contrast, in healthy controls, the TNF-α level was found elevated in A/G genotype, followed by A/A genotype and lowest in the carriers of G/G genotype. TGF-β, which is a suppressor and regulator for T cell response was found in less quantity in PTB cases as compared to healthy controls, indicating that in cases where T cell response was required, the concentration of cytokine was less, while in healthy controls, where a balance immune response was present and no further T cell response needed, the cytokine secreted in higher quantity. No difference in the concentration of TGF-β was observed between different genotypes of PTB cases. The concentration was almost equal in all the groups. While in controls, the level of TGF-β was varying in different genotypes. Highest concentration of TGF-β was found in the controls with A/G genotype, followed by A/A genotype and the lowest level was observed in the healthy controls with G/G genotype. Both TNF-α and TGF-β showed significant positive correlation with MCP-1. Our findings suggest that both the cytokines, IL-12 and IFN-γ of Th1 type, showed a positive correlation with MCP-1 and MCP-1 induced secretion of these cytokines in response to *M.tb* infection. During this process, the TGF-β level remains constant so that the T cell response reaches to its highest level.

5.3 MCP-1 and other cytokines secreted by PBMCs of PTB cases and healthy controls in response to WCL *M.tb* antigen

In vivo measurement of cytokines does not confirm that the increase or decrease in the level of particular cytokine, as compared to healthy controls, was due to infection of a specific pathogen, like *M.tb* because their serum concentration in cases is influenced by many other factors, apart from the pathogen and its antigens. To demonstrate the effect of *M.tb* antigens we did in vitro experiments by isolating PBMCs from 28 PTB cases and 18 healthy controls and stimulated it with WCL (whole cell lysate) antigen of *M.tb* and rIFN-γ. MCP-1 and other cytokines were measured in culture supernatant, collected after 24 hours.
In our study MCP-1 level was found to be higher in the culture supernatant of healthy controls than that of PTB patients in unstimulated condition as well as in response to stimulation with WCL *M. tb* antigen and rIFN-γ. Our findings are contradictory to that of the previous findings, where they reported that the basal MCP-1 level in PBMCs from tuberculosis patients was higher in comparison to healthy controls.

In healthy controls, *M. tb* antigen WCL and rhIFN-γ both induced the production of MCP-1, while in TB patients WCL antigen cause slight decrease in the production of MCP-1, while rhIFN-γ showed no effect on MCP-1 production. These findings are also contradictory to that of who reported that stimulation with *M. tb* and different components of *M. tb* induced the MCP-1 secretion. Hasan et al (2009) reported that *M. tb* induced higher MCP-1 production in PBMCs isolated from TB patients compared to tuberculin positive and negative healthy controls. Similarly Sterling et al (2007) in their study showed that TB patients had higher MCP-1 level than in tuberculin positive healthy controls, both in spontaneous condition and in response to LPS, LPS+ rhIFN-γ and PPD stimulation. It was also reported that CD14+ monocytes from TB patients expressed higher level of MCP-1 mRNA as well as MCP-1 protein spontaneously, than CD14+ monocytes from healthy tuberculin reactors. Further, CD14+ monocytes exposed to LPS antigen of *M. tb*, expressed higher amount of MCP-1 mRNA, while no MCP-1 transcripts was detected at unexposed level (Lin et al 1998). Whole blood stimulation with ESAT-6 and CFP-10 also induced higher MCP-1 production in PTB cases compared to healthy controls (Kellar et al 2011).

It is known that stimulation of *M. tb* and its components, like LPS, BCG, PPD and Ag85 complex induce the production of MCP-1 in vitro. While some negative reports are also available where they did not found any effect such as Lee et al (2008) in his study did not find any effect of PPD and 30kDa proteins on the in vitro production of MCP-1. Arias et al (2007) also showed that ManLam and LPS antigens of *M. tb* have no effect on the in vitro MCP-1 production, but PPD induced the production. In our study we used WCL (whole cell lysate) antigen of *M. tb* and observed that this antigen slightly decreased the production of MCP-1 in PBMCs of TB patients, but induced the production of MCP-1 in healthy controls. Analysis of the results on the basis of -2518A/G genotypes indicates that in PTB cases the MCP-1 concentration was higher in A/A genotype cases, while
lower in G/G genotype PTB cases in unstimulated condition and in response to WCL and rIFN-γ stimulation.

No study has been made so far on in vitro MCP-1 production in response to *M. tb* antigens in the subjects with -2518A/G polymorphism. Flores-villanueva et al (2005), however isolated PBMCs from healthy controls and stimulated with H$_{37}$Rv and compared the production of MCP-1 between G/G and A/A genotype subjects.

Our results in healthy controls are similar as reported by them. We found higher MCP-1 concentration in the controls, showing G/G genotypes, followed by A/G and A/A genotype controls in both unstimulated and in response to WCL antigen.

It is reported that IFN-γ induces production of MCP-1 in monocytes and endothelial cells (Penton-Rol et al 1998). Kawakami et al (1999) in their study demonstrated that IFN-γ induced the production of MCP-1 and other chemokines by macrophages, endothelial cells, keratinocytes and fibroblasts. A two to three fold increase in MCP-1 secretion was observed in response to IFN-γ (Marra et al 1993). In order to know the effect of IFN-γ on MCP-1 production, we stimulated the PBMCs of both PTB cases and controls with rhIFN-γ. We found that treatment with exogeneous rhIFN-γ has no effect on MCP-1 production in PTB cases, but increase the production in healthy controls. Effect of rhIFN-γ on MCP-1 production were similar when comparison was made at -2518A/G genotype basis in both patients and healthy controls.

We also measured IL-12p70, since it is a more reliable to measure biologically active IL-12 production. IL-12 was found to be highest in PTB cases as compared to healthy controls at the basal level and in response to WCL and rhIFN-γ. Stimulation with WCL slightly increased the production of IL-12p70, in PTB cases, while in controls, no effect was observed. In controls rhIFN-γ slightly increased the production of IL-12p70, but showed no effect in PTB cases. Our results support the findings of Sharma et al (2004) who reported that monocytes stimulated with *M. tb* antigens LAM, WCL, and CFP secret significantly higher IL-12 level compared to unstimulated cells from the patients.

Song et al (2000) also reported that induced IL-12p70 level to be very low in response to stimulation with *M. tb* antigens. It was also reported that macrophages stimulated with rMCP-1 also induced the expression of IL-12 both at mRNA level and at protein level.
No effect of CFP-10 and ESAT-6 were observe on the production of IL-12 in PTB cases and healthy controls (Kellar et al 2011). Analysis of results on the basis of -2518A/G genotype showed that A/A genotype PTB cases produced higher IL-12p70 in unstimulated condition as well as in response to stimulation with WCL and rhIFN-γ while G/G produced lowest and A/G produced intermediate level of IL-12p70. In healthy controls our results indicates that IL-12p70 level was slightly higher in the carrier of G/G genotype controls than A/A and A/G genotype healthy controls. Our findings are different from that reported by Flores-villanueva et al (2005) that G/G genotype controls have significantly lower IL-12p40 level than that of carrier of A/A genotype controls. In our study we observe a significant positive correlation between MCP-1 and IL-12p70, while Flores-villanueva et al (2005) reported significant negative correlation.

IFN-γ concentration was also found to be higher in healthy controls as compared to PTB cases in all studied conditions. In PTB cases, stimulation with WCL antigen slightly increased the production of IFN-γ, while in controls, significantly higher increase was observed in the IFN-γ production.

The effect of different M.tb components on the production of IFN-γ is different and found varying in several previous reports. Handzel et al (2007) reported that in vitro production of IFN-γ by PBMCs of PTB cases was reduced in response to lectins and PPD as compared to healthy controls. Fortes et al (2005) also found that IFN-γ level was slightly lower in PTB cases compared to healthy controls in response to PPD and Ag85 stimulation. While, Song et al (2000) observed similar results in response to 32kDa, M.tb antigen. M.tb stimulated IFN-γ level was lower in PTB cases compared to healthy controls (Hasan et al 2009). However, there are a few reports which showed significantly increased level of IFN-γ in response to different M.tb components.

It is reported that ESAT-6 antigen stimulate higher IFN-γ production in PTB cases as compared to healthy controls (Ulriches et al 2000, Ferrand et al 2005). Mattos et al (2010) and Kellar et al (2011) also found that ESAT-6 and CFP-10 proteins stimulate higher production of IFN-γ from PBMCs of PTB cases compared to healthy controls. Further, increased IFN-γ production was also observed in PTB cases compared to healthy

Our between and within the genotype analysis of -2518A/G polymorphism showed that IFN-\(\gamma\) level was higher in A/A genotype PTB cases than that of A/G and G/G genotype cases, but the differences are not significant. The concentration was slightly increased in response to WCL antigen in PTB cases with A/A and G/G genotypes, while, slightly decreased in A/G genotype PTB cases. Reverse results were observed in healthy controls where higher concentration was found in G/G genotype controls compared to A/G and A/A genotype controls. A/A genotype controls showed lowest concentration of IFN-\(\gamma\).

We also measured the TNF-\(\alpha\), another Th1 cytokine, which play important role in the immune response against tuberculosis. It is reported that TNF-\(\alpha\) also work as stimulator for MCP-1 production in different cell types (Karrer et al 2005, Bhattacharya et al 2001, Algood et al 2003). A spontaneous secretion or basal level of TNF-\(\alpha\) was found to be higher in PTB cases compared to healthy controls. Stimulation with WCL antigen of \textit{M.\textit{tb}} drastically increases the TNF-\(\alpha\) production in healthy controls, while, it is slightly decreased in PTB cases. rhIFN-\(\gamma\) has no effect on the production of TNF-\(\alpha\) in PTB cases, while in controls it increases the production of TNF-\(\alpha\). Our observations are contradictory to that of previous reports showing increased TNF-\(\alpha\) production in PTB cases compared to healthy controls in response to WCL, ESAT-6, WCS, CFP10 and ManLam antigens of \textit{M.\textit{tb}} (Sharma et al 2004, Mattos et al 2010, Dlugovitzky et al 2000, Majumdar et al 2007). While Kellar et al (2011) found no difference for TNF-\(\alpha\) level between PTB cases and controls and the production of TNF-\(\alpha\) in response to ESAT-6, CFP10 stimulation was very low. The relationship between MCP-1 and TNF-\(\alpha\) has been revealed in several reports. TNF-\(\alpha\) is found to be a very strong inducer for MCP-1 secretion and rMCP-1 induced the expression of TNF-\(\alpha\) at both transcriptional as well as at protein level (Karrer et al 2005, Bhattacharya et al 2001, Mazumdar et al 2008). In our study, we also found a significant positive correlation between MCP-1 and TNF-\(\alpha\) at the basal level. Stratification of PTB cases on the basis of \textit{MCP-1}, -2518A/G genotype showed that carrier of G/G genotype cases produced highest amount of TNF-\(\alpha\) than A/A and A/G genotype PTB cases, the lowest concentration was found in A/G genotype cases. In both, A/A and G/G genotype PTB cases, the concentration of TNF-\(\alpha\) decreased
in response to both WCL and rhIFN-γ stimulation. In healthy controls A/A genotypes produced higher quantity of TNF-α than A/G and G/G genotype controls. G/G genotype controls produced lowest TNF-α, both at basal level and in response to WCL and rhIFN-γ stimulation. WCL and rhIFN-γ stimulation induced production of TNF-α in all the genotypes.

We found TGF-β to be higher in controls compared to PTB cases at zero hour. Both, in patients and controls, after incubation the level of TGF-β goes down in unstimulated and in response to WCL and rhIFN-γ stimulation, although, the concentration was highest in healthy controls in all conditions. Our results about TGF-β also contradictory to the previously reported studies where it was found that PBMCs from PTB cases produced significantly more TGF-β than healthy controls in response to WCS antigens of M.tb (Dlugovitzky et al 2000). Blood monocytes from PTB cases released increased level of TGF-β, compared to healthy controls (Tossi et al 1995). Stratification of the results on the basis of MCP-1, -2518A/G showed almost equal concentration of TGF-β in the carriers of A/A and G/G genotype cases, while A/G genotype cases secreted slightly higher level of TGF-β at basal level. In response to WCL antigen, increase in the level of TGF-β was observed in G/G genotype PTB cases, while no effect was observed in A/A genotype cases and slightly decreased in A/G genotype cases. In healthy controls, higher concentration was found in G/G genotype controls, followed by A/G and A/A genotype controls. In A/A genotype controls, WCL and rhIFN-γ slightly increased the secretion of TGF-β, while in G/G genotype controls, they decreased the production and showed no effect in A/G genotype controls.

In the present study no significant correlation was observed between MCP-1 and TGF-β at basal level, but a positive correlation was observed after stimulation with WCL antigen of M.tb. Our report probably the first where we demonstrate the MCP-1 and other Th1 cytokines in the subjects with -2518A/G polymorphism.

### 5.4 Expression of MCP-1 and other chemokine genes in PTB cases and controls

We also studied the expression of RANTES (CCL5), MIP-1α (CCL3) and IL-8(CXCL8) chemokine genes because it was reported that IL-8 is responsible for neutrophil recruitment and necessary for granuloma formation, limits the growth of intracellular
M.tb and enhances the macrophage killing of M.tb. CCL5 and CCL3 both play important role in the recruitment, activation and proliferation of macrophages, however, considered as major chemokines involved in both, acute and chronic phase of inflammation and possibly participate in TB pathogenesis.

Expression of CCL3 gene was significantly higher in PTB cases compared to healthy controls in both unstimulated and in response to WCL antigen stimulation. The expression of CCL3 was found to decrease in response to WCL antigen stimulation in PTB cases, while slight increase was observed in healthy controls. Our results do not favour the previous findings of Saukkonen et al (2002) where they observed that stimulation of alveolar macrophages with M.tb, induce production of CCL3 more than the unstimulated. Zhu and Friedland (2006) also found similar results, like Saukkonen et al (2002), they observed higher CCL3 gene expression and protein production in response to M.tb stimulation. They also detected CCL3 during generation of granulomatous lesions. Hasan et al observed that M.tb and BCG both induced the production of CCL3 in PBMCs of PTB cases and healthy controls, but the difference was not significant between cases and controls (Hasan et al 2009). CCL3 was also found to be upregulated in M.tb infected macrophages when compared to uninfected macrophages (Volpe et al 2006). In multi nucleated giant cells, upregulated CCL3 gene expression was observed after stimulation with M.tb (Zhu and Friedland 2006). Whole blood stimulation with M.tb antigens ESAT-6 and CFP-10 release the higher CCL3 in TB patients compared to controls (Kellar et al 2011). Only in one study done by Lin et al (1998) no detectable quantity of CCL3 was found in alveolar macrophages cells stimulated with M.tb. Stratification of the results on the basis of -2518A/G genotype showed a significant difference in the expression of CCL3 gene between A/G & G/G and A/A & G/G genotype PTB cases in unstimulated condition, while no such difference was observed in stimulated condition. An uniform pattern was observed in the healthy controls in all the genotypes. A/A genotype controls showed higher fold change in both unstimulated and in response to WCL antigen, compared to A/G and G/G genotype controls. The difference was significant between all genotypes in unstimulated condition.

In our results we don’t find any significant difference in the expression of CCL5 gene between PTB cases and controls in both unstimulated and in response to WCL
stimulation. Saukkonen et al (2002) found higher CCL5 level in alveolar macrophages in response to *M. tb* infection than that of unstimulated macrophages (Saukkonen et al 2002). CCL5 was also found to be higher in BAL fluid in TB patients compared to uninfected controls (Miotto et al 2001, Kurashima et al 1997). While Lin et al (1998) in his study did not detect CCL5 in the alveolar macrophages cells infected with *M. tb* (Lin et al 1998). Plasma CCL5 level was observe to be higher in healthy controls compared to PTB cases (Almeida et al 2009).

No significant difference in CCL5 expression was observed between different genotypes of -2518A/G polymorphism in PTB cases. However, in controls, significant difference was found between all genotypes in unstimulated condition. In response to WCL antigen the expression of CCL5 was increased in all genotypes. The difference was also significant between different genotypes, except A/G & G/G.

Expression of IL-8 was significantly more in PTB cases compared to controls in both unstimulated and in response to WCL stimulation. WCL antigen slightly decreased the expression of IL-8 in PTB cases. Previously it was reported that exposure of *M. tb* and its components induced the IL-8 secretion and gene expression in macrophages and monocytes (Friedland et al 1992, Zhang et al 1995, Hasan et al 2009, Zhu et al 2006). *M. tb* stimulation of monocytes is also found to increase IL-8 secretion and increased gene expression (Zhu et al 2006). Serum IL-8 level was higher in PTB cases compared to healthy controls (Almeida et al 2009). IL-8 was also found to be upregulated in human macrophages infected with *M. tb*. The level of IL-8 mRNA was found higher in alveolar macrophages and in PBMCs from PTB cases compared to normal healthy controls (Huang et al 2013).

In both PTB cases and healthy controls higher expression was observed in A/A genotype, followed by A/G and G/G genotypes. The effect of WCL antigen was different, in PTB cases, it decreased the expression, while in healthy controls, it increases IL-8 expression. We also studied the MCP-1 expression level and found higher expression in PTB cases, compared to healthy controls in unstimulated condition, while lower in response to WCL stimulation than in healthy controls. In healthy controls, the expression was found higher in A/A genotype cases, followed by A/G and G/G genotype cases. The differences between various genotypes were found to be significant and WCL induced the expression
of MCP-1 in all genotypes in healthy controls. In PTB cases the expression was found to be highest in G/G genotype cases, followed by A/G and A/A genotype PTB cases. The antigen WCL decreased the expression of MCP-1 in A/G and G/G genotype cases.

As reported previously in several studies, we also observed higher expression of all these chemokines in PTB cases compared to healthy controls. Higher expression of these chemokines plays a crucial role in immune response against *M. tb* infection, by recruiting neutrophils, macrophages, monocytes etc. at the site of infection. These cells are primarily needed to control infection by formation of granuloma and secreting cytokines for the development of further immune response.

### 5.5 MCP-1 -2518A/G and -362G/C polymorphism in leprosy cases and healthy controls

Although a number of studies have reported the role of polymorphisms in cytokine genes, like TNF-α, IFN-γ, IL-12, IL-18, IL-10 etc. Study on MCP-1 gene polymorphism is almost unexplored in leprosy. As the *MCP-1* -2518A/G and -362G/C polymorphisms were reported to be significantly associated with tuberculosis and several other diseases, in our study, we made an investigation on its role in leprosy also. Our study is probably the first report in this respect. We found no difference in the distribution of genotypes and allele frequency between leprosy cases and healthy controls for -2518A/G polymorphism. Similarly, no difference was observed when comparison was made between different groups of leprosy cases. No difference was found when comparison was made between reactional leprosy cases and non reactional leprosy cases.

In case of -362G/C polymorphism, C/C genotype was found to be significantly associated with leprosy cases while G/C genotype was found to be associated with controls and may provide some protection against leprosy pathogenesis. Frequency of alleles were not different between leprosy cases and controls. When comparison was done between different leprosy groups and healthy controls, significant difference in the distribution of -362G/C genotypes was observed between BB leprosy cases & healthy controls and between LL leprosy cases & healthy controls. The results indicates that C/C genotype was associated with BB and LL leprosy cases and not with the other groups of leprosy cases. Significant difference was observed in the distribution of C/C, G/C and
G/G genotypes between BT and BB leprosy cases, but the allele frequency was not different. No difference was observed in the frequency of different genotypes and allele frequency of -362 G/C polymorphism between leprosy cases without reactions and leprosy cases with reactions. Haplotype analysis between -2518A/G and -362G/C polymorphisms showed that haplotype A-C and G-G was significantly associated with healthy controls and provides protection against leprosy.

5.6 Serum MCP-1 and other cytokines level in leprosy cases and healthy controls
Serum MCP-1, IL-12p70, IFN-γ, TNF-α and TGF-β was measured in 120 leprosy cases and 106 healthy controls. The level of MCP-1, IL-12p70, TNF-α and TGF-β was found to be significantly higher in leprosy cases compared to healthy controls. Only IFN-γ level was observed higher in healthy controls compared to leprosy cases. Our finding of higher MCP-1 level in leprosy cases is in concordance with the previous finding of Hasan et al (2005), where they found higher serum MCP-1 level in leprosy cases compared to healthy controls. While Mendonca et al (2010), in their study found no difference in the plasma MCP-1 level between leprosy cases and controls. Observation of high level of IL-12 is in agreement with the previous finding that serum IL-12 level was significantly elevated in leprosy cases as compared to healthy controls (Sallam et al 2014). Similarly, our results of high level of serum TNF-α is in line with the earlier reports by Costa et al (2013), Moubasher et al (1998) and Madan et al (2011), who also reported that the level of TNF-α decrease with MDT treatment. Although previous studies by Moubather et al (1998) and Madan et al (2011), reported high level of serum IFN-γ in leprosy cases, compared to healthy controls, we did not observe the same finding in our study. In our study, we reported the expression of serum TGF-β for the first time in leprosy. On the basis of serum cytokine data we can concluded that higher concentration of negative immunoregulatory molecules, like TGF-β, may contribute more in leprosy pathogenesis.

Analysis of results on the basis of MCP-1 -2518 A/G polymorphism shows that MCP-1 and TGF-β level was highest in A/A genotype, followed by A/G and G/G genotype leprosy cases, while reverse pattern was observed in healthy controls for MCP-1.

The level of IL-12p70 and TNF-α was found to be higher in G/G genotype followed by A/G genotype and lower in A/A genotype leprosy cases. Reverse pattern was observed in
healthy controls for both the cytokines. No uniform pattern of IFN-γ secretion was observed in both leprosy cases and healthy controls. On the basis of our results, we conclude that leprosy cases with A/A genotype has higher risk of development of disease as the levels of IL-12p70, IFN-γ and TNF-α, which are required for Th1 response, are lowered, while, immunosuppressive cytokine TGF-β level was increased. In leprosy cases we also observed a negative correlation between MCP-1 and IL-12p70, while a positive correlation was observed between MCP-1 and TGF-β, indicating an important role of MCP-1 in leprosy pathogenesis.

5.7 MCP-1 and other cytokines secreted by PBMCs of leprosy cases and healthy controls in response to WCS M.leprae antigen

PBMCs were isolated from 39 leprosy cases and 27 healthy controls and stimulated with whole cell sonicate (WCS) antigen of M.leprae and rIFN-γ. The level of MCP-1, IL-12p70, IFN-γ, TNF-α and TGF-β were measured in the culture supernatant, collected after 24 hours by ELISA, as described in methodology section. MCP-1 level was found to be significantly higher in healthy controls compared to leprosy cases in all studied conditions. MCP-1 production was slightly declined in response to stimulation with WCS antigen and rIFN-γ compared to the unstimulated condition but the difference was not significant. However, in controls, no effect of WCS antigen and rIFN-γ was observed. Our findings favours the previous reports of Hasan et al (2005), that spontaneous secretion of MCP-1 from monocytes of leprosy cases was significantly reduced as compared to healthy controls. Similarly, BCG and M.leprae induced MCP-1 secretion by monocytes was also lower in leprosy cases, compared to healthy controls (Hasan et al 2005). Previous report also showed that PBMCs from healthy controls infected with M.leprae induced the production of MCP-1, compared to non infected cells (Sinsimer et al 2010), but, in our study we observed no effect of WCS antigen on the production of MCP-1 in healthy controls. M.leprae antigen, PGL-1 was found to induce MCP-1 secretion, compared to unstimulated condition in PBMCs isolated from healthy donors (Manca et al 2012). Analysis done on the basis of MCP-1 -2518A/G polymorphism showed higher MCP-1 concentration in G/G genotype compared to A/G and A/A genotype leprosy cases in unstimulated as well as in response to WCS antigen. In
response to WCS stimulation the MCP-1 production goes down in all the genotypes in leprosy cases. While in controls also, higher MCP-1 concentration was measured in G/G genotype, followed by A/G and A/A genotype controls, but the effect of antigen was different in different genotype controls. In A/A and A/G genotypes, MCP-1 production was increased, while, in G/G genotype controls, MCP-1 production was reduced in response to WCS antigen. However, no significant difference was observed for MCP-1 level when comparison was made between and within different genotypes for different studied conditions.

IL-12p70 level was found to be higher in leprosy cases compared to healthy controls in all conditions, but the differences were not significant. No impact of WCS antigen on the production of IL-12p70 was observed while rIFN-γ slightly induced the production in leprosy cases and downregulate in healthy controls. Sinsimer et al (2010), reported undetectable amount of IL-12 in M.leprae infected PBMCs in comparison to the induction of BCG. However, Sampaio et al in his study report that stimulation of blood with synthetic peptides of M.leprae induced the production of IL-12p70 when compared with unstimulated condition (Sampaio et al 2012). Stratification of results as per -2518A/G polymorphism revealed slightly higher level of IL-12p70 in G/G genotype, followed by A/A and A/G genotype cases, while, in controls, higher concentration was found in A/A genotype, followed by A/G and G/G genotypes. No effect of WCS antigen was observed in the carrier of any genotype in both leprosy cases and healthy controls.

Concentration of IFN-γ was found to be higher in leprosy cases compared to healthy controls in all studied conditions, however, the difference not reached to a significant level. No effect of WCS antigen and exogeneous rIFN-γ on the production of IFN-γ was observed in both leprosy cases and healthy controls, the level remains constant as observed in spontaneous secretion.

Previously it was reported that stimulation with synthetic peptides induced the secretion of IFN-γ in TT/BT leprosy cases (Sampaio et al 2012). Manca et al (2012), found that the level of IFN-γ was below the detection limit in PBMCs of healthy controls stimulated with PGL-1 antigen. Higher level of IFN-γ was found in PB cases compared to MB cases and no difference was found between TT & BT leprosy cases and between BL and LL leprosy cases in the study done by Stefani et al (2009). Stratification of the results on the
basis of -2518 A/G showed higher IFN-γ level in A/A genotype controls, followed by A/G and G/G genotype controls in unstimulated condition as well as in response to WCS antigen. No effect of WCS antigen was observed on IFN-γ secretion in any of the genotypes. However, in leprosy cases, the pattern and effect of antigen were found to be different in different genotypes. In unstimulated condition, the level was found higher in A/G genotype cases, followed by A/A and G/G genotype cases. Production of IFN-γ increased in response to WCS antigen in A/A and G/G genotype cases, while decreased in A/G genotype leprosy cases. Similar effect of rIFN-γ was also observed in different genotypes.

TNF-α level was found to be higher in healthy controls at basal level, after incubation in unstimulated condition and in response to WCS antigen, but the differences are not significant. We observed that TNF-α production was induced by WCS antigen in both leprosy cases and healthy controls, but the level was more in healthy controls. However rIFN-γ slightly increased the production in both leprosy cases and controls.

TNF-α was found to be elevated in the PBMCs from leprosy cases in response to stimulation with sonicated M. leprae compared to control unstimulated cells in the study of Fulya et al (2006). PBMCs from healthy controls infected with M. leprae elicits low levels of TNF-α in comparison to BCG stimulated and unstimulated controls cells (Sinsimer et al 2010). PGL-1 M. leprae antigen elicited very low level of TNF-α in the PBMCS of healthy controls, compared to unstimulated control cells (Manca et al 2012). In monocytes of healthy controls no spontaneous expression of TNF-α was observed while stimulation with BCG and M. leprae induced the secretion of TNF-α. LL leprosy cases expressed undetectable amount of TNF-α (Hasan et al 2004). PBMCs from healthy controls and THP-1 cell line stimulated with PGL-1 alone did not induce the secretion of TNF-α, but, when mixed with M. leprae, TNF-α secretion was found to increase (Charlab et al 2001). Strong M. leprae induced TNF-α release was observed in mononuclear cell culture derived from ENL leprosy cases, compared to normal leprosy cases (Santos et al 1993). TNF-α release was found higher in PBMCs of tuberculoid leprosy cases, compared to lepromatous leprosy cases and between cases with reactions and normal leprosy cases (Barnes et al 1992). In leprosy cases, the highest TNF-α concentration was found in G/G genotype leprosy cases, followed by A/G and A/A genotype leprosy cases.
in unstimulated conditions. WCS antigen induced the production of TNF-α in all the genotypes in leprosy cases. While rIFN-γ decreases the level of TNF-α in A/A and G/G genotype, but was increased in A/G genotype leprosy cases.

In healthy controls, the level of TNF-α was found higher in A/A genotype, followed by G/G genotype and lower in A/G genotype controls in unstimulated condition. Production of TNF-α was found to increase in response to WCS antigen in all the genotypes. The rIFN-γ however slightly increased the production of TNF-α in A/A and G/G genotypes, but decreased in A/G genotype controls.

The level of TGF-β was found higher in healthy controls, compared to leprosy cases in all conditions. The concentration of TGF-β at basal level was found to be highest in both leprosy cases and controls than under incubation and stimulated conditions. WCS M.leprae antigen and rIFN-γ slightly increased the production of TGF-β in leprosy cases, while in healthy controls, WCS exerts no effect on the production of TGF-β and rIFN-γ slightly increased the production, but the differences are not significant.

The analysis showed that within the -2518A/G genotype, the level of TGF-β is high in G/G genotype leprosy cases, followed by A/A and lower in A/G genotype cases in unstimulated conditions. In response to WCS antigen, TGF-β production was slightly higher in A/A and A/G genotype cases, while slightly lower in G/G genotype leprosy cases. rIFN-γ induced the production of TGF-β in all genotype cases.

In healthy controls also, highest TGF-β level was detected in G/G genotype, followed by A/G and lower in A/A genotype controls in normal spontaneous secretion. Stimulation with WCS antigen induced TGF-β production in A/A genotype, while in A/G and G/G genotypes, it exerts negative effect and decreased the production of TGF-β. The rIFN-γ also decreased TGF-β production in G/G genotype controls, but increased the production in A/A and A/G genotype cases, however, the differences were not significant. TGF-β was reported to be higher in PBMCs from leprosy cases in response to stimulation with sonicated M.leprae and in unstimulated cells compared to controls (Fulya et al 2006). TGF-β level was found to be higher in PBMCs of BL leprosy cases, compared to BT leprosy cases in response to stimulation with sonicated M.leprae (Venturinij et al 2011).

No spontaneous expression of TGF-β was observed in the PBMCs of healthy controls, while, after stimulation with BCG and M.leprae, the expression was increased.
Monocytes from LL leprosy cases spontaneously expressed higher levels of TGF-β (Hasan et al 2004). TGF-β isoform staining in skin biopsies showed significantly higher expression in LL leprosy cases, compared to TT leprosy cases with indication that macrophages are the most common and strongest immunoreactives (Kiszewski et al 2003). High TGF-β level was detected in the monocytes of different leprosy cases, compared to healthy controls, except TT leprosy cases in both normal spontaneous secretion and in response to LPS and PGL-1 stimulation (Goulart et al 2000).

5.8 Expression of MCP-1 and other chemokine genes in leprosy cases and controls

We also evaluated the expression of MCP-1, RANTES (CCL5), MIP-1α (CCL3) and IL-8(CXCL8) genes at mRNA level in 20 leprosy cases and 10 healthy controls. Significant difference was observed between leprosy cases and controls for the expression of CCL5 gene in both unstimulated condition and in response to WCS M.leprae antigen. However, no effect of antigen was observed on the expression of CCL5 in both leprosy cases and controls. The levels of expression of MCP-1, MIP-1α (CCL3) and IL-8 genes were observed higher in healthy controls in unstimulated condition compared to leprosy cases, but, the differences are not significant. No significant effect of WCS antigen was observed on the expression of MCP-1, MIP-1α (CCL3) and IL-8 genes. For MCP-1 the expression was found to be higher in controls, compared to leprosy cases in unstimulated condition. Similar results were also observed at protein level secreted by PBMCs, but opposite results were observed at the serum level. Fulya et al (2006), in their study reported that monocytes from lepromatous cases, stimulated with MLS antigen of M.leprae, produced elevated level of IL-8 compared to control.

The mRNA expressions of MCP-1 and IL-8 were reported to be significantly downregulated in M.leprae infected THP-1 cells, compared to non infected cells. The results were also reproduced in nerve biopsies of leprosy cases compared to non leprosy peripheral neuropathy (Guerreiro et al 2013). MCP-1 immunoreactivity was also observed in the nerve biopsies of leprosy cases, compared to non leprosy nerve samples. The labelling was dominant in recruited macrophages and schwann cells found in leprosy affected nerves. Hasan et al (2004), reported that IL-8 secretion in response to BCG and
*M. leprae* was not significantly high compared to normal healthy controls and no spontaneous expression was observed in healthy controls (Hasan et al 2004). Results analysed on the basis of *MCP-1* -2518A/G polymorphism showed that *MCP-1* expression was highest in A/A genotype, followed by A/G and G/G genotypes in both leprosy cases and healthy controls. WCS antigen of *M. leprae* decreased the expression of *MCP-1* gene in all genotypes of leprosy cases and healthy controls. Significant difference was observed in the expression of *MCP-1* between different genotypes in unstimulated condition in leprosy cases only, but no such difference was observed in controls.

The serum CCL5 level was reported to be reduced in leprosy cases compared to healthy controls, while no difference was found in PBMCs of leprosy cases and healthy controls in response to stimulation with BCG and *M. leprae* (Hasan et al 2005). Interestingly no difference was observed between different genotypes for the expression of CCL5 gene, while in controls, the expression was highest in G/G genotype controls, followed by A/G and lowered in A/A genotype healthy controls. However, the differences between genotypes were not significant.

In case of CCL3, no difference was observed between leprosy cases and healthy controls. The pattern of expression of CCL3 was not constant in leprosy cases and controls and the differences were not significant between the genotypes.

Expression of IL-8 was found to be higher in A/A genotype cases followed by A/G and lower in G/G genotype leprosy cases in unstimulated condition, but a reverse pattern was observed in healthy controls. Decrease in the level of expression was observed in response to WCS antigen. But, the differences were not significant between different genotypes.

### 5.9 Identification of protein(transcription factor) by EMSA and MALDI-TOF

Rovin and Saxena (1999) first reported the -2518A/G regulatory region polymorphism and found that the -2518G allele is associated with increased *MCP-1* gene expression in response to stimulus with IL-1β in the A172 human glioblastoma cells. In contrast to the findings of Rovin and Saxena (1999), Kim et al (2002) and Wright et al (2008) in their study found reduced transcriptional activity associated with the mentioned polymorphism. Following this observation several reports become available, which indicate that -
2518G allele has been associated with increased MCP-1 expression level in plasma serum, CSF and urine in normal and pathological conditions (McDermott et al 2005, Cho et al 2004, Fenoglio 2004, Letendre et al 2004, Joven et al 2006), as well as in tissues, such as skin and liver (Karrer et al 2005, Muhlbauer et al 2003). However, several negative results are also available where they did not find any association of -2518G allele with increased serum MCP-1 levels and -2518A allele rather than G associated with higher serum/plasma MCP-1 concentration in different diseases (Kim et al 2002, Zietz et al 2005, van Wijik et al 2010). Several earlier studies suggested that the -2518A/G polymorphism mediates its effect via differential binding of various transcription factors and altered transcriptional activity. Thus, the contradictory observations of increase, decrease or no change still remains, regarding the role of this -2518G allele in the transcription of gene (Rovin and Saxena 1999, Kim et al 2002, Wright et al 2008). Several transcription factors have been demonstrated, which bind differentially, in response to different stimulation, to MCP-1 -2518A/G polymorphic site, including, STAT-1, IRF-1, Prep1/ Pbx complexes, PARP-1, (Gonzalez et al 2002, Mummidi et al 2009, Wright et al 2008, Nyquist et al 2010). So far, no acceptable molecular mechanism is available, which can satisfactorily explain the effect of -2518A/G polymorphism on the transcription of MCP-1 gene. Considering these things in our study we also tried to identify the protein involved in the mechanism. In the present study we observed an elevated level of MCP-1 in subjects with A/A genotype compared to subjects with G/G genotype.

However, as described in the methodology section, we designed oligonucleotides containing A and G sequences and performed EMSA experiments with the nuclear lysate prepared from THP-1 cell lines, stimulated with \textit{M.leprae} and \textit{M.tb} antigens. The results of EMSA experiments showed that a protein binds to both the oligonucleotides containing A and G sequences in all experimental conditions, such as unstimulated and stimulated with antigens. The protein bound to the sequences was isolated from the gel and identified as a transcription factor, the Zinc finger protein, by MALDI-TOF analysis. As the detected protein is capable of binding to -2518A as well as -2518G site, it indicates that possibly this protein binds to -2518 promoter region irrespective of any
specific base pair. Since we observed the similar expression of this protein with and without stimulation of antigens, it might be expressed constitutively.

Further studies are required to find out the reason behind the higher expression of serum MCP-1 level in A/A genotype cases rather than in G/G genotype, as observed in our study or higher level of MCP-1 in G/G genotype, compared to A/A genotype, as reported earlier in several studies, since multiple parameters may likely be involved, besides the molecular mechanism of transcriptional regulation of the gene.